Early infant HIV diagnosis and characterization of HIV drug resistance in Gauteng, South Africa

By

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DECLARATION OF ORIGINALITY

I, Odette Smit (Student Number:15187030), declare that this work was not copied or repeated from any other studies either from national or international publications. Procedures were carried out in accordance with the ethical rules as prescribed by the Faculty of Health Science Research Ethics Committee, University of Pretoria.

Signature: Date:

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EARLY INFANT HIV DIAGNOSIS AND CHARACTERIZATION OF HIV DRUG RESISTANCE IN GAUTENG, SOUTH AFRICA

By

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SUMMARY

Despite the high prevalence of the human immunodeficiency virus (HIV) in South African women of reproductive age, the South African (SA) Prevention of Mother-to-Child Transmission (PMTCT) programme has significantly reduced the incidence of new HIV infections in infants from >20% in 2004 to <2% and overall MTCT approximately >5%. The PMTCT programme, however, faces challenges in terms of early infant diagnosis (EID) because of HIV polymerase chain reaction (PCR) indeterminate results as well as HIV drug resistance (HIVDR) secondary to antiretroviral therapy (ART) exposure of mothers and infants. The National Health and Laboratory Services (NHLS) uses the Roche COBAS®AmpliPrep (CAP)/COBAS®TaqMan® (CTM) HIV-1 Qualitative Test (Roche Molecular Systems, Pleasanton, CA) (CAP/CTM) platform as part of EID. Recently, the Roche cobas® 6800/8800 System has been introduced to test HIV viral load and HIV DNA PCR for EID. The platform is already processing samples for HIV viral load; however, verification for HIV DNA PCR for EID with dried blood samples (DBS) is needed, especially for CAP/CTM HIV PCR indeterminate results (Cycle threshold [Ct]>33 with any relative fluorescent intensity [RFI] value or Ct≤33 and RFI <5 on the CAP/CTM,

Ct>38 on the Roche cobas® 6800/8800 System). In addition, HIVDR in newly diagnosed infants significantly limits treatment options. Therefore, the current study verified the Roche cobas® 6800/8800 System against the CAP/CTM system for the detection of HIV in EID and determined the HIVDR prevalence and profiles in infants ≤6 months, and how this affects current SA PMTCT and EID guidelines.

The study comprised 642 DBS samples (235 HIV PCR positive, 193 HIV PCR negative and 214 HIV PCR indeterminate) previously tested on the CAP/CTM assay. Overall, 99.6% (234/235) CAP/CTM HIV PCR positive samples remained positive, while 99.5% (192/193) HIV PCR negative samples remained negative with the Roche cobas® 6800/8800 System. The HIV PCR indeterminate results as detected by the CAP/CTM decreased from 100% (214/214) to 8.4% (18/214) with the Roche cobas® 6800/8800 System. The Roche cobas® 6800/8800 System had a specificity of 99.5% and a sensitivity of 99.6%, but this decreased to 96.3% and 90.8% when HIV PCR indeterminate results were included. The kappa value increased from 0.5, which signifies moderate agreement, to 0.9, which is excellent agreement, when RFI from the CAP/CTM was excluded for result determination. The overall agreement between the two assays, taking only cycle threshold values into account, was 93.8%.

As for HIVDR, mutations were detected in 42.9% (24/56) of infants ≤6 months. The most common non-nucleoside reverse transcriptase inhibitor (NNRTI) mutation causing high-level resistance was K103N (21.4% [12/56]), followed by Y181C and the NRTI mutation, M184V, both in 8.9% (5/56) of infants. Also, major protease inhibitor (PI) mutations, M46L and V82A were detected in one case each (1.8%).

In conclusion, the performance of the Roche cobas®6800/8800 System was comparable to the CAP/CTM; however, it detected fewer HIV PCR indeterminate results, thus potentially offering conclusive results in a larger proportion of infants. The detection of high levels of the NNRTI mutation, K103N, emphasises the need for constant surveillance since nevirapine is included as part of the SA PMTCT guidelines and the World Health Organization recommends that NNRTIs should be phased-out of as part of PMTCT once the resistance prevalence exceeds 10%.

PRESENTATIONS AND PUBLICATIONS

Smit O, Rossouw TM, Lukhwareni A. Assessment of Roche cobas®6800/8800 System for HIV early infant diagnosis [Oral presentation].Virology Africa 2020, Cape Town, South Africa. 10-14 February 2020.

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LIST OF ABBREVIATIONS

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
APV	Amprenavir
ART	Antiretroviral therapy
ARV	Antiretroviral
ATV	Atazanavir
AZT	Azidothymidine (also known as zidovudine)
CAP/CTM	Roche COBAS®AmpliPrep/COBAS®TaqMan® HIV-1 Qualitative Test
CCR5	C-C motif chemokine receptor type 5
CD	Cluster of differentiation
CRF	Circulating recombinant form
Ct	Cycle threshold
CXCR4	C-X-C motif chemokine receptor 4
d	Day
DBS	Dried blood spot
ddI	Didanosine
DOR	Doravirine
DTG	Dolutegravir
EID	Early infant diagnosis
EFV	Efavirenz
ETR	Etravirine
FDC	Fixed-dose combination
FP	False positive
FPV	Fosamprenavir
FN	False negative
FTC	Emtricitabine
HIV	Human immunodeficiency virus
HIVDR	Human immunodeficiency virus drug resistance
IDV	Indinavir
IN	Integrase
INSTI	Integrase strand transfer inhibitor
LIS	Laboratory Information System
LPV	Lopinavir
m	Month
MTCT	Mother-to-child transmission
NFV	Nelfinavir
NHLS	National Health Laboratory Service
NPV	Negative predictive value
NRTI	Nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
PCR	Polymerase chain reaction
PI	Protease inhibitor
PMTCT	Prevention of mother-to-child transmission
Pol	Polymerase
PPV	Positive predictive value
PR	Protease
PrEP	Pre-exposure prophylaxis
RFI	Relative fluorescence intensity
RPV	Rilpivirine
RT	Reverse transcriptase

RTV	Ritonavir
SA	South Africa
SOP	Standard operating procedures
TDF	Tenofovir disoproxilfumarate
TN	True negative
TP	True positive
WHO	World Health Organization

CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

Most human immunodeficiency virus (HIV) infections in infants occur via mother-to-child transmission (MTCT). Between 2010 and 2018, the prevention of MTCT (PMTCT) programme prevented about 1.4 million infections in children globally (UNAIDS, 2018c). In sub-Saharan Africa, the number of new HIV infections in children below the age of 14 years decreased from about 170 000 to 84 000 between 2010 and 2018 (HIV/AIDS, 2019). In South Africa (SA), between 2010 and 2018, the number of new HIV infections in children decreased from 28 000 to 14 000 because of a successful PMTCT programme (Avert, 2020a) .

The risk of infant HIV infections in SA through early MTCT has significantly decreased to less than 2% and overall MTCT approximately >5% since implementing the PMTCT programme but this has not, however, entirely prevented transmission (Ndirangu *et al.*, 2010, Sherman *et al.*, 2014, Shisana *et al.*, 2014, Zuma *et al.*, 2016). The use of antiretroviral therapy (ART) as part of PMTCT has made the laboratory diagnosis of new infections and HIV drug resistance (HIVDR) the subject of intense academic discussion (Maritz *et al.*, 2017).

Early infant diagnosis (EID) of HIV infection is complicated by the fact that infant exposure to ART reduces the levels of HIV RNA or proviral DNA, thus leading to indeterminate results (Mazanderani *et al.*, 2014). These infants are not linked to treatment centres and frequently become lost to follow up (Maritz *et al.*, 2017). The PMTCT programme also faces the challenge of HIVDR mutations caused by maternal and infant exposure to ART, which complicates and limits treatment options.

1.2 Human immunodeficiency virus

1.2.1 Classification

The HIV is a lentivirus and a member of the *Retroviridae* family. The *Retroviridae* family comprises enveloped viruses that can reverse transcribe their single-stranded RNA (ssRNA) genome into double-stranded DNA (dsDNA), and then incorporate the viral DNA into the host genome, thus forming a provirus (Waheed and Freed, 2010,

Gifford *et al.*, 2018). The virus can do this through the use of enzymes, specific to the *Retroviridae* family, known as reverse transcriptase (RT) and integrase (IN) (Gifford *et al.*, 2018).

There are two major groups of human lentiviruses, which originated from simian immunodeficiency viruses (SIVs) from nonhuman primates. The HIV type-1 (HIV-1) originated from a chimpanzee reservoir (SIVcpz), while HIV type 2 (HIV-2) originated from a sooty mangabey monkey virus (SIVsmm) (Fanales-Belasio *et al.*, 2010, Bächle *et al.*, 2016, Lewthwaite and Melhuish, 2018). HIV-1 and HIV-2 are related viruses, and they cause acquired immunodeficiency syndrome (AIDS) which decreases the immunity of the host and can eventually lead to death (Bagby *et al.*, 2015, Bächle *et al.*, 2016).

The HIV-1 is responsible for most cases of AIDS and is the cause of the HIV pandemic worldwide, while HIV-2 is predominantly limited to West Africa (McCutchan, 2006, Fanales-Belasio *et al.*, 2010, Bächle *et al.*, 2016). The major difference between HIV-1 and HIV-2 is that the latter is naturally resistant to all fusion inhibitors and non-nucleoside reverse transcriptase inhibitors (NNRTIs) and is also less transmittable with a lower likelihood of progression to AIDS (Nyamweya *et al.*, 2013, Triki *et al.*, 2018).

The HIV-1 is further classified in three subgroups, M (main, having clades A, B, C, D, F, G, H, J, and K), N (new), O (outlier) and P (derived from gorillas and thus far only a case reported in Cameroon and a case in a Cameroonian women living in France), while HIV-2 has subtypes A-G (Plantier *et al.*, 2009, Vallari *et al.*, 2011). When a cell becomes infected by two viruses of different subtypes, a hybrid mosaic virus can result from sharing genetic material, which the infected cell can shed and transmit (Fanales-Belasio *et al.*, 2010). These hybrid mosaic viruses are known as circulating recombinant forms (CRFs).

1.2.2 The HIV morphology

The complete spherical virion of HIV has a diameter of 100–120 nm. It comprises two ssRNA strands that are within the capsid, which is composed of 250 CA (p24) protein hexamers and also 12 CA pentamers (Fanales-Belasio *et al.*, 2010, Mattei *et al.*, 2016, Rankovic *et al.*, 2017). The virus is enveloped, surrounded by a lipid membrane making it more susceptible to the external environment, and therefore can easily be deactivated

when coming into contact with alcohol or other disinfectants (Figueroa *et al.*, 2017, Ghanim and Thwiny, 2018). The lipid bilayer membrane surrounding the virus is a dense truncated cone-shaped nucleocapsid (core). Myristoylation anchors the p17 matrix protein to the inside of the viral lipoprotein membrane (Gelderblom *et al.*, 1988). The p24 core antigen surrounds two copies of positive genomic ssRNA, which contains all the enzymatic machinery necessary for replication: RT (p66/p51), IN (p32) and PR (protease) (p11) (Kirchhoff, 2013). Accessory genes: *Vpu, Vif, Vpr and Nef*, and some cellular factors are also included (Faust *et al.*, 2017). Figure 1.1 illustrates the spherical HIV structure.

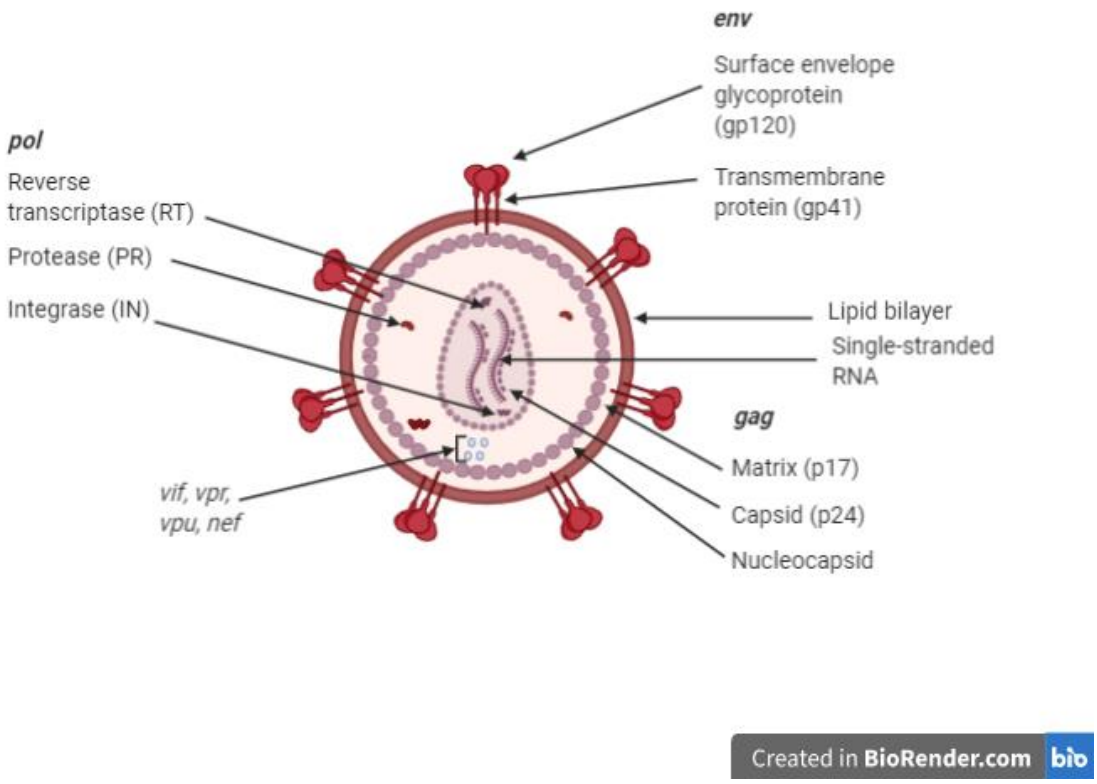

Figure 1.1: The HIV structure (Image created in BioRender.com)

1.2.3 Genetic structure of HIV

The HIV genome comprises structural, regulatory and accessory genes. The genome also comprises two identical ssRNA strands of about 9.2 kb that contain nine genes; three structural genes that are common to all retroviruses, such as group-specific antigen (*gag*), polymerase (*pol*) and envelope (*env*); and also six regulatory genes (i.e.

tat, rev, nef, vif, vpr, and *vpu*) which are depicted in Figure1.2 (Sierra *et al.*, 2005). The "classic" structure of the HIV genome is flanked by two long terminal repeat (LTR) regions and thus comprises of 5'LTR-gag-pol-env-LTR 3'. The LTR regions of the HIV genome are connected to the cellular DNA of the host cell after integration (Richetta *et al.*, 2019). The three structural genes (*gag, pol* and *env)* are the carriers of the required information for structural proteins necessary to form the new virus particles, while the six regulatory genes (*tat, rev, nef, vif, vpr,* and *vpu)* are necessary for the production of proteins which control the ability of HIV to infect a cell, and produce new copies of the virus, or cause disease (Rappaport *et al.*, 1994). Each of the regulatory genes also codes for accessory proteins, which enhances viral production and plays an essential role during virus replication (Malim and Emerman, 2008). The *vpu* accessory protein is unique to HIV-1, while *Vpx* is unique to HIV-2 (Sierra *et al.*, 2005). The protein product of the *tat* gene promotes the expression of the HIV genes, while the *rev* gene product ensures that correctly processed messenger RNA (mRNA) will be exported from the nucleus to the cytoplasm (Fanales-Belasio *et al.*, 2010, Kirchhoff, 2013).

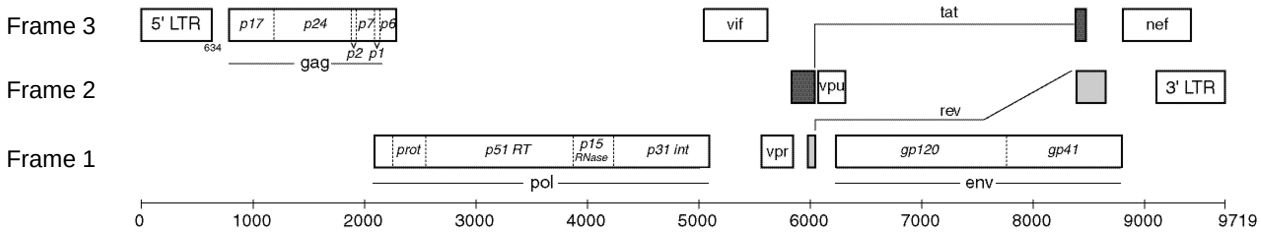

Figure 1.2: The genetic structure of HIV-1 (Image from Wikimedia Commons, the free media repository)

1.2.3.1 The group-specific antigen gene

The *pol* and the *gag* genes are translated from the same mRNA, but into different proteins through frameshifting (Sierra *et al.*, 2005). The frameshifting is essential for the production of *pol* gene products (PR, RT and IN) (Sierra *et al.*, 2005).

The *gag* gene encodes for both the structural proteins of the core and the matrix. The Gag protein is translated from viral RNA which contains a signal which shifts the

reading frame to -1 during translation, thus leading to expression of both a *gag* and *gag-pol* polyprotein (Wang *et al.*, 2019).

The Gag polyprotein is 55 kDa in size and is cleaved during viral maturation to produce structural proteins that will produce the mature virion upon their rearrangement (Sierra *et al.*, 2005). These structural proteins include the capsid protein (p24), the matrix protein (p17), the nucleoprotein (p7) and p6 (involved in the release of the virus particle). The *gag* gene alone can direct the synthesis, transport to the plasma membrane, and assembly of the structural precursor polyprotein Gag (Sierra *et al.*, 2005).

1.2.3.2 The envelope gene

Within the envelope gene is the code for the envelope glycoprotein gp160 precursor which is cleaved into surface glycoprotein gp120 and transmembrane glycoprotein gp41 which plays a role during host cell recognition and membrane fusion, respectively (Fanales-Belasio *et al.*, 2010, Checkley *et al.*, 2011). The gp120 protein is embedded in the viral lipid membrane and is primarily responsible for host cell receptor binding through host cell tropism to the cluster of differentiation (CD) 4 and co-receptors (C-C motif chemokine receptor 5 [CCR5] and C-X-C motif chemokine receptor 4 [CXCR4]) (Wilen *et al.*, 2012). The gp120 is structured into five conserved domains (C1-C5) that fold into a core and five highly variable domains (V1-V5) (Douglas *et al.*, 1997, Crublet *et al.*, 2008, Wang *et al.*, 2008).

1.2.3.3 The polymerase gene

The *pol* gene encodes the enzymes necessary for viral replication, which are originally synthesised as part of the large p160 (*gag-pol*) polyprotein precursor. During translation, a frameshift event on the p160 precursor leads to the production of enzymes: RT, PR and IN (Jacks *et al.*, 1988) (Figure1.3). The enzymes are discussed in the following sections.

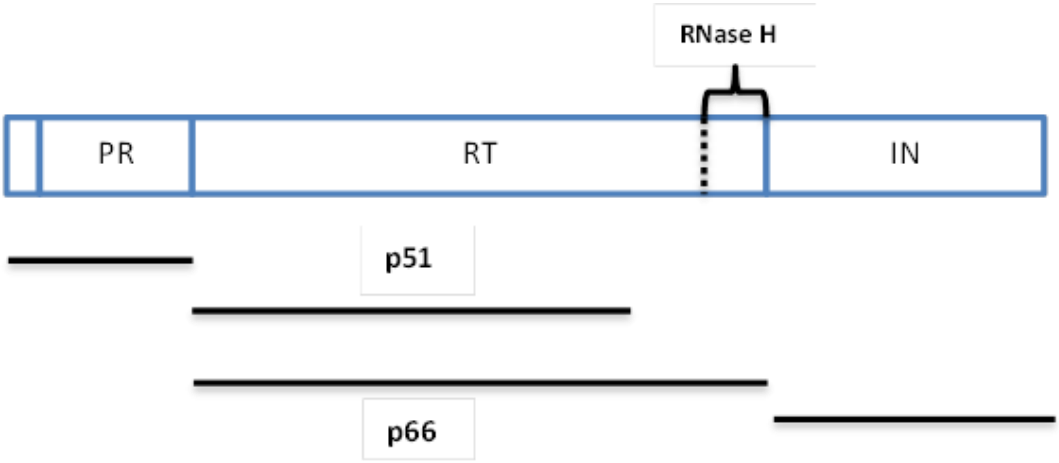

Figure 1.2: Schematic diagram of the HIV *polymerase* gene illustrating protease, reverse transcriptase and integrase (Image created in Microsoft Word 2010)

1.2.3.3.1 Reverse transcriptase

The RT enzyme is a heterodimer comprising the p66 (containing both polymerase and RNase H active sites which are necessary for the conversion of ssRNA into dsDNA upon entry into a host cell) and the p51 subunit, with the latter lacking the RNase H domain (Mulky *et al.*, 2004, Das and Arnold, 2013).

The p66 subunit (560 amino acids) comprises the DNA-binding groove and the active site while the p51 subunit (only the first 440 amino acid residues) does not display any enzymatic activity but functions as a scaffold for the p66 subunit (Shafer *et al.*, 2000a). The *pol* domain of the p66 subunit resembles a human right hand with fingers, a palm and a thumb and connection domains, which connects the polymerase domain (which serves as the primary location for nucleoside reverse transcriptase inhibitor [NRTI] or non-nucleoside reverse transcriptase inhibitor [NNRTI] mutations to occur) to the RNase H domain (Das and Arnold, 2013).

The p51 chain also resembles the hand structure; however, it differs in the spatial arrangement, having a rigid structure which structurally supports the p66 chain (Das and Arnold, 2013). Binding of an NNRTI in the binding pocket (Figure 1.4) will lead to a disruption in the conformational flexibility of the RT enzyme (Nizami *et al.*, 2016).

