Drug resistance mutations in newly diagnosed HIV-infected infants and in children and adolescents with virological failure on ART

Iqra Barakzai

Drug resistance mutations in newly diagnosed HIV-infected infants and in children and adolescents with virological failure on ART

by

Iqra Barakzai

Submitted in fulfilment of the requirements in respect of the

DOCTORAL DEGREE

PhD (Virology)

in the

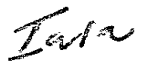
Division of Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein

Promoter: Professor Dominique Goedhals, Division of Virology, University of the Free State, Bloemfontein

December 2020

DECLARATION

I, Iqra Barakzai, declare that the thesis that I herewith submit for the Doctoral Degree in Virology at the University of the Free State, is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education."



Iqra Barakzai 8th December 2020

ALLAH (SWT), with all His wisdom, never closes a door of blessings for his servant, except that He opens for him two other doors of blessings

By Ibn Al Qayyim (RA)

DEDICATION

To my parents (Abdul Aziz Barakzai and Shenaaz Barakzai) and my husband (Sifiso Alfred Dlamini): Thank you for your support, love and understanding

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the Almighty Allah for granting me the opportunity to fulfil this research study

Secondly: I would like to sincerely thank the following individuals:

My family: Father (Abdul Aziz Barakzai), Mother (Shenaaz Barakzai), Husband (Sifiso Alfred Dlamini) and my Daughter (Amyrah Dlamini) for their constant motivation, love and support.

Prof Dominique Goedhals (supervisor), Division of Virology, University of the Free State/NHLS for her continuous academic guidance and emotional support throughout my PhD, for her endless patience, support, motivation and immense enthusiasm, without her guidance this journey may not have eventuated. I consider it an honour to have worked with such a tremendous supervisor.

Armand Bester for helping with statistical and data analysis and for continuous encouragement and endless support.

Dr Sabeeha Vawda for her continuous motivation and support.

My extended family (colleagues): Bonolo, Charles, Sindy, Devon and Marina for their endless support and motivation.

My friends: Adeola, Tsidy, Olga, Sarah and **Tumi** for their love, support and encouragement.

National Health Laboratory Sciences (NHLS) and Poliomyelitis Research Foundation (PRF) for the financial support received for my PhD degree.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS

%	Percentage
<	Less than
>	Greater than
±	Plus-minus sign
≤	Less than or equal to
≥	Greater than or equal to

ABBREVIATIONS

µL	Microliter
3TC	Lamivudine
ABC	Abacavir
ADR	Acquired drug resistance
AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
ARV	Antiretroviral
BLAST	Basic local alignment search tool
bp	Base pair
BWA	Burrows-wheeler alignment tool
CAEV	Caprine arthritis-encephalitis virus
CCR5	Chemokine receptor type 5
cDNA	Complementary DNA
CRFs	Circulating recombinant forms
d4T	Stavudine
DBS	Dried blood spot
ddl	Didanosine
ddNTPs	Dideoxynucleotidetriphosphates
DNA	Deoxyribonucleic acid
DTG	Dolutegravir
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
EIAV	Equine infectious anaemia virus
EID	Early infant diagnosis
ELISA	Enzyme-linked immunosorbent assay
F	Female
FIs	Fusion inhibitors

FIV	Feline immunodeficiency virus
FTC	Emtricitabine
Gb	Gigabyte
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLR	High level resistance
IC	Inhibitory concentration
INIs	Integrase inhibitors
IR	Intermediate resistance
LLR	Low level resistance
LPV/r	Lopinavir/ritonavir
M	Male
Mb	Megabyte
mins	Minutes
mL	Millilitre
mM	Millimolar
mRNA	Messenger RNA
MTCT	Mother to child transmission
NDOH	National department of health
ng	Nanogram
NGS	Next generation sequencing
NHLS	National health laboratory services
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
NTPs	Nucleotide triphosphates
NVP	Nevirapine
OH	Hydroxyl group
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDR	Pre-treatment drug resistance
PEP	Post-exposure prophylaxis
PHIV	Perinatal HIV
PIs	Protease inhibitors
PMTCT	Prevention of mother to child transmission
PrEP	Pre-exposure prophylaxis
RAL	Raltegravir
RCF	Relative centrifugal force
RNA	Ribonucleic acid
RPM	Revolutions per minute

RT	Reverse transcriptase
S	Susceptible
SIV	Simian immunodeficiency virus
SIVcpz	Simian immunodeficiency virus in chimpanzees
SIVgor	Simian immunodeficiency virus in gorillas
ssRNA	Single stranded RNA
TAM	Thymidine analog mutation
TB	Tuberculosis
TDF	Tenofovir
TDR	Transmitted drug resistance
UK	United Kingdom
UN	United Nations
UNAIDS	Joint United Nations Programme on HIV and AIDS
URFs	Unique recombinant forms
USA	United States of America
VL	Viral load
WHO	World health organisation
ZDV	Zidovudine

LIST OF PUBLICATIONS

PUBLICATIONS

1 Barakzai I, Bester PA and Goedhals D. Human immunodeficiency virus: review of a virus endemic in South Africa. (Manuscript in preparation).

2 Barakzai I, Bester PA and Goedhals D. Development and validation of a Sanger sequencing assay for HIV drug resistance testing of dried blood spot (DBS) samples. (Manuscript in preparation).

3 Barakzai I, Bester PA and Goedhals D. Pre-treatment drug resistant HIV in samples collected from the routine EID platform. (Manuscript in preparation).

4 Barakzai I, Bester PA and Goedhals D. Characterization of Sanger sequencing data collected from children and adolescents with virological failure while on ART. (Manuscript in preparation).

LIST OF CONFERENCE CONTRIBUTIONS

CONFERENCE CONTRIBUTIONS

1. Barakzai I, Bester PA and Goedhals D (2018) Development and validation of an HIV drug resistance assay using dried blood spot samples. Faculty Research Forum: 30th and 31st August 2018. Oral presentation.
2. Barakzai I, Bester PA and Goedhals D (2018) Development and validation of an HIV drug resistance assay using dried blood spot samples.7th Annual Free State Provincial Health Research Day, 8th to 9thNovember 2018. Oral presentation.
3. Barakzai I, Bester PA and Goedhals D (2019) HIV drug resistance in samples from the routine early infant diagnosis programme. Three Minute Thesis Competition: 23rd August 2019. Oral presentation.
4. Barakzai I, Bester PA and Goedhals D (2019) HIV drug resistance in samples from the routine early infant diagnosis programme. Faculty Research Forum: 29th and 30th August 2019. Oral presentation.
5. Barakzai I, Bester PA and Goedhals D (2019) HIV drug resistance in samples from the routine early infant diagnosis programme. 28th International Workshop on HIV Drug Resistance and Treatment Strategies, 16th to 18th October 2019. Oral and poster presentation.
6. Barakzai I, Bester PA and Goedhals D (2019) HIV drug resistance in samples from the routine early infant diagnosis programme. 8th Annual Free State Provincial Health Research Day, 7th to 8th November 2019. Oral presentation.

ABSTRACT

Introduction and aim: South Africa has never experienced an epidemic of the same extent and magnitude as the one with human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS).The roll out of antiretroviral therapy (ART) and other interventions, such as prevention of mother to child transmission (PMTCT), has greatly reduced the incidence of new HIV infections among children. However, early initiation of ART among children has increased their overall exposure to antiretrovirals (ARVs), resulting in an increased prevalence of acquired drug resistance (ADR) on treatment. Presence of drug resistant virus further complicates clinical management of HIV infection, resulting in fewer present and future treatment options. Therefore, drug resistance testing has been recommended in selecting appropriate treatment regimens among children and adolescents diagnosed with virological failure. The aim of the current study was to determine and characterise drug resistance mutations in newly diagnosed HIV-infected children and in children and adolescents with virological failure on ART.

Methods: To detect drug resistance mutations, the present study developed and validated a Sanger sequencing assay using dried blood spot (DBS) samples. The Sanger assay was used to perform HIV drug resistance testing on DBS samples testing positive on the early infant diagnosis (EID) platform. Lastly, a retrospective analysis was performed of resistance data from samples of children and adolescents submitted to the routine diagnostic laboratory.

Results: A high level of concordance was found among major mutations detected using the Sanger DBS and plasma assays. Since all the discordant mutations were either minor or existed as mixtures, the interpretation of resistance profiles was found to be identical. The DBS assay was then used to detect pre-treatment drug resistance (PDR) among samples from children on the EID platform. Overall, PDR was detected among 73% of the children, with the majority of the children showing resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs) (72%). Dual class resistance was most prevalent to NNRTIs and NRTIs (11% [33/308]), followed by NNRTIs + protease inhibitors (PIs) (0.6% [2/308]). Triple class resistance (NNRTI + NRTI + PI) was very rare and was only detected in two children. Protease resistance was very rare among the children and none of the children displayed high-level resistance against any PI. Results from children (0 to 9 years) and adolescents (10 to 19 years) with virological failure while on ART indicated that most of the children were resistant to NRTIs (86% [79/92]),

followed by NNRTIs (75% [69/92]) and PIs (28% [26/92]). Among adolescents, prevalence of resistance to NNRTIs (87% [146/167]) was the highest, followed by NRTIs (66% [111/167]) and PIs (30% [51/167]). Dual class resistance to NRTIs + NNRTIs was the most common among both children (44% [41/92]) and adolescents (38%, [63/167]). Triple class resistance (NNRTI+NRTI+PI) was detected in 18% of children (17/92) and 19% of adolescents (32/167).

Conclusion: The prevalence of infants, children and adolescents experiencing treatment failure due to the presence of transmitted, acquired and PDR is increasing. While the implementation of virological monitoring and genotypic testing play an essential role in identifying and managing drug resistance among children and adolescents experiencing treatment failure, these services are limited in many developing countries. The current study supports the implementation of HIV drug resistance testing for national surveys and optimal clinical management of HIV-infected children and adolescents.

Key words: Adolescents, ARVs, DBS, drug resistance, EID, HIV, PMTCT and Sanger sequencing

CHAPTER 1

LITERATURE REVIEW

1.1 Classification of HIV

On the basis of phylogenetic, morphological and biological properties, the human immunodeficiency virus (HIV) has been classified in the genus *Lentivirus* and the family *Retroviridae* (Costin, 2007). Lentiviruses are transmitted as enveloped, single-stranded, positive sense ribonucleic acid (ssRNA) viruses, which contain the enzyme reverse transcriptase (RT) and two identical copies of a linear RNA genome. Infections with lentiviruses demonstrate persistent viral replication resulting in a chronic state of the disease, with a period of clinical latency preceding the onset of clinical disease (Fanales-Belasio *et al.,* 2010). Lentiviruses are known for their immune-suppressive properties and include viruses such as simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), caprine arthritis-encephalitis virus (CAEV), and equine infectious anaemia virus (EIAV) (Costin, 2007).

As with all lentiviruses, HIV has a complex genomic structure, comprised of several accessory and regulatory genes. On the basis of genetic homologies reported among the genomic sequences of HI viruses, HIV-1 is easily distinguished from HIV-2 (Fanales-Belasio *et al.,* 2010). The first report of HIV-2 was published 1986, where a morphologically similar but antigenically distant virus was reported to cause AIDS and was mostly reported in western Africa (Clavel *et al.,* 1986; Sharp and Hahn, 2011; Bbosa *et al.,* 2019). The virus was found to be closely related to a simian virus that causes immunodeficiency in macaques but distantly related to HIV-1 (Chakrabarti *et al.,* 1987; Guyader *et al.,* 1987; Sharp and Hahn, 2011). HIV-1 is characterised by extensive genomic variability resulting in different variants, molecular subtypes and several recombinant forms of the virus, which are reported worldwide (Buonaguro *et al.,* 2007). This variability of the HIV-1 genome is a result of 1) the error-prone transcription mechanism of the RT enzyme resulting in an average of one substitution per genome replication, 2) the rapid viral replication rate in an infected individual, 3) host selective immune-pressure and 4) the occurrence of recombination events among different HIV strains in an infected individual (Fanales-Belasio *et al.,* 2010).

Due to this variability, HIV-1 strains are classified into four major phylogenetic groups, namely M (major), O (outlier), N (non-M/non-O) and P (Ayouba *et al.,* 2000; Buonaguro *et al.,* 2007; Tebit and Arts, 2011). Group M, which is associated with the majority of the HIV-1 cases worldwide, can be further subdivided into nine subtypes, known as clades (A, B, C, D, F, G, H, J and K) (Buonaguro *et al.,* 2007). Groups N and O are mostly confined to specific geographical settings, such as Cameroon, Gabon and other neighbouring countries (Peeters *et al.,* 1997). Phylogenetic studies revealed distinct genetic homologies found among groups M, N and O which are due to their close association with SIV, a member of the *Lentivirus* genus reported to cause immune-deficiency in chimpanzees (SIVcpz) (Gao *et al.,* 1999). A variant of group O, which is more closely related to SIV found in gorillas (SIVgor), has been reported from a Cameroonian women residing in France and has been designated to a new phylogenetic group, namely P (Plantier *et al.,* 2009; Tebit and Arts, 2011).

Occasionally, when an individual is infected with different viruses of two or more subtypes, these subtypes can infect the same cell, resulting in a combination of two different genomes (Buonaguro *et al.,* 2007; Fanales-Belasio *et al.,* 2010). These inter-subtype recombinant forms, known as circulating recombinant forms (CRFs), have been isolated from different individuals and are characterised by genome sequencing. The virus must be isolated from two different individuals infected with the same CRF and sequenced, in order to be classified as a CRF (Carr *et al.,* 1998; Bbosa *et al.,* 2019). The term cpx is used to identify a CRF when more than three subtypes are present (HIV sequence database, 2020). Latest reports indicate a total of at least 102 CRFs which have been identified, sequenced and updated in the Los Alamos National Laboratory HIV sequence database (HIV sequence database, 2020). The presence of CRFs and multiple subtypes in the same population can give rise to individuals being infected with different HIV-1 variants (Buonaguro *et al.,* 2007; Fanales-Belasio *et al.,* 2010). The combination of CRFs with other subtypes leads to the formation of several recombinant forms, which are known as unique recombinant forms (URFs).

1.2 Structure and replication of HIV

The structure of the virus is similar for HIV-1 and HIV-2 (Fanales-Belasio *et al.,* 2010). The retrovirus genome consists of two identical ssRNA molecules within the virion, enclosed by a lipid bilayer membrane that surrounds the nucleocapsid (Sierra *et al.,* 2005). The HIV-1 genome is characterised by the presence of structural and regulatory genes, such as *gag*, *env*,

pol, *tat*, *rev*, *vpr*, *vpu*, *vif* and *nef* genes (Costin, 2007). The genome organisation of HIV-1 differs from HIV-2 but the basic structure of the genome, such as the presence of structural genes (*gag*, *env* and *pol*), remains similar for all retroviruses and is shown in Figure 1.1 (Scarlata and Carter, 2003). The *gag* gene is responsible for the production of structural proteins of the core (p24, p7 and p6) and matrix (p17), while the *env* gene encodes for viral envelope glycoproteins (gp) (gp41 and gp120) which bind to host cell receptors (Fanales-Belasio *et al.*, 2010). The *pol* gene encodes for enzymes which are responsible for viral RNA replication within the host cell. These include the RT enzyme, which converts viral ssRNA into deoxyribonucleic acid (DNA), the integrase enzyme, which is responsible for the incorporation of viral DNA into the host chromosome, and the protease enzyme, which cleaves the viral protein precursors.

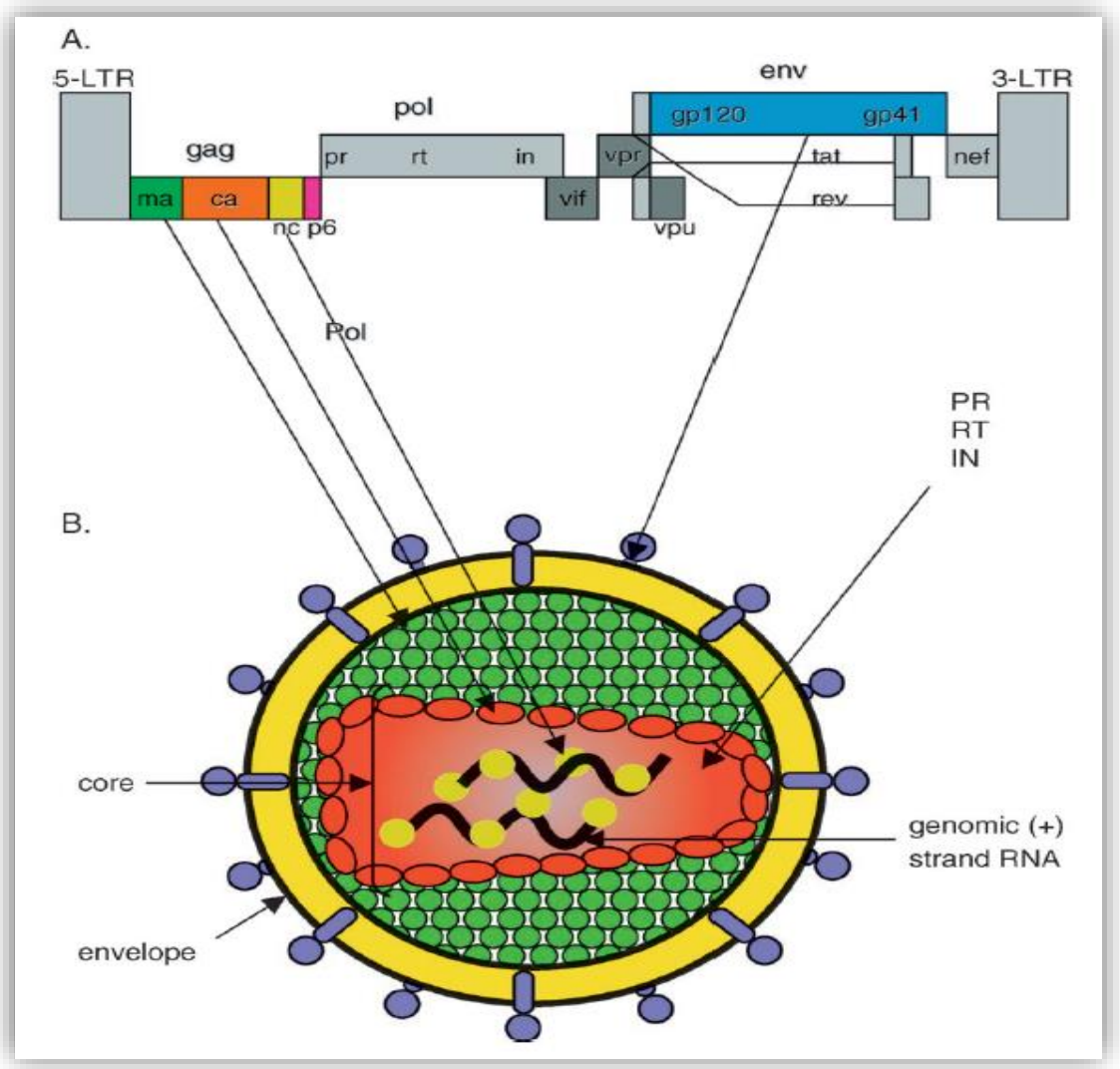


Figure 1.1 Structure of the HIV-1 particle [Scarlata and Carter, (2003) by permission of Elsevier]

Regulatory and accessory genes (*tat*, *rev*, *vpr*, *vpu*, *vif* and *nef*) play a key role in regulating the replication of virus particles inside the host cell (Emerman and Malim, 1998). These include 1) *tat* gene, which is responsible for regulating the production and expression of viral genes during replication, 2) *rev* gene encodes for Rev proteins, which are responsible for the transfer of genomic RNA from nucleus to cytoplasm, 3) *vpr* gene enables the transfer of reverse transcribed DNA into the nucleus, 4) *vpu* gene is responsible for the budding of the mature virions, 5) *vif* gene is responsible for increasing the efficiency of infection of progeny virus particles and 6) *nef* gene encodes for the Nef protein, which is responsible for down regulating the CD4 receptor activity on the cell surface enabling the budding of mature virions during the replication cycle (Fanales-Belasio *et al.*, 2010).