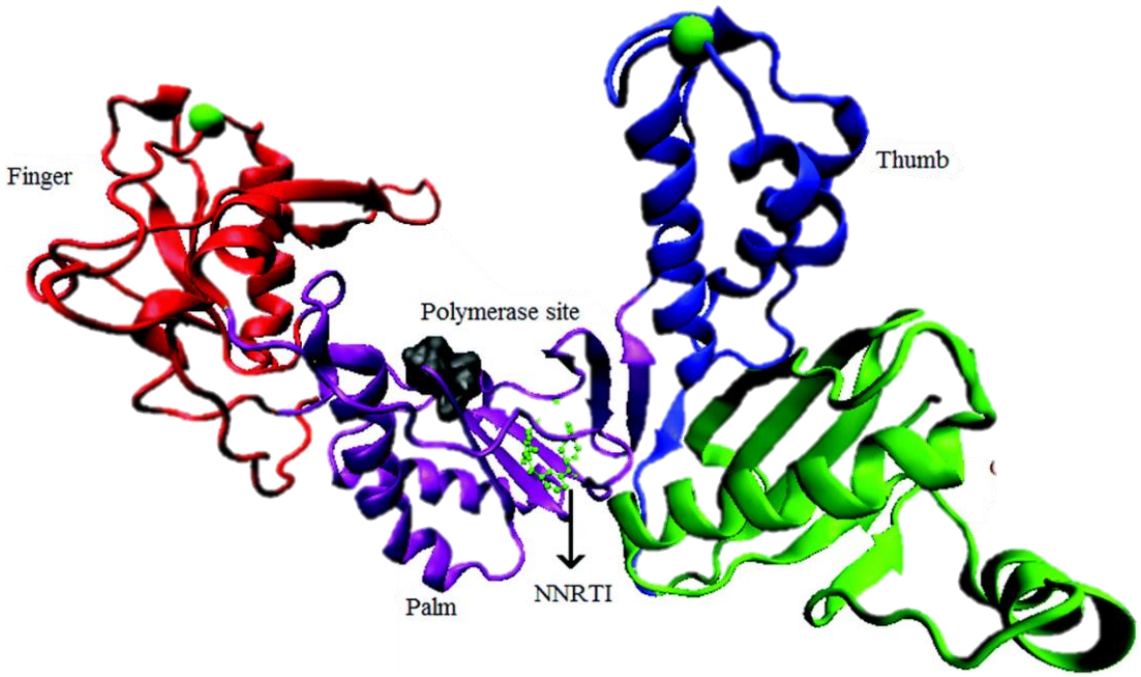

Figure 1.43: The "right hand" structure of the reverse transcriptase enzyme (image adapted from Nizami *et al*., 2016)

The viral RNA genome serves as a template for the RT enzyme to produce a minus-strand DNA, thus leading to the production of an RNA-DNA hybrid (Abbondanzieri *et al.*, 2008). The RNase H domain of the RT enzyme will cleave the RNA strand at multiple points, thus producing short RNA segments; two of which will serve as primers for the production of the plus-strand DNA (Abbondanzieri *et al.*, 2008).

The RT enzyme is error-prone, misincorporating about one mistake per 10^4 nucleotide incorporations, thus generating mutant HIV-1 proteins (including mutant RTs) which ultimately lead to NRTIs and NNRTIs resistance (Das and Arnold, 2013). The fingers and palm subdomains are most commonly associated with mutations that lead to NRTI and NNRTI resistance (Das and Arnold, 2013).

1.2.3.1.2 Protease

The PR enzyme is a C2 symmetric homodimer accompanied by a large substrate-binding pocket, covered by two “flaps” (glycine-rich-ß-hairpins) (Hornak *et al.*, 2006). The PR enzyme has two forms, namely the semi-open form and the closed form which is determined by the binding of a PR inhibitor (PI) (Figure 1.5) (Hornak *et al.*, 2006). Resistance to PIs arises from mutations within the “flaps” of the PR enzyme (Agniswamy *et al.*, 2016).

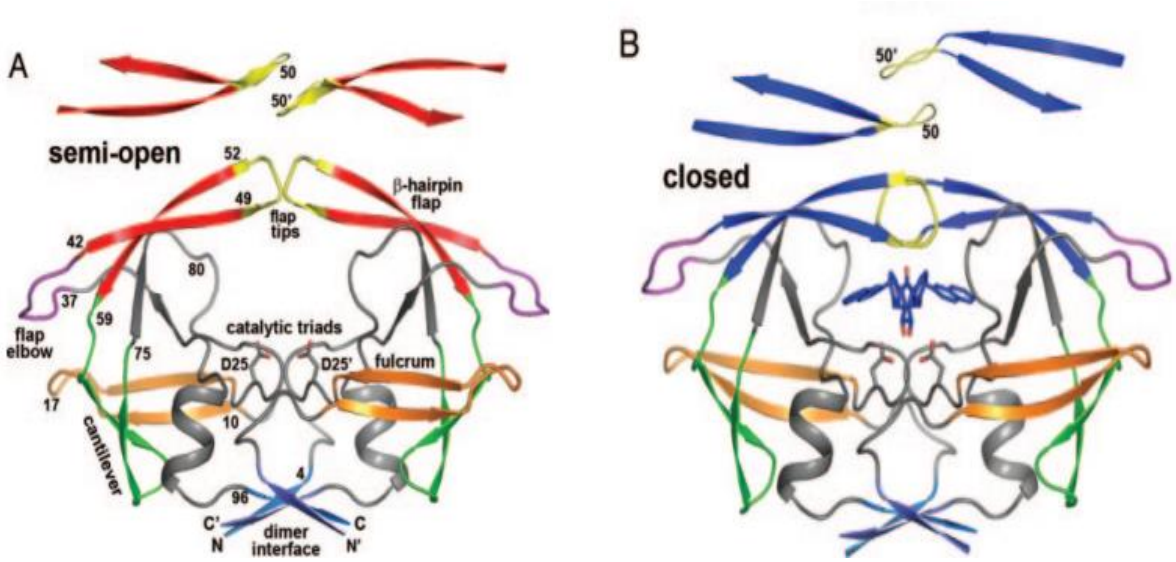

Figure 1.4: The structure of the protease enzyme (Hornak *et al.*, 2006)

The maturation of HIV-1 depends on the ability of the aspartic PR enzyme to proteolytically process the Gag and Gag-Pol polyproteins to release the structural proteins and necessary enzymes for viral assembly of an infectious particle (Kohl *et al.*, 1988, Könnyű *et al.*, 2013). The PR enzyme is assembled from two identical monomers of about 100 amino acids that each contributes an aspartate side chain to the active site (Wosicki *et al.*, 2019). The PR enzyme has a binding cleft that recognises and cleaves different sequences to produce the matrix, capsid, nucleocapsid and the PR, RT and IN proteins from the *gag-pol* polyprotein (Könnyű *et al.*, 2013, Weber *et al.*, 2015).

A single point mutation in the cleft of the PR enzyme can lead to drug-resistance, as mutations lead to reduced binding affinity between the drug and the mutant PR enzyme (Shafer *et al.*, 2000b, Dunn *et al.*, 2002). High-level resistance generally require the accumulation of multiple mutations within the PR gene (Weber *et al.*, 2015).

1.2.3.1.3 Integrase

The IN enzyme is a 32 kDa protein comprising 288 amino acids encoded by the 3' end of the HIV *pol* gene (Mohammed *et al.*, 2011). The IN enzyme is responsible for the insertion of HIV DNA into the chromosome of the infected cell (Delelis *et al.*, 2008). There are 3 functional domains within the IN enzyme, namely the catalytic core domain

(A), the N-terminal domain (B) and the C-terminal domain (C) (Figure 1.6) (Mohammed *et al.*, 2011, Shah *et al.*, 2014, Machado *et al.*, 2019).

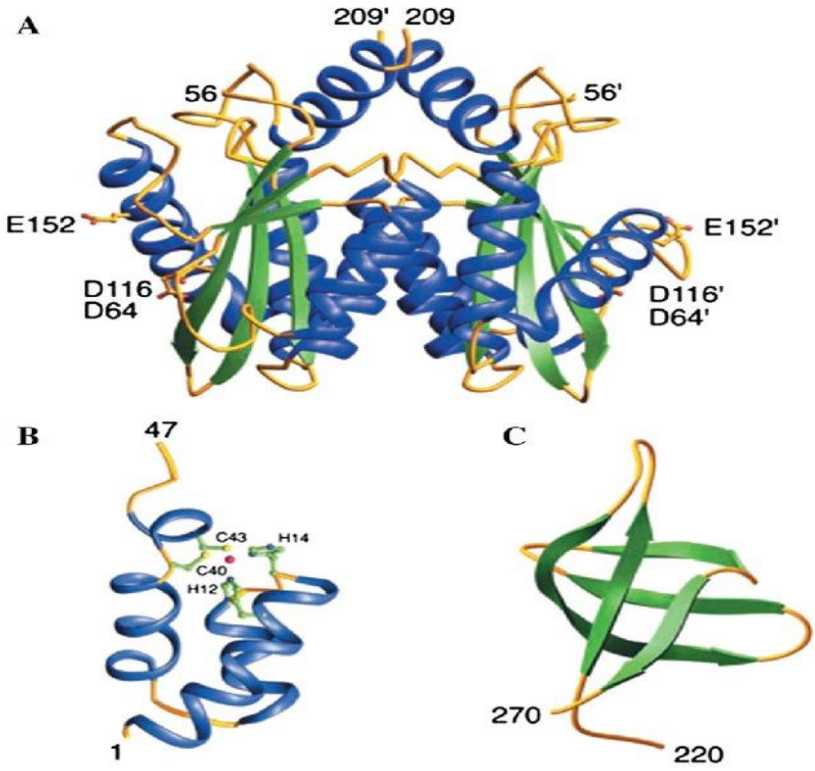

Figure 1.5: The structure of the integrase enzyme with the three domains (Shah *et al.*, 2014)

Integration by the IN enzyme is possible because of two major catalytic activities, namely 3'-processing and strand transfer. During 3'-processing, cleavage occurs at the terminal 3'-OH of the viral genome and strand transfer occurs via a trans-esterification mechanism (Delelis *et al.*, 2008). The ability of the enzyme to integrate the viral DNA into the host DNA is the reason it became an ideal target for drug design. The IN inhibitor drugs possess a two-metal-binding motif which attaches to the metals at the IN catalytic site, which is essential for its functioning, thus inhibiting the catalytic activities of the IN enzyme (Hightower *et al.*, 2011).

1.2.4 Replication

The replication process starts when the virion binds to a CD4 cell, and the appropriate co-receptor, resulting in fusion of the viral envelope and the cellular membrane, followed by the release of the viral nucleocapsid into the cytoplasm. The CD4 monomeric glycoprotein is found on the cell surface of about 60% of circulating T-lymphocytes, monocytes or macrophages, eosinophils, dendritic cells, and microglial

cells (Fanales-Belasio *et al.*, 2010). Once gp120 binds to the CD4 receptor, structural changes occur which allow membrane fusion to take place. The virus also binds to a CXCR4 or CCR5 co-receptor on the host cell and will enter the cell via membrane fusion with the help of the gp41 heterodimer (Golding *et al.*, 2002, Fanales-Belasio *et al.*, 2010).

After membrane fusion, viral core uncoating releases the viral RNA into the cytoplasm of the host cell (Fanales-Belasio *et al.*, 2010). The released viral RNA is then reverse transcribed by the RT enzyme to generate proviral DNA which is transported to the nucleus (Hu and Hughes, 2012). The IN enzyme then integrates the proviral DNA into the host genome where it undergoes transcription to produce mRNA (Lampejo and Pillay, 2013).

The mRNA is exported from the nucleus where it undergoes translation in order to produce the structural proteins which will assemble to form new viral particles (Fanales-Belasio *et al.*, 2010, Lampejo and Pillay, 2013). The new viral particles are then released from the host cell via budding and then mature with the help of the PR enzyme to form an infectious viral particle (Bounou *et al.*, 2002, McConville *et al.*, 2014). The replication process is summarised in Figure1.7 below.

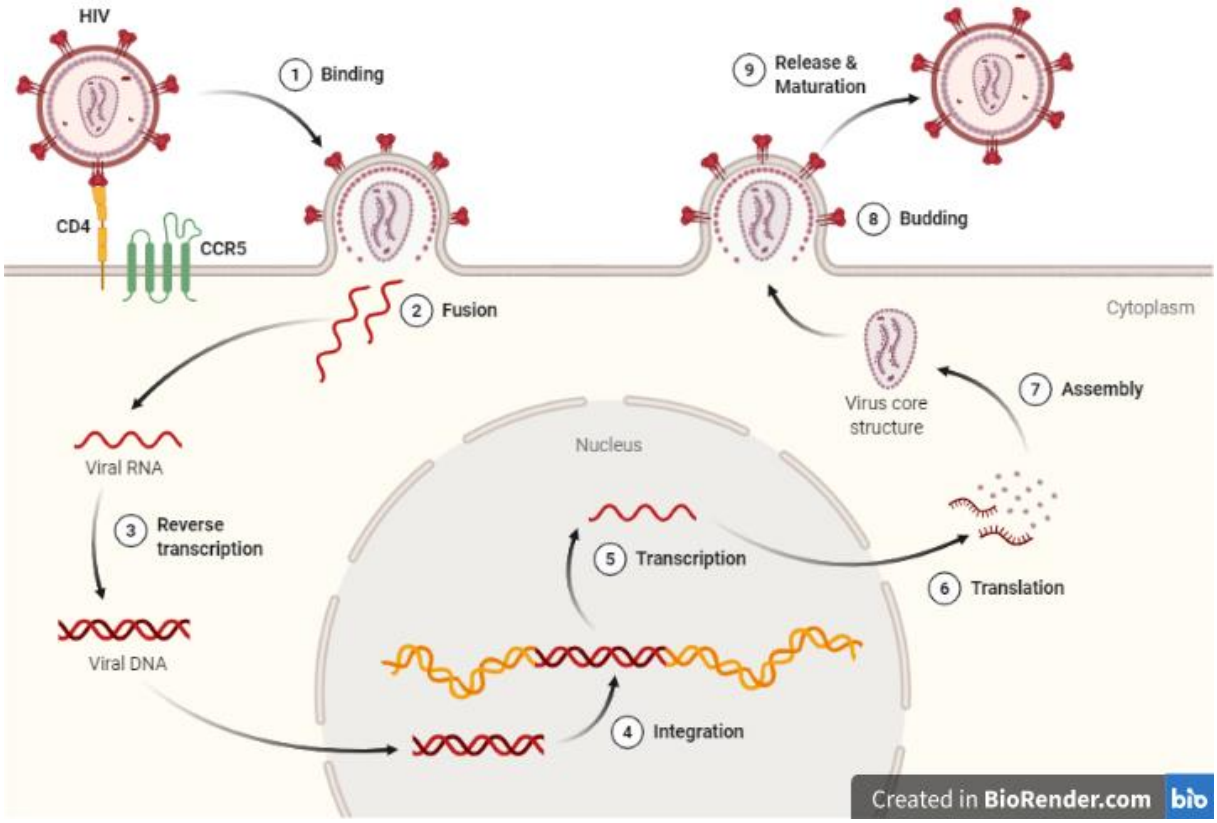

Figure 1.7: The replication cycle of HIV. The image illustrates the steps from host cell recognition to maturation of the virion (Image created in BioRender.com)

1.2.5 Disease profile

The HIV is transmitted through blood or other bodily fluids and can cause AIDS (Lewthwaite and Melhuish, 2018). Outbreak and transmission control of HIV is difficult, because of the delayed period between infection and diagnosis, population movement that can disperse HIV between different geographical regions, and the difficulty in identifying contacts of HIV-infected individuals who are at a high risk of acquiring the infection (Oster *et al.*, 2018).

There are different stages of HIV infection in untreated individuals. During the viral transmission stage, an HIV infection is acquired through sexual intercourse with an infected individual, exposure to infected blood, or via the perinatal route (Lewthwaite and Melhuish, 2018).

During the acute and early HIV infection stage, rapid viral replication and infection of CD4 cells will occur and the infected individual can be asymptomatic or can experience symptoms such as fever, lymphadenopathy, sore throat, rash, myalgia or arthralgia,

diarrhoea, and headaches (Silasi *et al.*, 2015, Sax, 2018). Seroconversion, the time when detectable antibodies against HIV antigens develop, will occur after HIV infection and can be detected by using nucleic acid amplification of viral RNA or by detecting the p24 viral core protein (Busch and Satten, 1997, Ling *et al.*, 2000, Fanales-Belasio *et al.*, 2010, Lewthwaite and Melhuish, 2018). After seroconversion, the viral set point (steady state of plasma viraemia) will be established at about 6 months of infection (Sax, 2018).

The chronic HIV stage (without AIDS) follows seroconversion and viral set point establishment but occurs before severe immune suppression (Sax, 2018). At this stage, the HIV-infected individual can be asymptomatic or can experience symptoms involving the skin or mucous membranes (Sax, 2018).

The final stage, AIDS and advanced HIV infection, occurs once the CD4 cell count has dropped drastically, thus allowing for multiple opportunistic infections to occur in the HIV-infected individual (Sax, 2018). Selinger and Katze (2013) generated a mathematical model of the stages of HIV infection as seen in Figure 1.8 (Selinger and Katze, 2013).

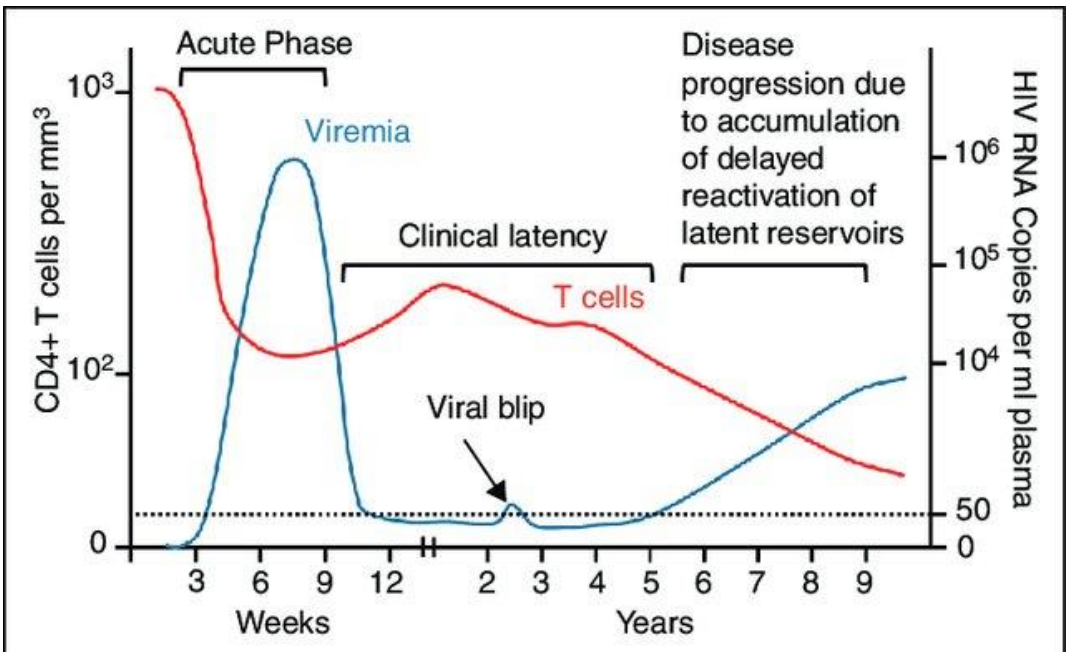

Figure 1.8: The different stages of HIV infection(Selinger and Katze, 2013). The viral blip is the short-termed viraemia above the level of detectability (Selinger and Katze, 2013).

1.2.6 HIV prevalence

In 2018, an estimated 1.7 million children below the age of 15 years were living with HIV and only 54% were accessing ART (UNAIDS, 2019b). Eastern and sub-Saharan Africa have the highest prevalence of HIV infections amounting to roughly 19.6 million people living with HIV and new infections of 800 000 in 2017, with 92 000 being children aged 0-14 years (UNAIDS, 2018b). The major cause of these new HIV infections is MTCT (Kityo *et al.*, 2017, Mutabazi *et al.*, 2017, Peltzer *et al.*, 2017).

South Africa has the highest incidence of HIV infection in the world and remains the country with the highest burden of people living with HIV (approximately 7.52 million in 2018). Most of the HIV infections in SA are in women with roughly one-fifth of women between the ages of 15–49 years (reproductive ages) being HIV positive (STATS-SA, 2018). In 2019, an estimated number of 340 000 children between 0 to 14 years of age were living with HIV in SA (UNAIDS, 2019a).

1.2.7 Mother-to-child transmission

Approximately 91% of the 1.4 million pregnant women living with HIV in 2017 were from sub-Saharan Africa (Bianchi *et al.*, 2019). Regarding HIV infections in infants, 90% of these infections occur because of MTCT transplacentally, during delivery, or through breastfeeding (UNICEF, 2018, Bianchi *et al.*, 2019).

About one-third of children born to HIV-positive mothers will acquire HIV, in the absence of PMTCT, with a third to half of these infections occurring through breastfeeding (Moland *et al.*, 2010). According to the PMTCT programmes in sub-Saharan Africa, HIV-infected mothers can either exclusively breastfeed their infant or exclusively formula feed their infant in the first 6 months of life (Moland *et al.*, 2010, Kafulafula *et al.*, 2014). The HIV transmission rate to infants for mothers practising exclusive breastfeeding while on ART is less than 1% (Nyoni *et al.*, 2019).

In sub-Saharan Africa, over 1 000 new-borns are infected with HIV per day because of MTCT (Hampanda, 2012). A nationally representative sample of SA HIV-exposed infants showed a 4.3% cumulative risk in MTCT at 18 months of age from 2012 to 2013, with 81% of all transmissions occurring by 6 months of age (Goga *et al.*, 2016a).

Compared to MTCT for infants born to mothers infected with HIV prior to pregnancy, the risk of MTCT is much higher when the mother becomes infected during pregnancy (Mandala *et al.*, 2019). If no transmission prevention interventions were implemented, the MTCT rate would be about 15%-25% during pregnancy or at delivery, while the rate during breastfeeding would be approximately 5%-20% (De Cock *et al.*, 2000, Ramraj *et al.*, 2018).

1.2.8 Prevention of mother-to-child transmission

The PMTCT programmes strive to prevent transmission of HIV to infants either *in utero*, during delivery or during breastfeeding. The annual global number of HIV infections has declined by about 35% from the year 2000 to 2015 because of proper prevention programmes and increased global coverage of PMTCT from 50% in 2010 to 77% in 2015 (World Health Organization, 2016b, Jordan *et al.*, 2017, UNAIDS, 2018b). By 2014, Botswana, Ghana, Namibia and Zambia had achieved about 90% PMTCT coverage, Rwanda achieved over 90% coverage and SA achieved over 80% coverage (World Health Organization, 2013, World Health Organization, 2014, Mutabazi *et al.*, 2017). In priority African countries MTCT has declined significantly via the PMTCT programme by 42% (Ndirangu *et al.*, 2010, Adetokunboh and Oluwasanu, 2016, Kharsany and Karim, 2016, Zuma *et al.*, 2016, UNAIDS, 2018d). Due to the PMTCT programme, SA has reduced early MTCT drastically from over 20% in 2014 to less than 2% in 2015 (Ndirangu *et al.*, 2010, Shisana *et al.*, 2014, Zuma *et al.*, 2016).

The risk of vertical HIV transmission has been reduced from about 40% to between 2-5% in some research and pilot settings in sub-Saharan Africa because of PMTCT measures such as ART (Tylleskär, 2008, Doherty *et al.*, 2009). In SA, the PMTCT programme reduced the rate of early infant transmission from almost 21% to about 1.5% between 2004 and 2015 (Sherman *et al.*, 2014, Moyo *et al.*, 2018a).

A nationally representative study conducted between 2012 and 2013 reported that the risk of early MTCT in SA has declined from 15% in 2009 to about 2.6% in 2012/2013 (Goga *et al.*, 2016b) and others have reported an 84% decrease in MTCT from 2009 to 2015, with approximately 330 000 infections prevented, secondary to increased coverage of the PMTCT programme in the country (World Health Organization, 2015, Mnyani *et al.*, 2020). Unfortunately, despite the wide availability of treatment and

although 88% of pregnant women were tested for HIV in 2018, only 87% of those living with HIV received ARVs to reduce the risk of MTCT (Avert, 2020b).

The World Health Organization (WHO) PMTCT guidelines have evolved from Option A (different ART regimens given to mothers depending on their immunological profile) in 2010 to Option B (triple ART for all HIV-infected pregnant and breastfeeding mothers) in 2013 and finally to Option B+ (lifelong ART irrespective of CD4 count or WHO staging) in 2015 (Moyo *et al.*, 2018b). The latter comprises lifelong treatment with a combination of 3 antiretrovirals (ARVs) regardless of the CD4 count or the clinical staging of the patient (World Health Organization, 2012). Table 1.1 illustrates the different options (Kim *et al.*, 2013).

Table 1.1: Summary of the different WHO Options for PMTCT (Kim *et al.*, 2013)

WHO option	Women-Treatment	Women-Prophylaxis	Breastfeeding Infants - Prophylaxis
A *(Requires CD4)*	CD4≤350: Triple ART for life	CD4>350: Zidovudine (AZT) from 14 weeks' gestation, single-dose nevirapine (sdNVP) at onset of labour, and AZT/3TC for 7 days postpartum	Daily NVP from birth to 1 week after all exposure to breast milk has ended
B *(Requires CD4)*	CD4≤350: Triple ART for life	CD4>350: Triple ART from 14 weeks' gestation until 1 week after all exposure to breast milk has ended	Daily NVP from birth to 6 weeks
B+ (*CD4 not required)*	All women: Triple ART for life	Not applicable	Daily NVP from birth to 6 weeks

Abbreviations: 3TC (lamivudine), ART (antiretroviral), AZT (zidovudine), NVP (nevirapine)

High-risk infants who are exclusively breastfed should additionally receive AZT for the first 6 weeks of life and NVP for at least 12 weeks. NVP should only be stopped about 4 weeks after the mother stopped breastfeeding. Exclusively formula-fed infants should receive AZT for 6 weeks and NVP for 6 weeks (South African National Department of Health, 2019).

Although the success of the PMTCT programme has led to major reductions in vertical transmission of HIV in most middle- to high-income countries with some countries eliminating MTCT, HIV in sub-Saharan Africa remains a threat to new-borns (Inzaule *et al.*, 2018a, Yah and Tambo, 2018).

The reasons behind this are late or missed HIV diagnosis, incident HIV infection in pregnant women, low uptake or postponed treatment initiation, insufficient adherence to ART, late diagnosis of HIV-exposed infants or loss to follow-up (Inzaule *et al.*, 2018a, Yah and Tambo, 2018). Although the SA PMTCT programme significantly reduced morbidity and mortality in HIV exposed infants, there were still about 92 000 children below the age of 15 years that acquired HIV infection in 2017 (UNAIDS, 2018b).

1.2.9 Treatment of HIV

There are five main classes of ARVs that target different steps of HIV replication. This includes the entry inhibitors, RT inhibitors, IN strand transfer inhibitors (INSTIs), and the PIs. Entry inhibitors, like maraviroc, prevent the entry of an HIV particle into the host cell by blocking binding of the HIV gp120 to the CCR5/CXCR4 co-receptor of the host cell, while the fusion of the HIV particle with the host cell can be inhibited by using fusion inhibitors, like enfuvirtide (Arts and Hazuda, 2012).

The RT inhibitors comprise the NRTIs and the NNRTIs. The NRTIs are nucleoside analogues that lack a 3' hydroxyl group, thus leading to DNA chain termination, while the NNRTIs inactivate the RT enzyme by binding directly to a catalytic pocket on the enzyme (Arts and Hazuda, 2012).

The INSTIs, like raltegravir and dolutegravir (DTG), prevent the HIV genome from integrating into the host genome by inhibiting the IN enzyme. The PIs, like lopinavir (LPV), inhibit the protease enzyme, thus preventing the cleavage of the viral polyprotein precursors, which ultimately inhibits viral maturation (Arts and Hazuda, 2012). Table 1.2 summarises the different drug classes and their targets.

Table 1.2: The different HIV drug classes, their targets and examples of each class

Drug class	Target	Portion of replication cycle affected	Examples
Entry inhibitors	gp41, CCR5/CXCR4 co-receptor	Viral entry	Enfuvirtide (targets gp41) Maraviroc (targets CCR5/CXCR4 co-receptor)
NRTIs	Reverse transcriptase	Reverse transcription	TDF, AZT, ABC, 3TC, FTC
NNRTIs	Reverse transcriptase	Reverse transcription	EFV, NVP
INSTIs	Integrase	Integration	Raltegravir, DTG
PIs	Protease	Maturation of the viral particle	LPV/r

Abbreviations: 3TC (lamivudine), ABC (abacavir), AZT (zidovudine), DTG (Dolutegravir), EFV (efavirenz), FTC (emtricitabine), INSTI (integrase strand transfer inhibitor), LPV/r (ritonavir boosted lopinavir), NNRTI (non-nucleoside reverse transcriptase inhibitor), NRTI (nucleoside reverse transcriptase inhibitor), NVP (nevirapine), PI (protease inhibitor)

Up to recently, the standard first-line regimen for HIV treatment for adults, as recommended by the WHO, comprised an NNRTI and two NRTIs (Doherty *et al.*, 2013, Phillips *et al.*, 2018). The NNRTI usually used was EFV and the NRTIs used were usually either lamivudine (3TC) or emtricitabine (FTC) together with TDF (Ramjan *et al.*, 2014, Gregson *et al.*, 2016, World Health Organization, 2016a, Ford *et al.*, 2018).

Dolutegravir (DTG) is a new IN inhibitor that was introduced in SA, in combination with TDF and 3TC (UNAIDS, 2017, Dorward and Hamers, 2019). An HIV synthesis model study in which this newly introduced regimen was compared to the previous regimen consisting of TDF, 3TC or FTC, and the NNRTI, EFV, predicted that the DTG-based regimen was superior as it would be cost-saving and would improve both health outcomes and viral suppression, and hence reduce MTCT (Phillips *et al.*, 2019). DTG is dosed once daily, but because of drug interactions between DTG and rifampicin (used for tuberculosis treatment), patients should receive twice daily doses of DTG when these medications are used together (South African National Department of Health, 2019).

According to the SA PMTCT guidelines of 2019, women who are newly initiating ART should receive a fixed-dose combination of TDF, 3TC and DTG, unless they are in the first trimester of pregnancy, in which case they should still receive the older TDF, FTC, EFV combination (South African National Department of Health, 2019). Women who

are no longer in the first trimester of pregnancy and who are already on ART should only be switched to DTG if their HIV viral load (VL) is <50 copies/mL (South African National Department of Health, 2019). Due to the concern that DTG used during the first six weeks of pregnancy could cause neural tube defects in new-borns, pregnant women should only consider switching to DTG after the neural tubes have closed after the first six weeks of gestation and should be well informed about this risk (South African National Department of Health, 2019).

Recent WHO guidance recommends that the INSTI, DTG, replace EFV in all adults newly initiated on ART. Second-line treatment (INSTI based regimen) for adults comprises a change of NRTI in a PI-based regimen, and usually consists of AZT, 3TC and LPV/ritonavir (LPV/r) (South African National Department of Health, 2015).

The recommended HIV treatment regimen for children is as follows; AZT or Abacavir (ABC) along with 3TC and LPV/r for children younger than 3 years old; ABC along with 3TC and EFV or DTG for children between 3-10 years of age; and TDF along with either 3TC or FTC and EFV or DTG for children older than 10 years of age (Ford *et al.*, 2018, South African National Department of Health, 2019).

The treatment regimen for pregnant women who are not currently on ART and who might be at risk of transmitting HIV to their infants should start with a fixed-dose combination (FDC) comprising TDF, 3TC (or FTC) and DTG upon their first antenatal care visit after being informed about the risk of neural tube defects with DTG use. According to the latest guidelines, these women should receive an immediate dose of TDF, 3TC and DTG as a FDC tablet along with a single dose of NVP (South African National Department of Health, 2019). One of the major problems faced by the PMTCT programme and ultimate elimination of HIV transmission is HIVDR (Ngo-Giang-Huong and Aghokeng, 2019). The problem with the use of EFV or NVP is that it has a low genetic barrier to resistance so mothers have an increased risk of developing HIVDR against these drugs, which can be transmitted to the infant and complicate infant treatment (Ngo-Giang-Huong and Aghokeng, 2019, Nyakato *et al.*, 2020). Because the pre-treatment drug resistance percentage reaches 10%, the SA guidelines considered replacing NNRTIs as a first-line treatment option with DTG (World Health Organization, 2017a).

1.2.9.1 HIV drug resistance

The HIV-1 has an immensely rapid replication rate and uses the RT enzyme which lacks proofreading abilities, thus HIV mutations are a common occurrence (Sanjuán *et al.*, 2010, Lauring *et al.*, 2013, Cuevas *et al.*, 2015, Silvestri and Cartwright, 2016, Lewthwaite and Melhuish, 2018). Some mutations are usually silent; however, some mutations can lead to HIVDR if they occur within the protein sequences coding for the RT enzyme, the IN enzyme, the PR enzyme or the gp41 transmembrane protein (Wensing *et al.*, 2016).

Mutations of the HIV genome that occur in ART naïve individuals will usually disappear while the wild-type virus will predominate; however, if mutations occur in a patient using ART, the ART exposure will give the drug-resistant virus a selective advantage (Das and Arnold, 2013, Adams, 2019).

The problem with the first-line regimen used for HIV treatment in adults until recently was the inclusion of NNRTIs commonly associated with treatment failure (EFV and NVP) and drug resistance because of their low genetic barrier, requiring a single point mutation for drug resistance to occur (Havlir *et al.*, 1996, Whitcomb *et al.*, 2002, Ren and Stammers, 2008, Rhee *et al.*, 2015, Gupta *et al.*, 2018, Steinberg *et al.*, 2018). An analysis by Gupta et al., (2018), determined the NNRTI resistance in 2016 to be 11% in southern Africa, 10.1% in eastern Africa and 7.2% in western and central Africa (Gupta *et al.*, 2018). A pooled analysis of pre-treatment HIV drug resistance in ART-naïve South African adults between 2000 and 2016 observed a 11% annual increase in odds of pre-treatment drug resistance, primarily because of NNRTI-resistance, which potentially occurred with the increased coverage of ART (UNAIDS, 2018a, Chimukangara *et al.*, 2019).

1.2.9.2 HIV drug resistance in children

Drug resistance to HIV can be acquired because of inadequate blood levels, secondary to poor adherence to treatment, drug-drug interactions, etc., or resistance can be transmitted (Adams, 2019). Children are at risk of both transmitted and acquired HIVDR. During pregnancy, mothers with HIVDR strains can transmit these strains to the infant while exposure of the infant to ART used as PMTCT can select for mutations. HIVDR complicates the choice of prophylaxis or treatment to use for the infant.

Between 2014 and 2017, a systematic literature review of pre-treatment HIVDR in an African setting documented high levels for children starting ART, mostly in infants (over 50% of infants whose mothers were following the PMTCT guidelines had NNRTI resistance) (Inzaule *et al.*, 2019).

According to the WHO 2018 progress report, 6 out of 11 countries that were implementing national HIVDR surveys in 2017 found that the prevalence of pre-treatment HIVDR to NNRTIs (EFV or NVP) exceeded 10% (World Health Organization, 2018a). A systematic review and meta-regression analysis found that the prevalence of pre-treatment NNRTI resistance is growing annually by 23% in southern Africa and 17% in eastern, western and central Africa (Gupta *et al.*, 2018). A nationally representative HIVDR survey among children under the age of 18 months reported that during 2014 to 2016 the prevalence of NNRTI resistance was up to 63.7% in SA infants diagnosed with HIV during EID (World Health Organization, 2017b).

A single dose of NVP is given to the baby shortly after birth as PMTCT of HIV-1 (Guay *et al.*, 1999, Eshleman *et al.*, 2005, Johnson *et al.*, 2005, Loubser *et al.*, 2006, Flys *et al.*, 2007, Hauser *et al.*, 2011). However, single-dose NVP is associated with the selection and transmission of NVP-resistant HIV strains (Eshleman *et al.*, 2001, Micek *et al.*, 2014, Kanthula *et al.*, 2017). The WHO therefore recommends PI-based regimens for infants because of the risk of HIVDR from the failed first-line prophylaxis used for PMTCT; however, it is difficult to fully implement PI-based regimens in sub-Saharan Africa because of the cost of second-line therapy (Estill *et al.*, 2016, World Health Organization, 2016a).

A review on the ARV resistance patterns in children with HIV infection found that the K103N mutation was more common than mutations at position 181 in SA, which could be because of the predominant use of EFV-based ART and the earlier implementation of the WHO Option B+ in the SA PMTCT programme (Nuttall and Pillay, 2019).

1.2.10 Early infant diagnosis of HIV

In infants, HIV progresses rapidly, thus early diagnosis and treatment are important (Violari *et al.*, 2008, Bourne *et al.*, 2009, Innes *et al.*, 2014, Technau *et al.*, 2017b, Veldsman *et al.*, 2018). Diagnosis of infants less than 18 months old depends on

molecular assays, such as the polymerase chain reaction (PCR), as serology is inaccurate because of the presence of maternal antibodies (Penazzato *et al.*, 2014). While molecular assays are the definite diagnostic method to use, they are complicated by HIV PCR indeterminate results (results that are neither clearly positive nor negative). Since infants without a firm diagnosis often become lost to follow-up, due to weak mechanisms to track mothers and infants who do not return for testing at 6 weeks of age, and have a high mortality, EID in SA is of great importance (Violari *et al.*, 2008, Penazzato *et al.*, 2014, Mazanderani *et al.*, 2016, Essajee *et al.*, 2017, Moyo *et al.*, 2018b, Bianchi *et al.*, 2019, Mpinganjira *et al.*, 2020). As per NHLS EID testing guidelines, a confirmatory sample should be requested whenever HIV PCR indeterminate results are detected, in order to repeat the test and also HIV viral load. However, viral load might be undetectable due to ART exposure lowering viraemia below the detection level (South African National Department of Health, 2019).

Indeterminate HIV-1 PCR results in HIV-exposed infants may occur because of low levels of HIV viraemia and exposure to treatment because of PMTCT (both maternal ART and infant ARV prophylaxis) (Mazanderani *et al.*, 2016, Maritz *et al.*, 2017). The purpose of the EID programme is to detect HIV in infants shortly after infection so that ART can be initiated as soon as possible with the aim of decrease morbidity and mortality in infants (Violari *et al.*, 2008, Mazanderani *et al.*, 2018).

Despite SA implementing routine birth testing for all HIV exposed infants in June 2015 instead of targeted birth EID in high-risk infants, loss to follow-up and resultant infant mortality and morbidity remain a concern (Technau *et al.*, 2017a, Inzaule *et al.*, 2018a, Kalawan *et al.*, 2020). Loss-to-follow-up occurs because of multiple factors, including personal factors (poor understanding of the importance of ART, side effects, poverty), family factors (lack of support from family), community factors (stigma of living with HIV), and health system factors (distance to the healthcare facilities, poor attitudes of healthcare workers) (Mpinganjira *et al.*, 2020). Infants with perinatal HIV-1 infection experience high rates of early mortality, peaking at approximately 2-3 months of age, with about 50% of infected children without therapy dying by 2 years of age, which reiterates the importance of EID (Newell *et al.*, 2004, Bourne *et al.*, 2009, Mofenson *et al.*, 2020).

The SA EID program was revised in 2019 and all HIV-exposed infants should be tested for HIV by PCR at birth to identify possible in-utero HIV transmission and a follow-up PCR at 10 weeks and 6 months and HIV serology testing for all children at 18 months (and those testing HIV positive should be confirmed with an HIV PCR test if they are 18-24 months old), where infants previously were tested at birth, 10 weeks, 14 to 18 weeks and 18 months (South African National Department of Health, 2019). Testing for HIV is also recommended post-weaning for all HIV exposed infants and children, with the test method dependant on the age at time of weaning. A major achievement of SA EID programmes and early ART initiation is a 76% reduction in early infant mortality and 75% reduction in HIV progression (Violari *et al.*, 2008, Bourne *et al.*, 2009, Bianchi *et al.*, 2019).

1.2.10.1 Dried blood spot samples

As part of the EID programme in sub-Saharan Africa, dried blood spot (DBS) samples are sent from health care facilities (which often wait to send samples in batches) to referral diagnostic laboratories which can use nucleic acid testing (Manumbu *et al.*, 2015). Once the samples are received, the laboratories will process them and return the results as paper forms, SMSs, or via electronic databases (Bianchi *et al.*, 2019).

There are several advantages regarding the use of DBS, most notably the ease of collection, storage and transport. In terms of DBS sample collection, a healthcare worker uses a sterile needle to prick the heel of an infant to collect a droplet of blood on filter paper. The filter paper is then left to dry at room temperature, after which it is sent to a diagnostic laboratory for analysis (Nys *et al.*, 2017). These samples can be stored at room temperature (for up to 14 days) without compromising integrity and this also simplifies the transportation to laboratories for testing (Nyambi *et al.*, 1994, Sherman *et al.*, 2005, World Health Organization, 2020). Transport of DBS samples is also easier than the transport of plasma, because of DBS samples do not have the risk of sample leakage (Sherman *et al.*, 2005, Stevens *et al.*, 2008). The collection of DBS is thus less traumatic than venepuncture, uses a smaller volume of blood, and is cost-effective in terms of storing and transport (World Health Organization, 2010).

The use of DBS samples is suitable for EID HIV PCR assays and while other virological assays, like viral quantification, can be done using DBS samples, it is not ideal because of decreased sensitivity (World Health Organization, 2010). Regarding the storage of

DBS after routine diagnosis, these samples can be stored for up to four weeks at room temperature with desiccant, however, genotyping efficiency might be reduced (Parry *et al.*, 2014) .

1.2.10.2 Molecular assays

As a gold standard for paediatric HIV diagnosis worldwide and in SA, EID programmes make use of a direct detection method, such as a PCR, in order to detect viral nucleic acids instead of antibodies. The WHO recommends HIV PCR testing for HIV-exposed infants at birth and 6 weeks of age (World Health Organization, 2016a). The SA EID guidelines have recommended the use of HIV-1 PCR on whole blood samples at birth and at 10 weeks of age for diagnosis and screening since 2015, while HIV-1 viral load assays are used on plasma to monitor treatment (South African National Department of Health, 2019).

The PCR method involves the exponential amplification of a single copy of a DNA sequence to generate multiple copies of that particular DNA segment. If the specific DNA sequence was present, it would be amplified and reported as an HIV PCR positive result, while cases where the specific DNA sequence was absent will be reported as HIV PCR negative. However, some borderline cases are reported as being HIV PCR indeterminate, thus being neither clearly positive nor clearly negative. Indeterminate HIV PCR results can either be true low-positive results or non-specific amplification signals (Maritz *et al.*, 2017).

The positive predictive value (PPV) of a diagnostic assay decreases as the incidence of HIV in infants falls because of improved access to ART (Dunning *et al.*, 2017). The problem with a decreased PPV is that a larger proportion of HIV-negative infants might receive false-positive results, thus leading to infants unnecessarily starting ART (Dunning *et al.*, 2017). On the other hand, a diagnostic assay should also not miss cases of true infection.

In order to prevent missed diagnostic opportunities, it is advised that healthcare facilities should not rely on targeted testing (testing only self-reported or documented HIV-exposed infants) as some HIV-infected mothers lack proper documentation of their HIV status (Woldesenbet *et al.*, 2015). As such, additional testing is essential over the

period of HIV exposure, and the EID programme should not be regarded as a one-test process (World Health Organization, 2018b).

1.3 Other platforms and point-of-care tests used for EID

The conventional EID cascade starts with an infant presenting for HIV testing. After sample collection, sample packaging and transport to a conventional/referral laboratory that will test the sample and send the result back to the requesting facility follows. The requesting facility will then log the result and discuss it with the caregiver of the infant. Within the NHLS, resulting is performed by the testing laboratory.

In order to improve the turnaround time from sample collection to result, introducing point-of-care (POC) nucleic acid testing for HIV EID could potentially reduce the turnaround time, allow for earlier ART initiation in HIV-positive infants and potentially decrease the rate of loss to follow-up (Essajee *et al.*, 2015, Bianchi *et al.*, 2019). An advantage of POC systems is the little training required for their use: non-laboratory personnel can operate it within about 90 minutes. Rapid results could lead to accelerated clinical decision-making (Bianchi *et al.*, 2019).

Two examples of POC systems include the Xpert HIV-1 Qual (Cepheid; Sunnyvale, CA) used for infant diagnosis of HIV and the m-PIMA HIV-1/2 Detect (Abbott Laboratories; Lake Forest, IL, USA) which have sensitivities of 98.69% and 98.67% respectively. Both have a specificity of 100% which makes it comparable to conventional EID systems like the Roche CAP/CTM (World Health Organization, 2016c, World Health Organization, 2016d, Bianchi *et al.*, 2019). The Xpert HIV-1 Qual (Cepheid; Sunnyvale, CA) can process plasma (for HIV viral load testing) and EDTA or DBS samples (for EID) and perform RNA extraction, purification, reverse transcription and real-time quantification within 90 minutes (Ceffa *et al.*, 2016). A disadvantage observed when verifying the Xpert HIV-1 Qual (Cepheid; Sunnyvale, CA) was that the analytical sensitivity when using plasma was better compared to DBS or whole blood (Gous *et al.*, 2016, Bwana *et al.*, 2019). Using POC systems therefore requires more research in terms of clinical utility, reliability of results, and cost-effectiveness (Laurence *et al.*, 2008, Stevens *et al.*, 2014, Agutu *et al.*, 2019).

Other platforms used worldwide for EID include the Hologic Panther (Hologic Inc., Manchester, UK), the Abbott m2000sp Automated Sample Preparation System and

Abbott Real-time HIV-1 assay which is an automated system that can perform automated nucleic acid extraction, reagent dispensing and real-time amplification of HIV-1 in plasma or DBS samples (Marconi *et al.*, 2009, May *et al.*, 2018).

The SA National Health Laboratory Service (NHLS) uses the Roche COBAS®AmpliPrep (CAP)/COBAS®TaqMan® (CTM) HIV-1 Qualitative Test version 2 (Roche Molecular Systems, Branchburg, NJ) (CAP/CTM) assay and has recently introduced the Roche cobas® 6800/8800 System, which offers increased automation.

1.4.1 The Roche CAP/CTM assay

The CAP/CTM is an automated real-time amplification assay with a sensitivity and a specificity of 221.8 copies/mL (limit of detection) and 99.9%, respectively, when testing DBS for HIV-1 (Templer *et al.*, 2016). The CAP/CTM can run 72 samples in an 8-hour shift and requires sample pre-sorting (Cobb *et al.*, 2017).

The assay is well suited for EID testing in the SA context as it is a high-throughput, automated system, optimized to yield comparable amplification efficiencies for different HIV-1 group M subtypes and can test both whole EDTA blood and DBS samples (Roche®COBAS®AmpliPrep/COBAS®TaqMan HIV-1 Qual Test, v2.0 [package insert] Branchburg, NJ, Roche, 2014). The CAP/CTM reports indeterminate results as "detected", but these are distinguished from true positive results by having a cycle threshold (Ct) value of over 33 and/or relative fluorescence intensity (RFI) value above 5 according to the NHLS national EID SOP.