The HIV replication cycle can be divided into the following steps

1) fusion of the virus particle to the cell membrane, 2) entry of the virus particle, including uncoating and release of the viral RNA genome, 3) reverse transcription, 4) transport of the proviral DNA into the nucleus, 5) integration of the proviral DNA into the host genome, 6) transcription, 7) synthesis of the new viral proteins, 8) assembly of the progeny virus and 9) budding of the immature virion from the host cell membrane (Herrera-Carrillo and Berkhout, 2015) (Figure 1.2). The infection begins with the fusion of the virus particles with the host cell membrane, an interaction which is mediated by viral gp 41 and gp 120 (Fanales-Belasio *et al.*, 2010). These proteins are essential for the recognition of host cell receptors and the entry of the viral genome into the host cell cytoplasm.

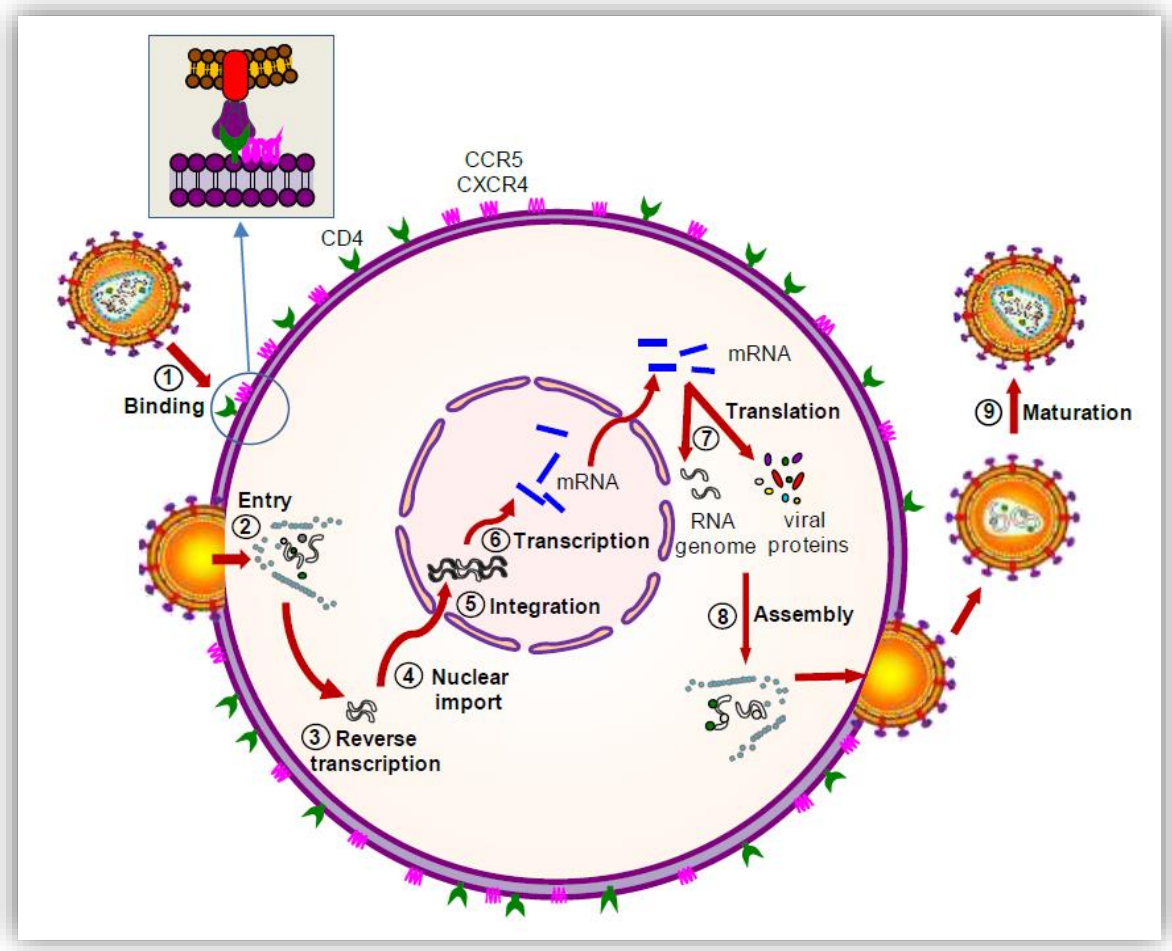


Figure 1.2 **The HIV replication cycle [Herrera-Carrillo and Berkhout, (2015) under the creative commons attribution license (https://creativecommons.org/licenses/by/4.0/)]**

Following membrane fusion, the viral RNA is released into the host cell cytoplasm, where it is then transcribed into proviral DNA by the enzymatic activity of RT (Garcia and Gaynor, 1994). The nuclease activity of RT degrades the RNA template and accessory genes, such as *vpr* and *vif* mediate the entry of the DNA into the host cell nucleus. The transcription of viral RNA into DNA is prone to error and every time the virus genome is transcribed, on average one mutation is introduced (Clavel and Hance, 2004). These errors are mostly base substitutions but other mutations such as duplications, insertions and recombinations, can also occur. The high levels of viral replication and turnover combined with the error prone mechanism of RT enzyme occurring during each cycle of viral replication results in a complex mixture of quasispecies within infected individuals, which can include resistant strains that are selected out in the presence of ART.

Enzymatic activity of integrase regulates the insertion of the proviral DNA into the host cell chromosome (Garcia and Gaynor, 1994). The proviral DNA is transcribed into messenger RNA (mRNA) inside the host cell (Chun *et al.*, 1997). The transcription process involves the synthesis of regulatory HIV proteins, such as Tat and Rev, which mediate the transcription of RNA transcripts, expression of structural and enzymatic genes, inhibition of host cell regulatory proteins and thus promote the formation of progeny virus particles. The viral mRNA then migrates into the host cell cytoplasm, where the structural proteins for progeny viruses are synthesized. The protease enzyme cleaves the precursor molecules, which are essential for the formation of mature progeny virions. The new virus particle migrates towards the host cell surface and is cleaved by the enzyme protease, thus enabling the formation of progeny virions, which bud through the host cell membrane.

1.3 Epidemiology and transmission of HIV

According to the Joint United Nations Programme on HIV and AIDS (UNAIDS), an estimated 38 million people were living with HIV in 2019, with the highest prevalence in Eastern and Southern Africa (UNAIDS, 2020). Heterosexual transmission is predominant and more women as compared to men are infected with HIV in sub-Saharan Africa (UNAIDS, 2020). According to the annual national antenatal sentinel HIV prevalence survey conducted in South Africa, HIV prevalence among pregnant women has remained stable at around 30%, similar to the previous decade (National Department of Health, 2017). The mortality rate for infants has decreased from 55.5 deaths per 1,000 live births in 2002 to 23.6 deaths per 1,000 births in 2020 (Statistics South Africa, 2020). This has mostly been due to the expansion of the prevention of mother to child transmission (PMTCT) programme, which has played a crucial role in reducing the maternal and infant morbidity and mortality rates across the country.

HIV has spread across the globe, with deaths caused by HIV reported at around 2.3 million in 2005, decreasing to 1.6 million in 2012 and 690,000 in 2019 (Maartens *et al.*, 2014; UNAIDS, 2020). The decrease was a result of access to antiretroviral therapy (ART) as 68% of adults and 53% of children had access to ART as reported by the World Health Organisation (WHO) (World Health Organisation, 2020). Access to ART has increased the global prevalence of HIV, which rose from 31 million in 2002 to 35.3 million in 2012 and 38 million in 2019 and decreased the global incidence from 3.3 million in 2002 to 2.3 million in 2012 and 1.7 million in 2019 (UNAIDS, 2013; Zaidi *et al.*, 2013; UNAIDS, 2020). The global increase in HIV

prevalence was a result of individuals infected with HIV living longer as compared to the era before access to ART.

The global decrease in HIV incidence is mainly a result of a reduction in heterosexual transmission (Maartens *et al.,* 2014). The United Kingdom (UK), the United States of America (USA) and many countries in Europe have documented a high incidence of HIV in people who are in a same sex relationships despite access to ART (Birrell *et al.,* 2013; UNAIDS, 2013). This high incidence in these individuals is driven by a number of factors which include inaccessibility of ART, increased risk of transmission through sexual contact and stigma related to these individuals restricting the access to treatment (Beyrer *et al.,* 2013).

According to UNAIDS, the incidence of HIV among children decreased by 52% from 310,000 in 2010 to 150,000 in 2019 due to the increased access to ART for PMTCT (UNAIDS, 2020). However, the overall prevalence of HIV still remained high among children as compared to adults. A better understanding of HIV transmission has resulted in the development of appropriate interventions which can prevent mother to child transmission (MTCT) of HIV (Maartens *et al.,* 2014).

The MTCT of HIV can occur 1) *in utero* (foetus mostly infected during the third trimester of pregnancy), 2) intrapartum (infants infected around or during labour or delivery) and 3) post natal (breastfeeding) (Lehman and Farquhar, 2007). *In utero* transmission has been considered as the least common route (5% to 10% of infants annually) for MTCT of HIV and while the majority of cases have been reported during the third trimester of pregnancy, reports regarding the foetus being infected as early as eight weeks have also been documented (Lewis *et al.,* 1990; Milligan and Overbaugh, 2014). A large proportion of infants are infected intrapartum, mostly around the time of delivery due to breaches caused in the maternal-infant blood barrier resulting in the exchange of maternal and foetal blood, also known as placental microtransfusions (Kaneda *et al.,* 1997). Intrapartum MTCT affects around 30% to 50% of infants born to HIV positive women (Lehman and Farquhar, 2007; Milligan and Overbaugh, 2014). Postnatal transmission (breastfeeding) also contributes to the transmission of HIV, resulting in approximately 40% of infant infections reported. Viral RNA levels detected in breast milk typically correlate with the level of plasma viral load (VL), however, they are approximately 100-fold lower than the plasma VL (Lewis *et al.,* 1998; Milligan and Overbaugh, 2014).

1.4 Treatment and prevention

Combination ART for the treatment of HIV infected individuals has transformed HIV from a progressive infection with a fatal outcome, into a chronic and manageable disease (Maartens *et al.*, 2014). The ART is potent and capable of reducing HIV VL concentrations to undetectable levels within a few weeks of treatment initiation and inducing immune system recovery (as indicated by a rise in the CD4 cell count) (Kaufmann *et al.*, 2003; Mocroft *et al.*, 2007). Several antiretrovirals (ARVs) that inhibit the replication of HIV at different stages of the virus replication cycle are currently available (Walker *et al.*, 2014) (Figure 1.3). At least six classes of ARVs are currently available, which include 1) nucleoside reverse transcriptase inhibitors (NRTIs), 2) non-nucleoside reverse transcriptase inhibitors (NNRTIs), 3) protease inhibitors (PIs), 4) integrase inhibitors (INIs), 5) fusion inhibitors (FIs) and 6) chemokine receptor type 5 (CCR5) antagonists (Maartens *et al.*, 2014).

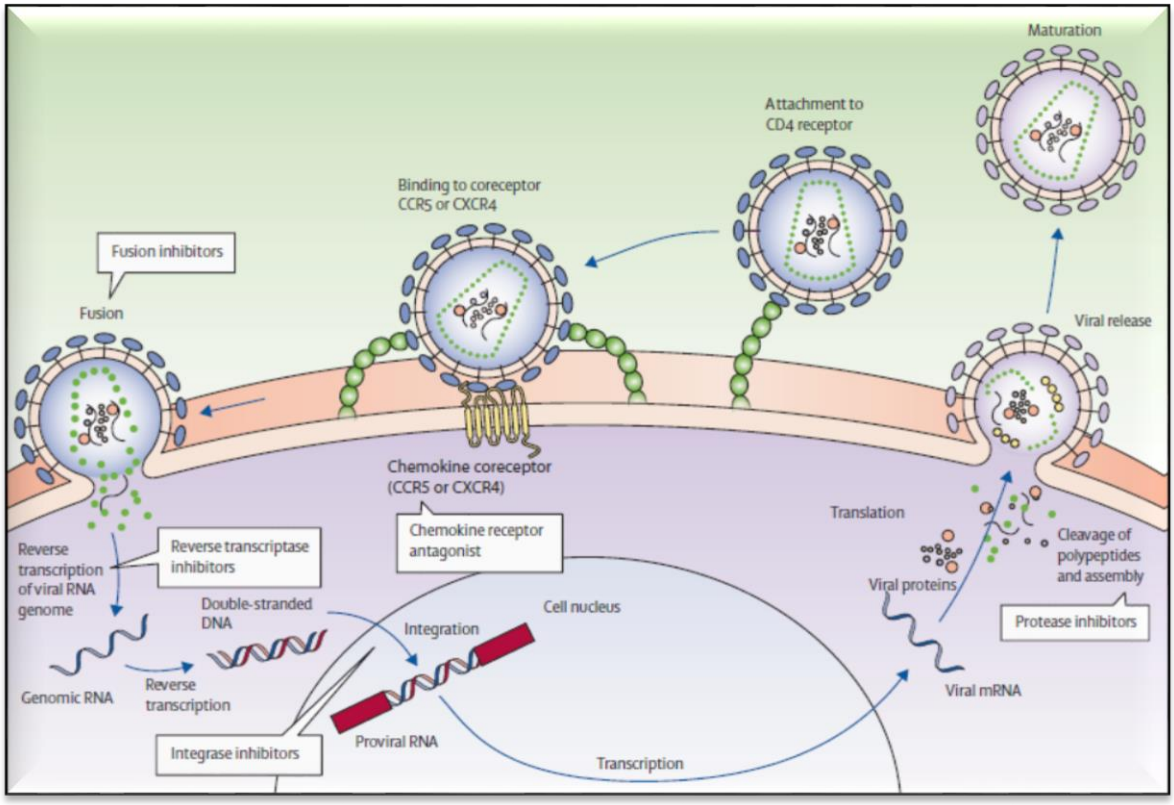


Figure 1.3 The HIV life cycle displaying the sites of action of different classes of ARVs [Walker *et al.* (2014) by permission of Elsevier]

The WHO guidelines for the treatment of HIV include the use of tenofovir (TDF) + lamivudine (3TC) or emtricitabine (FTC) + dolutegravir (DTG) for adolescents and adults (World Health Organisation, 2019a). For children, WHO recommends the use of abacavir (ABC) + 3TC + DTG and for neonates atazanavir (ATV) + 3TC + raltegravir (RAL) is recommended. In 2015, the National Department of Health (NDOH) guidelines recommended the use of ABC + 3TC + lopinavir/ritonavir (LPV/r) for children < 3 years or older children weighing < 10 kg (National Department of Health, 2015b). For children, between 3 and 10 years of age weighing more than 10 kg or for adolescents between 10 to 15 years of age and weighing < 40 kg, ABC + 3TC + EFV was recommended. For adolescents > 15 years of age and adults weighing > 40 kg, TDF + 3TC (or FTC) + EFV was recommended. The current standard first line ART regimen is ABC + 3TC + LPV/r (Kaletra) for infants ≥ 4 weeks of age (and ≥ 42 weeks of gestational age) and weighing ≥ 3 kg (National Department of Health, 2020). For preterm neonates and neonates with birth weight < 3 kg, the use of zidovudine (ZDV) + 3TC + nevirapine (NVP) plus additional advice from an expert is recommended. Infants with virological failure on a first-line regimen are recommended for resistance testing and if PI resistance is detected, then advice from an expert is recommended before switching to a second-line or INI-based regimen (containing DTG). As a child reaches 20 kg and is less than 10 years of age, they are switched to ABC + 3TC + DTG. The transition requires VL < 50 copies/mL. As the weight increases to 35 kg and more than 10 years of age, with adequate renal function, they are transitioned to TDF + 3TC + DTG. Children and adolescents with virological failure, which is defined as VL of more than 1,000 RNA copies/mL at two separate occasions, are switched to a second line regimen guided by resistance testing and an expert opinion.

Commencement of ART leads to a decrease in the plasma VL concentration to a level which is below the limit of detection for most commercial assays (Maartens *et al.*, 2014). In most people, this decrease in the VL is reported within three months of initiation of ART, however, the recovery of the host immune response is slower and variable among individuals. According to a study conducted by Tuboi and colleagues (2007) in low-income countries, a successful virological and CD4 cell count response was reported in 56% of patients in response to ART at six months, 19% of patients reported a virological response without a rise in a CD4 cell count and 15% of patients had an increase in the CD4 cell count without a subsequent virological response. The 19% of patients who did not have a rise in CD4 cell count despite showing a

virological response were associated with several risk factors and were at a high risk of opportunistic infections.

To combat the high maternal and infant mortality rate associated with HIV infection, remarkable progress has been made in reducing the risk of perinatal HIV (PHIV) transmission (National Department of Health, 2010b; Maartens *et al.,* 2014). In 2009, appropriate interventions to improve the access to ART in specific populations such as pregnant women, HIV exposed infants and individuals with tuberculosis (TB) and HIV co-infection were introduced across the country. The intervention addressed individuals with a CD4 count of ≤ 350 cells/mm^3 and resulted in an estimated 2.6 million individuals being initiated on ART by 2014 (National Department of Health, 2015a). The current NDOH guidelines recommend that all people living with HIV are eligible to initiate ART irrespective of the age, CD4 cell count and clinical stage (National Department of Health, 2020). According to the WHO programme for PMTCT of HIV, the aim is to reduce the risk of PHIV transmission to less than 5% through the use of appropriate ART given to HIV infected pregnant women (World Health Organisation, 2006; World Health Organisation, 2010).

In South Africa, single dose NVP was regarded as the standard PMTCT regimen for HIV infected pregnant women from 2003 (Meyers *et al.,* 2007). Although NVP is regarded as a simple and cost effective regimen for PMTCT, some studies have reported its efficacy at between 30% and 50% (Guay *et al.,* 1999). However, in studies conducted in urban facilities where infants were given formula feed, the PHIV transmission rate with single dose NVP was reported to be as low as 9% (Sherman *et al.,* 2004; Coetzee *et al.,* 2005). The addition of ZDV further reduced the PHIV transmission in infants to less than 2% (Lallemant *et al.,* 2004; Dabis *et al.,* 2005; Leroy *et al.,* 2005). Thus, according to the WHO guidelines, countries with a high rate of maternal and infant mortality have been advised to use combination therapy to reduce the risk of PHIV transmission among HIV-infected pregnant women and children (World Health Organization, 2005; World Health Organisation, 2010; World Health Organisation, 2019a). The PMTCT programme was first introduced in South Africa in 2002 (Ibeto *et al.,* 2014). In 2005, pregnant women in South Africa with a CD4 count of ≤ 200 cells/µl were eligible for ART and in 2008, pregnant women with a CD4 count of > 200 cells/µl were offered combination therapy of AZT from 28 weeks and a single dose of NVP in labour, while infant prophylaxis included a single dose of NVP with AZT. The fixed dose combination was introduced into South Africa in 2013, consisting of the three ARVs which form part of the first-

line regimen (TDF, 3TC or FTC and efavirenz [EFV]) and is recommended for HIV-infected pregnant women to lower the pill burden and to improve adherence and retention on ART (National Department of Health, 2015b). The CD4 count for initiation of ART was increased from ≤ 350 cells/µl to ≤ 500 cells/µl and the PMTCT programme adopted an option B approach, which stated that ART is recommended for every pregnant and breastfeeding woman regardless of the CD4 count or the clinical presentation of HIV infection (National Department of Health, 2015b; World Health Organisation, 2019a).