A disadvantage of the CAP/CTM assay is its limited specificity because of genetic diversity or genotype inclusivity (only targets HIV-1 Group M subtypes A-H, Group O) (Pas *et al.*, 2010, Zhao *et al.*, 2019, Roche Molecular Diagnostics, 2021). The CAP/CTM is currently used for EID within the SA NHLS (Maritz *et al.*, 2014).

1.4.2 The Roche cobas® 6800/8800 System

Roche Molecular Diagnostics has introduced the Roche cobas® 6800/8800 System to test HIV viral load and recently, in 2019, HIV DNA PCR for EID. While the platform is already processing samples for HIV viral load, verification for HIV DNA PCR for EID with DBS needs to be established, especially for samples with HIV PCR indeterminate results.

The Roche cobas® 6800/8800 System makes use of a universal sample preparation method and reagents in order to isolate, purify, and extract the total nucleic acids in the sample rather than using a favoured nucleic acid isolation process in order to achieve testing efficiency (Cobb *et al.*, 2017). Samples do not require any pre-sorting, because the system sorts the samples by itself, which can save a lot of time (Cobb *et al.*, 2017).

Regarding the cycling conditions during amplification of a specific target, the Roche cobas® 6800/8800 System uses the same temperature and number of cycles for all targets, thus assay-specific setup plays a role in primer and probe design rather than in the PCR profile (Cobb *et al.*, 2017).

The Roche cobas® 6800/8800 System can run a maximum of 960 samples in an 8-hour shift and can run up to three different assays simultaneously, thus making it an optimal system to include in a diagnostic setting (Cobb *et al.*, 2017). The system makes use of different modules (Figure 1.9) in order to reduce the number of hands-on tasks required to process samples (Roche Molecular Systems cobas® 6800/8800 System [package insert], Branchburg, NJ). Reagents (kits and controls) can be stored within an on-board refrigerator, further decreasing the time taken to unload and reload the system (Cobb *et al.*, 2017). The samples are loaded on to the Roche cobas® 6800/8800 System through the sample supply module. The system contains a barcode scanner, which scans the samples as they enter the transfer module where the system adds the controls and transfers the samples from sample tubes to a processing plate. Sample extraction occurs in the processing modules, followed by analysis in the analytical modules, which contain light cyclers.

In a study that compared the Roche cobas® 6800/8800 System to the Hologic Panther platform, the former had a shorter turnaround time (almost two hours shorter), fewer hands-on time and user interaction and also used fewer reagents and made less waste (Aretzweiler *et al.*, 2019).

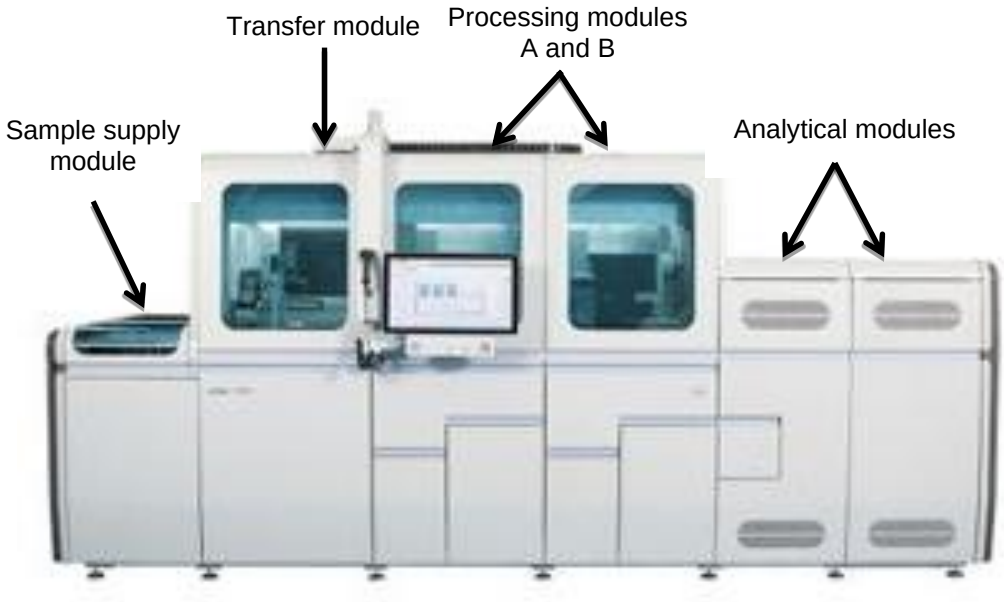

Figure 1.9: The Roche cobas® 6800/8800 System

1.5 Rationale for study

South Africa has the largest burden of people living with HIV infection (7.52 million in 2018) in the world, with most of these infections occurring in women and children. There are about 360 000 HIV-exposed babies born every year who require EID to determine if they have been infected with the virus (Maritz *et al.*, 2017). Despite the high HIV infection rate, the SA PMTCT programme has improved significantly and has reduced the incidence of new early HIV infections in infants from >20% in 2004 to <2% in 2015. While this is a notable success of the PMTCT programme, the challenge of a high prevalence of HIV among childbearing women remains with acquisition of HIV infection during pregnancy posing an exceptionally high risk of infant infection. In addition, exposure of mothers and infants to ART as part of PMTCT pose a risk for HIVDR. In SA, >50% of newly-diagnosed infants aged <18 months have been reported to have drug-resistant strains (Kuhn *et al.*, 2014, Jordan *et al.*, 2017). Another study surveyed HIVDR in 2014 to 2016 among children under the age of 18 months and reported a prevalence of NNRTI resistance was up to 63.7% in infants diagnosed with HIV during EID (World Health Organization, 2017b). Given these high levels of HIVDR, the WHO recommends the use of PIs as part of first-line paediatric ART to optimise treatment response (World Health Organization, 2017b).

Another challenge that the SA PMTCT programme faces is the EID of HIV infection in HIV-exposed infants because of HIV PCR indeterminate results. In SA the current platform for EID is the Roche CAP/CTM, which is a qualitative nucleic acid amplification test, which detects HIV-1 RNA and proviral DNA using DBS (Maritz *et al.*, 2017). Due to DBS samples having potentially low concentrations of the HIV-1 target, assays with high sensitivity and specificity are required. Conventional PCR generally has a lower sensitivity and specificity compared to real-time PCR, thus a highly optimized conventional PCR assay is of importance when used for sequencing. High level of PMTCT ART exposure for infants during the time of testing can lead to suppression of viraemia in the infected infants and thus a loss of detectability, which leads to uncertain results, known as HIV PCR indeterminate results. The HIV PCR indeterminate results pose a major problem for EID in SA, since weak mechanisms to track mothers and infants who do not return for testing at 6 weeks of age lead to loss to follow-up (Essajee *et al.*, 2017, Moyo *et al.*, 2018b). Testing for HIV in exposed new-born infants is

imperative as early treatment can reduce mortality and limit the viral reservoir size. Timely and conclusive diagnosis and linkage to care are therefore important.

The PCR methods used for EID have sensitivities and specificities close to 100%; however, limitations still exist (Stevens *et al.*, 2008, Technau *et al.*, 2017b). A specific concern is a decrease in the PPV of these tests because of the low prevalence of HIV infection in infants (Maritz *et al.*, 2017). In addition, as mentioned earlier, high levels of ART exposure in infants during the time of testing can lead to suppression of viraemia in the infected infants and thus a loss of detectability, which leads to indeterminate results (Technau *et al.*, 2017b). While an association between ART and indeterminate HIV-1 PCR results has been suggested, further research into the diagnostic accuracy of EID assays is required to ensure that infants receive a timely and conclusive diagnosis and are linked to proper care (Sutcliffe and Moss, 2015, Technau *et al.*, 2017b).

Therefore, the current study aimed to verify the Roche cobas® 6800/8800 System against the CAP/CTM system for detection of HIV in EID and compare indeterminate results. In addition, to determine HIVDR prevalence and profiles in infants who tested HIV positive or indeterminate upon EID and describe how this can affect current SA PMTCT and EID programmes.

Therefore, the objectives of the study were:

- To verify the Roche cobas® 6800/8800 System (Roche Molecular Systems, Branchburg, NJ) for HIV in EID
- To process samples with previous HIV PCR indeterminate results as obtained by the CAP/CTM HIV-1 Qualitative Test (Roche Molecular Systems, Branchburg, NJ) and compare to the Roche cobas® 6800/8800 System (Roche Molecular Systems, Branchburg, NJ)
- To amplify and sequence the HIV PCR positive samples using an HIVDR in-house assay targeting both the protease and reverse transcriptase genes
- To amplify and sequence the HIV PCR indeterminate samples using an HIVDR in-house assay targeting both the protease and reverse transcriptase genes
- To determine the prevalence of HIVDR and mutation patterns to three drug classes, namely NRTIs, NNRTIs and PIs, in infants ($\leq$6 months of age) newly diagnosed with HIV infection.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Study design

The study was part of an ongoing main study with ethics approval number 266/2018 to assess HIVDR in infants ≤6 months. This was a cross-sectional study that comprised two components: (i) assessment of the new platform (Roche cobas® 6800/8800 System) for HIV EID, and comparison of HIV PCR negative, positive and indeterminate results as obtained from the CAP/CTM system.
(ii) Determination of HIVDR profiles in infants from Tshwane and surrounding metropoles. All available samples with sufficient DBS spot after all routine diagnosis and quality controls were done, were included.

The Roche cobas® 6800/8800 System assessment

The study used samples previously tested for HIV PCR with the CAP/CTM as part of the routine NHLS EID program. This included HIV PCR positive, negative, and indeterminate results. All samples were from infants <18 months of age as per the policy of SA EID guidelines within the NHLS.

HIV Drug resistance

The study was part of an ongoing laboratory-based, cross-sectional analysis of the HIVDR profiles of infants in the mean age group ≤6 months. Samples were remnant DBS from EID at the NHLS-Tshwane Academic Division (TAD), Department of Medical Virology, University of Pretoria, collected from June 2018 until September 2020 through the national EID program.

The samples were processed at the NHLS-TAD, Department of Medical Virology, University of Pretoria, for routine diagnosis. Remnant DBS available after all diagnostic, clinical, and quality assurance tests had been performed were used for the study. The samples were stored at room temperature without desiccant for a minimum of one month. Demographic and clinical information such as age, ART regimen, time on ART, and the latest viral load measurements, were collected from laboratory request forms or the electronic laboratory information system, where available.

2.2 Ethical approval

Ethics approval was obtained from the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria (ethics approval number 406/2019) (Appendix C). Permission was also obtained from NHLS-TAD to access remnant HIV dried blood spot samples and patients' demographic information when required.

2.3 Study population

2.3.1 Verification of the Roche cobas® 6800/8800 System for HIV EID

Statistical determination of sample size

The main sample size calculation was performed on the primary objective, namely verification of the Roche cobas® 6800/8800 System (Roche Molecular Systems, Pleasanton, CA). The sensitivity of the CAP/CTM is 98% and with 95% confidence, a sample of at least 640 cases (including excess in case of invalids) was needed to produce a two-sided confidence interval width of 0.02, i.e. within 2% accuracy of CAP/CTM sensitivity. The study consecutively collected all available positive, indeterminate and negative samples with sufficient DBS spots, resulting in 642 DBS samples (214 HIV PCR indeterminate, 235 HIV PCR positive and 193 HIV PCR negative) that were previously tested with the CAP/CTM platform. The minimum sample number requirement for statistical significance was thus met.

2.3.2 Determination of HIVDR profile

Statistical determination of sample size

For estimating the proportion of HIVDR cases, an expected proportion of 0.25 was assumed and a sample of at least 147 would estimate this population to an accuracy of 0.05 with 95% confidence and 80% power. For the HIVDR study, 147 randomly-selected HIV PCR positive samples were used, thus the minimum sample size requirement was met. Most HIV PCR positive samples came from Tshwane and the surrounding metropoles. Out of interest, 45 HIV PCR indeterminate were also tested. No formal sample size calculation was performed for this exploratory component of the study.

2.3.3 Statistical analysis:

2.3.3.1 Specificity and sensitivity

Specificity is the ability of an assay to identify the true negatives, and thus specificity can be defined as the true negative (TN) rate. The WHO states that an assay used for EID must have a specificity of at least 98% (World Health Organization, 2016a).

The sensitivity is the ability of an assay to identify the presence of the virus in samples from infants who are truly infected, thus the true positive (TP) rate. According to the WHO, an assay used for EID must have a sensitivity of at least 95% (World Health Organization, 2016a). Because the Roche cobas® 6800/8800 System classifies indeterminate samples as "detected", these samples were interpreted as positive. The following formulas were used for these calculations (Lalkhen and McCluskey, 2008):

Sensitivity = $\frac{TP}{TP+FN}$

Specificity = $\frac{TN}{TN+FP}$

Positive predictive value (PPV) = $\frac{TP}{TP+FP}$

Negative predictive value (NPV) = $\frac{TN}{TN+FN}$

TP = true positive; FN = false negative; TN = true negative; FP = false positive

For the calculation of the Kappa statistic, the Stata16 statistical software (StataCorp LLC) was used.

2.4 Laboratory methods

2.4.1 Verification of the Roche cobas® 6800/8800 System against CAP/CTM

The Roche cobas® 6800/8800 System is an HIV-1 dual-target assay targeting the highly conserved region of the *gag* gene and LTR in the HIV-1 genome, and the highly conserved LTR gene of the HIV-2 genome. The assay uses two probes to detect HIV-1, but does not discriminate its subtypes: group M, O, N and P. A third probe is for detection of HIV-2, but cannot discriminate between HIV-2 group A and group B. Two dyes are used in the test to distinguish between HIV-1 and HIV-2 genotypes: HIV-1 probes are tagged with FAM dye while HIV-2 is labelled with HEX. The assay includes

armoured RNA as an internal control (Roche Molecular Systems cobas® 6800/8800 System [package insert], Pleasanton, CA).

Table 2.1 summarises the manufacturer's specification and key performance of the CAP/CTM and the Roche cobas® 6800/8800 System (Roche Molecular Systems cobas® 6800/8800 System [package insert], Pleasanton, CA; Roche Molecular Systems COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 [package insert], Pleasanton, CA).

The CAP/CTM also targets the *gag* and *LTR* region of the HIV-1 genome (Roche Molecular Systems COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 [package insert], Pleasanton, CA).

Table 2.1: Key performance features of the CAP/CTM and the Roche cobas® 6800/8800 System
DBS= dried blood spot, SPER= Specimen Pre-Extraction Reagent, N/A= not applicable

Platform	Key features			
	Sample type	**EDTA plasma**	**Serum**	**DBS**
CAP/CTM	Minimum amount of sample required	70 uL	N/A	70 uL
	Sample process volume	1 mL	N/A	1000uL
	Analytical sensitivity	20 copies/mL	N/A	300 copies/mL
	Specificity	99.9%	N/A	99.9%
	Groups/subtypes	HIV-1M (subtype A-H, CRF01_AE), HIV-1O, HIV-1N		
	Sample type	**EDTA plasma**	**Serum**	**DBS**
Roche cobas® 6800/8800 System	Minimum amount of sample required	650uL	650uL	70uL (Per DBS) 1150uL SPER
	Sample process volume	500uL	500uL	850uL
	Analytical sensitivity	HIV-1M: 12.6 copies/ml HIV-2: 27.9 copies/mL	HIV-1M: 12.1 copies/mL HIV-2: 23.4 copies/mL	HIV-1M: 255 copies/mL HIV-2: 984 copies/mL
	Specificity	100% (one-sided 95% confidence interval: 99.5%)	100% (one-sided 95% confidence interval: 99.5%)	100% (one-sided 95% confidence interval: 99.5%)
	Groups/subtypes	HIV-1M (A-D, F-H, J, K, CRF01_AE, CRF02_BF, CRF14_BG), HIV-1O, HIV-1N HIV-2 (A and B)		

The results obtained, both *non-reactive* and *reactive*, from the Roche cobas® 6800/8800 System (Roche Molecular Systems, Pleasanton, CA) were analysed in order to determine its performance compared to the CAP/CTM (Roche Molecular Systems, Pleasanton, CA) for HIV-1 diagnosis. The CAP/CTM results are interpreted as per the NHLS standard operating procedure (SOP) as positive, negative, or indeterminate (Table 2.2). The interpretation of these results is based on RFI and Ct values. The CAP/CTM includes armoured RNA with identical binding sites to the HIV-1 target, but a unique probe-binding region, as an internal control (Mossoro-Kpinde *et al.*, 2016).

Table 2.2: The NHLS result interpretation for the CAP/CTM and Roche cobas® 6800/8800 System

System	Cycle threshold value	Relative Fluorescent Intensity value	Result
CAP/CTM	0	-	Negative
	≤33	≥5	Positive
	>33	Any value	Indeterminate
	≤33	<5	
Roche cobas® 6800/8800 System	0	N/A	Negative
	≤38	N/A	Positive
	>38	N/A	Indeterminate

N/A= Not applicable

The procedure for using the Roche cobas® 6800/8800 System (Roche Molecular Systems, Pleasanton, CA) is summarised in Figure 2.1. A single circular spot is released from the DBS card and transferred to a tube to which 1150 µL Specimen Pre-extraction Reagent is added, and incubated for 10 minutes at 56°C in the Eppendorf Thermomixer. Finally, the tube is transferred to the Roche cobas® 6800/8800 System where it undergoes real-time amplification. The results are interpreted as "reactive" for positive results and "non-reactive" for negative results. Indeterminate results are reported as "reactive", however, they are deemed indeterminate if the Ct value exceeds 38 according to the cut-off criteria within the NHLS. The NHLS determined through a receiver operating characteristic curve that a Ct=37 was the optimal cut-off, however, increasing to Ct=38 increases sensitivity (while decreasing specificity).

Figure 2.1: The basic steps of the Roche cobas® 6800/8800 System (Roche Molecular Systems, Pleasanton, CA) (Image created in Microsoft Word 2010)

2.4.1.1 Intra and inter assay reproducibility

The intra-assay reproducibility of the Roche cobas® 6800/8800 System was determined using 5 samples processed in triplicate within the run, and the Ct values per Roche cobas® 6800/8800 System were compared.

The inter-assay reproducibility of the Roche cobas® 6800/8800 System was determined using 3 samples processed in three days, and the Ct values were compared.

2.4.1.2 Indeterminate samples

Indeterminate HIV PCR results comprise over 16% of all samples, positive, indeterminate and negative samples collectively (Mazanderani *et al.*, 2016). An HIV PCR indeterminate result, as defined by the CAP/CTM NHLS EID SOP, is a sample either with a Ct ≤33 and an RFI ≤5, or a Ct>33 and an RFI >5 or <5 (Table 2.2). A total of 214 samples with CAP/CTM HIV PCR indeterminate results were processed on the Roche cobas® 6800/8800 System to determine the Ct values from this assay and the true status of CAP/CTM HIV PCR indeterminate result. However, confirmatory results for the indeterminate HIV PCR samples were not available on the NHLS system at the time. The Roche cobas®6800/8800 defines a positive result as a sample

with a Ct≤38 and an HIV PCR indeterminate result as a sample with a Ct>38 as per the NHLS HIV PCR SOP.

2.4.1.3 Follow-up confirmation results of CAP/CTM indeterminate samples

The confirmation results for only 30 HIV PCR indeterminate samples were available, potentially due to loss to follow-up, and compared to the result obtained by the Roche cobas® 6800/8800 System. The confirmation results were obtained through the NHLS TrakCare system.

2.4.2 HIV drug resistance detection

2.4.2.1 Extraction of dried blood spots

Total nucleic acid was extracted from DBS using the NUCLISENS® EASYMAG® platform (BioMerieux, Inc., Durham, NC) as per the manufacturer's instructions. The principle of this extraction is as follows: samples are lysed overnight at 4ºC during incubation with guanidinium hydrochloride, thereafter target nucleic acid is captured by magnetic silica particles. The nucleic acid is purified by several wash steps conducted by the NUCLISENS® EASYMAG® (BioMerieux, Inc., Durham, NC) magnetic device that attracts magnetic silica. The nucleic acid is released during a heating step from the magnetic silica. In the final step, a magnetic device separates the magnetic silica from the eluate, resulting in a 50 µL volume of the eluate. The eluate was then used for PCR or stored at -80°C for later use.

2.4.2.2 Amplification

A PCR targeting the *pol* gene was employed with primers targeting the protease (PR, codons 1–99) and reverse transcriptase (RT, codons 1–250) genes to amplify about 1080 base pairs (bp) as previously described (Zhou *et al.*, 2011). For full primer sequences, see Appendix A (Zhou *et al.*, 2011).

Briefly, two oligonucleotides that are mixed at a ratio of 1:1 (w/w) were used as the forward primer for the one-step RT-PCR (Appendix A). One-step RT-PCR was performed in a 50 µL reaction, which comprised 10 µL of RNA or total nucleic acid extracts, 0.16 µM each of primers PRTM-F1 and RT-R1, and 0.5 µL SuperScript™ III one-step RT/Platinum® Taq High Fidelity Enzyme Mix and 1x reaction buffer mixture containing Mg2+ and deoxyribonucleotide triphosphates (dNTPs) (Invitrogen, Carlsbad, CA). The RT-PCR conditions were as follows: an initial cycle RT step at 50°C for 45 min and 94°C for 2 min and followed by 40 cycles of PCR at 94°C for 15 sec, 50°C for 20 sec, 72°C for 2 min and an extension at 72°C for 10 min. For nested PCR, 4 µL of RT-PCR product was added to a 50 µL reaction containing 0.12 µM of each of the inner primers PRT-F2 and RT-R2, 1x GeneAmp Gold Buffer II, 2 mM $MgCl_2$, 400 µM each dNTP and 2.5 U of AmpliTaq Gold LD DNA polymerase (Applied Biosystems, Foster City, CA). After initial denaturation at 94°C for 4 min, 40 cycles of PCR were performed in the Applied Biosystems SimpliAmp Thermal Cycler (Applied

Biosystems, Foster City, CA) with the PCR conditions as 94°C for 15 sec, 57°C for 20 sec and 72°C for 2 min and following an extension at 72°C for 10 min.

About a 1080 bp segment of the 5' region of the *pol* gene was generated by a two-step RT and nested PCR. Five μL of the amplified products were run at 100V on a 2% ethidium bromide-stained agarose gel for 60 minutes in a 1x TAE buffer (Promega Corp., Madison, WI) alongside the GeneRuler 1kb DNA ladder (ThermoFischer Scientific, Waltham, MA). Expected bands were viewed using the Gel Doc XR+ System (Bio-Rad Laboratories, Inc., Rosebank, Sandton).

2.4.2.3 In-house PCR sensitivity

Plasma was used to evaluate the assay sensitivity and was compared with DBS. The assay used is part of the NHLS HIV drug resistance routine testing assay.

2.4.2.4 Direct Sanger sequencing

The 1080 bp fragment was purified with the GeneJET PCR Purification kit (ThermoFisher Scientific, Waltham, MA) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). This was sent to Inqaba Biotec (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) for sequencing.

2.4.2.5 Sequence analysis

Sequence data were assembled and analysed with Sequencher 4.10.1 (Gene codes corporation, Ann Arbor, MI) and submitted to the Stanford HIV drug resistance database tools (HIVdb Program, Genotypic Resistance Interpretation Algorithm) (http://sierra2.stanford.edu/sierra/servlet/JSierra) for quality assessment and drug resistance prediction. The Stanford HIV database tool is an online program for HIV-1 subtyping and drug susceptibility prediction. The HIVdb program assesses how active an ARV is likely to be against a particular mutant virus compared with that ARV's activity against a wild type virus. When combined with a sound understanding of the principles of ARV therapy, the interpretation and associated comments help health care providers better understand the results of HIV-1 genotypic resistance tests.

Using the total drug score, the program reports one of the following 5 levels of inferred drug resistance: susceptible, potential low-level resistance, low-level resistance,

intermediate resistance, and high-level resistance. The analysis comprised of a description of the prevalence of drug resistance mutations and the prevalence of high, intermediate, and low levels of resistance for each relevant ARV medication as determined by the Stanford drug resistance score. The mutations profiles were classified according to patterns for PR and RT genes.

2.4.3 Contamination controls

2.4.3.1 Roche cobas® 6800/8800 System

The risk of contamination of samples can only occur during the transfer of DBS to tubes to which SPER is added since these steps are the only hands-on steps. Clean forceps were used to transfer the DBS to the tube (a new forceps per DBS) to decrease the risk of contamination. After this, samples were loaded onto the Roche cobas® 6800/8800 System.

The Roche cobas® 6800/8800 System utilises two positive controls (HIV-1M/HIV-2[+] C and HIV-1O [+] C) and a normal human plasma negative control. Both the target and internal control (IC) sequences are simultaneously amplified using a universal PCR amplification profile with pre-defined temperatures and several cycles. Instead of deoxythimidine triphosphate (dTTP), the master mix contains deoxyuridine triphosphate (dUTP) which is incorporated into the newly-synthesised DNA amplicon. The AmpErase enzyme is also included in the master mix which eliminates any contaminating amplicons from previous PCR runs during the initial thermal cycling step. The AmpErase enzyme is inactivated once exposed to temperatures above 55°C, thus will not eliminate any newly-formed amplicons (Roche Molecular Systems cobas® 6800/8800 System [package insert], Branchburg, NJ).

2.4.3.2 HIVDR quality controls

Master mixes for the in-house PCR rounds were prepared in a clean room (containing a UV light). The RNA extract was transferred to PCR tubes in an extraction cabinet. Positive and negative controls were added to each PCR to determine whether possible contamination occurred. In Figure 2.2 below, the basic steps that were followed for the in-house PCR assay for the detection of HIVDR are summarised. A distance matrix and a phylogenetic tree were generated using MEGA X (Molecular Evolutionary

Genetics Analysis program) with bootstrapping to determine whether contamination occurred during the process up to sequencing.

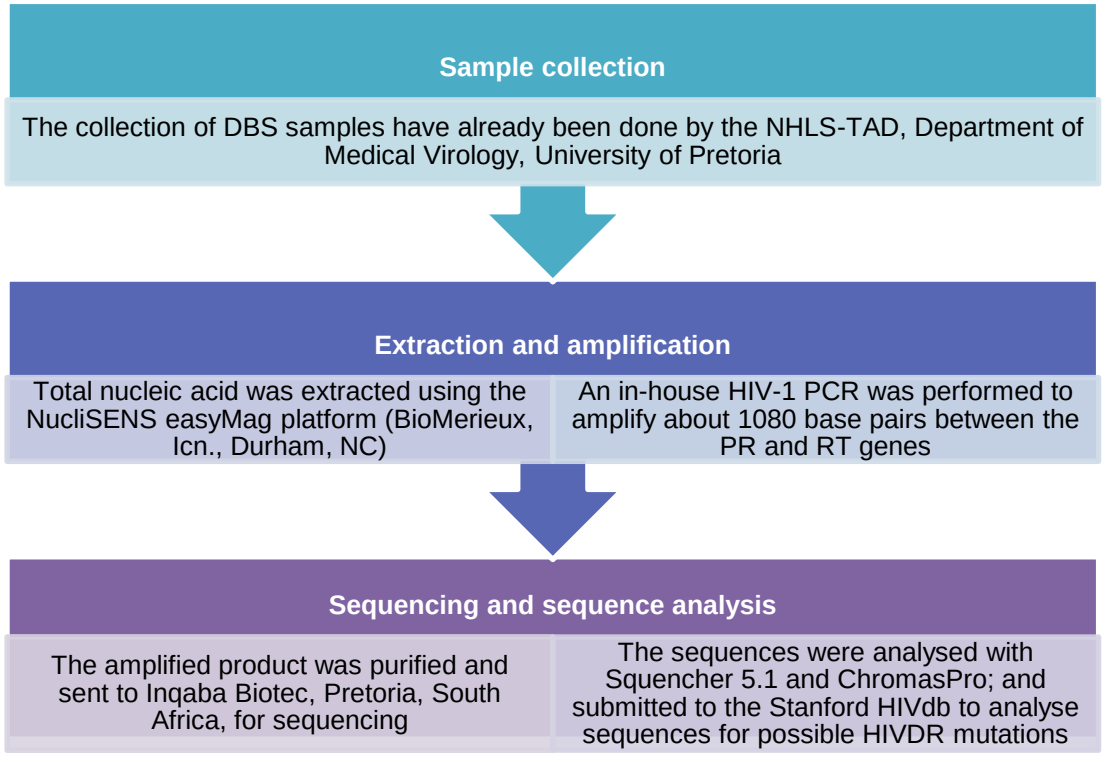

Figure 2.2: Flow diagram of the basic methodology that was followed for the in-house HIVDR PCR assay

CHAPTER THREE: RESULTS

3.1 Overall Roche cobas® 6800/8800 System versus CAP/CTM platform analysis

In order to verify the Roche cobas® 6800/8800 System for HIV in EID, 642 DBS samples (235 HIV PCR positive, 193 HIV PCR negative and 214 HIV PCR indeterminate) that had previously been tested on the CAP/CTM platform as part of NHLS routine EID were tested on the Roche cobas® 6800/8800 System.

The comparative results for the 642 samples tested with the Roche cobas® 6800/8800 System (HIV PCR positive, HIV PCR negative or HIV PCR indeterminate) and the CAP/CTM are summarised in Figure 3.1.

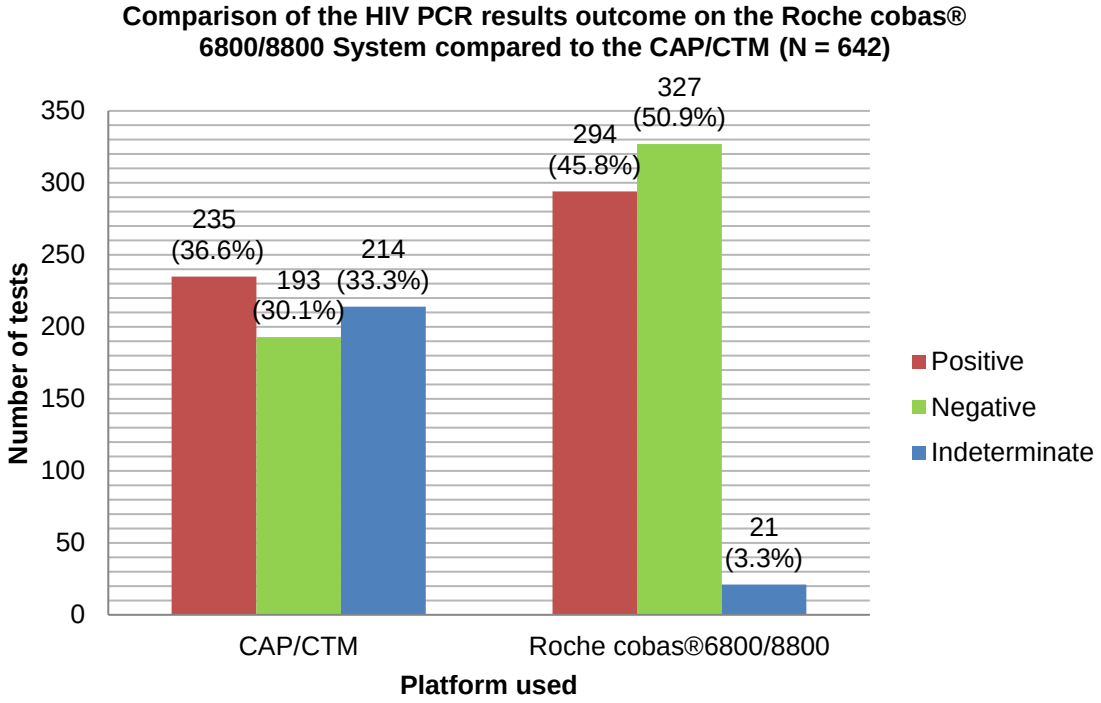

Figure 3.1: Comparison of the HIV PCR results outcome on the Roche cobas® 6800/8800 System compared to the CAP/CTM

3.1.1 HIV PCR positive CAP/CTM samples

Overall, 99.6% (234/235) CAP/CTM HIV PCR positive samples tested HIV PCR positive with the Roche cobas® 6800/8800 System. The 2/234 were Roche cobas® 6800/8800 System HIV PCR indeterminate samples with the following HIV PCR positive CAP/CTM Ct and RFI values, 32.3 and 6.9; 32.4 and 5.4, while the Roche

cobas® 6800/8800 System indeterminate results had Ct values of 38.8 and 38.2. The remaining 0.4% (1/235) became HIV PCR negative on the Roche cobas® 6800/8800 System. The HIV PCR negative sample had a previous CAP/CTM Ct and RFI value of 33.0 and 5.6.

The overall HIV PCR positives increased by 9.2% from 36.6% (235/642) to 45.8% (294/642) as detected with the cobas® 6800/8800 System, with most of the increase attributed to samples that have previously tested HIV PCR indeterminate on CAP/CTM (Figure 3.1).

3.1.2 HIV PCR negative CAP/CTM samples

Of the CAP/CTM HIV PCR negative samples, 99.5% (192/193) remained HIV PCR negative on the Roche cobas® 6800/8800 System, while one sample tested HIV PCR indeterminate with a Ct value of 39 on the Roche cobas® 6800/8800 System.

3.1.3 HIV PCR indeterminate CAP/CTM samples

A total of 214 HIV DBS samples considered CAP/CTM HIV PCR indeterminate were tested with the Roche cobas® 6800/8800 System. The HIV PCR indeterminate results, as detected by CAP/CTM, is defined as samples with Ct values ≤33 and RFI <5 or CT >33 with RFI ≥5 or <5 (NHLS SOP). The Roche cobas®6800/8800 defines an HIV PCR indeterminate result as a sample with a Ct>38 (NHLS SOP).

Overall, the HIV PCR indeterminate results, as detected by the cobas® 6800/8800 System, was 3.3% (18 previously indeterminate + 2 previously positive+ 1 previously negative, thus 21/642), while the HIV PCR negative results increased by 20.8% from 30.1% (193/642) to 50.9% (327/642), with most of the increase attributed to samples that have previously tested HIV PCR indeterminate on CAP/CTM (Figure 3.1).

The number of HIV PCR indeterminate results as detected by CAP/CTM decreased significantly from 100% (214/214) to 8.4% (18/214) with the Roche cobas® 6800/8800 System (Figure 3.1). This was a decrease of 92.1%.

A total of 29.0% (62/214) CAP/CTM HIV PCR indeterminate samples tested HIV PCR positive with the Roche cobas® 6800/8800 System, while 8.4% (18/214) remained HIV PCR indeterminate with a Ct value >38. The remaining 62.6% (134/214) tested

HIV PCR negative with the Roche cobas® 6800/8800 System. These results are summarised in Figure 3.2. A total of 34.0% (21/62) CAP/CTM HIV PCR indeterminate samples that tested HIV PCR positive with the Roche cobas® 6800/8800 System had an RFI <5, with a Ct value <33.0 (mean Ct = 34.6, minimum to maximum value range 18.6-41) on the CAP/CTM. Of the samples that were CAP/CTM HIV PCR indeterminate with both an RFI <5 and a Ct >33.0, 17.0% (29/166) were HIV PCR positive with the Roche cobas® 6800/8800 System.

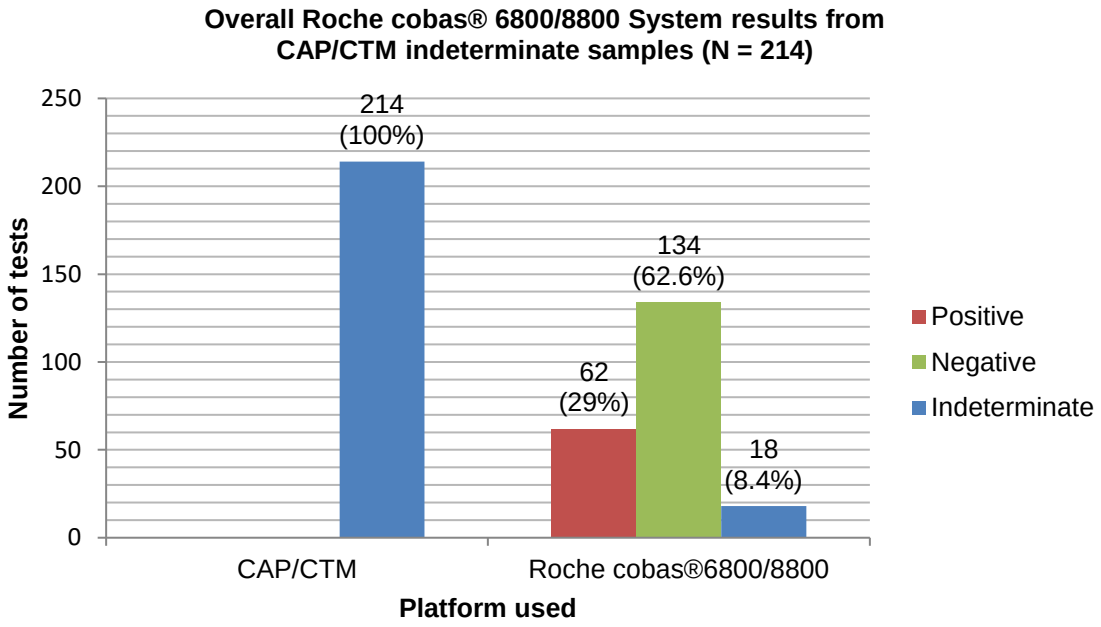

Figure 3.2: Overall Roche cobas® 6800/8800 System results from CAP/CTM indeterminate samples

The distribution of Ct and RFI values for the HIV PCR indeterminate results varied accordingly (Table 3.1). The 29.0% (62/214) that tested HIV PCR positive with the Roche cobas® 6800/8800 System had a mean Ct value of 34.7 (minimum to maximum value range 19.5-39.5) with a mean RFI of 2.5271 (minimum to maximum value range 0.3-11.6). The 8.4% (18/214) that remained HIV PCR indeterminate with the Roche cobas® 6800/8800 System had a mean Ct value of 35.3 (minimum to maximum value range 33.5-40.7) and a mean RFI of 2.5 (minimum to maximum value range 0.4-5.1), and lastly, the 62.6% (134/214) that tested HIV PCR negative with the Roche cobas® 6800/8800 System had a mean Ct value of 34.5 (minimum to maximum value range 18.6 - 41.3) with a mean RFI of 2.7 (minimum to maximum value range 0.3-5.1). A Wilcoxon signed-rank test showed that the difference in Ct and RFI values between

CAP/CTM HIV PCR indeterminate results and their Roche cobas® 6800/8800 System results were statistically significant (Z=-12.654, p<0.000).

Table 3.1: The CAP/CTM Ct value ranges for indeterminate results versus the Roche cobas® 6800/8800 System results

Roche cobas®6800/8800 results	Mean Roche cobas®6800/8800 Ct value	Mean CAP/CTM Ct value	Mean CAP/CTM RFI
Positive	30.5 (35.7, 37.4)*	34.7 (33.4, 37.8)*	2.5 (0.7, 3.5)*
Indeterminate	38.8 (38.3, 39.3)*	35.3 (32.2, 35.1)*	2.5 (2.7, 4.3)*
Negative	N/A**	N/A**	N/A**

*Interquartile range (quartile 1, quartile 3)
**HIV PCR negative results do not have Ct and RFI values

3.1.4 HIV PCR confirmed results of previously CAP/CTM indeterminate results

When a sample tests HIV PCR indeterminate, an HIV PCR and viral load must be repeated urgently (South African National Department of Health, 2019). Only 30/214 HIV PCR indeterminate samples had confirmatory CAP/CTM results from the NHLS diagnostic laboratory information system. Half of these samples (50.0% [15/30]), detected as Roche cobas® 6800/8800 System HIV PCR negative, were also confirmed as HIV PCR negative on repeat CAP/CTM testing, while 36.7% (11/30) Roche cobas® 6800/8800 System HIV PCR positive samples were confirmed as such.

Two of the eleven CAP/CTM positive samples that were Roche cobas® 6800/8800 System HIV PCR indeterminate with Ct values >38.0 (38.9, 40.1) were both confirmed as HIV PCR positive. The Roche cobas®6800/8800 System was in 87.6% concordance with conformation results regarding previously HIV PCR indeterminate results.

Discordant HIV PCR results between the Roche cobas® 6800/8800 System and confirmatory testing results were observed in four samples 13.3% (4/30). One discordant case was previously CAP/CTM HIV PCR indeterminate with a Ct value of 33.3, and RFI of <5 from the NHLS Laboratory Information System (LIS), however, this sample tested HIV PCR positive on the Roche cobas® 6800/8800 System with a

Ct value of 36.0. This sample was confirmed as HIV PCR negative upon follow-up from the NHLS LIS as per guideline testing.

A second discordant case had an HIV PCR negative result on the Roche cobas® 6800/8800 System, while the confirmation CAP/CTM result remained HIV PCR indeterminate.

The remaining two discordant cases had HIV PCR negative Roche cobas® 6800/8800 System results, with HIV PCR positive confirmation results and HIV viral loads of 128 000 and 183 000 copies/mL respectively. These results are summarized in Table 3.3 and Figure 3.2.

Table 3.2: Roche cobas® 6800/8800 System and confirmatory testing results for CAP/CTM HIV PCR indeterminate samples

Roche cobas® 6800/8800 System result	Confirmation testing result	Total cases (%)
Negative	Negative	15 (50.0%)
Indeterminate	Positive	2 (6.7%)
Negative	Indeterminate	1 (3.3%)
Positive	Negative	1 (3.3%)
Positive	Positive	11 (36.7%)
Total		**30 (100.0%)**

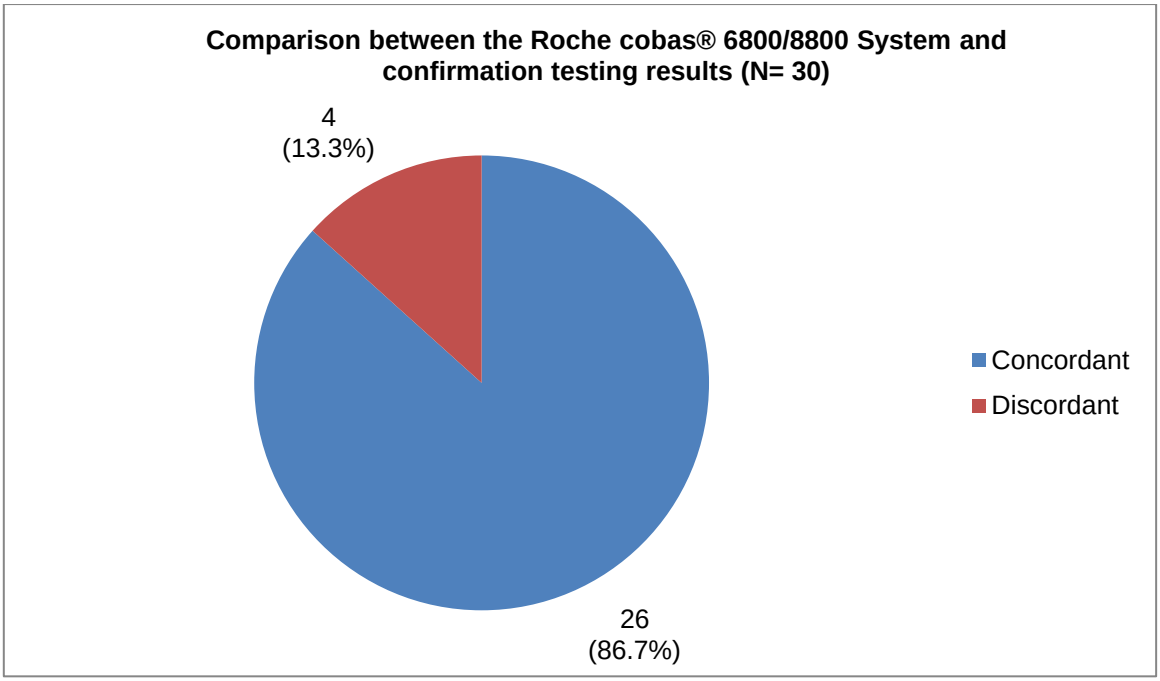

Figure 3.3: Comparison between the Roche cobas® 6800/8800 System and confirmatory testing results

3.1.5 Specificity and sensitivity

A total of 428 samples (235 HIV PCR positive and 193 HIV PCR negative on CAP/CTM) were used to determine the sensitivity, specificity, PPV and NPV, when the CAP/CTM HIV PCR indeterminate samples were excluded (Table 3.3). The true positive (TP) value, defined as the number of samples that tested HIV PCR positive on the CAP/CTM and either HIV PCR positive or indeterminate on the Roche cobas® 6800/8800 System, was 234, while the true negative (TN) value, defined as the number of samples that tested HIV PCR negative on both platforms, was 192.

The false negative (FN) value was one and defined as samples that tested HIV PCR positive with the CAP/CTM but Roche cobas® 6800/8800 System HIV PCR negative. The false positive (FP) value was 1 and defined as samples that had an HIV PCR negative result with the CAP/CTM but was HIV PCR indeterminate with the Roche cobas® 6800/8800 System (Table 3.3).

Table 3.3: CAP/CTM HIV PCR positive and negative results versus Roche cobas® 6800/8800 System results

CAP/CTM results	Roche cobas® 6800/8800 System results		
	Positive	**Negative**	**Total**
Positive	234 (TP)	1 (FN)	**235**
Negative	1 (FP)	192 (TN)	**193**
Total	**235**	**193**	**428**

Abbreviations: FN (false negative), FP (false positive), TN (true negative), TP (true positive)

The specificity of the Roche cobas® 6800/8800 System determined only using the CAP/CTM HIV PCR positive and negative results was found to be 99.5% with a sensitivity of 99.6%. The following formulas were used during these calculations:

$$\text{Sensitivity} = \frac{TP}{TP+FN} = \frac{234}{234+1} = 0.9957, \text{ thus a sensitivity of } 99.6\%$$

$$\text{Specificity} = \frac{TN}{TN+FP} = \frac{192}{192+1} = 0.9948, \text{ thus a specificity of } 99.5\%$$

$$\text{PPV} = \frac{TP}{TP+FP} = \frac{234}{234+1} = 0.9957, \text{ thus } 99.6\%$$

$$\text{NPV} = \frac{TN}{TN+FN} = \frac{192}{192+1} = 0.9948, \text{ thus } 99.5\%$$

The RFI influences the CAP/CTM results as per NHLS EID SOP; however, the current Roche cobas® 6800/8800 System does not have RFI as part of results and only uses Ct values. When including the CAP/CTM HIV PCR indeterminate samples without regard for the RFI values, samples with CAP/CTM Ct values of ≤33.0 were classified as HIV PCR positive, while CAP/CTM Ct of >33.0 were classified as HIV PCR negative. They were compared with the Roche cobas® 6800/8800 System HIV PCR positive or negative results, based solely on Ct values.