The risk of PHIV transmission to infants is estimated at 25% at the time of delivery in the absence of appropriate intervention within the PMTCT programme (Kourtis *et al.,* 2006). The risk factors associated with the transmission of HIV include 1) women with high VL, 2) more than 36 weeks of gestational age at initiation of ART, and 3) acute primary HIV infection during pregnancy (Kourtis *et al.,* 2006; Meyers *et al.,* 2007). Furthermore, women who have acquired acute HIV infection during pregnancy might not be identified as eligible for PMTCT due to the window period associated with serological diagnosis and will be at a higher risk of transmitting HIV to infants if not retested during late pregnancy (Garcia *et al.,* 1999). Recommended interventions with the PMTCT programme have decreased the risk of HIV transmission to infants by ten fold and complete eradication of MTCT of HIV has been achieved in some countries, such as Belarus, Armenia and the Republic of Moldova (Maartens *et al.,* 2014; UNAIDS, 2016b). Although HIV transmission to infants occurs mostly through breastfeeding, exclusive breastfeeding for up to the first six months of life is associated with a lower risk of HIV transmission as compared to mixed formula and breastfeeding (Coutsoudis *et al.,* 1999; Iliff *et al.,* 2005). Breastfeeding for an extensive period of time is considered a norm in most low-income countries, where an estimated risk of 40% is reported for the transmission of HIV to infants without the use of appropriate interventions (Maartens *et al.,* 2014).

The use of combination ART among HIV-infected pregnant women is regarded as a key factor in the PMTCT. According to the current guidelines available for HIV-exposed infants, breastfed infants of HIV positive mothers are administered NVP and ZDV as compared to only NVP, which was administered in the early days of the PMTCT programme (National Department of Health, 2015b; National Department of Health, 2019; National Department of Health, 2020). Furthermore, NVP and ZDV were also recommended for HIV-infected pregnant women during labour but recently it was superseded by combination ART for

pregnant women (Maartens *et al.*, 2014). Combination ART is more efficient in preventing HIV transmission to infants and has the additional advantages of reducing sexual HIV transmission and reducing morbidity and mortality.

Treatment with ARVs will not reach the goal of elimination of MTCT of HIV unless access to antenatal care, HIV testing and counselling and PMTCT interventions are increased in areas of high HIV prevalence (Wettstein *et al.*, 2012). In low and middle income countries, women often do not have access to antenatal care during pregnancy and a lack of PMTCT interventions has been reported (Maartens *et al.*, 2014). Some studies have reported a lack of adherence of HIV-infected pregnant women to ART, which increases the risk of transmission to infants (Nachega *et al.*, 2012). Due to the high prevalence of HIV in South Africa, more interventions are needed to reduce the incidence of HIV among young women, which will eventually lead to a lower prevalence of HIV among women and children, resulting in a reduced rate of morbidity and mortality due to HIV across the country (Maartens *et al.*, 2014).

1.5 Categories of HIV drug resistance

The use of combination ART has proven to be very effective in controlling the replication of HIV and prolonging the survival of the individuals infected, however, the response can be compromised by the development of resistance to ARVs (Palella Jr *et al.*, 1998; Ledergerber *et al.*, 1999; DeGruttola *et al.*, 2000). A combination of ARVs from different drug classes is needed to combat resistance and subsequently decrease the viral replication in an infected individual (Clavel and Hance, 2004). In addition, appropriate interventions are needed to control transmission of resistant strains, since these resistant strains often exhibit resistance to several classes of ARVs and the presence of cross-resistance (to other ARVs of the same class) has been reported. Three major categories of HIV drug resistance have been documented, namely primary or transmitted resistance, secondary or acquired resistance and pre-treatment drug resistance (PDR) (World Health Organisation, 2017a).

1.5.1 Primary or transmitted resistance

Primary or transmitted resistance implies that a virus has developed resistance to ARVs during combination ART and has been transmitted either through sexual transmission or MTCT (Clavel and Hance, 2004). Challenges in detecting and defining transmitted resistance include difficulty in directly comparing epidemiological studies, differing antiretroviral treatment strategies which impact on the rates of transmitted resistance, and methods used to interpret and thus define resistance and distinguish it from naturally occurring polymorphisms (Booth and Geretti, 2007). Several drug resistance surveillance platforms, such as the IAS-USA drug resistance mutation panel and the drug resistance surveillance lists by WHO and European Centre for Disease Prevention and Control are commonly used to define and report transmitted drug resistance (World Health Organisation, 2009; European Centre for Disease Prevention and Control, 2019; Wensing *et al.,* 2019). While resistant strains which are acquired through primary transmission are prone to revert to wild type over a period of time in the absence of drug pressure, resistance mutations such as M184V are more likely to revert as compared to K103N due to higher fitness costs (Tang and Shafer, 2012).

In a comparison of the transmitted drug resistance prevalence globally in ART naïve individuals between 2014 and 2019, the prevalence ranged from 4.1% in Southeast Asia, to 8.5% in Europe, 9% in Latin America/Caribbean and 14.2% in North America (Rhee *et al.*, 2020). Between 2012 and 2018, surveys conducted in nine countries in sub-Saharan Africa to determine drug resistance in newly diagnosed infants ≤ 18 months of age, from the early infant diagnosis (EID) platform reported that over half of the infants carried drug resistant HIV, mostly resistant to NNRTIs (efavirenz and nevirapine) (World Health Organisation, 2017a). Resistance to NRTIs also exceeded 10% in some countries (World Health Organisation, 2017a).

Therefore, in such settings where baseline drug resistance testing is not recommended or feasible, the surveillance of primary drug resistance is of particular interest to identify populations which are at high risk of acquiring transmitted resistance and for whom initial ART needs to be modified and baseline resistance testing needs to be considered (Bennett, 2006).

1.5.2 Secondary or acquired resistance

Secondary or acquired resistance implies development of resistance in the presence of ART within an infected individual (Clavel and Hance, 2004). Acquired resistance is characterised by the presence of high levels of viral replication in an infected individual. In the chronic phase of HIV, the VL in an infected individual is relatively stable, which indicates that a balance is maintained between the infection of new target cells and clearance of the old infected cells. The half-life of infected cells is relatively short, indicating that HIV infects new target cells at a very high rate maintaining the balance of the infected cells (10^7 to 10^8) (Ho *et al.,* 1995; Wei *et al.,* 1995; Perelson *et al.,* 1996).

1.5.3 Pre-treatment drug resistance

PDR refers to patients who carry drug resistant strains of HIV before initiation of first-line ART (Boender *et al.,* 2015). According to the WHO, PDR is detected among ART naïve-people who are initiating first-line ARV treatment or in patients with prior ART exposure who are initiating or re-initiating first-line ART (World Health Organisation, 2017a). Therefore, it can result from either transmitted or acquired drug resistance or both.

PDR can contribute to the increasing rates of treatment failure and thus compromise the effectiveness of first-line ARV regimens (Hamers *et al.,* 2012). The increase in PDR is largely attributed to an increase in NNRTI resistance (Hamers *et al.,* 2011; Gupta *et al.,* 2009; Gupta *et al.,* 2012). Previous studies conducted in sub-Saharan Africa have reported that treatment failure on a first-line NNRTI regimen was significantly higher in children as compared to adults (Kamya *et al.,* 2007). According to the HIV drug resistance report published by the WHO, an increase in PDR to NNRTIs from 62.7% between 2014 and 2016 to 63.7% in 2019 was reported among infants who were starting first-line ART (World Health Organisation, 2017b; World Health Organisation, 2019b).

Additionally, studies conducted in resource limited settings have reported an increase in virological failure due to PDR and due to limited resources, such as limited VL monitoring and absence of genotypic resistance testing, switching to second-line regimens in such settings is mostly based on CD4 cell count and clinical criteria (Hamers *et al.,* 2012; Phanuphak *et al.,* 2014; Kantor *et al.,* 2015). This approach can lead to incorrect management of patients, due

to switching to a second-line regimen without documented virological failure or continuation of a failing treatment regimen in the presence of a resistant virus (Sigaloff *et al.,* 2011a).

1.6 Factors influencing selection of HIV drug resistance

The use of ART in the presence of viral replication results in the selection of resistant strains containing mutations, which exhibit resistance to ARVs (Maartens *et al.,* 2014). Sub-optimal adherence is the major risk factor, which is associated with the development of resistance among HIV-infected individuals (Gardner *et al.,* 2009). Several differences among classes of ARVs for the selection of resistant variants have been reported (Maartens *et al.,* 2014). Resistant variants of HIV in which a single mutation can confer high level resistance to ARVs, such as EFV, FTC, 3TC, NVP and RAL have been documented. In comparison, in other ARVs such as the PIs, resistant variants develop slowly and several mutations are required before efficacy is lost to a specific ARV or class of ARVs.

HIV-infected children are more prone to the selection of resistant variants as compared to adults (Chen and Aldrovandi, 2008). High levels of plasma VL have been reported among children and therefore more potent ART is required to achieve subsequent virological suppression. Presence of decreased antiviral immune responses and lower ARV absorption place children at a higher risk of acquiring HIV resistant variants. Furthermore, not all ARVs which are administered in adults are approved for usage in children and not all of them are available in paediatric formulation (Sánchez and Holguín, 2014). In addition, ART in the form of PMTCT given to mothers and as prophylaxis to infants can select for resistant variants in infected infants (Persaud *et al.,* 2007). These children require long-term ART and additional factors, such as 1) sub-optimal or non-adherence to ART, 2) decreased levels of ARVs in plasma and 3) inadequate dosage guidelines for different age groups of children, can further select for resistant variants of HIV among these children (Clavel and Hance, 2004; Zoufaly *et al.,* 2013). Additionally, challenges, such as 1) poor palatability, 2) regimen complexity, 3) swallowing of medication and 4) storage and transportation of ARVs, which leads to poor adherence to ART among HIV infected children have been reported (Schlatter *et al.,* 2016).

PDR variants of HIV are more common in children and studies have reported a two-fold higher risk of virological failure among children after five years of ART as compared to adults (Zhao *et al.,* 2011; Musiime *et al.,* 2013). According to a study conducted by Kempf and colleagues

(2004), ART regimens based on NNRTIs or unboosted PIs are more susceptible to resistance as compared to boosted PIs. Boosted PIs are reported to have a high genetic barrier to resistance and also provide a protective mechanism in which resistance is less likely to evolve to other ARVs (NRTIs) taken in addition to boosted PIs. The protective mechanism can be explained by the low VL or viraemia achieved among individuals taking PI-based ART.

In many European countries, the use of genotyping has become an essential tool to guide clinicians (European AIDS Clinical Society, 2019). The European AIDS Clinical Society (EACS) guidelines recommend resistance testing in the following cases: 1) treatment-naïve individuals with acute or recent infection, 2) treatment failure, 3) pregnant women and paediatric patients when initiation or change in treatment is considered due to detectable VL and 4) pre and post-exposure prophylaxis. Due to the high cost and the complexity of the procedure associated with resistance genotyping, its implementation is limited in low-income countries (Nasir *et al.,* 2017). The South African National Department of Health (NDOH) recommends resistance testing in children with virological failure on a PI or INI-based regimen, particularly in children who previously received an unboosted PI or INI and in children who were treated for TB while on a PI-based regimen (National Department of Health, 2015b; National Department of Health, 2020).

1.7 Laboratory assays for detection of HIV drug resistance

The selection of ART to suppress HIV replication in infected individuals continues to be challenging due to the potential of HIV to develop resistance to ARVs and also to develop cross resistance to ARVs of the same class (Mayer *et al.,* 2001). Development of cross resistance to other ARVs has a significant impact on the efficacy of future ARV regimens. Several resistance assays have been developed to improve the efficacy of ART selection and to assess the potential of alternative regimens in the presence of resistance (Clavel and Hance, 2004). Several studies have demonstrated that resistance testing helps in identifying resistance associated mutations and improving the virological response to optimized therapy (Durant *et al.,* 1999; Baxter *et al.,* 2000; Clevenbergh *et al.,* 2000; Meynard *et al.,* 2002; Tural *et al.,* 2002). There are two types of resistance testing which are currently available, namely phenotypic testing and genotypic testing (Mayer *et al.,* 2001).

1.7.1 Phenotypic testing

Phenotypic testing involves assessing the growth of a virus in the presence of ARVs to provide a direct measure of ARV resistance (Mayer *et al.*, 2001). These assays use recombinant DNA technology to measure the ability of HIV to replicate in the presence of ARVs against which the resistance is being determined. Therefore, the results obtained from phenotypic resistance testing include the total effect of any and all mutations present.

The advantages of phenotypic testing include determination of phenotypic susceptibility to different ARVs and usefulness in providing accurate determination of ARV susceptibility, particularly when the virus being tested has a complex mutational profile. The disadvantages include 1) culture requires six weeks, 2) labour intensive, 3) time consuming, 4) more costly than genotypic assays and 5) lack of ARV selective pressure during stock generations that can favour the outgrowth of a certain virus population other than the original virus population of interest. Due to more recent advances in phenotypic assays, such as the use of second-generation phenotypic assays, recombinant virus assays have replaced the slow culture based technique, thus improving the turn around time (Marino-Merlo *et al.*, 2018). Two standardised phenotypic assays are commercially available to test ARV susceptibility, namely Antivirogram® and Phenosense™, and were first described by Hertogs *et al.* (1998) and Petropoulos *et al.* (2000).

1.7.2 Genotypic testing

Genotypic testing involves determination of the nucleotide sequence of the region of the HIV genome which confers phenotypic resistance (Mayer *et al.*, 2001). These assays identify resistance associated mutations, particularly in the coding region of the proteins targeted by ARVs. After sequencing the region of interest, the sequences are compared with a database consisting of resistance associated mutations and the presence or absence of any ARV resistance is determined. The advantages of genotypic testing include, 1) less costly, 2) shorter turn-around time and 3) better efficacy in detecting evolving resistance (Mayer *et al.*, 2001; Tang and Shafer, 2012). The disadvantages include 1) cannot reproducibly generate results for VL < 500 copies/mL and 2) only assess known drug resistance mutations, whereas phenotypic testing can pick up effects of mutations not yet known to be associated with drug resistance

and 3) the complexity of the interpretation of the resistance mutation profile (Mayer *et al.*, 2001; Tang and Shafer, 2012). Some of the well described mutations can alter phenotypic susceptibility and cause cross resistance to other ARVs (Hanna and Richard, 1999). Several mutations, such as RT associated mutation L74V associated with didanosine (ddl) resistance, M184V with 3TC resistance and Y181C with NVP resistance, can alternatively restore the susceptibility of HIV to ZDV even in the presence of several ZDV resistance associated mutations (Mayer *et al.*, 2001). The common approaches for genotypic testing involve the use of dideoxyterminator (Sanger) sequencing (Tang and Shafer, 2012).

1.7.2.1 Sanger sequencing

This specific genotypic assay relies on detecting resistance mutations in the known targets of ART, such as protease, RT, integrase and glycoproteins (gp41) (Tang and Shafer, 2012). The technique is more commonly known as dideoxyterminator sequencing, which is based on dideoxynucleotide triphosphates (ddNTPs) being used as DNA chain terminators in comparison to the normal nucleotide triphosphates (NTPs) which are found in the DNA chain (Karger and Guttman, 2009). These ddNTPs share similar properties with normal NTPs with the exception of a hydrogen group on the 3′ carbon instead of a hydroxyl group (OH) which is found in NTPs. Upon addition in the DNA chain, these modified ddNTPs block the addition of normal NTPs, since the phosphodiester bond cannot form between the ddNTPs and the normal NTPs, thus resulting in termination of the DNA chain.

The technique has been modified with the use of automated sequencing, which enables the sequencing of more DNA in a single tube within a short period of time (Walker and Lorsch, 2013). Additionally, the four ddNTPs are labelled with different colour dyes to allow detection of each ddNTP present in a tube. As the DNA is allowed to separate on a single lane and each of the four dyes fluoresce at a different wavelength, the laser is thus used to measure and identify the presence of each dye at its unique wavelength. The peaks are then displayed in chromatographic format, which indicates the different coloured peaks corresponding to the position of specific nucleotides.

Sanger sequencing has been considered as a gold standard for detecting resistance mutations to the commonly used ARVs for treating HIV (Lapointe *et al.*, 2015). The advantages include the 1) lower cost as compared to NGS and 2) easy to interpret resistance profiles with the use

of a standard database, such as the Stanford HIV Drug Resistance Database (Rhee *et al.,* 2003; Shafer, 2006; Ekici *et al.,* 2014). However, the technique has a number of disadvantages, such as 1) limited in throughput and requires the use of modern technology and equipment 2) lacks the ability to detect minority variants and 3) not practical to be used for massive sequencing projects (Inzaule *et al.,* 2016).

1.8 Problem identification

The South African HIV epidemic is characterized by the high HIV prevalence and incidence rates observed over the past decade (Statistics South Africa, 2019). The HIV incidence rate reflecting the rate of new infections per 1,000 population is estimated at 3.98 (3.58 to 4.42) (UNAIDS, 2019c). The overall HIV prevalence rate among the South African population was recorded as 13.5%, with an estimate of 7.97 million people living with HIV in 2019 (Statistics South Africa, 2019). In addition to adults, children are severely affected by the disease (Marais *et al.,* 2007). According to UNAIDS, an estimated 260,000 children (0 to 14) were living with HIV in South Africa in 2018, of whom 63% had access to treatment (UNAIDS, 2018). The country has the largest ART programme in the world, providing ARV treatment to over 4 million individuals and it has greatly increased the national life expectancy of HIV-positive individuals (UNAIDS, 2016a). Furthermore, interventions such as PMTCT have greatly reduced the vertical transmission of HIV (Anderson *et al.,* 2020).

While the use of ARVs has greatly reduced the number of new HIV infections, it has increased the duration of exposure to ARVs, resulting in an increase in the prevalence of HIV drug resistance. Drug resistance is a result of high genetic variability of HIV which results from 1) the error prone mechanism of RT, 2) recombination of viral variants infected within the same cell and 3) accumulation of proviral variants, which were acquired during the course of the initial infection (Coffin, 1995; Mansky, 1996; Levy *et al.,* 2004; Abram *et al.,* 2010). The selection of these drug resistant variants depends upon the replication of virus in response to sub-optimal treatment, acquisition of a particular drug resistance mutation and the effect of the resistance mutation on viral replication (fitness of the virus) and drug susceptibility (Clutter *et al.,* 2016). For some of the ARVs, a single mutation is enough to cause resistance, while for others, multiple mutations are required. The number of mutations required to impair the viral replication or fitness of the virus defines the particular ARV's genetic barrier to resistance. The mutations can be categorised as primary mutations, which directly reduce the susceptibility to

a particular ARV, or accessory mutations, which enhance the fitness of the variants with primary mutations or which further reduce the susceptibility to ARVs. Hence, the detection of these mutations in HIV-positive individuals is of utmost importance to determine appropriate treatment regimens.

Standard genotypic drug resistance testing involves the use of Sanger sequencing to determine drug resistance mutations in the RT, protease and integrase enzymes of the *pol* region of the HIV genome. The sequencing is performed directly on amplified polymerase chain reaction (PCR) products from cDNA, which has been reverse transcribed from plasma RNA. The continuous replication of the virus combined with the error prone mechanism of RT leads to a heterogeneous viral population inside HIV-positive individuals, resulting in more than one nucleotide (mixtures) present on a Sanger sequencing electropherogram. Hence, standardization is required within laboratories to accurately identify mixed bases and assess sequence quality using software based technology (Shafer *et al.,* 2000; Woods *et al.,* 2012). Therefore, the Stanford HIV drug resistance database is used as a standard online genotypic programme to interpret sequences and to identify relevant mutations within the *pol* region (Rhee *et al.,* 2003; Shafer, 2006). Drug resistance variants, which are present at below 20%, are however missed by Sanger sequencing and it has been identified as a disadvantage to using the assay (Clutter *et al.,* 2016). Ultra-sensitive NGS technologies are then recommended to detect these low abundance drug resistant variants. However, due to the high cost and complexity involved with the usage of NGS techniques, the technology has mostly been limited to research settings.

The use of Sanger sequencing to determine resistance mutations is of utmost importance in children experiencing treatment failure. The early initiation of ART in children has dramatically reduced mortality in newly diagnosed infants but has subsequently increased the emergence of drug resistance in the paediatric population (Violari *et al.,* 2008; Steegen *et al.,* 2014). Since children face life-long treatment, it is essential to identify appropriate treatment regimens and to ensure that they are maintained on first-line and second-line treatment regimens for as long as possible (Steegen *et al.,* 2014). Several studies conducted in South Africa have highlighted the drug resistance profiles of children failing ART (Van Zyl *et al.,* 2009; Wallis *et al.,* 2009; Barth *et al.,* 2011; Taylor *et al.,* 2011; Orrell *et al.,* 2013). There has been a shift towards older children, as these perinatally infected children age towards adolescence (Johnson *et al.,* 2012; Anderson *et al.,* 2020). Previous studies have highlighted

that adolescents in South Africa are a unique group with its own challenges, such as facing a change in treatment as they grow older, accessing treatment, adherence to treatment and retention in HIV-care and transition programmes (Bock *et al.,* 2008; Nachega *et al.,* 2009; Bygrave *et al.,* 2012; Nglazi *et al.,* 2012; Govindasamy *et al.,* 2015; Brown *et al.,* 2018). Furthermore, additional factors, such as psychological changes, greater peer influence, stigma associated with HIV, treatment fatigue, high pill burdens and disclosure issues are some of the many factors associated with high treatment and virological failure reported in adolescents (Sohn and Hazra, 2013).

Previous studies conducted in sub-Saharan Africa have reported that newly diagnosed infants and adolescents have a higher rate of treatment failure (Bock *et al.,* 2008; Nachega *et al.,* 2009; Bygrave *et al.,* 2012; Nglazi *et al.,* 2012; Govindasamy *et al.,* 2015; Brown *et al.,* 2018; Maskew *et al.,* 2019). Among infants, the risk factors such as 1) previous exposure to PMTCT regimens, 2) young age at ART initiation and 3) lower adherence to ART due to difficulty in swallowing of the medication makes them more susceptible to virological failure (Davies *et al.,* 2011; Chiappini *et al.,* 2012; Bunupuradah *et al.,* 2015; Rasoolinejad *et al.,* 2019). Among adolescents, 1) lower CD4 count before ART initiation, 2) higher VL at ART initiation, 3) previous ART regimens and 4) lower adherence leads to increased virological failure (Bunupuradah *et al.,* 2015; Kahana *et al.,* 2015; Collins *et al.,* 2017). Adolescents are divided into two groups, namely PHIV and behaviourally infected adolescents (Crowley *et al.,* 2020). Adolescents who were infected perinatally have previous exposure to PMTCT regimens as an infant and are heavily treatment experienced, which makes them more susceptible to treatment failure as compared to newly infected adolescents (Bunupuradah *et al.,* 2015; Kahana *et al.,* 2015; Collins *et al.,* 2017). The increased number of previous treatment regimens leads to an increased number of resistance mutations and an increased risk of virological failure (Anderson *et al.,* 2020). However, recent studies regarding the prevalence of resistance mutations in PHIV and behaviourally infected adolescents, particularly from South Africa, is lacking.

1.9 Aims and objectives

The aim of the study was to investigate drug resistance mutations in newly diagnosed HIV-infected infants and in children and adolescents with virological failure on ART.

The study had the following objectives:

1) To develop and validate a Sanger sequencing assay for the detection of HIV drug resistance mutations from dried blood spot (DBS) samples
2) To perform the Sanger sequencing for HIV drug resistance on samples testing positive by HIV PCR on the routine early infant diagnosis (EID) platform
3) To characterize Sanger sequencing data collected from children and adolescents with virological failure while on ART

1.10 Structure of the thesis

The thesis is presented as a series of research articles and will be tailored for submission to specific scientific journals. Chapter 1 provides a focused overview of HIV literature, with specific reference to epidemiology, ARV treatment, drug resistance and the usage of molecular testing to detect specific drug resistance mutations. Chapter 2 reports the validation of a Sanger sequencing assay for the detection of resistance mutations using DBS samples. A plasma based assay in use in the diagnostic laboratory was used as the comparator assay. Following validation of the assay, chapter 3 describes the use of the Sanger sequencing assay to detect PDR in samples which were collected from HIV positive infants on the routine EID platform. The National Department of Health (NDOH) recommends the use of EID testing for infants born to HIV positive mothers. Chapter 4 reports the characterization of Sanger sequencing data which was collected from children and adolescents, aged 0-19 years, with virological failure while on ART. While I carried out all the laboratory work and data analysis for chapters 2 and 3, routine genotypic testing had already been completed for the samples included in chapter 4 and therefore data collection and analysis was performed for this study. Finally, chapter 5 provides the discussion and overall conclusions derived from the thesis and highlights possible themes for future research.

CHAPTER 2

DEVELOPMENT AND VALIDATION OF A SANGER SEQUENCING ASSAY FOR HIV DRUG RESISTANCE TESTING OF DRIED BLOOD SPOT SAMPLES

2.1 Introduction

South Africa is known to have one of the worst epidemics of HIV globally (UNAIDS, 2019a). The combination of antiretrovirals (ARVs) referred to as highly active antiretroviral therapy (ART) has dramatically reduced HIV associated mortality, mother to child transmission (MTCT) of HIV and the adult HIV incidence (Rhee *et al.*, 2015a). While a number of ARVs has been released in the past decade, the usage of first line regimens containing nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) has remained the mainstay of treatment in Southern Africa until the introduction of an integrase inhibitor, dolutegravir, in 2020 (Pau and George, 2014; National Department of Health, 2020). A major barrier to successful treatment with ARVs is the emergence of resistant variants (Brumme and Poon, 2017).

An estimated 30% of individuals receiving antiretroviral therapy (ART) will develop resistance to NRTIs and NNRTIs during treatment, resulting in virological failure (Barth *et al.*, 2010; Aghokeng *et al.*, 2013; McMahon *et al.*, 2013; Rhee *et al.*, 2015b). Emergence and transmission of these drug resistant variants leads to poor treatment outcomes and increased mortality (DeGruttola *et al.*, 2000; Hogg *et al.*, 2006; Brumme and Poon, 2017). In addition, individuals infected with drug resistant HIV are at a high risk of developing resistance to the remaining ARVs in the combination (Brumme and Poon, 2017). Therefore, in many European countries, drug resistance testing is recommended for individuals failing their first line regimen and is guided by virological response (European AIDS Clinical Society, 2019).

HIV drug resistance testing involves the use of genotyping assays to interpret drug susceptibility using the sequence changes in the viral genes targeted by ARVs (Johnson *et al.*, 2013; Brumme and Poon, 2017). These viral genes are amplified from extracted HIV ribonucleic acid (RNA) by PCR and sequenced, mostly using an automated Sanger sequencing instrument (Cockerill, 1999; Brumme and Poon, 2017; Lapointe *et al.*, 2015). Genomic regions targeted by ARVs, such as protease, RT and integrase are amplified and sequenced resulting

in a single consensus sequence per sample. These sequences represent the dominant HIV quasispecies within the sample. The sequences are then analysed for mutations by interpretative algorithms, such as rule-based algorithms developed by Stanford HIV drug resistance database, which examines the sequences for known or possible resistance mutations within the sample (Rhee *et al.,* 2003; Shafer, 2006).

Traditionally, plasma has been the sample of choice for monitoring HIV RNA viral load (VL) and drug resistance testing (Jordan *et al.,* 2012; Singh *et al.,* 2017). However, there are a number of disadvantages associated with the use of plasma in resistance testing, which include collection, centrifugation/separation and appropriate transportation of viral plasma to diagnostic laboratory services (Lew *et al.,* 1998; Sebire *et al.,* 1998; Amellal *et al.,* 2008; Singh *et al.,* 2017). In addition, venipuncture necessary for the collection of plasma is difficult in certain population groups, such as infants (Singh *et al.,* 2017). Thus, the use of viral plasma for resistance testing will be less feasible in areas which lack laboratory infrastructure, trained personnel and appropriate storage and transportation to maintain the integrity of viral RNA (Lew *et al.,* 1998; Sebire *et al.,* 1998; Amellal *et al.,* 2008; Singh *et al.,* 2017). The use of dried blood spots (DBS) has been introduced as an alternative to plasma in resistance testing (Parry *et al.,* 2014; Singh *et al.,* 2017). Preparation of DBS cards requires a small amount of whole blood, which can easily be obtained from infants (Snijdewind *et al.,* 2012). The cards allow for convenient transportation and storage of samples. Studies have reported that DBS cards have been found to be more reliable in maintaining the stability of viral RNA, deoxyribonucleic acid (DNA), antibodies and other proteins for an extensive period of time as compared to serum or plasma (Singh *et al.,* 2017).

Several studies have indicated a high level of concordance among genotyping assays using DBS cards and the results were found to be similar when compared with plasma samples, using both in-house and commercially available assays (Parry *et al.,* 2014; Singh *et al.,* 2017). An in-house Sanger sequencing HIV drug resistance assay is currently in use in the diagnostic laboratory of the Division of Virology, National Health Laboratory Services (NHLS), Universitas Academic Laboratories to determine the presence of drug resistance mutations in HIV infected patients but the assay has only been validated on plasma samples. The aim of the study was therefore to develop and validate a Sanger sequencing assay for the detection of HIV drug resistance mutations using DBS samples.

2.2 Materials and methods

2.2.1 Sample collection and processing

The study was performed in the Division of Virology, University of the Free State/NHLS, Bloemfontein, South Africa. As per National Department of Health (NDOH) guidelines, during the study period HIV drug resistance testing was performed at the Universitas Academic Laboratories for any patient failing a PI-based regimen with a viral load of above 1,000 copies/mL on two separate occasions (National Department of Health, 2020). The viral load testing was performed using a reverse transcription RealTime PCR assay manufactured by Abbott (Abbott RealTime HIV-1, Abbott Molecular). Aliquots of 70 µℓ of EDTA blood were spotted onto perforated DBS specimen collection cards (Lasec) and were left to dry overnight at ambient temperature before storage in individual zip lock bags containing silica gel desiccant (Lasec) at -80°C.

2.2.2 Optimisation of RNA extraction using DBS samples

The assay was optimised to use DBS cards instead of plasma for RNA extraction using the NucliSENS®EasyMAG® magnetic silica based extraction technique. Briefly, two spots containing 140 µL of whole blood were added to 2 mL of NucliSENS® lysis buffer (BioMérieux) and were left on a thermomixer (5350BJ536916, Eppendorf, Thermomixer compact, Merck) at room temperature at 300 revolutions per minute (RPM) for 2 hours. After the lysis step, the samples were centrifuged (MBB14A088, Microfuge®16 Centrifuge, Beckman Coulter®) for 4 minutes (mins) at 4 000 *x g* and the supernatant was transferred to 1.7 mL microcentrifuge tubes. A 50 µL volume of magnetic silica (BioMérieux) was added to the samples and placed in the NucliSENS®EasyMAG®Extraction system (02370; BioMérieux) according to the manufacturer's instructions. Nucleic acid was eluted in 25 µL of NucliSENS®Extraction buffer 3 and was transferred to 1.7 mL microcentrifuge tubes and was stored at -80°C until further processing.

2.2.3 Nested PCR assay

PCR amplification was performed to detect drug resistance mutations in the RT and protease regions found in the *pol* gene of the HIV genome. The position of amino acid residues targeted for protease was 1 to 99 and for RT 1 to 240. The primers used were previously validated for

an in-house assay used to detect drug resistance mutations from plasma. The four primers validated for the plasma assay were F2 and R3, which were designed in-house, and PolM4 and AV150, which had been previously published (Vergne *et al.*, 2000). The primers were synthesized by Integrated DNA Technologies (Whitehead Scientific) and are indicated in Table 2.1. The nucleotide position on HXB2 was determined using Los Alamos National Laboratory HIV sequence database (HIV sequence database, 2020).

Table 2.1 Oligonucleotide sequences and concentrations of the primers used in the first and second round of the nested PCR assay for the detection of the RT and protease regions of HIV

Primer	Oligonucleotide Sequence (5'---3')	Primer	HXB2 nucleotide position	Target	Amplicon size (bp)	Conc (µM)
	First round PCR assay					
F2	GTTGGAAATGTGGAAAGGAAG	Sense	2027-2047	Protease and RT	1866	20
PolM4	CTATTAGCTGCCCCATCTACATA	Antisense	3870-3892			
	Second round PCR assay					
AV150	GTGGAAAGGAAGGACACCAAATGAAAG	Sense	2036--2062	Protease and RT	1760	20
R3	ACAAACTCCCACTCAGGAATC	Antisense	3779-3799			

Bp Base pair Conc Concentration PCR Polymerase chain reaction
RT Reverse transcriptase

A one-step RT-PCR assay was used to generate complimentary DNA (cDNA) and to perform the first round of amplification, using the SuperScript™ III One-Step RT-PCR System with Platinum®Taq High Fidelity (Themofisher Scientific). The master mix for the first round PCR was prepared according to the manufacturer's instructions, as shown in Table 2.2. The PCR programme for the thermocycler (ProFlex™ Base PCR System, S/Ns: 297802347, Applied Biosystems®, Life Technologies™) included an initial incubation step of 50ºC for 30 mins and activation step of 94ºC for 2 mins, followed by 2 cycles of denaturation at 94ºC for 15 seconds (sec), annealing at 61ºC for 30 sec, extension at 68ºC for 2 mins, 14 cycles (decrease annealing temperature with 0.5°C per cycle) of denaturation at 94ºC for 15 sec, annealing at 60ºC for 30 sec, extension at 68ºC for 2 mins, 34 cycles of denaturation at 94ºC for 15 sec, annealing at 57.5ºC for 30 sec, extension at 68ºC for 2 mins and a final extension step at 68ºC for 7 mins.

Table 2.2 Reagent components used in the first round PCR assay for the detection of the RT and protease regions of HIV

Reagents	Volume (µL)	Final concentration
SuperScript™ III RT/Platinum™ Taq High Fidelity Enzyme Mix	0.5	2 U
2x PCR buffer and reaction mix (containing 0.4 mM of dNTPs and 2.4 mM $MgSO_4$)	12.5	1.2 mM of $MgSO_4$ and 200 µM of dNTPs
Primer F2	0.5	20 µM
Primer PolM4	0.5	20 µM
Nuclease free water	1	1mM
Template RNA	10	<1 µg RNA
Total volume	25	

dNTPs deoxyribonucleotide triphosphate PCR Polymerase Chain Reaction RNA Ribonucleic acid RT Reverse transcriptase

The second round PCR assay was performed using KOD Hot Start DNA Polymerase (Novagen®, Merck). The reagent composition for the PCR assay was prepared according to the manufacturer's instructions. The conventional PCR was performed with an optimised concentration of 10 mM of each primer in the reaction mixture. The PCR assay reaction composition is shown in Table 2.3.

Table 2.3 Reagent components used in the second round PCR assay

Reagents	Volume (µL)	Final concentration
KOD Hot Start DNA Polymerase(1 U/µL)	1	0.02 U/µL
10x PCR buffer	5	1x
Primer R3	1	10 mM
Primer AV150	1	10 mM
25 mM$MgSO_4$	2	1.5 mM
2mM dNTPs	5	0.2mM each
Nuclease free water	20	-
Template from 1st round of PCR assay	15	<1 µg cDNA
Total volume	50	

DNA Deoxyribonucleic acid dNTPs Deoxyribonucleotide triphosphate PCR Polymerase Chain Reaction

The PCR programme for the thermocycler (ProFlex™ Base PCR System, S/Ns: 297802347, Applied Biosystems®, Life Technologies™) included an initial activation step of 95ºC for 2 mins, followed by 30 cycles of denaturation at 95ºC for 20 sec, annealing at 57.5ºC for 10 sec, extension at 70ºC for 1 hour 20 mins and a final extension step at 70ºC for 5 mins.

2.2.4 Agarose gel electrophoresis

The amplicons of the nested PCR assay were separated at 90 Volts for 40 mins using a 0.8% Seakem® agarose gel (Lonza) in a 1× Tris-acetic acid-ethylenediamine tetraacetic acid (EDTA) (Merck) buffer, stained with 2 µL of loading dye. The loading dye was prepared with 4.7 µL GelRed (BioTium) per 750 µL loading dye. A 100 base pair (bp) DNA ladder (GeneRuler DNA Ladder Mix, Thermo Fisher Scientific) was included as a molecular weight marker on each gel. The DNA ladder (GeneRuler DNA Ladder Mix, Thermo Fisher Scientific) was prepared using the GeneRuler DNA Ladder Mix with the loading dye containing GelRed (BioTium). The target products for the nested PCR assay were 1760 bp in size and the visualisation of the bands was performed using an Ultraviolet Transilluminator (TFM-26, Ultraviolet product).

2.2.5 Purification of the PCR products

The amplicons were purified using the PureLink® PCR Purification Kit (Invitrogen™, Life Technologies™, Thermo Fisher Scientific) as per the manufacturer's instructions. Briefly, 4 volumes of the PCR binding buffer (B2) with isopropanol were added to one volume of the PCR product (50 µL) and mixed well. The sample was pipetted into a PureLink®Spin Column in a collection tube and was centrifuged (0023081, Eppendorf AG, Centrifuge 5417C, Lasec) at > 10,000 *x g* for 1 min and the flow-through was discarded. The column was re-inserted into the collection tube and 650 µL of the wash buffer (W1) with ethanol was added. The column was centrifuged (0023081, Eppendorf AG, Centrifuge 5417C, Lasec) at > 10,000 *x g* for 1 min, the flow-through was discarded and the column was placed back in the same collection tube. The column was centrifuged (0023081, Eppendorf AG, Centrifuge 5417C, Lasec) at approximately 16,000 *x g* for 3 mins. The column was then placed into a 1.7 mL clean elution tube and 50 µL of the elution buffer was added to the centre of the column. The column was incubated at room temperature for 1 min. Following the incubation, the column was centrifuged (0023081, Eppendorf AG, Centrifuge 5417C, Lasec) at approximately 16,000 *x g* for 2 mins.

2.2.6 Measurement of the DNA concentrations and dilutions

The DNA concentrations of the purified PCR products were measured using the NanoDrop 2000 Spectrophotometer (Serial number: E107, 2000 Spectrophotometer, Thermo Fisher Scientific). A 3 µL volume of the purified PCR product was pipetted onto the measurement pedestal and the DNA concentration was measured at the absorbance of 260 nm. The 260/280 nm and 260/230 nm ratio of absorbance were checked to confirm the purity of DNA and to determine the presence of any co-purified contaminants. The samples were normalized to a concentration of 20 ng/µL by making dilutions in nuclease-free water. The samples were stored at -20°C until further processing.

2.2.7 Sanger sequencing

The sequencing primers were designed and validated previously in an in-house assay. The primers were synthesized by Integrated DNA Technologies (Whitehead Scientific) and are indicated in Table 2.4. Sequencing was performed using the BigDye Terminator V3.1 Sequencing Kit (Applied Biosystems™, Themo Fisher Scientific). The assay was performed with an optimised concentration of 3.2 mM of each primer in the reaction mixture.