The sample size increased to 642 after this classification (Table 3.4). The definitions for the TP, TN, FP and FN remained the same as for those in Table 3.3. The TP value increased from 234 to 267 and the TN value increased from 192 to 335. The FP value increased from 1 to 13 and the FN value increased from 3 to 27 (Table 3.4).

Table 3.4: Overall results from the verification of the Roche cobas® 6800/8800 System

CAP/CTM results	Roche cobas® 6800/8800 System results		
	Positive	**Negative**	**Total**
Positive	267 (TP)	27(FN)	**294**
Negative	13(FP)	335 (TN)	**348**
Total	**280**	**362**	**642**

Abbreviations: FN (false negative), FP (false positive), TN (true negative), TP (true positive)

When the CAP/CTM HIV PCR indeterminate samples without RFI were included, the specificity was 96.3%, with a sensitivity of 90.8% (Table 3.7). The values from Table 3.4 were used in the following calculations to determine the specificity, sensitivity, PPV and the NPV:

$$\text{Sensitivity} = \frac{TP}{TP+FN} = \frac{267}{267+27} = 0.9082, \text{ thus a sensitivity of } 90.8\%$$

$$\text{Specificity} = \frac{TN}{TN+FP} = \frac{335}{335+13} = 0.9626, \text{ thus a specificity of } 96.3\%$$

$$\text{PPV} = \frac{TP}{TP+FP} = \frac{267}{267+13} = 0.9536, \text{ thus } 95.4\%$$

$$\text{NPV} = \frac{TN}{TN+FN} = \frac{335}{335+27} = 0.9254, \text{ thus } 92.5\%$$

To determine the agreement between the two assays, the Kappa statistic was calculated using Stata 16 statistical software **(**StataCorp LLC). The Kappa calculation when RFI values were included was 0.5, thus 66.6% in agreement, while when the RFI values were excluded the Kappa value was 0.9, thus 93.8% in agreement. The standard error of Kappa was 0.019 with a 95% confidence interval from 0.8 to 0.9. Once again, when excluding RFI values, samples with CAP/CTM Ct values of ≤33.0 were classified as HIV PCR positive, while CAP/CTM Ct of >33.0 were classified as HIV PCR negative.

The results from all the statistical analyses (sensitivity, specificity, PPV, NPV and agreement) are summarised in Table 3.5.

Table 3.5: Sensitivity, specificity, PPV, NPV and agreement between the assays

Statistic	Excluding RFI	Including RFI
Sensitivity	99.6%	90.8%
Specificity	99.5%	96.3%
Positive predictive value	99.6%	95.4%
Negative predictive value	98.5%	92.5%
Agreement (Kappa statistic)	93.8%	66.6%

3.1.6 Intra and inter assay reproducibility:

During intra- and inter-reproducibility analysis, sample results that were previously obtained from the CAP/CTM were used, thus these analyses were only performed on the Roche cobas® 6800/8800 System. The CAP/CTM Ct and RFI values for the samples thus remained the same, while only the Ct values for the Roche cobas® 6800/8800 System varied.

Regarding intra-reproducibility analysis, using five samples processed in triplicate within the run, all CAP/CTM HIV PCR positive results remained cobas® 6800/8800 System HIV PCR positive and the CAP/CTM HIV PCR negative result remained Roche cobas® 6800/8800 System HIV PCR negative. Table 3.6 summarises these results.

Only one indeterminate sample was only included for inter-assay testing, thus the reproducibility was nearly 100% in terms of positive and negative HIV PCR results.

Table 3.6: Intra-reproducibility results for the Roche cobas® 6800/8800 System

Sample ID	CAP/CTM HIV-1 Qualitative Test Results			Roche cobas® 6800/8800 System	
	Results	***Ct value***	***RFI***	***Results***	***Ct value***
A00166452	**Positive**	23.4	10.2	**Positive**	29.7
A00166452 #2				**Positive**	30.0
A00166452 #3				**Positive**	29.0
B01104371	**Positive**	22.9	11.4	**Positive**	29.0
B01104371 #2				**Positive**	29.0
B01104371 #3				**Positive**	28.8
C04117298	Negative			Negative	
C04117298 #2				Negative	
C04117298 #3				Negative	
D00362396	**Positive**	21.3	11.6	**Positive**	26.6
D00362396 #2				**Positive**	27.0
D00362396 #3				**Positive**	27.0
E00696726	**Positive**	20.5	9.9	**Positive**	27.0
E00696726 #2				**Positive**	25.5
E00696726 #3				**Positive**	26.5

Abbreviations: Ct (cycle threshold), RFI (relative fluorescence intensity)

The scatter plot (Figure 3.4) illustrates the distribution of the Roche cobas® 6800/8800 System Ct values of each of the samples (labelled as 1 to 5 for samples A to E) when performed on the Roche cobas® 6800/8800 System in triplicate. Sample 3 (C04117298) was used as an HIV PCR negative control, thus had no Ct value (or Ct=0).

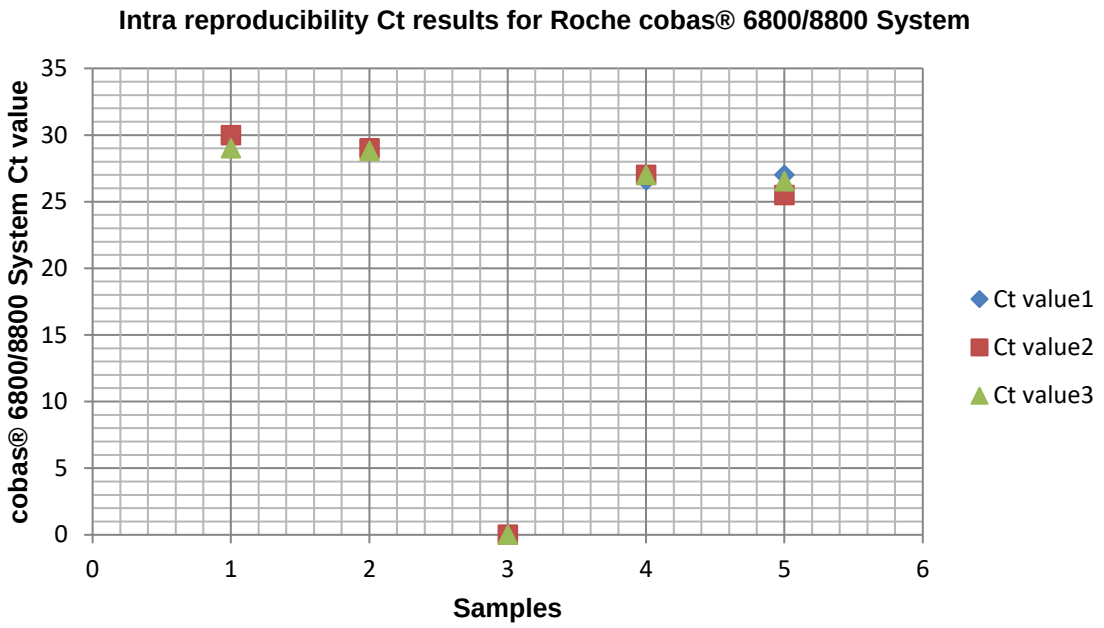

Figure 3.4: Intra-assay reproducibility Ct results for the Roche cobas® 6800/8800 System. The Ct value ranges for the samples when performed in replicate (Ct values 1 to 3) are illustrated.

Regarding the inter-reproducibility analysis, determined using three samples processed in three days, one sample was HIV PCR indeterminate on the CAP/CTM because of the RFI value being <5, but the Ct value was <33. This HIV PCR indeterminate sample tested HIV PCR positive on the Roche cobas® 6800/8800 System with an average Ct value of 32.14 (Ct ranging from 31.87 to 32.33). Regarding the other two samples used for inter-reproducibility testing, the Ct value on the Roche cobas® 6800/8800 System was approximately 5 values (range 5.28-5.33) higher than the Ct produced by the CAP/CTM. Table 3.7 summarises these results.

Table 3.7: Inter-reproducibility results for the Roche cobas® 6800/8800 System

Days of testing	Sample ID	CAP/CTM HIV-1 Qualitative Test			Roche cobas® 6800/8800 System	
		Results	Ct value	RFI	Results	Ct value
1	F00164995	Positive	21	9.9	Positive	26.1
	G00484876	Positive	25.7	12.2	Positive	31.6
	H00301853	Indeterminate	27.4	1.7	Positive	31.9
2	F00164995	Positive	21	9.9	Positive	26.1
	G00484876	Positive	25.7	12.2	Positive	30.6
	H00301853	Indeterminate	27.4	1.7	Positive	32.2
3	F00164995	Positive	21	9.9	Positive	26.8
	G00484876	Positive	25.7	12.2	Positive	30.7
	H00301853	Indeterminate	27.4	1.7	Positive	32.3

Abbreviations: Ct (cycle threshold), RFI (relative fluorescence intensity)

These samples (Table 3.7) were run on the Roche cobas® 6800/8800 System over the course of three days. The Ct values obtained per day are illustrated in the scatterplot and followed the same trend per day (Figure 3.5).

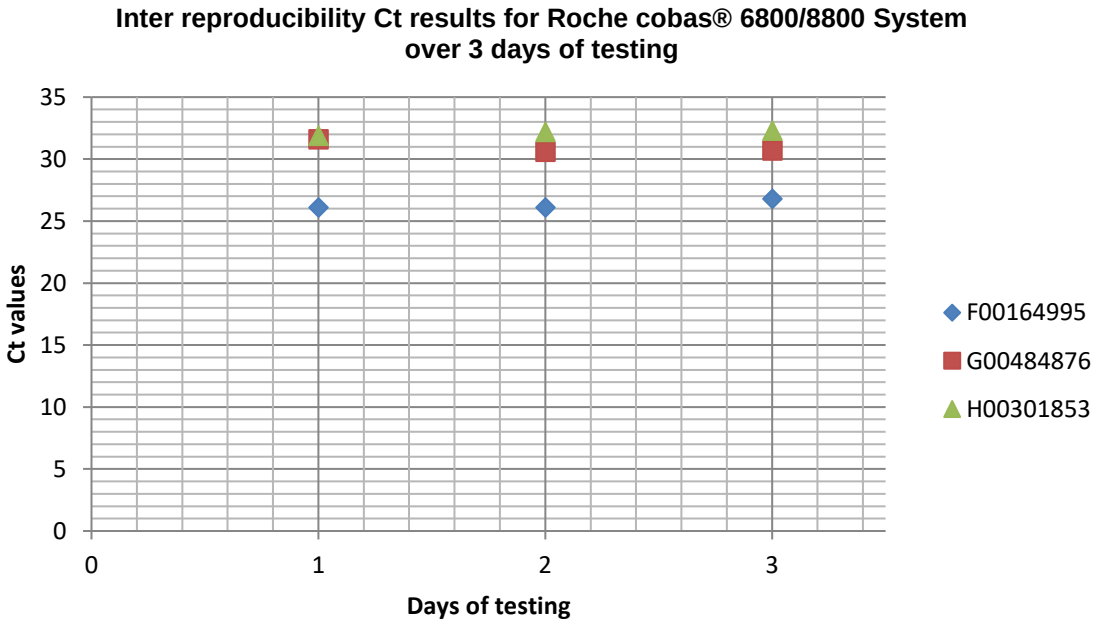

Figure 3.5: Inter-assay reproducibility Ct results for the Roche cobas® 6800/8800 System over three days

3.2 The HIV drug resistance results

One hundred and ninety-two samples, 147 HIV PCR positive and 45 HIV PCR indeterminate, were amplified with the in-house PCR assay targeting HIV *pol* genes (PR and RT). Of these, 102 samples failed amplification (of which 42 were HIV PCR indeterminate) while 90 passed amplification (of which only three were HIV PCR indeterminate). Only 56 amplicons were successfully sequenced, while the others failed (of which nine were CAP/CTM indeterminate), even upon repeat amplification, potentially because of low amplicon concentrations.

Of the 56 HIV PCR samples sequenced, the overall age of the infants ranged from 0 days to 18 months and 5 days. A total of 76.8% (43/56) were <4 months of age, and 23.2% (13/56) were ≥4 months, with 61.5% (8/13) of this latter group ≤6 months of age. Thus, 91.1% (43+8= 51/56) of the infants were ≤6 months of age.

Nine HIV PCR indeterminate samples (the three that passed amplification along with six that had very weak amplification due to very low DNA concentration) were included; however, these samples failed sequencing. Samples that were sequenced came from hospitals in Tshwane and the surrounding metropoles (Hammanskraal, Tembisa and Brits), as shown in Figure 3.6.

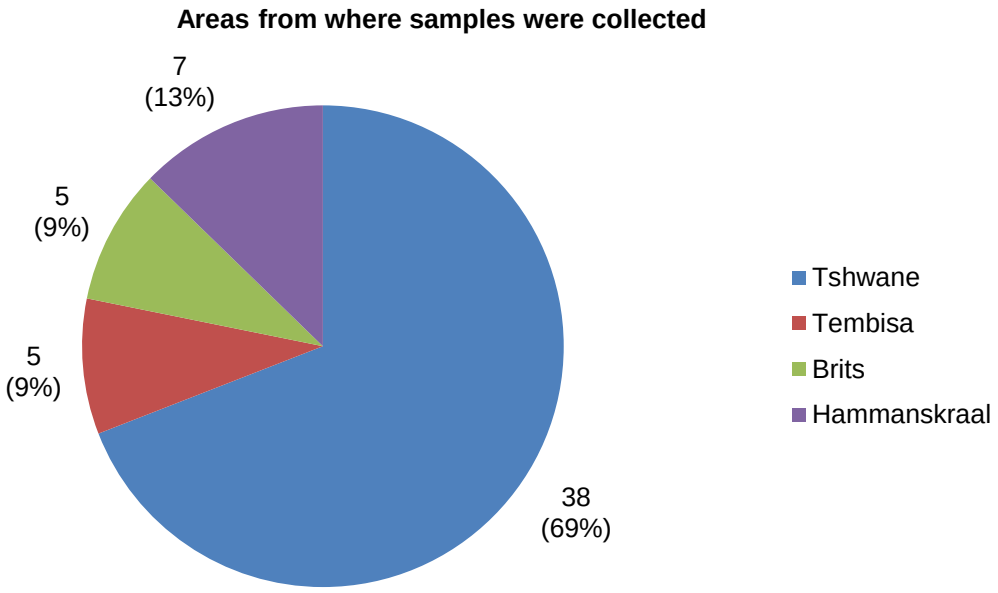

Figure 3.6: The different areas from where samples were collected

3.2.1 Gel electrophoresis

The target gene for sequencing was about 1080 bp in size. As seen in the representative gel of results obtained from the in-house PCR in Figure 3.7, the molecular marker is indicated as “MM”, the HIV PCR positive control lane is indicated as “+” while the HIV PCR negative control lane is indicated as “-”. All the lanes between MM and + contained samples. HIV PCR positive samples had amplification bands of 1080 bp (Figure 3.7).

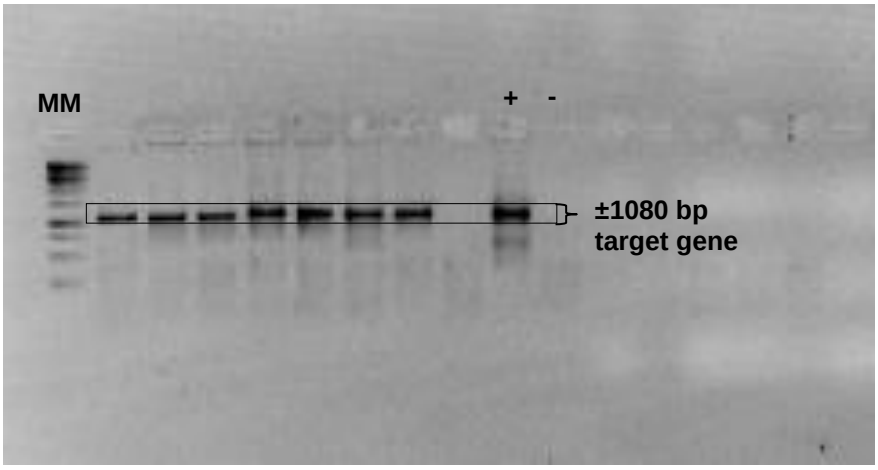

Figure 3.7: Agarose gel electrophoresis PCR results

3.2.2 HIVDR results interpretation using the Stanford HIVdb

The sequences obtained from Inqaba were analysed using Sequencher 4.10.1 (Gene Codes Corporation), after which they were submitted to the Stanford HIVdb (available at https://hivdb.stanford.edu/hivdb/by-mutations/) for quality and drug resistance-associated mutation profiles. Figure 3.8 is an example of the reports obtained from the Stanford HIVdb. The report also shows the susceptibility or the levels of resistance (susceptible, low, intermediate or high) to the different ART.

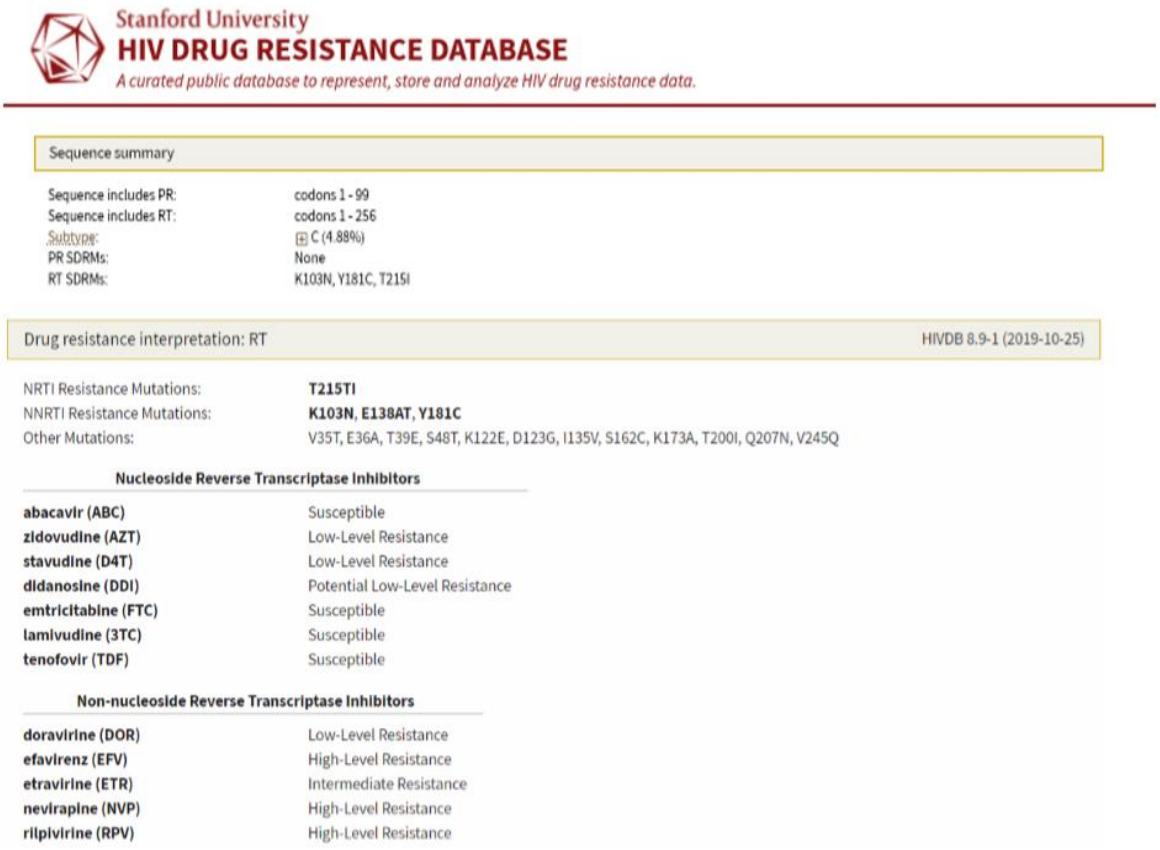

Figure 3.8: Screenshot from the Stanford HIVdb of the resistance mutations found in a sequence and susceptibility to the different antiretroviral medications

3.2.3 Mutation analysis with Stanford HIVdb

HIVDR-associated mutations were detected in 42.9% (24/56) of infants. The most common drug resistance mutation was K103N, detected in 21.4% (12/56) of infants. This mutation is associated with high-level resistance to the first-generation NNRTI's (Table 3.8). Other mutations commonly detected were the NRTI-associated mutation, M184V, and the NNRTI-associated mutation, Y181C, both at 8.9% (5/56).

Also, 57.1% (32/56) of the samples had polymorphic mutations which, on their own, do not lead to a reduction in drug susceptibility. Polymorphic mutations are defined as mutations that usually have little effect on ARV susceptibility when they occur in the absence of other drug resistance mutations. The most common polymorphic mutation was the PI-associated mutation, K20R, which was found in 28.6% (16/56) of the infant samples.

Table 3.8 illustrates the number of patients with specific resistance-associated mutations in the three different classes of ARVs (labelled “cases”) and also the percentages (out of the 56 sequenced samples). The Stanford HIVdb scores are also shown. According to the Stanford HIVdb, scores less than 10 indicate drug susceptibility; scores between 10 and 14 indicate potential low-level resistance; scores between 15 and 29 indicate low-level resistance; scores between 30 and 59 indicate intermediate resistance. A score of 60 or greater indicates high-level resistance. A negative score indicates increased susceptibility conferred by the mutation concerned. The polymorphic K20R mutation was not included in this table, as this mutation did not occur in combination with any non-polymorphic mutations.