Table 2.4 Oligonucleotide sequences and concentrations of the primers used in the Sanger sequencing assay for the detection of the RT and protease region from HIV-infected patients

Primer	Oligonucleotide Sequence (5'---3')	Primer	Target	Conc (mM)
1	ACCTACACCTGTCAACATAATTG	Sense	Protease and RT	3.2
2	TGTCAATGGCCATTGTTTAACCTTTGG	Antisense		
3	CACCAGGGATTAGATATCAATATAATGTGC	Sense	Protease and RT	3.2
4	CTAAATCAGATCCTACATACAAGTCATCC	Antisense		
M	TCCCTCAGATCACTCTTTGGCAACGAC	Sense	Protease and RT	3.2
Y	GTGTCTCATTGTTTATACTAGG	Antisense	Protease and RT	3.2

RT Reverse transcriptase Conc Concentration

The reagent composition for the sequencing assay was prepared according to the manufacturer's instructions. Briefly, using a 96-well reaction plate, 2 µL of the diluted sample

was added to 8 µL master mix for each of the six primers. The sequencing assay reaction composition is shown in Table 2.5.

Table 2.5 Reagent components used in the Sanger sequencing assay for each of the six different primers

Reagents	Volume (µL)	Final concentration
BigDye™ Terminator Ready Reaction Mix	1	
BigDye™ Terminator 5x Sequencing Buffer	2	5x
Primers (1,2,3,4,M or Y)	1	3.2 mM
Nuclease free water	4	-
Template (Purified PCR product)	2	4 ng/µL
Total volume	10	

The plate was sealed using thermo stable adhesive plate film (Optical Adhesive Cover, Abbott) and placed in the thermocycler (ProFlex™ Base PCR System, S/Ns: 297802347, Applied Biosystems®, Life Technologies™). The sequencing programme (ProFlex™ Base PCR System, S/Ns: 297802347, Applied Biosystems®, Life Technologies™) included an initial incubation step of 96ºC for 1 min, followed by 25 cycles of denaturation at 96ºC for 10 sec, annealing at 50ºC for 5 sec and extension at 60ºC for 4 mins.

2.2.8 Purification of the sequencing reactions

The sequencing reactions were purified using the ethanol/EDTA precipitation technique. One mL of nuclease free water (Invitrogen™, Ambion™, Themo Fisher Scientific) was added to 500 µL of 125 mM EDTA (pH 8.0) in a 2 to 1 ratio and 15 µL of the solution was added to each well. Following the EDTA solution, 60 µL of 96% to 100% cold ethanol (Merck) was added to each well. The ethanol was stored at -20°C. The plate was covered using a clean silicon cover and was briefly vortexed and placed at -20°C for 5 mins. The 96-well reaction plate was then centrifuged (CE 113, Digicen 21R, Orto Alresa) at 2,200 *x g* for 80 mins at 4°C. After 80 mins, the silicon cover was removed and the plate was inverted on absorbent paper without dislodging the pellet. The reaction plate was then briefly centrifuged upside down on the absorbent paper until the centrifuge reached a relative centrifugal force (RCF) between 120 and 200 *x g*. A volume of 200 µL of cold 70% ethanol (Merck) was added to each well. The reaction plate was centrifuged at 2,200 *x g* for 40 mins at 4°C. The silicon cover was removed and the plate was inverted on absorbent paper without dislodging the pellet. The reaction plate

was then briefly centrifuged upside down on absorbent paper until the centrifuge reached a RCF between 120 and 200 *x g*. The 96 well reaction plate was then placed in a thermocycler (ProFlex™ Base PCR System, S/Ns: 297802347, Applied Biosystems®, Life Technologies™) at 94°C for 25 to 40 sec with the thermocycler lid kept open to make sure any remaining ethanol was evaporated. Finally, 10 µL of HiDi™formamide (Applied Biosystems™, Themo Fisher Scientific) was added to each well and was covered with a clean silicon cover. The sequencing reactions were denatured at 95°C for 5 mins and were immediately placed on ice.

2.2.9 Data analysis

Sequencing of the HIV *pol* gene was performed using the 3500 and 3500XL ABI genetic analysers (Applied Biosystems™, Themo Fisher Scientific). The chromatograms obtained from the sequencer were checked for the sequence quality and mixed bases using a sequence analysis tool; Recall (beta v3.05) (Woods *et al.,* 2012). The quality of the sequences was inspected, checked and verified using Recall. The position of the mixed bases was inspected and verified using the quality of the base call. Sequence editing was performed according to Recall instructions i.e for green: no editing was required, orange: editing required for marked bases only, red and black: the sequences were rejected. Minority variants were called by Recall if present at 17.5% or more with at least bidirectional coverage and the sequences were then exported as text files and were analysed using the Stanford HIV Drug Resistance Database (version 8.9-1) (Rhee *et al.,* 2003; Shafer, 2006). Drug resistance associated mutations in the protease and RT regions and their respective resistance profiles were recorded. The subtype for the validation samples was determined using the Stanford HIV Drug Resistance Database (version 8.9-1) (Rhee *et al.,* 2003; Shafer, 2006). Phylogenetic analysis was performed using the in-house PhyloPi software and phylogenetic trees were constructed to check for any contamination among the samples (Bester *et al.,* 2019). For the validation parameters, alignments of the samples were analysed using percentage identity matrix from Clustal W 2.1 software available at EMBL-EBI to determine the similarity of sequences at the nucleotide level (Sievers *et al.,* 2011; Sievers and Higgins, 2018, https://www.ebi.ac.uk/). The gold standard was an in-house Sanger sequence assay already validated on plasma samples. Mutation differences which were only observed in mixtures and had no clinical impact on patient treatment were accepted. Results were summarised by frequencies and percentages and means, median, range or percentiles. Subgroup comparisons were performed using differences in means, medians or percentiles.

2.2.10 Validation of the DBS Sanger sequencing assay

According to the WHO guidelines (2012) for validation of DBS HIV drug resistance assay, the in-house Sanger sequencing was validated using different parameters to ensure the assay was producing reliable results. These parameters included 1) amplification sensitivity, 2) linearity, 3) accuracy, 4) precision and 5) reproducibility.

2.2.10.1 Amplification sensitivity

The amplification sensitivity was performed to identify the lowest VL required to successfully perform the assay. The minimum required copy number for the current in-house Sanger sequencing assay on plasma is 1,000 RNA copies/mL. Previous studies have reported a similar copy number (VL 1,000 copies/mL) for a DBS assay (Smit *et al.,* 2014; Zhang *et al.,* 2018). However, some studies have reported reduced sensitivity in samples with VL < 10,000 copies/mL (Masciotra *et al.,* 2007; Parry *et al.,* 2014). Therefore, in the current study the samples were divided in three categories 1) 1,000 – 5,000, 2) 5,000 – 10,000 and 3) > 10,000 copies/mL to accurately identify the minimum VL required for reproducible amplification and sequencing. Negative samples (n=12) were interspersed with the positive samples. The acceptance criterion was that more than 85% of the validation samples should be successfully amplified and more than 90% of these amplified samples should be successfully sequenced. The negative samples should not show any amplification. Positive samples which failed amplification or sequencing were repeated once. Samples for which the second attempt failed were considered to be unsuccessful.

2.2.10.2 Linearity

Assessment of sequence similarity was performed by diluting a known sample (including mixtures) over a specific range of copy numbers. These copy numbers were 1) 100,000, 2) 50,000, 3) 10,000, 4) 5,000 and 5) 1,000. Samples which failed on the first attempt were repeated once and considered to be unsuccessful if the second attempt failed.

2.2.10.3 Accuracy

This was to determine the accuracy of the assay to detect similar mutations when compared to the gold standard. Twelve samples without replicates were tested. The acceptance criterion was that more than ≥ 98% similarity must be observed when compared with the reference sequence at the nucleotide level for all specimens.

2.2.10.4 Precision

Precision reflects the intra-assay variability in the sequencing results obtained using the assay. Five replicates of three specimens were processed in a single run to ensure that the assay produces similar results for each specimen. Five DBS spots from three specimens were prepared and RNA was extracted, amplified and sequenced from each of these replicates. The sequences were then compared at the nucleotide level to determine the similarity of the sequences. The acceptance criterion was that ≥ 90% of specimens must show ≥ 98% identity between replicates at nucleotide level.

2.2.10.5 Reproducibility

Reproducibility refers to the inter-assay variability in the sequencing results. Five replicates of three specimens were processed in five separate runs on different days. As for precision, five DBS spots from three specimens were prepared and processed from RNA extraction. The acceptance criterion was that ≥ 90% of specimens must show ≥ 98% identity between replicates at nucleotide level.

2.3 Ethics

Ethics approval was obtained from the Health Sciences Research Ethics Committee of the University of the Free State (UFS-HSD2017/0633). Additional approval was obtained from the Free State DOH and NHLS.

2.4 Results

2.4.1 Amplification sensitivity

The amplification sensitivity of the assay was determined using a total of 55 samples over a pre-determined VL range. All samples were subtype C. Table 2.6 shows the different VL categories tested to determine the amplification sensitivity of the assay.

Table 2.6 Amplification and sequencing analysis for DBS samples tested for the amplification sensitivity

Viral load range copies/mL	Number of samples tested	Number of samples amplified (%)	Number of samples sequenced (%)*
1 000 to 5 000	20	13 (65)	11 (85)
5 000 to 10 000	20	18 (90)	17 (94)
> 10 000	15	14 (93)	14 (100)

*The sequencing percentages are calculated with the number of successfully amplified samples as the denominator since sequencing can only be performed on samples which are amplified

The assay achieved minimum amplification rate of ≥ 90% for samples with VL between 5,000 and 10,000 and for samples with VL > 10,000 copies/mL. A low amplification success rate (65%) was observed for samples with VL between 1,000 and 5,000 copies/mL. The samples which failed to amplify had VL ranging from 1,140 to 2,645 copies/mL. The two samples that could not be amplified between 5,000 and 10,000 copies/mL had a VL of 6,868 and 8,700 copies/mL. The only sample that could not be amplified in the category of samples withVL of > 10,000 copies/mL had a VL of 11,561 copies/mL. No amplification was observed in the negative samples.

2.4.2 Linearity

The different copy numbers which were used to determine the linearity of the assay are displayed in Table 2.7. No amplification was obtained for one of the samples (10,000) and the sample was repeated without success. The sequence similarity was compared using an identity matrix from Clustal W 2.1 software available at EMBL-EBI to determine the similarity of sequences at the nucleotide level (Sievers *et al.,* 2011; Sievers and Higgins, 2018, https://www.ebi.ac.uk/).

Table 2.7 **Sequence similarity at the nucleotide level for four sample dilutions with copy numbers ranging from 1,000 to 100,000 copies/mL, for assessment of linearity**

100,000	**50,000**	**5,000**	**1,000**
98.3	98.4	100.0	98.3
100.0	98.8	98.3	98.2
98.8	100.0	98.4	99.1
98.2	99.1	98.3	100.0

2.4.3 Accuracy

A total of 12 samples which were sent for HIV drug resistance testing were used to determine the accuracy of the assay, since the plasma genotype for these respective samples had already been performed and was used as the gold standard in this study to compare the results obtained from the DBS assay. The VL of these samples ranged from 3,336 to 142,686 copies/mL. All of the samples were genotyped except one sample with a VL of 6,877 copies/mL. The sequence similarity at the nucleotide level between plasma and DBS samples is shown in Table 2.8.

Table 2.8 **Sequence similarity at the nucleotide level between DBS and plasma assay determined using Clustal W 2.1**

1	**2**	**3**	**4**	**5**	**6**
98%	99%	99%	99%	99%	99%
7	**8**	**9**	**10**	**11**	**12**
98%	99%	98%	99%	98%	AF

AF Amplification failure

The assay achieved minimum sequence similarity of ≥ 98% at the nucleotide level for all the samples.

The detailed resistance mutations and the interpretation of resistance profiles for accuracy samples are shown in Appendices 1a and 1b. A total of 156 mutations were found in the protease region among the matched plasma and DBS samples, with no discordant major mutations noted. Only two discordant minor protease mutations were found, both of which were present as mixtures, with an additional K45KR mutation in a plasma sample and an M36MI mutation in a DBS sample. A total of 216 RT mutations were found among plasma

and DBS samples, with no discrepancies noted amongst the major mutations. The discordant mutations included nine minor mutations in DBS samples (G93GE, I202V, V60VI, V106VG, Q145QR, P157PS, Q182QP, L209LP and L210LP) and one minor mutation in a plasma sample (A98AS). The majority of these mutations were mixed codons where the minority population may have been missed due to PCR amplification bias, due to being close to or below the Sanger sequencing cut off of approximately 20%, or due to proviral DNA in the DBS specimens, particularly in samples with low viraemia. Since all the discordant mutations were minor and no discrepancies were found among the major protease and reverse transcriptase mutations, the interpretation of the resistance profiles were found to be identical for both the sample types. These minor mutation differences therefore would have no clinical impact on the patient treatment.

2.4.4 Precision

The three samples had a VL of 25,011, 60,514 and 240,517 copies/mL (Table 2.10). The results of the five replicates were aligned using Clustal W 2.1 software available at EMBL-EBI and the resulting identity matrix is shown in Table 2.10 (Sievers *et al.*, 2011; Sievers and Higgins, 2018, https://www.ebi.ac.uk/).

Table 2.9 Sequence similarity at the nucleotide level for five replicates of three specimens to assess precision

Specimen 1 (VL 25,011)

1a	**1b**	**1c**	**1d**	**1e**
100.0	98.4	98.5	98.3	98.5
98.4	100.0	98.7	98.6	98.7
98.5	98.7	100.0	98.4	98.7
98.3	98.6	98.4	100.0	98.8
98.5	98.7	98.7	98.8	100.0

Specimen 2 (VL 60,514)

2a	**2b**	**2c**	**2d**	**2e**
100.0	98.9	99.0	99.1	99.3
98.9	100.0	98.7	99.4	99.2

99.0	98.7	100.0	99.3	99.5
99.1	99.4	99.3	100.0	99.6
99.3	99.2	99.5	99.6	100.0

Specimen 3 (VL 240,517)

3a	**3b**	**3c**	**3d**	**3e**
100.0	99.7	99.6	99.5	99.6
99.7	100.0	99.9	99.8	99.9
99.6	99.9	100.0	99.7	99.8
99.5	99.8	99.7	100.0	99.9
99.6	99.9	99.8	99.9	100.0

The assay achieved minimum sequence similarity of ≥ 98% between all replicates for all three specimens at nucleotide level, therefore meeting the acceptance criteria for precision. The mean and the median of the nucleotide similarities for the precision of the assay was 99%.

The detailed resistance mutations and the interpretation of resistance profiles for precision samples are shown in Appendix 2a. A total of 29 minor mutations and no major mutations were detected in the protease region. The minor mutations found among the replicates included 3 discordant mutations. Two of the discordant mutations (I13IL and G49K) occurred in one replicate each of sample 1, while the third discordant mutation (T12TS) was detected in a single replicate of sample 3. For the RT region, a total of 17 major mutations were detected, with 4 discordant mutations noted. One of the discordant mutations (K219KR) was detected in three replicates of sample 1. The remaining three discordant mutations (A62AV, V75VIM and Y115YF) were each detected in three replicates of sample 2. A total of 46 minor mutations were detected. Two discordant mutations were found. One of the discordant mutations (I5IV) was detected in one replicate of the sample 1, while the second discordant mutation (S68SN) was detected in four replicates of sample 2. All of the above mentioned additional major and minor mutations were found in mixtures except for one major (K219R) and one minor mutation (I5IV). The mutations had no impact on the resistance profiles which were found to be similar among all five replicates of the three samples.

2.4.5 Reproducibility

Five replicates of three specimens were processed in five separate runs on different days. The same three samples used for precision were used to assess reproducibility. The identity matrix following alignment using Clustal W 2.1 is shown in Table 2.11 (Sievers *et al.*, 2011; Sievers and Higgins, 2018).

Table 2.10 Similarity matrix from the Clustal W 2.1 alignment of the five replicates of three specimens for assessment of reproducibility

Specimen 1 (VL 25,011)

1a	**1b**	**1c**	**1d**	**1e**
100.0	99.0	98.9	98.4	98.9
99.0	100.0	98.9	98.3	99.0
98.9	98.9	100.0	98.0	99.1
98.4	98.3	98.0	100.0	98.2
98.9	99.0	99.1	98.2	100.0

Specimen 2 (VL 60,514)

2a	**2b**	**2c**	**2d**	**2e**
100.0	99.4	99.2	99.4	99.0
99.4	100.0	99.0	99.0	99.0
99.2	99.0	100.0	99.0	99.2
99.4	99.0	99.0	100.0	99.0
99.0	99.0	99.2	99.0	100.0

Specimen 3 (VL 240,517)

3a	**3b**	**3c**	**3d**	**3e**
100.0	99.8	99.9	99.5	99.6
99.8	100.0	99.9	99.5	99.6
99.9	99.9	100.0	99.6	99.7
99.5	99.5	99.6	100.0	99.7
99.6	99.6	99.7	99.7	100.0

The assay achieved minimum sequence similarity of ≥ 98% between replicates at nucleotide level for all samples tested. The mean and the median nucleotide similarities for the reproducibility of the assay was 99%.

The detailed resistance mutations and the interpretation of resistance profiles for reproducibility samples are shown in Appendix 2b. A total of 26 minor protease mutations and no major protease mutations were identified in the protease region. No discrepancies were found amongst the minor protease mutations. For the RT region, a total of 18 major mutations were detected, including 3 discordant mutations. Each of these three discordant mutations (K219KR, A62AV and V108VI) were detected in two replicates of the respective samples. A total of 46 minor mutations were identified, with only one discordant mutation (K20KR) noted in one replicate of a sample. One of the replicates of sample 1 detected a stop codon (I167X) which is not unexpected due to the presence of proviral DNA in DBS derived genotypes. These mutations had no impact on the resistance profiles of the replicates and the resistance profiles among all 5 replicates of each sample were found to be identical.

2.5 Discussion

The use of DBS has been considered as an alternative specimen type for HIV drug resistance genotyping, surveillance and monitoring in areas which lack the necessary infrastructure for collection and transport of plasma (McNulty *et al.,* 2007). Several studies have demonstrated a high concordance rate (78% to 94%) in identifying mutations among plasma and whole blood specimens spotted on DBS cards in both treatment naïve and treatment exposed patients (Bertagnolio *et al.,* 2010; Johannessen *et al.,* 2010; Rottinghaus *et al.,* 2012; Monleau *et al.,* 2014).

In the current study, the in-house HIV drug resistance assay currently in use in the NHLS Virology diagnostic division, which was already validated on plasma samples, was tested on DBS cards to identify mutations in the *pol* region consisting of the RT and protease enzymes. The assay was validated against different parameters to ensure that it produces reliable results and was compared with the in-house Sanger sequencing assay. High amplification sensitivity (≥ 90%) was observed in samples with VL between 5,000 and ≥ 10,000 copies/mL. Previous studies conducted in Europe and North America have also reported a high amplification rate found using DBS specimens ranging from 79% to 95% (Bertagnolio *et al.,* 2007; Masciotra *et*

al., 2007; McNulty *et al.,* 2007; Hallack *et al.,* 2008; Youngpairoj *et al.,* 2008; Hearps *et al.,* 2010; Johannessen *et al.,* 2010).