Table 3.8: The different mutations found per drug class

Mutations per ARV class	Cases	Percentage*	Stanford HIVdb Scores							
PIs			ATV/r	FPV/r	IDV/r	LPV/r	NFV	SQV/r	TPV/r	DRV/r
• **L10L/F/V**	1	1.8%	0	15	10	5	15	0	0	5
• **M46L**	1	1.8%	10	10	10	10	20	10	10	0
• **T74T/A/P/S**	1	1.8%	10	10	10	5	20	10	25	5
• **V82A**	1	1.8%	15	15	30	30	30	15	0	0
NRTIs			ABC	AZT	D4T	DDI	FTC	3TC	TDF	
• **M41L**	1	1.8%	5	15	15	10	0	0	5	
• **A62V**	1	1.8%	5	5	5	5	5	5	5	
• **K65R**	1	1.8%	45	-15	60	60	30	30	60	
• **T69D**	1	1.8%	0	0	10	30	0	0	0	
• **V75V/I**	1	1.8%	5	5	5	5	5	5	5	
• **M184V**	5	8.9%	15	-10	-10	10	60	60	-10	
• **T215 revertants**	4	7.1%	5	20	20	10	0	0	5	
NNRTIs			DOR	EFV	ETR	NVP	RPV			
• **A98G**	1	1.8%	15	15	10	30	15			
• **K101E**	1	1.8%	15	15	15	30	45			
• **K103R+V179D/E**	1	1.8%	0	20	0	20	15			
• **K103N**	12	21.4%	0	60	0	60	0			
• **V106M**	4	7.1%	50	60	0	60	0			
• **E138A/T**	1	1.8%	0	0	10	0	15			
• **Y181C**	5	8.9%	10	30	30	60	45			
• **G190A**	1	1.8%	0	45	10	60	15			
• **H221H/Y**	1	1.8%	15	10	10	15	15			
• **P225H**	1	1.8%	30	45	0	45	0			
• **F227L**	1	1.8%	50	15	0	30	0			

* Percentage is calculated using the 56 sequenced samples as denominator

Abbreviations: 3TC (lamivudine), ABC (abacavir), ARV (antiretroviral), ATV/r (ritonavir boosted atazanavir), AZT (Zidovudine), d4T (stavudine), ddI (didanosine), DOR (doravirine), DRV/r (ritonavir boosted darunavir), EFV (efavirenz), ETR (etravirine), FPV (fosamprenavir), FTC (emtricitabine), IDV/r (ritonavir boosted idinavir), LPV/r (ritonavir boosted lopinavir), NFV (nelfinavir), NPV (nevirapine) NNRTI (non-nucleoside reverse transcriptase inhibitor), NRTI (nucleoside reverse transcriptase inhibitor), PI (protease inhibitor), RPV (rilpivirine), SQV/r (ritonavir boosted saquinavir), TDF (tenofovir disoproxilfumarate), TPV/r (ritonavir boosted tipranavir)

From Table 3.8, the most important mutations (i.e. the non-polymorphic mutations which on their own or with other mutations can lead to reduced drug susceptibility as per Stanford HIVdb) per drug class were the PI mutations M46L and V82A, the NRTI mutation, M184V, and the NNRTI mutations K103N and Y181C. To determine the possible transmission route for these mutations (transplacentally, through breastfeeding or via neonatal use of AZT or NVP), the ages of these infants were collected from the NHLS database (Table 3.9). Ages of infants who did not have any HIVDR from sequencing results ranged from 0 days to about 18 months.

Table 3.9: The ages of infants harbouring the most important mutations

Drug class	Mutation	Age
PI	M46L	0d
	V82A	2m 12d
NRTI	M184V	2m 8d, 2m 18d, 4m 29d, 11m 29d
NNRTI	K103N	0d, 1m 2d, 1m 19d, 2m 8d, 2m 9d, 3m 18d (in two infants), 3m 30d, 4m 3d (in two infants), 4m 29d, 6m 5d
	Y181C	1m 2d, 2m 14d, 3m 22d, 4m 3d, 4m 3d

Abbreviations: d (days), m (months), NNRTI (non-nucleoside reverse transcriptase inhibitor), NRTI (nucleoside reverse transcriptase inhibitor), PI (protease inhibitor)

The PI mutations, M46L and V82A, occurred each on their own (no accessory or polymorphic mutations), while the K103N and Y181C occurred together in 5.4% (3/56) of infants and the M184V and K103N mutations occurred together in only 1.8% (1/56) of the infants. No infants harboured a combination of the K103N, Y181C and M184V mutations.

When examining whether mutations to the different drug classes occurred alone or in combination with other drug class resistance mutations, most cases (13/56 - 23.2%) only had NNRTI mutations (Table 3.10).

Table 3.10: The different drug class mutation combinations per sample

Resistance to:	Cases	Percentage (%)
PIs only	2	3.6%
NRTIs only	1	1.8%
NNRTIs only	13	23.2%
NNRTIs and NRTIs	7	12.5%

Abbreviations: NNRTI (non-nucleoside reverse transcriptase inhibitor), NRTI (nucleoside reverse transcriptase inhibitor), PI (protease inhibitor)

3.2.4 Contamination detection

Samples were analysed with distance matrix and phylogenetic analysis on MEGA X (Molecular Evolutionary Genetics Analysis program) to detect any contamination. Contamination is deemed to be present when sequences are closely related to each other (distance <0.02) and have ≥98% similarity. All the samples had scores above 0.02 for distance matrix analysis (see Appendix B) and less than 98% similarity between sequences (Figure 3.9).

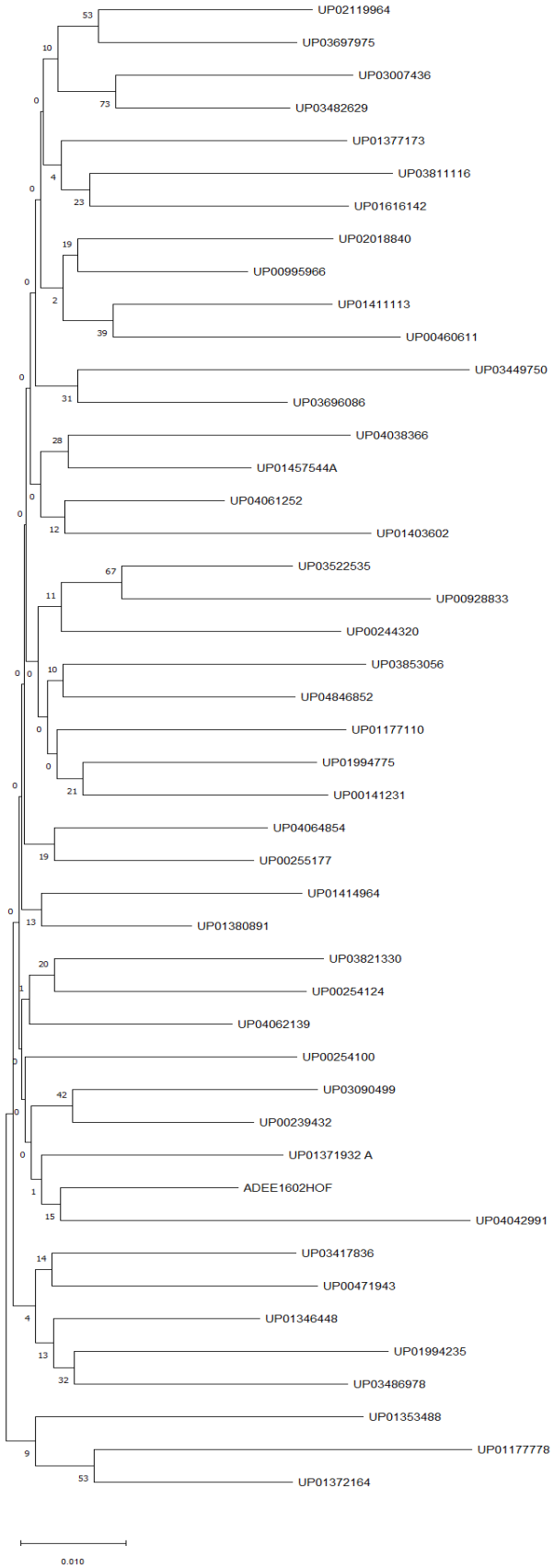

Figure 3.6: MEGA X phylogenetic analysis of samples for contamination detection

CHAPTER FOUR: DISCUSSION

The success of the PMTCT programme has led to major reductions in the vertical transmission of HIV in most countries; however, HIV transmission in sub-Saharan Africa remains a continual threat to new-borns (Inzaule *et al.*, 2018a, Yah and Tambo, 2018). Multiple factors have been identified as some reasons behind the failure of the PMTCT programme in sub-Saharan Africa, such as late or missed HIV diagnosis, incident HIV infection in pregnant women, low uptake or postponed treatment initiation, insufficient adherence to ART, late diagnosis of HIV-exposed infants or loss to follow-up (Inzaule *et al.*, 2018a, Yah and Tambo, 2018).

Also of importance is accurate and timely EID, especially because HIV PCR indeterminate results impact the immediate initiation and linkage to treatment and care of infants (Inzaule *et al.*, 2018a, Haeri Mazanderani, 2018). The HIV PCR indeterminate results are influenced by the RFI values when using the CAP/CTM platform, as per the NHLS EID CAP/CTM SOP. Given the limitations of the CAP/CTM platform, the main aim of the current study was to verify the Roche cobas® 6800/8800 System against the CAP/CTM system for detection of HIV in EID and to compare indeterminate results. In the current study, the Roche cobas®6800/8800 System had a sensitivity of 90.8% and a specificity of 96.3% when HIV PCR indeterminate results were included. However, the sensitivity and specificity were much higher (99.6% and 99.5% respectively) when HIV PCR indeterminate results were excluded, thus emphasising the challenges arising from HIV PCR indeterminate results. Most importantly, the HIV PCR indeterminate results decrease the ability of an EID assay to detect true HIV PCR positive and true HIV PCR negative cases. Hence, better EID diagnostic assays are needed that can improve the detection of HIV by being highly specific and sensitive, while not including RFI as part of the interpretation of results. This is, however, complicated by the high levels of PMTCT ART exposure in infants during the time of testing that can affect the level of viraemia (Mazanderani *et al.*, 2014, Maritz *et al.*, 2017, Mazanderani *et al.*, 2018).

Laboratory diagnosis of HIV from DBS using molecular assays has become a standard practice in children below 18 months of age. The minimum recommended DBS sample volume is 70 uL. The concentration of the HIV-1 target can therefore be low, leading to HIV PCR indeterminate or false negative results (Phillips *et al.*, 2015).

Because of the potential low concentration of virus, a diagnostic assay would require high sensitivity and specificity for the detection and quantification of HIV (Zakaria *et al.*, 2016). Accordingly, the WHO recommends that EID assays should have a sensitivity of at least 95% and a specificity of 98% (World Health Organization, 2016a).

When HIV PCR indeterminate results were excluded in the current study, the specificity and sensitivity were 99.5% and 99.6%, respectively, and thus met the WHO requirements of EID assays.

It was also observed that excluding the RFI value and classifying HIV PCR indeterminate samples as HIV PCR positive or HIV PCR negative based on Ct values alone, had a major positive impact on the agreement between the CAP/CTM and the Roche cobas®6800/8800 System. This is because the kappa value increased from 0.5, which is moderate agreement, to 0.9, which is regarded as excellent agreement, when RFI was excluded. The overall agreement between the two assays, taking only Ct values into account, was 93.8%.

Some infants receive an indeterminate result when only the RFI failed the criteria, thus these infants need to return for follow-up testing. This creates the possibility of loss to follow-up. The Roche cobas®6800/8800 System relies solely on Ct values, thus has the potential to reduce HIV PCR indeterminate results and prevent loss to follow-up. When RFI values were excluded from both EID assays, more conclusive infant results were obtained with 46% HIV PCR positive, 51% HIV PCR negative and only 3% HIV PCR indeterminate results. More infants can therefore be linked to care immediately after an HIV PCR positive diagnosis. This observation is in agreement with previous studies that also observed the problem that RFI values pose regarding linking HIV-infected infants to care as soon as possible (Inzaule *et al.*, 2018a, Haeri Mazanderani, 2018).

Other studies have suggested that lowering the RFI cut-off value might decrease the number of indeterminate results; however, insufficient data on this is available, and certain samples were classified as HIV PCR negative although their Ct values were lower than those of samples that had HIV PCR positive results (Maritz *et al.*, 2012). A study examining the cost-effectiveness and accuracy of conducting pooled PCR testing on DBS samples observed that a subset of samples initially termed

indeterminate with a CAP/CTM Ct ≥32 (the specific study worked with a Ct cut-off value of 32 versus the NHLS cut-off of 33) or an RFI ≤5 were later proven to be positive (Van Schalkwyk *et al.*, 2019). All these findings from previous studies and the current study illustrate the problem that RFI values pose in terms of result classification. Correct classification of results in exposed new-born infants is imperative as early treatment can reduce mortality and limit the viral reservoir size, which is an important determinant of viral blips and low-level viraemia, and could have implications for future cure strategies (Jain *et al.*, 2013, Bachmann *et al.*, 2019).

Regarding the overall results of the Roche cobas®6800/8800 System based on the CAP/CTM HIV PCR indeterminate samples, 62.6% (134/214) tested HIV PCR negative, 29.0% (62/214) tested HIV PCR positive while only 8.4% (18/214) remained HIV PCR indeterminate on the Roche cobas®6800/8800 System. The drastic decrease in HIV PCR indeterminate results from 33.3% (214/642) on the CAP/CTM to 3.27% (21/642) on the Roche cobas®6800/8800 System illustrates how the new technology, which relies solely on Ct values for result interpretation, can decrease confusion during EID, thus leading to conclusive results, which will limit the number of infants lost to follow-up. This is because infants who receive indeterminate results need to return for a confirmation HIV PCR test until a conclusive diagnosis can be made. Unfortunately, most of these indeterminate results had no record of a final or confirmatory HIV PCR diagnosis within the NHLS LIS to compare with the Rochecobas®6800/8800 System results. Since 2012, indeterminate HIV PCR results comprise over 16% of all samples (positive, indeterminate and negative samples collectively), which represents a significant proportion of infants that can potentially be lost to follow-up (Mazanderani *et al.*, 2016).

Confirmatory HIV PCR results of the previously CAP/CTM HIV PCR indeterminate samples were only available for 30 samples of which 86.7% (26/30) sample results on the Roche cobas®6800/8800 System were concordant with the confirmation HIV PCR results. Two samples that were Roche cobas®6800/8800 System HIV PCR negative but turned out HIV PCR positive upon confirmatory CAP/CTM testing (with viral loads of 128 000 and 183 000 copies/mL respectively) could be explained either by the timing of the confirmation HIV PCR (which might have required a new sample to be taken) or by the lower sensitivity in terms of viral load because of the lower sample

volume of DBS versus that of plasma samples (Garrido *et al.*, 2009, Fajardo *et al.*, 2014, Phillips *et al.*, 2015).

A confirmatory HIV PCR test is done sometime after the initial HIV PCR, thus the opportunity for PMTCT through breastfeeding between the different tests exists. One sample that tested Roche cobas®6800/8800 System HIV PCR negative, tested HIV PCR indeterminate upon confirmation testing. The confirmation PCR was repeated on three different occasions and the result remained HIV PCR indeterminate. When dealing with EID challenges, mechanisms of virological control (ART and immunological factors) need to be considered as a lower viral load might be related to indeterminate results (Technau *et al.*, 2017b). Other studies have also noted cases that remain HIV PCR indeterminate upon confirmation testing, thus these infants were closely monitored and treatment was interrupted under clinical supervision (Technau *et al.*, 2017b). The conclusive results of the infants from the Johannesburg study could not be determined as the required length of time for monitoring after treatment cessation is unknown (Sutcliffe *et al.*, 2015, Technau *et al.*, 2017b).

In the Johannesburg study, seven out of 19 infants (36.8%) with initial HIV PCR indeterminate results received a positive diagnosis upon repeat testing, thus HIV PCR indeterminate cases cannot be ignored (Technau *et al.*, 2017a). As per Mazanderani *et al*., (2016), infants with HIV PCR indeterminate results should be closely monitored and screened until a conclusive diagnosis can be made and all precautions should be taken not to lose these infants to follow-up. According to the SA PMTCT guidelines, infants with indeterminate results should be managed as follows: If the infant had a prior HIV PCR positive or indeterminate result, the infant must be treated as HIV infected and ART must be initiated. If the infant was indeterminate, but had a detectable viral load, the infant must be treated as HIV infected and ART must be initiated. If the infant had a prior HIV PCR negative result and undetectable viral load, the HIV PCR and HIV viral load need to be repeated. If the situation persists after repeat testing, the infant requires further HIV testing as per the PMTCT guidelines (South African National Department of Health, 2019).

There was 99.5% and 98.7% agreement between the CAP/CTM and the Roche cobas®6800/8800 System for HIV PCR positive or HIV PCR negative results, respectively. A sample that tested Roche cobas®6800/8800 System HIV PCR positive

but was HIV PCR negative upon confirmatory CAP/CTM testing could potentially be because of exposure of the infant to an ART regimen, thus leading to viral suppression below the detection level.

In a previous study where the Roche cobas®6800/8800 System was compared to CAP/CTM, results between the two platforms in terms of HIV-1 RNA quantification had good correlation (R^2 of 0.98, Deming regression line slope of 0.94), with the Roche cobas®6800/8800 System yielding slightly higher results (Wirden *et al.*, 2017). In the Wirden *et al*., (2017), study only 6.5% of samples had discrepancies; however, these minor differences observed between the assays would not have affected the treatment decisions for HIV-infected individuals (Wirden *et al.*, 2017).

In the current study, 99.6% (234/235) of the samples that tested CAP/CTM HIV PCR positive remained HIV PCR positive on the Roche cobas®6800/8800 System. Two of these samples were HIV PCR indeterminate on the Roche cobas®6800/8800. Regarding CAP/CTM HIV PCR negative samples, all but one sample (192/193) tested Roche cobas®6800/8800 System HIV PCR negative. One sample tested Roche cobas®6800/8800 System HIV PCR indeterminate with a Ct value of 39. This occurrence is possible as HIV PCR indeterminate results are neither clearly positive nor clearly negative (Maritz *et al.*, 2014, Mazanderani *et al.*, 2016, Mazanderani *et al.*, 2018). As such, these patients are requested to send another sample for confirmatory HIV PCR and viral load. Most of the HIV PCR indeterminate results have lower viral loads and, as such, are missed or detected as HIV PCR negative or HIV PCR positive at higher Ct values. The HIV PCR negative result could not be confirmed to be truly negative as the follow-up sample result was not available on the NHLS LIS at the specific point of time. Confirmatory results were only available for 30 samples on the NHLS LIS. Discordance results from samples confirmed on NHLS LIS between CAP/CTM and the Roche cobas®6800/8800 System was only observed in 13.3% (4/30) of cases. It should be noted that some samples that were tested in 2019 on the Roche cobas®6800/8800 System had been stored at room temperature since 2018, thereby potentially compromising integrity and could potentially explain the discordance.

The overall number of samples detected as HIV PCR positive on the Roche cobas®6800/8800 System was 45.8% (294/642) versus 36.6% (235/642) on

CAP/CTM, while the overall number of HIV PCR negative samples detected were 50.9% (327/642) versus 29.9% (192/642) on CAP/CTM. The Roche cobas®6800/8800 System will thus return definitive results in a larger proportion of infants, thus more infants can be timeously placed on ART, fewer infants will be incorrectly placed on ART or less would be incorrectly termed indeterminate and be potentially lost to follow-up.

The intra- and inter-reproducibility of the Roche cobas®6800/8800 System was almost 100.0% for positive and negative samples. A sample that tested HIV PCR indeterminate on the CAP/CTM solely because of an RFI value<5 was detected as HIV PCR positive on the Roche cobas®6800/8800 System. This is because of the Roche cobas®6800/8800 System not including the RFI value as a determining factor for result interpretation.

The Roche cobas®6800/8800 System also processed 93 samples (excluding controls) in three hours versus the CAP/CTM processing 22 samples (excluding controls) in six hours. The Roche cobas®6800/8800 System allows for 960 samples to be processed in an eight-hour shift versus the CAP/CTM that can only process 72 samples in this time (Cobb *et al.*, 2017).

Based on the high level of concordance between the Roche cobas®6800/8800 System and the confirmatory PCR results, the new platform proves to be reliable in terms of result determination. In combination with its lower turnaround time, the capacity of samples that can be processed in this time and the decrease in HIV PCR indeterminate results, the Roche cobas®6800/8800 System proves to be an excellent platform for EID in resource-limited settings with high levels of loss to follow-up.

The secondary aim of the current study was to determine the HIVDR prevalence and profiles in infants who tested HIV PCR positive or indeterminate upon EID and describe how this can affect the current SA PMTCT and EID programmes. In this study, of the 56 samples successfully amplified and sequenced, major HIV mutations associated with drug resistance were detected in 42.9% (24/56) with the most common mutation being K103N (21.4%) that confers resistance to the first-generation NNRTI class. The K103N mutation therefore has specific significance for the use of NVP as

part of the SA PTMTC programme (South African National Department of Health, 2019).

Although PMTCT has decreased the overall rate of MTCT of HIV, there remains a portion of infants who become infected and harbour HIVDR in early life. The proportion of infants with HIVDR has been estimated to be between 35% and 64% in the African region (Jordan *et al.*, 2017, Inzaule *et al.*, 2018a, Inzaule *et al.*, 2018b). The HIVDR-associated mutations could either have been transmitted directly from a mother with HIVDR or could have been acquired because of intra-uterine and postnatal exposure to ART as part of the PMTCT programme. Infants with HIVDR are at higher risk of virological failure and death (Violari *et al.*, 2012, Sebunya *et al.*, 2013, World Health Organization, 2018a, Hunt *et al.*, 2019). Due to low viral load sample quality and storage, there was a significant drop-out of samples that could not be amplified, thus prevalence in this study was lower than expected. The samples used for this study were remnant DBS from the NHLS after all routine diagnosis, thus these samples were stored for a minimum of months at room temperature without desiccant which could have affected sample quality.

The most prevalent mutations in the current study were the NNRTI mutations, K103N (21.4%) and Y181C, and the NRTI mutation, M184V (the latter two at 8.9%). This is in agreement with other SA surveillance studies that found the K103N and Y181C mutations to be the most common mutations among South African infants (Persaud *et al.*, 2011, Hunt *et al.*, 2019). A nationally representative HIVDR survey from SA reported a 63.7% (95% CI, 59.0-68.4) prevalence of NNRTI resistance among infants <18 months old diagnosed with HIV through the EID programme from 2014 to 2016 (World Health Organization, 2017b). This high prevalence supports the WHO 2016 recommendation of starting all children <3 years on a PI-based regimen regardless of NNRTI exposure (World Health Organization, 2016a, World Health Organization, 2017b). Another study conducted in Johannesburg, SA, found the most common HIVDR mutations to be the K103N (NNRTI) at 42% and the M184V (NRTI) at 54% in 96 samples of a population ranging from two to 66 years of age, thus focusing on an older population compared to the current study that focused on infants ≤6 months old (Obasa *et al.*, 2020). The occurrence of these mutations within an older population,

including mothers of reproductive age, poses a risk of being eventually transmitted to infants.

The K103N mutation is selected by NVP and EFV, and leads to reduced susceptibility to these NNRTIs, and is estimated to be prevalent worldwide in 40%-60% of adults receiving treatment with NNRTI-resistant viruses (Jackson *et al.*, 2000, Lai *et al.*, 2016). A previous study conducted by Gilead Sciences 934, observed that approximately 84% of EFV resistant cases had the K103N mutation (Adams *et al.*, 2010). In another study conducted by Flys et al. (2007), women with subtype C HIV receiving sdNVP for 6-8 weeks had the highest mean level of K103N mutations (approximately 41.7% found in subtype A, 55.3% in subtype D and 69.8% in subtype C). Other studies have also determined that the K103N mutation is the most common mutation to emerge from women who received single-dose NVP as part of PMTCT (Flys *et al.*, 2005, Flys *et al.*, 2007, Chimukangara *et al.*, 2019).

Variants containing the K103N mutation can persist at low levels for years in patients infected with these strains while not using ART. In those who discontinue NNRTI-based regimens, the efficacy of NNRTI-based regimens for future use (for treatment of their HIV-1 infection) could potentially be reduced (Nolan *et al.*, 2002, Palmer *et al.*, 2006, Flys *et al.*, 2007).

In the current study, approximately one in five infants had the K103N mutation. The high prevalence of the K103N mutation can be explained by the early introduction of WHO option B+ in South Africa, leading to earlier and longer exposure to EFV-based regimens which potentially lead to selection of the K103N mutation (Nuttall and Pillay, 2019). This mutation also develops at a higher frequency in women with subtype C or D HIV versus those with subtype A (Flys *et al.*, 2006, Wallis *et al.*, 2010). In this study, all samples were from subtype C. This is because of subtype C being the major cause of the HIV epidemic in South Africa (Harmelen *et al.*, 1999, Skhosana *et al.*, 2015).

In the current study, the age of infants with the K103N mutation ranged from 0 days to 6 months. It can be speculated that the mutation could have either arisen because of the mother failing an EFV-based ART regimen or having been exposed to NVP,

especially sdNVP in the past, or by the infant's exposure to NVP for the first six weeks postpartum, or during breastfeeding.

Another NNRTI drug resistance-associated mutation detected was Y181C, which is found in 15%-25% of NNRTI-resistant viruses globally and is selected by NVP, etravirine (ETR), EFV and rilpivirine (RPV) use, reducing susceptibility to these NNRTIs. It does not, however, reduce susceptibility to doravirine (DOR) when present on its own (Maïga *et al.*, 2010, Lai *et al.*, 2016, Saravanan *et al.*, 2017).

The Y181C mutation was the second most common NNRTI resistant mutation detected in this sample at 8.93% (5/56). Because of South Africa being a resource-limited country, NVP and EFV use was predominant in the past, thus explaining the occurrence of the Y181C mutation in infants (Flys *et al.*, 2006, L'homme *et al.*, 2008).

The age of infants that harbour the Y181C mutation ranged from 1 month and 2 days to 4 months and 3 days. This mutation could have been transferred from the mother to her infant during breastfeeding or the infant failed the neonatal NVP-based regimen.

A total of 5.4% (3/56) of the infants had both the K103N and the Y181C mutation. Because of these mutations, these infants will have high-level resistance to EFV and NVP-based regimens, and should thus be initiated on a PI-based or alternatively on a DTG-based regimen once they meet the age and weight requirements.

Although the NNRTI mutations K103N and Y181C might not seem to be a problem for the newly introduced DTG-based regimen, these mutations might confer resistance to INSTIs (Li and De Clercq, 2016). Under the selective pressure of raltegravir, these two NNRTI mutations can rescue the replicative fitness of viruses with IN drug-resistant mutations (Hu and Kuritzkes, 2014, Li and De Clercq, 2016). According to the VIKING trial, DTG only failed to inhibit raltegravir-resistant viruses involving the Q148 pathway when the Q148HKR mutation was associated with two secondary mutations, one of which was the NNRTI E138K mutation (Castagna *et al.*, 2014, Malet *et al.*, 2015).

Thus far, there is no evidence that the K103N or the Y181C mutations are associated with DTG resistance (Siedner *et al.*, 2020). Although a recent study by Siedner et al. published in 2020, observed that NNRTI mutations affected the susceptibility of DTG,

their observation may be because of INSTI drug pressure leading to higher replication fitness of NNRTI mutant viruses (Clutter *et al.*, 2016, Siedner *et al.*, 2020).

The most common NRTI drug-associated mutation detected was M184V, which is selected by the NRTIs 3TC/FTC, ABC and didanosine (ddI), leading to high-level resistance to 3TC/FTC and low-level resistance to ABC and ddI (Keulen *et al.*, 1996, Miller *et al.*, 2000). Usually, the M184I mutation will emerge before M184V; however, M184V has higher fitness, thus out-competes M184I (Keulen *et al.*, 1997). The M184V was present in 8.9% of infants which is comparable to another study which detected this mutation in 9.5% of South African children ≥18 months old (Jordan *et al.*, 2017). Studies also showed that primary IN resistance mutations can occur in treatment-experienced patients with pre-existing M184V mutations (White *et al.*, 2015, Fulcher *et al.*, 2018). The M184V has a score of 60 against both 3TC and FTC, which is considered high-level resistance. This is of a benefit because it increases susceptibility to other ARVs (e.g. AZT and TDF), therefore suppressing viral replication, thus 3TC/FTC can be recycled more often as part of PI based ART (Wolf *et al.*, 2003, Nel *et al.*, 2020)

The ages of the infants who harbour the M184V mutation ranged from 2 months and 8 days to 11 months and 29 days. Because either 3TC or FTC forms part of all current treatment regimen, also those given to women of childbearing potential, this mutation arose from failure of this regimen and could have been transferred to the infant during breastfeeding.

The PI mutations detected in this study were M46L and V82A and are described as multi-drug resistant mutations which affect the binding affinity of an inhibitor to the active site of the PR enzyme indirectly by reducing hydrophobic interactions or by strengthening interactions with a substrate (Kovalevsky *et al.*, 2006, Meher and Wang, 2015). According to the HIV Stanford Drug resistance database, the non-polymorphic M46L mutation leads to reduced susceptibility to PI drugs available in SA such as indinavir (IDV), LPV and nelfinavir (NFV) and is selected through the use of any of these PIs and confers as score of 10 against LPV/r which is considered as potential low-level resistance (Rhee *et al.*, 2003).

The non-polymorphic V82A mutation occurs in the active site of the PR enzyme and directly interacts with PI inhibitors amprenavir (APV) and ritonavir (RTV) (Dirauf *et al.*, 2010). From the Stanford University HIV Drug Resistance Database, the V82A mutation is selected for through the use of IDV and LPV and confers a score of 30 against LPV/r which is considered as intermediate resistance (Rhee *et al.*, 2003).

The infants that had these PI mutations most likely acquired the mutation through MTCT from mothers who were failing or exposed to second-line therapy. The infant harbouring the M46L mutation was 0 days old upon testing, thus the transmission occurred via MTCT either transplacentally or during labour because of the mother failing ART. The infant harbouring the V82A mutation was 2 months and 12 days old upon testing, thus this infant could have acquired the resistance via MTCT during breastfeeding. Although these mutations were only present in two infants, the occurrence can complicate the choice of therapy for these infants once they start with second-line therapy. In a study that focused on individuals from Johannesburg, SA, aged two to 66 years who are on a PI boosted treatment, the most common major circulating PI mutations observed were M46L and V28A, which were present in 13% of the 96 sequences they had analysed (Obasa *et al.*, 2020). Therefore, since these mutations are present in adults, they can potentially be transmitted to infants. This could potentially explain the occurrence of these mutations in the infants from the current study. The low occurrence of PI mutations within SA infants is in agreement with another SA study which detected PI mutations in only 2/220 samples (Hunt *et al.*, 2019).

Nine CAP/CTM HIV PCR indeterminate samples were included for sequencing; however, these samples could not be sequenced. A potential explanation for this occurrence could be a low viral load, or these samples could actually have been weak HIV PCR positive.

A major cause of HIVDR is non-adherence or interrupted use of ART. Multiple factors can play a role when it comes to ART non-adherence, such as personal factors (poor understanding of the importance of ART, side effects, poverty), family factors (lack of support from family), community factors (stigma of living with HIV), and health system factors (distance to the healthcare facilities, poor attitudes of healthcare workers) (Mpinganjira *et al.*, 2020).

Women also face specific challenges when pregnant and breastfeeding, such as poverty, lack of support from marriage partners or family members and distance to healthcare facilities, which could predispose them to non-adherence or loss to follow-up (Mpinganjira *et al.*, 2020). It is critical that attention is paid to these factors in order to find strategies to support women during this difficult time in order to limit non-adherence and treatment failure.

Significance of findings:

The results from this study illustrate the importance of developing newer technologies for EID in order to improve diagnosis of infants and linkage to care as soon as possible. The decrease in the amount of samples resulting in indeterminate HIV PCR results will help ease the interpretation of results as an indeterminate HIV PCR result must be differentiated as false-positive or true-positive by longitudinal follow-up.

According to the WHO, once the prevalence of pre-treatment drug resistance reaches 10%, NNRTIs must be removed from the country's first-line therapy, however there remains a small percentage of infants with PI resistance, thus continual HIVDR surveillance is of importance (World Health Organization, 2017a).

In this study, the infants had mutations which could affect all potential ART regimens available in the public sector. This emphasizes the need for an evolving PMTCT guideline to alleviate potential infant morbidity and mortality. There were also 57.1% of infants who only had polymorphic resistance mutations, which is selected by ART use. The most common polymorphic mutation observed in this study was the K20R mutation (present in 28.6%) within the PR region. The K20R mutation is of concern as it increases replication fitness of HIV when in combination with other PI mutations, however, this mutation did not occur in combination with other mutations in this study (Arias *et al.*, 2018).

This study showed that HIVDR screening plays an important role in informing effective therapy; however, the question of cost-effectiveness of HIVDR screening is of concern. A study conducted in Kenya predicted that pre-treatment HIVDR screening could improve the rates of suppression of viraemia among women and decrease both HIV transmission and the prevalence of drug resistance; however, in the Kenyan setting, screening would not be cost-effective (Duarte *et al.*, 2020).

Regarding the transitioning from an EFV-based regimen to a DTG-based regimen, this transition will be beneficial regardless of the level of NNRTI resistance, and it will also lower the risk of HIVDR selection since DTG has a much higher genetic barrier to resistance than EFV (Phillips *et al.*, 2018). The predicted benefits of DTG-based regimens will be most efficient in populations with high levels of NNRTI resistance (such as the population from this study), especially if these regimens prove to be both cost-effective and cost-saving in the sub-Saharan setting (Phillips *et al.*, 2018).

The prevalence of the NNRTI mutations K103N and Y181C along with the prevalence observed from other studies support the phase-out of NNRTI-based regimens and also supports the WHO 2016 recommendation of switching children <3 years to a PI-based regimen (World Health Organization, 2016a).

Strengths and limitations:

One of the main strengths of the study is that it comprised a large sample size thus estimates made can be considered reliable and generalisable to similar settings. A single operator performed the Rochecobas®6800/8800 System studies thus all samples were handled in the same way. This study is one of few that included such a young age group for HIVDR determination in Tshwane and surrounding metropoles.

However, there were limitations to the study as well. Regarding the verification of the Roche cobas®6800/8800 System, not all confirmatory results for previously CAP/CTM HIV PCR indeterminate results could be obtained due to restriction of access to follow-up results. In addition, samples were remnant DBS samples that had been stored at room temperature for long durations of time, which could have affected the sample quality, thus explaining the low amplification rate and low number of samples successfully sequenced. Optimally, these samples should be stored at -20°C in order to conserve sample integrity.

For the HIVDR studies, a major limitation for the determination of drug resistance profiles in the infants was that resistance profiles and ART regimens used by the mothers and infants were unknown. By knowing the resistance and treatment status of the mother, we could have determined whether the resistance found in the infants was because of transmission of resistant variants from the mother or because of selection of resistance secondary to the infants' ART exposure.

Finally, only the PR and RT regions were sequenced, and not the IN region. At the time of the study, the PMTCT programme had not yet started transitioning to an INSTI-based regimen and circulating levels of INSTI resistance was believed to be negligible (South African National Department of Health, 2019).

In order to eliminate MTCT, it is important to use effective ART regimens in HIV-infected individuals in order to suppress viral loads to undetectable levels. Once the viral loads of HIV-infected women of childbearing potential become undetectable, HIV can no longer be transmitted to infants (Townsend *et al.*, 2014, Wessels *et al.*, 2020).

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

The performance of the Roche cobas®6800/8800 System was verified to be comparable to the CAP/CTM; however, it produced fewer indeterminate results, thus potentially lowering the number of infants lost to follow-up. From this study it could be seen how the RFI value used in the CAP/CTM platform complicates the interpretation of the results and should preferably not be used as a determining factor. The Roche cobas®6800/8800 System was also time-efficient and processes 93 samples per run in 3 hours compared with the CAP/CTM, which only processed 22 samples in 6 hours. These advantages, together with the good concordance between the Roche cobas®6800/8800 System results and the confirmatory CAP/CTM testing results, lead this study to conclude that the Roche cobas®6800/8800 System could improve the SA EID programme. When considering the limitations, it is recommended that DBS samples need to be stored at -20°C if sample integrity needs to be ensured over a short time period (two to three months) or at -80°C for a long time period.

According to the findings from this study, the K103N, M184V and Y181C mutations were the most prevalent HIVDR mutations in infants ≤6 months from Gauteng, SA and this finding was in agreement with other studies. Because these mutations significantly lower the efficacy of the antiretroviral drugs, such as NVP, EFV, 3TC, FTC and ABC, recommended in the SA PMTCT and infant treatment guidelines, this is of concern. There is also the concern that these mutations could affect the efficacy of the newly-introduced DTG, thus future studies need to be conducted to determine the possibility of DTG failure in the presence of these pre-existing circulating NNRTI and NRTI mutations.

The study contributes to the 90-90-90 targets from the UNAIDS, which aims to have 90% of all children living with HIV diagnosed, 90% of those diagnosed HIV-positive receiving treatment, and finally 90% of those children receiving treatment achieving viral suppression by 2020, in a number of ways. Firstly, it provides data on an alternative assay for EID with accurate, yet fewer indeterminate results. Secondly, it provides valuable information about HIVDR profiles circulating in SA infants in the PMTCT programme, which can be used to inform guidelines and treatment decisions.

Continued surveillance of HIVDR in both pregnant women and infants is of importance to ensure that all HIV-exposed infants receive effective treatment. Better linkage between clinical and laboratory data can improve both treatment and research as knowing which ART a women was exposed to could determine the cause of HIVDR in their infants, and knowing to which ART the infant will have reduced susceptibility will lead to improved treatment option decisions.

To improve EID and PMTCT, samples with HIV PCR indeterminate results pose a major problem because of weak mechanisms to track mothers and infants who do not return for testing. As such, new assays which are more specific need to be developed. It is thus of importance to detect HIV as soon as possible in infants and also to determine the regimen that would be most effective in order to decrease infant morbidity and mortality. This study aimed to aid in the fight against infant HIV-associated morbidity and mortality by evaluating newer technology to be used for EID and determining circulating HIVDR mutations within this population.

CHAPTER SIX: REFERENCES

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APPENDIX A

List of the primers used in the optimised HIVDR in-house assay

Primer name	Sequence (5′→3′)	Location(based on HXB2)	Purpose
PRTM-F1*	F1a-TGAARGAITGYACTGARAGRCAGGCTAAT F1b-ACTGARAGRCAGGCTAATTTTTTAG	2057–2085 2068–2092	RT-PCR, one of mixture components
PRTM2-F1	TAGGGA RAATYTGGCCTTCC	2090–2109	Rescue RT-PCR primer
RT-R1	ATCCCTGCATAAATCTGACTTGC	3370–3348	RT-PCR
PRT-F2	CTTTARCTTCCCTCARATCACTCT	2243–2266	Nested PCR & sequencing
RT-R2	CTTCTGTATGTCATTGACAGTCC	3326–3304	Nested PCR & sequencing
SeqF3	AGTCCTATTGARACTGTRCCAG	2556–2577	Sequencing
SeqR3	TTTYTCTTCTGTCAATGGCCA	2639–2619	Sequencing
SeqF4	CAGTACTGGATGTGGGRGAYG	2869–2889	Sequencing
SeqR4	TACTAGGTATGGTAAATGCAGT	2952–2931	Sequencing

*: PRTM-F1 is a mixture of primers F1a and F1b at a ratio of 1:1 (w/w).

APPENDIX B

MEGA X Distance matrix

Species 1	Species 2	Distance
UP01380891 A	UP00114879 A	0.037
UP01380891 A	UP00114879 A(2)	0.037
UP01380891 A	UP01377173 A	0.044
UP00114879 A	UP01377173 A	0.052
UP00114879 A(2)	UP01377173 A	0.052
UP01380891 A	UP01376885 A	0.044
UP00114879 A	UP01376885 A	0.052
UP00114879 A(2)	UP01376885 A	0.052
UP01380891 A	UP01371932 A	0.037
UP01377173 A	UP01371932 A	0.052
UP01376885 A	UP01371932 A	0.052
UP01380891 A	UP01346448 A	0.039
UP00114879 A	UP01346448 A	0.042
UP00114879 A(2)	UP01346448 A	0.042
UP01377173 A	UP01346448 A	0.052
UP01376885 A	UP01346448 A	0.052
UP01371932 A	UP01346448 A	0.042
UP00114879 A	UP03777520 A	0.037
UP00114879 A(2)	UP03777520 A	0.037
UP01377173 A	UP03777520 A	0.044
UP01376885 A	UP03777520 A	0.044
UP01371932 A	UP03777520 A	0.037
UP01346448 A	UP03777520 A	0.039
UP01380891 A	UP03486978 A	0.048
UP00114879 A	UP03486978 A	0.056
UP00114879 A(2)	UP03486978 A	0.056
UP01377173 A	UP03486978 A	0.062
UP01376885 A	UP03486978 A	0.062
UP01371932 A	UP03486978 A	0.056
UP01346448 A	UP03486978 A	0.044
UP03777520 A	UP03486978 A	0.048
UP01380891 A	ADEE1602HOF	0.039
UP00114879 A	ADEE1602HOF	0.034
UP00114879 A(2)	ADEE1602HOF	0.034
UP01377173 A	ADEE1602HOF	0.046
UP01376885 A	ADEE1602HOF	0.046
UP01371932 A	ADEE1602HOF	0.034
UP01346448 A	ADEE1602HOF	0.039
UP03777520 A	ADEE1602HOF	0.039
UP03486978 A	ADEE1602HOF	0.045
UP01380891 A	UP01994235	0.054
UP00114879 A	UP01994235	0.055
UP00114879 A(2)	UP01994235	0.055

UP01377173 A	UP01994235	0.065
UP01376885 A	UP01994235	0.065
UP01371932 A	UP01994235	0.055
UP01346448 A	UP01994235	0.049
UP03777520 A	UP01994235	0.054
UP03486978 A	UP01994235	0.051
ADEE1602HOF	UP01994235	0.056
UP01380891 A	UP01994775	0.041
UP00114879 A	UP01994775	0.049
UP00114879 A(2)	UP01994775	0.049
UP01377173 A	UP01994775	0.058
UP01376885 A	UP01994775	0.058
UP01371932 A	UP01994775	0.049
UP01346448 A	UP01994775	0.046
UP03777520 A	UP01994775	0.041
UP03486978 A	UP01994775	0.054
ADEE1602HOF	UP01994775	0.048
UP01994235	UP01994775	0.055
UP01380891 A	UP02018840	0.039
UP00114879 A	UP02018840	0.053
UP00114879 A(2)	UP02018840	0.053
UP01377173 A	UP02018840	0.052
UP01376885 A	UP02018840	0.052
UP01371932 A	UP02018840	0.053
UP01346448 A	UP02018840	0.048
UP03777520 A	UP02018840	0.039
UP03486978 A	UP02018840	0.059
ADEE1602HOF	UP02018840	0.041
UP01994235	UP02018840	0.062
UP01994775	UP02018840	0.053
UP01380891 A	UP02119964	0.037
UP00114879 A	UP02119964	0.051
UP00114879 A(2)	UP02119964	0.051
UP01377173 A	UP02119964	0.051
UP01376885 A	UP02119964	0.051
UP01371932 A	UP02119964	0.051
UP01346448 A	UP02119964	0.046
UP03777520 A	UP02119964	0.037
UP03486978 A	UP02119964	0.057
ADEE1602HOF	UP02119964	0.042
UP01994235	UP02119964	0.068
UP01994775	UP02119964	0.052
UP02018840	UP02119964	0.049
UP01380891 A	UP03007436	0.045
UP00114879 A	UP03007436	0.059
UP00114879 A(2)	UP03007436	0.059
UP01377173 A	UP03007436	0.053
UP01376885 A	UP03007436	0.053
UP01371932 A	UP03007436	0.059
UP01346448 A	UP03007436	0.058

UP03777520 A	UP03007436	0.045
UP03486978 A	UP03007436	0.064
ADEE1602HOF	UP03007436	0.048
UP01994235	UP03007436	0.071
UP01994775	UP03007436	0.059
UP02018840	UP03007436	0.051
UP02119964	UP03007436	0.054
UP01380891 A	UP03090499	0.049
UP00114879 A	UP03090499	0.051
UP00114879 A(2)	UP03090499	0.051
UP01377173 A	UP03090499	0.054
UP01376885 A	UP03090499	0.054
UP01371932 A	UP03090499	0.051
UP01346448 A	UP03090499	0.061
UP03777520 A	UP03090499	0.049
UP03486978 A	UP03090499	0.061
ADEE1602HOF	UP03090499	0.044
UP01994235	UP03090499	0.070
UP01994775	UP03090499	0.061
UP02018840	UP03090499	0.058
UP02119964	UP03090499	0.060
UP03007436	UP03090499	0.057
UP01380891 A	UP03417836	0.045
UP00114879 A	UP03417836	0.055
UP00114879 A(2)	UP03417836	0.055
UP01377173 A	UP03417836	0.062

APPENDIX C

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

20 July 2020

Approval Certificate
Annual Renewal

Ethics Reference No.: 406/2019
Title: Early infant HIV diagnosis and characterization of HIV drug resistance in Gauteng, South Africa

Dear Miss O Smit

The **Annual Renewal** as supported by documents received between 2020-06-22 and 2020-07-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2020-07-15.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2021-07-20.
- Please remember to use your protocol number (406/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Announcement and instruction to all researchers

from the Faculty of Health Sciences Research Ethics Committee

This is an update following previous instructions from the Research Ethics Committee, accounting for recent announcements by government in relation to COVID-19 on 24 May 2020.

All researchers must minimise the risk of transmission at research sites and in studies involving human participants approved by the Research Ethics Committee. To this end,

1) all non-therapeutic or non-interventional research data gathering involving contact with human participants remain suspended, with the exception of studies involving telephonic or other online/remote methods of data collection;
2) research that is entirely situated in a laboratory is permitted provided that COVID-19 precautionary measures are in place;
3) research that is merely utilising existing records or data is permitted provided that COVID-19 precautionary measures are in place
4) emergency research related to COVID-19 is permitted after ethics approval;
5) everyone should endeavour protecting research participants, personnel and students in reducing the risk of transmission of COVID-19.

For therapeutic and clinical research trials:

1) each research study or study site must maintain a plan to minimise exposure to COVID-19 risk for all parties involved in the study, including but not limited to research participants, researchers and student researchers;
2) Whenever feasible, in-person visits should be substituted with telephonic visits;
3) Principal investigators and study sites should maintain measures to ensure that there is no interruption of required medication/essential treatment and monitoring of adverse events;
4) Researchers and study sites should develop a 'COVID-19' template register in case retrospective contact tracing becomes necessary;
5) New enrolments into clinical trials remain suspended. Potential exceptions to this announcement should be discussed with the chair or a deputy chair of the REC;
6) Serious adverse events at an UP-site should be reported on the PeopleSoft system within 72 hours as usual.

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