Despite the high similarity observed among HIV genotypes generated from plasma and DBS specimens, a number of limitations are associated with the use of DBS cards. The low volume of blood in a DBS specimen leads to a smaller quantity of RNA present in the sample, which can lead to reduced sensitivity of the assay as compared to plasma samples (Singh *et al.,* 2017). Studies conducted by Parry *et al.* (2014) and Masciotra *et al.* (2007) have reported that yield from DBS specimens with a plasma VL of < 10,000 copies/mL can be as low as 50% to 55%. This could be of great clinical significance, since HIV infected patients with VL between 500 and 1,000 copies/mL have been reported to harbour resistance mutations similar to patients with high viraemia (Prosperi *et al.,* 2011; Gonzalez-Serna *et al.,* 2014; Santoro *et al.,* 2014). Due to the lower sensitivity of DBS specimens in patients with low viraemia, the presence of resistance mutations in these samples might go undetected, thus preventing early treatment control and eventually leading to treatment failure (Singh *et al.,* 2017). In the current study, low amplification sensitivity (65%) was observed in samples with a VL of between 1,000 and 5,000 copies/mL. All of the samples (n=7) in this VL range that could not be amplified had a VL of < 3,000 copies/mL. Johannessen and colleagues (2010) reported that the reduced sensitivity has been associated with samples with a lower viral burden. A study conducted by Hallack *et al.* (2008) reported that 90% of the samples with a VL of ≥ 6,000 copies/mL were genotyped as compared to 58% of the samples with a VL of < 6,000 copies/mL with the use of the Trugene® HIV-1 genotyping assay. Another study conducted by Youngpairoj *et al.* (2008) reported that genotyping was only successful in 8% of the samples with a VL of < 10,000 copies/mL as compared to 81% of the samples with a VL of ≥ 10,000 copies/mL using the commercial ViroSeq™ kit. However, in the second study the samples were retested using an in-house nested RT-PCR assay, which resulted in the successful amplification of 95% of the samples with a VL of ≥ 10,000 copies/mL (Youngpairoj *et al.,* 2008). In the current study, the in-house nested PCR assay was used for amplification, which resulted in the successful amplification rate of ≥ 90% for samples with VL above 5,000 copies/mL. As all samples in the 3,000 – 5,000 copies/mL range were successfully amplified, the lower VL limit for the assay is likely in this range. Overall, the assay displayed low sensitivity for samples with VL between 1,000 and 5,000 copies/mL, which requires additional optimisation and further studies are required to improve the sensitivity of the DBS assay. Plasma therefore remains the specimen of choice for HIV drug resistance testing, particularly in a diagnostic setting, due to

the ability to reliably amplify and sequence samples with VL between 1,000 and 5,000 copies/mL.

Another major challenge associated with the use of DBS specimens is the presence of proviral DNA from peripheral blood mononuclear cells (PBMC), which can impact HIV DNA sequences (Johannessen *et al.,* 2010). Previous studies have reported the presence of proviral DNA ranging from 33% to 80% in a nested RT-PCR assay from treatment naïve as well as treatment exposed patients (Ziemniak *et al.,* 2006; Masciotra *et al.,* 2007; Bertagnolio *et al.,* 2007; McNulty *et al.,* 2007; Hearps *et al.,* 2010). However, despite the presence of proviral DNA, the results found from plasma and DBS specimens were found to be highly similar, which was in agreement with the results of the current study. The degree of interference caused by the proviral DNA may vary depending on several factors, such as CD4 cell counts, VL, disease status and treatment characteristics of the specific population (Escaich *et al.,* 1992; Chun *et al.,* 1997; Bruisten *et al.,* 1998; McNulty *et al.,* 2007). Previous studies have reported that high level of concordance have been found between plasma and DBS samples particularly from patients experiencing virological failure with VL ≥ 1,000 copies/mL and found more detectable mutations in plasma as compared to DBS, while the opposite has been encountered in patients who have undergone treatment interruptions who tend to have more detectable mutations present in the DBS as compared to plasma (Bertagnolio *et al.,* 2010; Johannessen *et al.,* 2010; Rottinghaus *et al.,* 2012; Monleau *et al.,* 2014).

The contribution of proviral DNA and impact on the resulting HIV genotype is likely influenced by the patient's VL (Johannessen *et al.*, 2010). Furthermore, in patients with low viraemia, interference caused by archived proviral DNA has been reported to be higher as compared to patients with high viraemia (Parry *et al*., 2014). Previous studies have reported high concordance among proviral DNA and RNA genotypes, with strong concordance reported with samples with high viraemia and weak concordance reported among samples with low viraemia (Wirden *et al.,* 2011; Derache *et al.,* 2015).

As the virus population is typically heterogeneous within individuals, it is possible to detect the presence of more than one nucleotide, more commonly known as a mixture, at a given position on Sanger sequencing electropherograms. These mixtures are commonly recognised as low-frequency variants, which can be present in a minority in a given viral population. A study conducted by Zhou *et al.* (2011) has reported a number of discrepancies found in

replicates when comparing an optimised in-house assay with the commercially available TRUGENE® assay. However, despite a number of minor differences reported due to the presence of mixed bases among the replicates, sequence identity among the replicates ranged from 98.22% to 99.65%. The results are in agreement with the current study, which found ≥ 98% identity among replicates analysed for the precision and reproducibility parameters of the assay. Although discrepancies were found among the replicates, the interpretation of the resistance profiles was found to be identical among the replicates. Furthermore, the results indicated that the sequences generated by Sanger sequencing were found to be highly reproducible.

In conclusion, monitoring of ART with VL and drug resistance testing has been the standard of care to detect treatment failure and to design and establish effective ARV regimens (Hammer *et al.,* 2008). However, in the past genotypic drug resistance testing has only been available in developed countries and has been limited in countries which lack the necessary infrastructure (Johannessen *et al.,* 2010). Since the demand for ART has been increasing in developing countries, the need for monitoring VL and resistance testing has increased in these settings (Calmy *et al.,* 2007; Johannessen *et al.,* 2010). Therefore, the use of DBS specimens as an alternative to plasma will improve the ease of sample collection and transport in resource limited settings, especially in infants and young children where a lack of phlebotomy skills may limit access to laboratory testing. Supporting the previous evidence with the results of the current study, HIV drug resistance could easily be detected using DBS specimens in these settings.

CHAPTER 3

PRE-TREATMENT HIV DRUG RESISTANCE IN EARLY INFANT DIAGNOSIS SAMPLES IN CENTRAL SOUTH AFRICA

3.1 Introduction

South Africa has been recognised as one of the countries with the largest number of people living with human immunodeficiency virus (HIV) (estimated 7.7 million) (UNAIDS, 2019b). The country accounts for a third of all the new HIV infections reported in Southern Africa (UNAIDS, 2017). In 2018, an estimated 240,000 new HIV infections were reported and a total of 71,000 South Africans died due to acquired immune deficiency syndrome (AIDS) related illnesses (Hunt *et al.,* 2015; Statistics South Africa, 2018). Consequently, the government supports the largest antiretroviral (ARV) treatment programme in the world and the success of the programme has been evident, since it has caused a significant increase in life expectancy for HIV-positive individuals and has dramatically reduced AIDS related deaths with a 50% decrease from 140,000 deaths in 2010 to 71,000 deaths in 2018 (UNAIDS, 2018). In addition, a decrease in the infant mortality rate has been noted from 58 deaths per 1,000 births in 2002 to 36.4 deaths per 1,000 births in 2018 (Hunt *et al.,* 2015; Statistics South Africa, 2018).

The large scale roll-out of antiretroviral therapy (ART) has been accompanied by increasing levels of HIV drug resistance, which can compromise the success of ART (Hamers *et al.,* 2011; Gupta *et al.,* 2012; Hunt *et al.,* 2015; Boerma *et al.,* 2016). Drug resistance is a result of mutations within the viral enzymes, such as protease and reverse transcriptase (RT), which can reduce susceptibility to ARVs (Hunt *et al.,* 2015). Factors such as poor adherence to ARVs, lower concentrations of ARVs used and switching among the regimens can lead to emergence of drug resistant variants (Hunt *et al.,* 2015). HIV drug resistance can occur in individuals experiencing regimen failure, known as acquired drug resistance (ADR) and can be transmitted to ARV naïve individuals, known as transmitted drug resistance (TDR) (Hunt *et al.,* 2015).

A third form of drug resistance is known as pre-treatment drug resistance (PDR) (Boerma *et al.,* 2016). PDR can result from 1) transmission of drug resistant HIV from an individual who has ADR due to selective pressure of ARVs, 2) transmission of drug resistant HIV from an ART naïve individual infected with primary drug resistant HIV or 3) ADR resulting from

previous exposure to ARVs used for treatment or prevention (Wittkop *et al.,* 2011; Hamers *et al.,* 2012; Chimukangara *et al.,* 2019). PDR is commonly associated with virological failure, mostly on first-line ART.

Drug resistant HIV can be transmitted from mother to child (MTCT) during pregnancy, intrapartum or post-partum (breastfeeding) (Delaugerre *et al.,* 2009; Inzaule *et al.,* 2018a). The prevention of mother to child transmission (PMTCT) services have been a huge success but although the number of new infections in children has decreased, there is a risk of transmitting drug resistant HIV to the children who become HIV positive (Salou *et al.,* 2016). According to the World Health Organisation (WHO) (2018), newly diagnosed HIV infected children are at a higher risk of carrying drug resistant HIV than adults. The WHO further reported that one out of every two of these children carry drug resistant HIV, mostly resistant to non-nucleoside reverse transcriptase inhibitors (NNRTIs) (nevirapine [NVP] or efavirenz [EFV]) (World Health Organisation, 2018).

In 2008, the national PMTCT policy recommended d4T + 3TC + EFV/NVP for mothers with a CD4 count of < 200 cells/mm^3 or ZDV therapy from 28 weeks of gestation with single dose NVP in labour for those not qualifying for lifelong ART based on their CD4 count (National Department of Health, 2008). In 2010, option A was introduced for mothers with a CD4 count of < 350 cells/mm^3, which recommended lifelong ART consisting of TDF + 3TC/FTC + NVP or AZT + 3TC + NVP (National Department of Health, 2010a). Those women not qualifying for lifelong ART received ZDV prophylaxis offered from 14 weeks of gestation with single dose NVP and 3 hourly ZDV in labour and single dose of TDF or FTC postpartum. In 2013 and 2015, option B and B+ were introduced, which recommended lifelong ART for all HIV positive pregnant and lactating women regardless of their CD4 cell count (National Department of Health, 2013; National Department of Health, 2015b). The current National Department of Health (NDOH) guidelines recommend the use of NVP, which is given to infants at birth and then daily for 6-12 weeks to prevent MTCT of HIV and in high risk infants, AZT is given for six weeks (National Department of Health, 2015b; National Department of Health, 2019).

NVP, which is a potent NNRTI, commonly selects for resistance mutations associated with the enzyme RT (Palmer *et al.,* 2006; Battini and Bollini, 2019). The most common mutations are K103N and Y181C, which cause resistance to NVP and cross resistance to other NNRTIs (Palmer *et al.,* 2006; Battini and Bollini, 2019). These mutations have a limited effect on the

replicative ability of the virus and can therefore persist long after discontinuation of NVP therapy (Palmer *et al.,* 2006). Additionally, the presence of the V106M mutation in HIV-1 virus exposed to efavirenz can cause cross resistance to other NNRTIs (Brenner *et al.,* 2003; Wainberg and Brenner, 2012; Etta *et al.,* 2017).

The NDOH guidelines recommend early infant diagnosis (EID) testing for children born to HIV positive mothers, which includes the use of dried blood spots (DBS) to perform HIV polymerase chain reaction (PCR) testing at birth and 10 weeks of age (National Department of Health, 2019). Due to the transfer of antibodies from mother to child, EID is performed with direct detection techniques, such as the nucleic acid testing which involves the use of PCR (Mazanderani *et al.,* 2018). Repeat samples for a positive result have been recommended by NDOH, although newly diagnosed children are started on ART as soon as the first PCR is positive to limit the reservoir size (National Department of Health, 2019).

As the scale up of PMTCT services continues, including EID testing and ART, it is critical to determine the underlying patterns of HIV drug resistance among newly diagnosed children (Fitzgerald *et al.,* 2013; Salimo *et al.,* 2015). Plasma has been the specimen of choice for resistance testing using Sanger sequencing, but due to the inherent requirement to perform phlebotomy and maintain the integrity of HIV RNA, the use of DBS has been considered as an alternative specimen (Salimo *et al.,* 2015). Integrity of HIV RNA in plasma is maintained by usage of nucleic acid stabilizers, transport of specimens in a cooler box, rapid centrifugation and separation of plasma and assessment of collection times to provide results in a timely manner (Puren *et al.,* 2010). The DBS cards can easily be collected using a heel-prick from infants and children and can be transported and stored at ambient temperature for up to 14 days until transferred to -80°C (Salimo *et al.,* 2015; World Health Organisation, 2012). Previous studies in adults have reported a high rate of concordance in detecting drug resistance mutations using plasma and DBS cards in treatment naïve and exposed patients (Bertagnolio *et al.,* 2010; Johannessen *et al.,* 2010; Rottinghaus *et al.,* 2012; Monleau *et al.,* 2014). In addition, a number of paediatric studies have been previously published to assess the use of DBS cards for detecting drug resistance mutations (Salimo *et al.,* 2015; Zhang *et al.,* 2018).

Limited information is available regarding the presence of drug resistance variants among the paediatric population in sub-Saharan Africa (Salimo *et al.,* 2015). A study conducted by Boerma *et al.* (2016) reported an increase in the PDR profiles among children living in sub-

Saharan Africa. The study further reported a PDR prevalence of 42.7% among children exposed to PMTCT as compared to 12.7% in children unexposed to PMTCT which had greatly increased over the years to 56.1% in children exposed to PMTCT and 33% and 23.5% of children with unknown or no exposure to PMTCT (Jordan *et al.,* 2017).

The presence of PDR in PMTCT unexposed children highlights the possibility of children acquiring TDR virus from mothers failing ART (Boerma *et al.,* 2016; Poppe *et al.,* 2017). Therefore, the WHO has recommended the use of protease inhibitors (PIs) in children as their first line ART regardless of the exposure to PMTCT (World Health Organisation, 2019a). However, due to the increased cost involved with PIs and large scale drug stock outs, many children in developing countries are still initiated on NNRTI based regimens, which greatly affects the treatment outcomes among these children (Boerma *et al.,* 2016; Poppe *et al.,* 2017; World Health Organisation, 2017a).

A study conducted by Hunt *et al.* (2019) compared the prevalence of drug resistance found among children who were enrolled in three PMTCT surveys conducted in 2010, 2011-2012 and 2012-2013. These surveys measured MTCT of 3.5%, 2.7% and 2.6% respectively, while drug resistance was lower in 2010 (37%) as compared to 2011 (64%) and 2012 (63%). However, further studies are required to determine the current PDR prevalence of HIV circulating among the paediatric population across the country. The aim of this study was therefore to determine the prevalence of PDR in samples collected from the routine EID platform.

3.2 Materials and Methods

3.2.1 Sample collection and processing

This was a cross-sectional study which was performed at the Division of Virology, University of the Free State/National Health Laboratory Service (NHLS), Bloemfontein. During February 2018 to April 2019, a total of 521 samples from HIV-infected children submitted for EID HIV PCR testing were collected from the Virology laboratory of the NHLS at Universitas Academic Laboratories, Bloemfontein. The laboratory receives samples from the Free State, Northern Cape and North West provinces of South Africa. The EID testing is performed on children born to HIV-positive mothers from birth until the age of 18 months. At the time of the present study, NDOH guidelines recommended infant post-exposure prophylaxis (PEP) consisting of

6 to 12 weeks of NVP, with high risk infants receiving NVP + zidovudine (ZDV) (National Department of Health, 2015b). ART consisting of abacavir (ABC) + lamivudine (3TC) + lopinavir/ritonavir (LPV/r) was initiated as a first-line regimen for infants as soon as the first PCR was positive, however, a second PCR sample was required to confirm the positive result (National Department of Health, 2015b). Inclusion criteria for the collection of study samples consisted of any sample from children under the age of 18 months testing HIV PCR positive for the first time on routine EID testing. Exclusion criteria consisted of 1) samples from children above the age of 18 months 2) insufficient samples 3) infants with a previous positive PCR result and 4) children which had documented prior exposure to ART (other than PMTCT). The request forms sent for laboratory testing (EID platform) were examined to check the patient history on ART and were excluded if previous exposure to ART (other than PMTCT) was indicated. Both DBS and whole blood samples in ethylenediamine tetraacetic acid (EDTA) tubes were included. Samples were collected as residual DBS specimens after the EID testing had been performed or, in-case of whole-blood, 70 µL of EDTA blood was spotted onto perforated DBS specimen collection cards (Lasec®, South Africa) and were left to dry overnight at ambient temperature, before storage in individual zip lock bags containing silica gel desiccant (Lasec®, South Africa) at -80°C.

3.2.2 Nucleic acid extraction and nested PCR assay

Nucleic acid extraction was performed using the NucliSENS®EasyMAG®extraction system (02370; BioMérieux). The assay was optimised to use DBS cards (Lasec®, South Africa) instead of plasma for RNA extraction. Two spots containing 140 µL of whole blood were used to extract RNA using the NucliSENS®silica based extraction technique, as described in Section 2.2.2. After the extraction, RNA was eluted in the NucliSENS®Extraction buffer 3 and was stored at -80°C until further processing.

Following extraction, a nested PCR assay was performed to detect mutations in the RT and protease regions of the *pol* gene of the HIV-1 genome. The position of amino acid sequences targeted for protease was 1 to 99 and for RT enzyme 1 to 240 residues. The SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity (Thermofisher Scientific) was used for the first round of amplification, while the nested PCR assay was performed using KOD Hot Start deoxyribonucleic acid (DNA) Polymerase (Novagen®, Merck). The primers for the first round of PCR and nested PCR assay were synthesized by Integrated

DNA Technologies (Whitehead Scientific) and are indicated in Table 2.1. The procedure for the nested PCR assay is described in detail in Section 2.2.3.

The PCR amplicons were analysed using a 0.8% Seakem® agarose gel (Lonza) in a 1× Tris-Acetic Acid-EDTA (Merck) buffer, stained with 2 µL of loading dye containing GelRed (BioTium). A 100 base pair (bp) deoxyribonucleic acid (DNA) ladder (GeneRuler DNA Ladder Mix, Thermo Fisher Scientific) was included as a molecular weight marker on each gel. Analysis of the PCR assay amplicons including the target visualisation are described in detail in Section 2.2.4.

The nested PCR assay amplicons were purified using the PureLink® PCR Purification Kit (Invitrogen™, Life Technologies™, Thermo Fisher Scientific) according to the manufacturer's instructions as indicated in Section 2.2.5 and DNA concentrations of the purified PCR products were measured using the NanoDrop 2000 Spectrophotometer (Serial number: E107, 2000 Spectrophotometer, Thermo Fisher Scientific) as indicated in Section 2.2.6.

3.2.3 Sanger sequencing

Sanger sequencing was performed using sequencing primers synthesized by Integrated DNA Technologies (Whitehead Scientific) as indicated in Table 2.4. The sequencing assay is outlined in Section 2.2.7. The Ethanol/EDTA precipitation technique was used for the purification of the sequencing reactions as described in Section 2.2.8.

3.2.4 Data analysis

Sequencing of the HIV-1 *pol* gene was performed using the 3500 and 3500XL ABI genetic analysers (Applied Biosystems™, Themo Fisher Scientific). The chromatograms obtained from the sequencer were checked for sequence quality and mixed bases using a sequence analysis tool, Recall (beta v3.05) (Woods *et al.,* 2012). The sequences were then exported as text files and were analysed using the Stanford HIV Drug Resistance Database, including determination of the HIV-1 subtype (version 8.9-1) (Rhee *et al.,* 2003; Shafer, 2006). Drug resistance associated mutations in protease and RT regions and their respective resistance profiles were recorded. Phylogenetic analysis was performed using the in-house PhyloPi software and phylogenetic trees were constructed to identify any contamination among the samples (Bester *et al.,* 2019). Results were summarised by frequencies and percentages and means, median, range or percentiles. Subgroup comparisons were performed using differences in means, medians or percentiles.

3.3 Ethics

Ethics approval was obtained from the Health Sciences Research Ethics Committee of the University of the Free State (UFS-HSD2017/0633). Additional approval was obtained from the Free State DOH and NHLS.

3.4 Results

The sample group was comprised of a total of 521 DBS samples, which were collected from children who tested positive for HIV on the routine EID platform. The median age of the children was 8 months (range less than a day old to 17 months). Among the 521 samples, 71% (372/521) were successfully amplified using the nested PCR assay and 83% (308/372) were sequenced using Sanger sequencing. The samples which could not be amplified were repeated using a different DBS spot from the same card to ascertain the possibility of amplifying such samples; however, no additional samples were successfully amplified. A number of factors could have contributed to the inability to amplify these samples including compromised quality of the specimens resulting in the degradation of the total nucleic acid present in the sample, low VL due to exposure to PMTCT, insufficient blood volume collected on the DBS, and the lower sensitivity of the drug resistance assay compared to the EID PCR. The limit of detection

for the EID assay is approximately 300 copies/mL, which is much lower than the minimum copy number required for successful amplification and sequencing on the validated DBS drug resistance assay (65% amplification sensitivity for VL between 1,000 and 5,000 copies/mL and ≥ 90% for samples with VL above 5,000 copies/mL) (COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Qualitative Test, Version 2.0). VL testing was not performed on the samples as the majority had insufficient spots remaining following EID testing and repeat drug resistance testing, and a validated DBS VL assay is not available in the laboratory. No patterns were noted in the transit times, storage times or geographic origin of samples in relation to amplification failure. Figure 3.1 displays the flow-diagram for the study workflow.

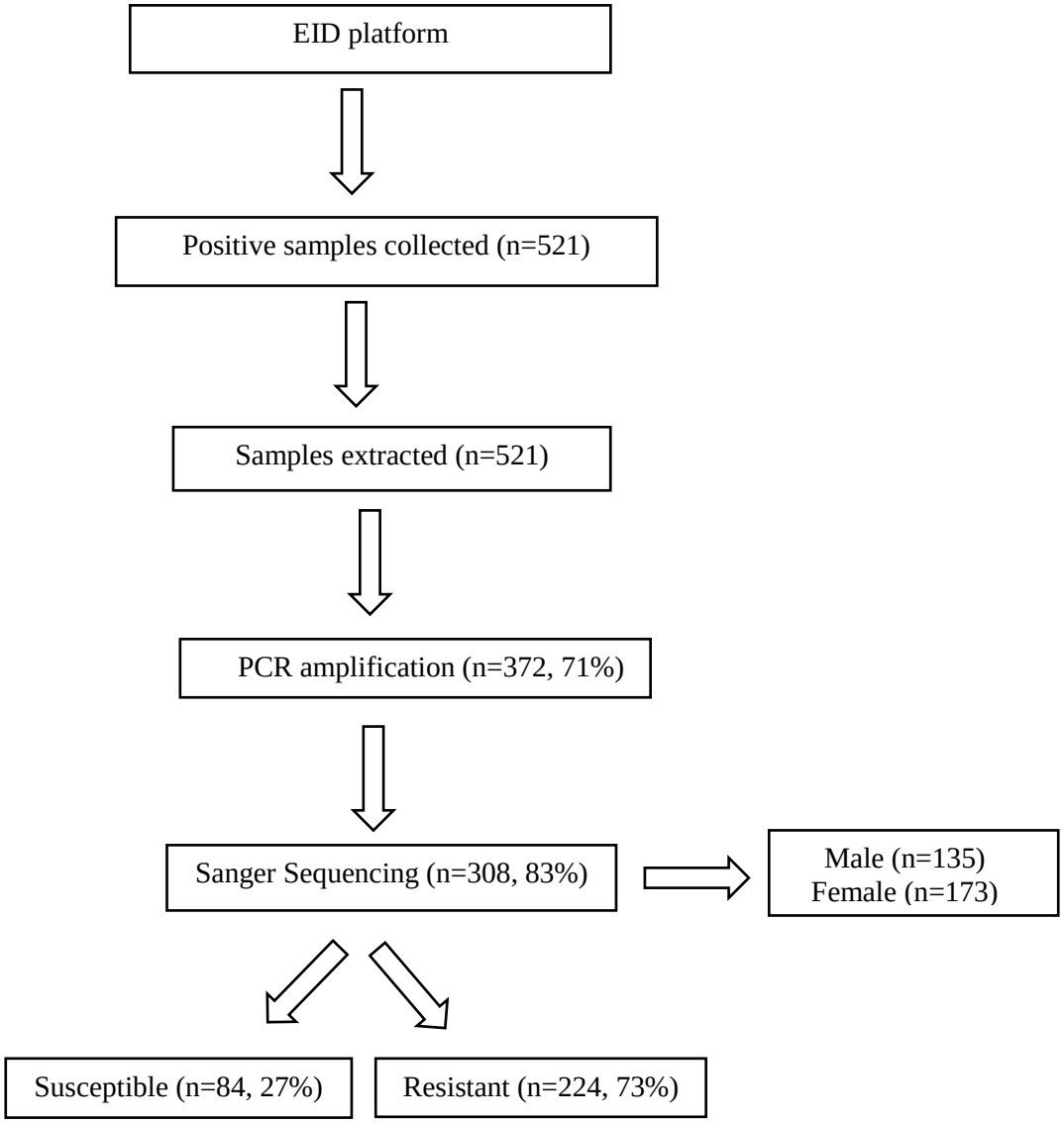


Figure 3.1 Flow-diagram indicating the study workflow

All of the HIV-1 sequences were identified as subtype C. Drug resistance testing indicated that 27% (84/308) of the samples were fully susceptible, while resistance was found in 73% (224/308) of the samples. Three of the samples with susceptible drug resistance profiles (1% [3/308]) had minor mutations which caused no change in the resistance profiles of the different classes of antiretrovirals tested. These minor mutations were M184MK (NRTI), Y188YD (NNRTI) and Q58E (an accessory PI mutation). The age of the children for these respective samples was 0 days, 5 months and 12 months. Information regarding the maternal and neonatal ARV prophylaxis was not available in the laboratory records for the majority of samples tested. The samples were therefore analyzed based on the type of drug resistance mutation and their associated resistance profile detected using the Stanford HIV drug resistance database (Rhee *et al.*, 2003; Shafer, 2006).

Table 3.1 indicates the number of drug resistance mutations found in samples collected from the EID platform. NNRTI resistance was detected in 72% (222/308) of the children, NRTI resistance in 11.7% (36/308) and PI resistance in 1.6% (5/308).

Table 3.1 Number of resistance mutations by drug category found in paediatric samples collected from the routine EID platform

Resistance category	Number of sample with DRM (n=308)	Number of mutations (%)
At least one NNRTI DRM	222	72
At least one NRTI DRM	36	11.7
At least one PI DRM	5	1.6
Dual class resistance		
NNRTI + NRTI	33	10.7
NNRTI + PI	2	0.6
Triple class resistance		
NNRTI + NRTI + PI	2	0.6

DRM Drug resistance mutation NNRTI Non-nucleoside reverse transcriptase inhibitor NRTI Nucleoside reverse transcriptase inhibitor PI Protease inhibitor

In those children with resistant drug profiles, the prevalence of NNRTI mutations (99%, 222/224) was the highest, followed by nucleoside reverse transcriptase inhibitor (NRTIs) (16%, 36/224) and PIs (2%, 5/224). The prevalence of dual resistance to NNRTI and NRTI (15%) was the highest, followed by NNRTIs + PIs (0.9%). None of the children harboured dual resistance to NRTIs + PIs. Triple class resistance (NNRTI + NRTI + PI) was detected in two children. Both of the children were ≤ 1 month, with one of the children only a day old and the other one month old.

Among the different classes of ARVs, high-level resistance was only detected to NNRTIs and NRTIs (Table 3.2). High-level resistance to NRTIs was considerably lower as compared to NNRTIs and was mostly found to emtricitabine (FTC) (8%) and 3TC (8%) and less so to ABC (4%) and tenofovir (TDF) (3%). None of the samples displayed high-level resistance to ZDV, however, two of the children displayed intermediate resistance to ZDV, while low-level resistance was found in eight children.

Table 3.2 HIV drug resistance profiles among children who tested positive on the routine EID platform

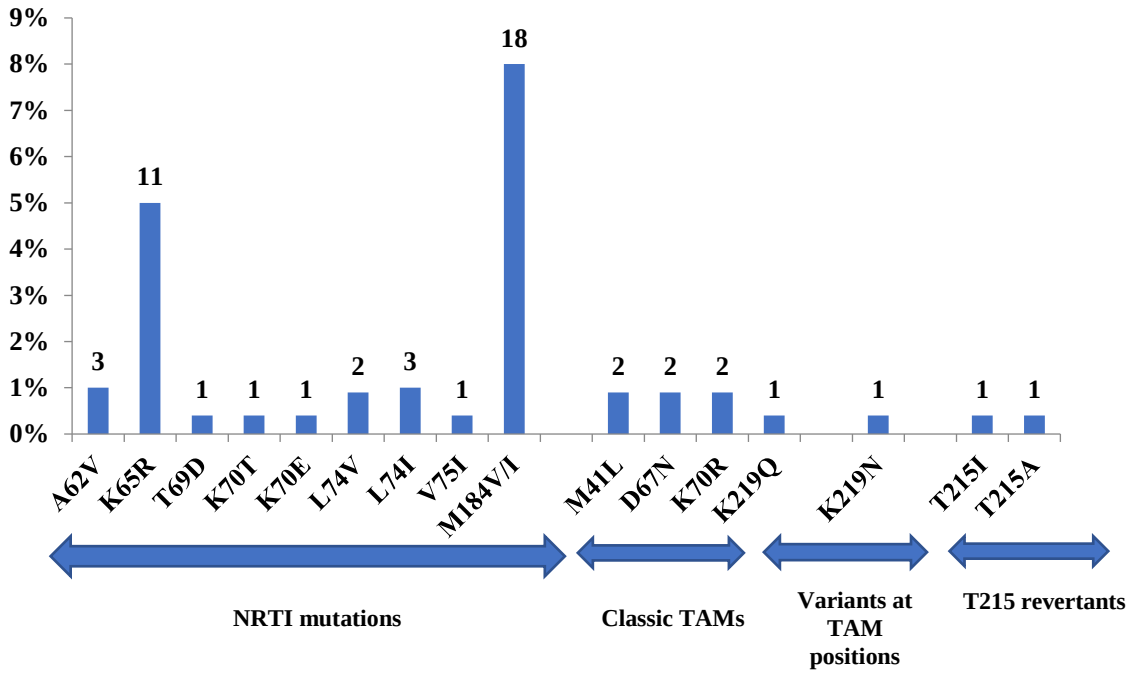
Antiretrovirals (n=224)	Susceptible n (%)	High-level resistance n (%)	Intermediate n (%)	Low-level resistance n (%)
NRTIs				
ABC	198 (88.4)	10 (4.5)	6 (2.6)	10 (4.5)
ZDV	214 (95.5)	0 (-)	2 (0.9)	8 (3.6)
FTC	200 (89.3)	18 (8)	5 (2.2)	1 (0.5)
3TC	200 (89.3)	18 (8)	5 (2.2)	1 (0.5)
TDF	211 (94.2)	6 (2.7)	5 (2.2)	2 (0.9)
NNRTIs				
DOR	109 (48.7)	21 (9.4)	48 (21.4)	46 (20.5)
EFV	12 (5.3)	188 (84)	15 (6.7)	9 (4)
NVP	11 (5)	203 (90.6)	5 (2.2)	5 (2.2)
ETR	127 (56.7)	5 (2.2)	42 (18.8)	50 (22.3)
RPV	127 (56.7)	30 (13.4)	32 (14.3)	35 (15.6)
PIs				
ATV	220 (98.2)	0 (-)	0 (-)	4 (1.8)
DRV	222 (99.1)	0 (-)	0 (-)	2 (0.9)
LPV	219 (97.8)	0 (-)	1 (0.4)	4 (1.8)

3TC Lamivudine ABC Abacavir ATV Atazanavir DRV Darunavir
DOR Doravirine EFV Efavirenz ETR Etravirine FTC Emtricitabine
LPV Lopinavir NVP Nevirapine NNRTI Non-nucleoside reverse transcriptase inhibitor
NRTI Nucleoside reverse transcriptase inhibitor PI Protease inhibitor RPV rilpivirine
TDF Tenofovir ZDV Zidovudine

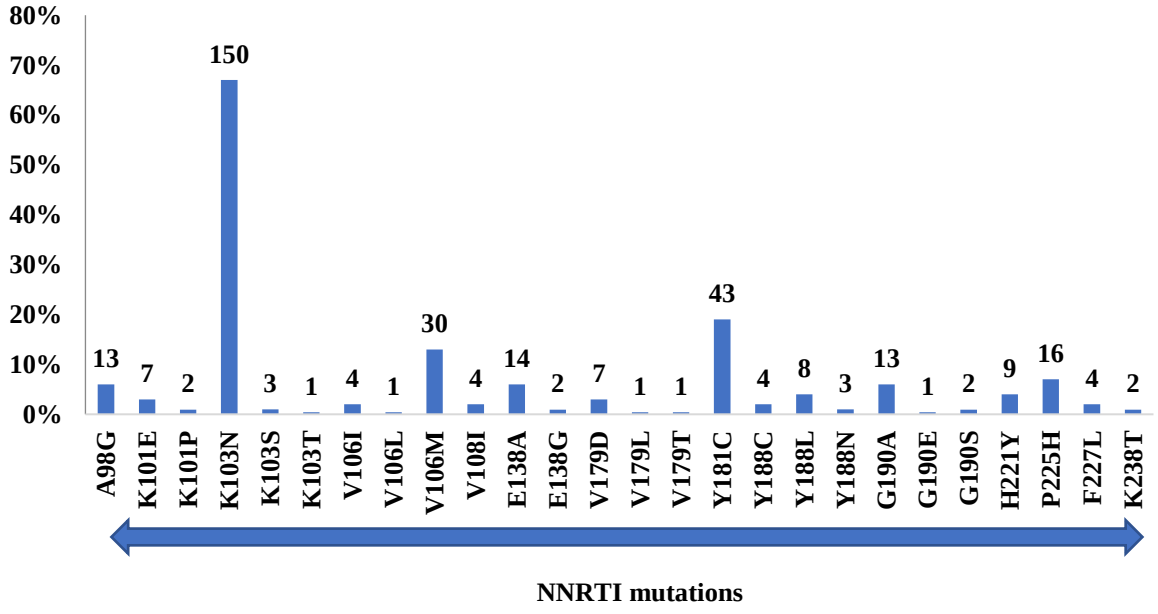
Due to the low-genetic barrier to resistance, high-level resistance was mostly found against the first-generation of NNRTIs, such as NVP (91%) and EFV (84%). High-level resistance to the second-generation of NNRTIs was considerably lower as compared to first-generation NNRTIs, with etravirine (ETR) at 2% and rilpivirine (RPV) at 13%. Interestingly, high-level resistance was also detected to the new FDA approved NNRTI doravirine (DOR) (9%) but was much lower than first generation NNRTIs.

Protease resistance was very rare among the children. None of the children displayed high-level resistance against any PI. Among the five children with PI resistance, four children displayed low-level resistance to atazanavir (ATV) and LPV/r, while the remaining one was

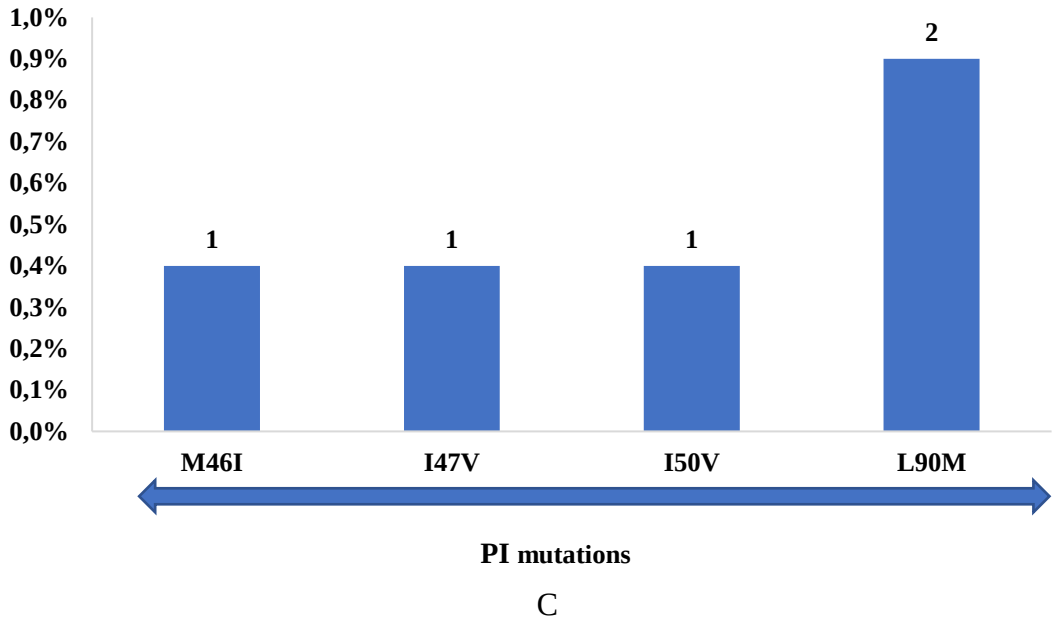
susceptible to ATV, displayed intermediate resistance to LPV/r and low level resistance to darunavir (DRV). Among the four children with low-level resistance to ATV and LPV/r, three were susceptible to DRV, while one displayed low-level resistance to DRV. The ages of these children was 1 day, 1 month and 17 months, while two of the children were less than a day old. Figure 3.2 displays the mutations found among the different classes of ARVs from children included in the study population.



A



B



C

Figure 3.2 **HIV drug resistance mutations identified in children included in the study population to (A) NRTIs, (B) NNRTIs and (C) PIs**

Among the NRTIs, the most common mutation was M184V/I (8%), which was detected in 18 children. The age of these children ranged from 0 days to 7 months. It was followed by K65R (5%), which was detected in 11 children (age ranged from 0 days to 7 months). Other NRTI mutations, such as T69D (0.4%), K70T (0.4%) and K70E (0.4%), existed in combination with other NRTI mutations and caused intermediate to potential low-level resistance to NRTIs. Classic TAMs, such as M41L (0.9%), D67N (0.9%), K70R (0.9%) were detected in two children each, while K219Q (0.4%) was found in one child, respectively.

Among the NNRTIs, the most common mutation was K103N (67%), which was detected in 150 children. It was followed by Y181C (19%), which was detected in 43 children. The third most common mutation among the NNRTIs was V106M (13%), which was detected in 30 children. Other NNRTI mutations, including A98G (6%), E138A (6%), G190A (6%), H221HY (4%) and P225H (7%) existed in combination with other NNRTI mutations and caused intermediate to potential low-level resistance to NNRTIs.

Among the PIs, the most common mutation was L90M, which was detected in two children, followed by M46I, 147V and I50V which were detected in one child each.

3.5 Discussion

The emergence of drug resistance mutations has remained a major threat to successful treatment in paediatric HIV, in particular where adherence and drug options are limited for sub-Saharan African children (Sigaloff *et al.,* 2012b; Fokam *et al.,* 2013; World Health Organisation, 2013; Fokam *et al.,* 2018). Thus novel strategies are required to limit the spread of HIV drug resistance among newly diagnosed children born to HIV-positive mothers (World Health Organisation, 2012; Paredes *et al.,* 2013; Fokam *et al.,* 2018). According to the nationally representative South African PMTCT surveys, MTCT has decreased over the years, from 3.5% in 2010, to 2.7% in 2011-2012, 2.6% in 2012-2013, 1.1% in 2015-2016 and 0.9% in 2016-2017 measured as the *in utero* paediatric HIV infection rate per 100,000 live births (Goga *et al.,* 2012; Goga *et al.,* 2015; Goga *et al.,* 2016; Goga *et al.,* 2018; Hunt *et al.,* 2019). However, the prevalence of resistance to NNRTIs observed among newly diagnosed children has continued to increase over the years.

In the current study, PDR was determined in samples collected from the routine EID platform. The overall PDR among children was 73% (224/308), with the majority of the children harbouring resistance to NNRTIs (72% [222/308]), particularly to NVP and EFV. The current PDR observed among children (73%) was higher than previous national PMTCT surveys, where resistance levels of 37% (2010), 64% (2011), 63% (2012) and 68.7% (2014) were recorded (Goga *et al.,* 2012; Goga *et al.,* 2015; Goga *et al.,* 2016, Jordan *et al.,* 2017). These findings suggest an increase in the drug resistance levels in children over the years, which currently stands at 73% as observed in the present study. Furthermore, the findings are higher than national PMTCT programmes conducted in sub-Saharan Africa which reported 60% in Togo and 63% in Zimbabwe, while even lower resistance rates were documented in Nigeria (48%) and Swaziland (35%) (Penazzato, 2013; Salou *et al.,* 2016; Fokam *et al.,* 2018).

The increase in NNRTI resistance in children coincides with the change in the national PMTCT policy for HIV positive mothers and HIV-exposed children. For HIV-exposed infants, national PMTCT policy recommended single dose NVP and 7 or 28 days of infant ZDV in 2008 and in 2010 under option A, daily NVP for 6 weeks if not breastfed or until one week after cessation of breastfeeding (National Department of Health, 2008; National Department of Health, 2010a). The change from single dose NVP to 6 weeks of NVP prophylaxis for HIV-exposed children has likely contributed to the increase in the prevalence of NNRTI resistance observed

over the years. Additionally, HIV-infected mothers are recommended to breastfeed the children, leading to prolonged exposure, which might have played a significant role in the increase of NNRTI resistance.

In the current study, high-level NVP resistance was detected in 66% (203/308) of children, accounting for 91% of the resistant samples (203/224). This has been observed among other studies, which reported 27% prevalence of NVP associated mutations in children exposed to single dose NVP and 60% among children with extended exposure to NVP prophylaxis (Hunt *et al.,* 2011; Salou *et al.,* 2016). A study conducted by Kanthula *et al.* (2017) compared the prevalence and persistence of NNRTI resistant mutations among HIV-infected children who were given single dose NVP as compared to extended exposure prophylaxis of NVP for 6 weeks. The study reported that the prevalence of NNRTI mutations was higher in older children (> 50%) and these mutations dominated the child's quasispecies (Kanthula *et al.,* 2017). A decrease in NNRTI mutations was rarely observed in the cohort even with children followed up until at least 3 years of age (Kanthula *et al.,* 2017). Previous studies have reported that the K103N mutation has a minor effect on viral replication fitness and the mutant virus has similar replicative ability to wild type (Wang *et al.,* 2010).

Furthermore, dual class resistance i.e resistance to both NNRTIs and NRTIs (11% [33/308]) was the most prevalent, followed by NNRTIs +PIs (0.6% [2/308]). All the NRTI resistance existed in combination with NNRTI resistance except one sample (8 months), which had a single NRTI mutation (T215I). The mutation is classified as a T215 revertant which can lead to development of T215Y/F due to a single base change. T215Y/F is a non-polymorphic thymidine analog mutation (TAM) and is selected mostly by thymidine analogs, such as ZDV and d4T (stavudine) (Violin *et al.*, 2004; Van Laethem *et al.*, 2007; Mitsuya *et al.*, 2008; Pingen *et al.,* 2013). These TAMs result in reduced susceptibility to NRTIs by facilitating nucleotide excision. Triple class resistance (NNRTI+NRTI+PI) was very rare and was only detected in two children.

Overall, among the NRTIs, the most common mutation was M184V/I (6% 18/308), which was detected in 18 children. These children displayed high level resistance to 3TC (6% 18/308) and FTC (6% 18/308). It was followed by K65R (3% 11/308), which was detected in 11 children but high-level resistance to TDF was only detected in 6 children, as the remaining five children had both M184V and K65R mutations which resulted in high-level resistance to 3TC, FTC and

ABC but intermediate resistance to TDF. Interestingly, one of the children (2 months) with high-level resistance to TDF harboured multiple NRTI mutations, including A62V**,** K65R**,** K70T and M184I, resulting in high-level resistance to TDF. These findings suggest pre-treatment NRTI resistance which could be linked to exposure to maternal ARVs. This is worrisome as the response to INI-based regimens which rely on an active NRTI backbone has not been established in the presence of high-level NRTI resistance, which could potentially lead to infants sub-optimally responding to treatment resulting in virological failure (National Department of Health, 2019). Furthermore, the results support a study conducted by Hunt *et al.* (2019), which compared the findings from three PMTCT surveys conducted nationwide and reported dual class resistance (NRTIs and NNRTIs) of 5% among infants aged 4 to 8 weeks. The most common mutation among these infants was M184V/I which were detected in 6 samples (Goga *et al.,* 2012; Goga *et al.,* 2015; Goga *et al.,* 2016; Hunt *et al.,* 2019).

To address HIV drug resistance among children, since 2013, WHO has recommended the use of PIs as first-line therapy among children under the age of 3 years (World Health Organization, 2019a). As with many other countries in sub-Saharan Africa, this recommendation has been adopted in South Africa and the current paediatric treatment guidelines recommend the use of ABC + 3TC + LPV/r for children ≥ 4 weeks of age (and ≥ 42 weeks of gestational age) and weighing ≥ 3 kg (National Department of Health, 2019). For preterm neonates and neonates with birth weight < 2.5 kg, the use of AZT + 3TC + NVP plus additional advice from an expert is recommended. Resistance testing is recommended for children with virological failure on first-line regimens to ascertain the possibility of treatment adherence issues and if PI resistance is detected, then advice from an expert is recommended before switching to second-line or integrase-based regimens. The prevalence of protease resistance in the current study was very low and none of the children displayed high-level resistance to any PI, with intermediate resistance in one child and low-level PI resistance detected in four children. This indicates the probability of continued efficacy of the current first line regimen recommended for children under the age of 3 years, even in children with high level NNRTI resistance. However, a previous study conducted by Kanthula *et al.* (2017) suggested that NNRTI resistance could last for at least 2 years and could re-emerge after a period following selection pressure by NNRTIs, which could likely have an effect on long term treatment options in children with PDR acquired from maternal ARVs or following PMTCT prophylaxis.

The study was limited by the unavailability of maternal and infant treatment history, prior ART exposure in infants, lack of information on infant prophylaxis and treatment after birth and individual factors, such as CD4 cell count and duration of infection, which were not available for the majority of the children and was therefore not analysed. Furthermore, the mothers and children were not followed prospectively, which makes it difficult to determine the time of onset of HIV drug resistance i.e whether the resistance was transmitted from mother to child or acquired due to drug selection pressure during the administration of prophylaxis or treatment. Additionally, the number of samples collected as DBS or whole blood was not recorded. Lastly, Sanger sequencing was used for testing of drug resistance mutations on DBS specimens. More sensitive technologies, such as the use of next-generation sequencing could possibly provide additional information such as the presence of drug resistant variants and minority sequences among children.

In conclusion, PMTCT has caused a considerable decrease in the MTCT rate of HIV infections over the years. However, the rate of PDR among children who become infected due to failure of PMTCT prophylaxis is increasing, which will lead to higher morbidity and mortality in the absence of optimal paediatric regimens. A high rate of PDR was found in the current study with most children harbouring NNRTI resistance. Previous studies have indicated the presence of PDR in children unexposed to PMTCT, which indicates the inadequacy of using PMTCT history as a means of ruling out PDR among children (Inzaule *et al.*, 2018b). Furthermore, the findings from the present study support the use and implementation of PIs as first-line therapy for children < 3 years as suggested by WHO and the current NDOH guidelines (National Department of Health, 2019; World Health Organization, 2019a).

CHAPTER 4

HIV DRUG RESISTANCE IN CHILDREN AND ADOLESCENTS WITH VIROLOGICAL FAILURE WHILE ON ART

4.1 Introduction

In 2018, an estimated 260,000 children (0 to 14 years of age) were living with HIV in South Africa, of whom 63% had access to treatment (UNAIDS, 2018; UNAIDS, 2019b). Due to the roll out of antiretroviral therapy (ART) and interventions such as prevention of mother to child transmission (PMTCT) programmes, the number of new HIV infections among children has dramatically decreased (28,000 in 2010 to 14,000 in 2018) and an increased life-expectancy of perinatally HIV infected children has been reported (UNAIDS, 2018). As the burden of paediatric HIV tends to decrease, the focus is shifting towards older children, as these children age towards early adolescence (Johnson *et al.,* 2012; Anderson *et al.,* 2020).

In 2016, around 1.2 million adolescents (10 to 19 years) were living with HIV in South Africa (Statistics South Africa, 2018). Despite the widespread availability of ART, access to treatment among adolescents has been limited (Maskew *et al.,* 2019). The prevalence of HIV among adolescent girls has reported to be the highest of demographic group (Human Sciences Research Council, 2018). Several factors, such as poor adherence to antiretroviral (ARV) treatment, accumulation of drug resistance mutations and socio-economic difficulties have resulted in increased treatment failure among adolescents (Anderson *et al.,* 2020). Furthermore, it is difficult to distinguish between PHIV and behaviourally acquired HIV infection among adolescents (Lam *et al.,* 2017). A study conducted by Judd *et al.* (2017a) reported a high risk of triple class virological failure among adolescents infected with PHIV. Furthermore, a study conducted in the United Kingdom (UK) reported a decline in CD4 counts in adolescents prior to their adult care transition, with mean rates of decline between 3-30 cells/mm^3 per year, thus highlighting the vulnerability of this age group with regard to poor adherence (Judd *et al.*, 2017b).

The probability of virological suppression and long-term treatment success has been reported to be lower in children and adolescents as compared to adults (Fokam *et al.,* 2019). Younger children tend to have high pre-ART viral load (VL), resulting in an increased risk of treatment

failure. The presence of a high VL on ART is likely a result of sub-therapeutic drug concentrations owing to the limited availability of paediatric drug formulations, differences in pharmacokinetics among adults and children, continuous body weight changes and poor adherence (Abrams *et al.,* 1998; Menson *et al.,* 2006). Furthermore, lack of treatment monitoring combined with advanced immune-suppression further complicates treatment outcomes among children (Sutcliffe *et al.,* 2008; Bratholm *et al.,* 2010). As these children age towards adolescence, achieving adherence becomes a bigger challenge due to several practical and social barriers, such as stigma related to HIV, disclosure of HIV status and adverse treatment effects (Simoni *et al.,* 2007).

Early treatment initiation among HIV-infected children has greatly reduced mortality in the paediatric population (Violari *et al.,* 2008). However, it has dramatically increased the number of children failing first-line ART with a subsequent increase in drug resistance at an early age (Steegen *et al.,* 2014). Since treatment for HIV is life-long, maintaining children on first-line and second-line treatment becomes a priority before resorting to third-line treatment options (Steegen *et al.,* 2014). Several studies conducted in South Africa have reported the presence of drug resistance mutations among children failing ART (Van Zyl *et al.,* 2009; Wallis *et al.,* 2009; Barth *et al.,* 2011; Taylor *et al.,* 2011; Orrell *et al.,* 2013). These studies reported similar resistance profiles in the reverse transcriptase (RT) region, such as the presence of M184V and K103N mutations, as compared to adults (Wallis *et al.,* 2009; Barth *et al.,* 2011; Orrell *et al.,* 2013). However, a higher prevalence of protease mutations has been reported in children as compared to adults (Van Zyl *et al.,* 2009; Wallis *et al.,* 2009; Taylor *et al.,* 2011; Orrell *et al.,* 2013).

Currently, the National Department of Health (NDOH) recommends the use of two nucleoside reverse transcriptase inhibitors (NRTIs) plus a protease inhibitor (PI) for ART in children weighing 3 to 20 kg (National Department of Health, 2020). A combination of abacavir (ABC) + lamivudine (3TC) + lopinavir/ritonavir (LPV/r) has been used for children ≥ 4 weeks of age, and ≥ 42 weeks gestational age and weighing < 20 kg (National Department of Health, 2020). These recommendations are in line with World Health Organisation (WHO) guidelines for first-line ART in children weighing < 20 kg (World Health Organization, 2019a). The use of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in first-line ART has been restricted in children since the implementation of NNRTIs given as single dose nevirapine (NVP) for PMTCT (Moorthy *et al.,* 2009). Exposure to NVP as part of the PMTCT for a longer period

of time increases the risk of children acquiring virus resistant to NNRTIs, thus leading to treatment failure (Moorthy *et al.,* 2009). The current NDOH guidelines recommend the use of NVP, which is given to infants at birth and then daily for 6 weeks or until the cessation of breastfeeding (National Department of Health, 2020). Timely virological monitoring and genotypic testing to determine drug resistance mutations have been identified as essential services to reduce the rate of treatment failure among children and adolescents (Anderson *et al.,* 2020). Identification of resistance mutations will aid in selecting appropriate treatment regimens among children and adolescents experiencing treatment failure (Anderson *et al.,* 2020).

Information regarding the long-term treatment outcomes, including the virologic and immunologic outcomes, in this group of highly treatment experienced children and adolescents is lacking (Anderson *et al.,* 2020). Several studies have reported that virological and immunological criteria, such as VL and CD4 cell count monitoring, which are required to detect treatment failure among children and adolescents are limited in most resource limited settings (Kantor *et al.,* 2009; Van Oosterhout *et al.,* 2009; Mutwa *et al.,* 2014). The limitation of fewer treatment options for this age group combined with the acquisition of drug resistant virus leads to poor clinical outcomes and reduced chance of survival (Muri *et al.,* 2017). Limited information is available regarding drug resistance mutations among children and adolescent with virological failure while on ART (Anderson *et al.,* 2020). The aim of this study was therefore to analyze Sanger sequencing data collected from children and adolescents experiencing virological failure while on ART.

4.2 Materials and methods

4.2.1 Sample collection

This was a retrospective study which was performed at the Division of Virology, University of the Free State/National Health Laboratory Service. The diagnostic Virology laboratory at NHLS Universitas Academic Laboratories performs the routine HIV drug resistance testing using an in-house nested PCR and Sanger sequencing assay on samples from patients failing ART. The assay targets amino acids 1 to 99 of protease and 1 to 240 of RT, as described in detail in Chapter 3. The laboratory receives samples from the Free State, Northern Cape and North West provinces of South Africa. The drug resistance testing was performed on patients with virological failure, which is defined as an HIV VL > 1,000 RNA copies/mL on two

separate occasions, while receiving PI-based ART (National Department of Health, 2020). Data for all samples received between June 2016 and December 2019 for children and adolescents ages 0 to 19 years were retrieved from the laboratory information system. Exclusion criteria included samples from adults above the age of 19 years and samples with unsuccessful PCR amplification or sequencing.

4.2.2 Data analysis

HIV drug resistance data was retrieved from the National Health Laboratory Services (NHLS) Trakcare laboratory system. Sierra web services tool (SierraPy version 0.2.1) from Stanford HIV Drug Resistance Database (version 8.9-1) was used to analyse the data (Rhee *et al.,* 2003; Shafer, 2006). The subtype for the samples was determined using the Stanford HIV Drug Resistance Database (version 8.9-1) (Rhee *et al.,* 2003; Shafer, 2006). Samples were divided into two groups namely children (0 to 9 years) and adolescents (10 to 19 years) depending on their age. Drug resistance associated mutations in the protease and RT regions and their respective resistance profiles were recorded. Results were summarised by frequencies and percentages and are shown as tables and figures. Subgroup comparisons were performed using differences in means, medians or percentiles.

4.3 Ethics

Ethics approval was obtained from the Health Sciences Research Ethics Committee of the University of the Free State (UFS-HSD2017/0633). Approval was obtained from the Free State DOH and the NHLS.

4.4 Results

The study data included a total of 295 samples which were sequenced for HIV drug resistance testing between June 2016 and December 2019. The information regarding the current and previous regimens of patients was recorded from the request forms sent to the laboratory for drug resistance testing. Where no information was provided on the request form, the pathologists attempted to contact the submitting clinician to request the treatment history and documented this information. Among the 295 samples, 34% (101/295) were from children,

while 66% (194/295) of the samples were from adolescents. The median age of the children was 4.5 years and for adolescents was 14.5 years and 53% from each group were female.

Among children, 9% (9/101) were fully susceptible, while resistance was detected in 91% (92/101) of children. Regarding the current treatment regimens of children at the time of resistance testing, 91% (91/101) of the children were on PI based regimen, followed by NNRTI based at 4% (4/101). Two of the children only had a single NRTI (3TC) recorded and were likely on a holding regimen, while four of the children had no current regimen recorded. Among adolescents, 14% (27/194) of the samples were susceptible and resistance was detected in 86% (167/194) of the samples. The majority of adolescents were receiving PI based regimens (90% [174/194]), followed by 5% (10/194) on NNRTI based regimen, two were on NRTI, while eight had no regimen reported.

HIV drug resistance was identified in the samples of 92 children and 167 adolescents. Among children with resistance (n=92) on a current PI based regimen (n=81), 86% (70/81) showed resistance to NRTIs, 74% (60/81) had resistance to NNRTIs, and 31% (25/92) had resistance to PIs. Among children receiving a NNRTI based regimen (n=4), all except one child had resistance to NRTIs, all children had resistance to NNRTIs, and no PI resistance was detected. Among adolescents (n=167) with resistance on a current PI based regimen (n=150), 64% (96/150) had resistance to NRTIs, 83% (125/150) had resistance to NNRTIs, and 30% (45/150) had PI resistance. Among adolescents receiving a NNRTI based regimen (n=11), 8 adolescents had resistance to NRTIs, all adolescents had resistance to NNRTIs, and only one adolescent had resistance to PIs. Table 4.1 displays the current and previous regimens used for children and adolescents included in the study population.

Table 4.1 The current and previous antiretroviral regimens used for children and adolescents with resistance

Type of regimen	Children (n=92) (%)	Adolescents (n=167) (%)
Current regimen		
3TC + ABC + EFV	3 (3.2)	4 (2.4)
FTC + TDF + EFV	0	3 (1.8)
Other NNRTI regimen	1 (1.1)	4 (2.4)
3TC + ZDV + LPV/r	17 (18.5)	87 (52.1)
3TC + ABC + LPV/r	61 (66.3)	42 (25.1)
Other LPV/r regimen	3 (3.2)	12 (7.2)
Other ATZ/r regimen	1 (1.1)	9 (5.4)
Only 3TC indicated	2 (2.2)	0
No current regimen recorded	4 (4.4)	6 (3.6)
Previous regimen		
3TC + ABC + EFV	5 (5.4)	37 (22.1)
3TC + d4T + EFV	0	14 (8.4)
FTC + TDF + EFV	0	4 (2.4)
Other NNRTI regimen	2 (2.2)	23 (13.8)
3TC + ZDV + LPV/r	1 (1.1)	4 (2.4)
Other LPV/r regimen	5 (5.4)	10 (6)
Other ATZ/r regimen	0	2 (1.2)
No previous regimen recorded	79 (85.9)	73 (43.7)

3TC Lamivudine ABC Abacavir ATZ/r Atazanavir/ritnonavir d4T Stavudine
EFV Efavirenz FTC Emtricitabine LPV/r Lopinavir/Ritonavir TDF Tenofovir
ZDV Zidovudine

Table 4.2 below indicates the frequency of drug resistance mutations found among the children and adolescents included in the study. Among children (n=92), resistance to NRTIs (86%) was the highest, followed by NNRTIs (75%) and PIs (28%). Among children with PI resistance (28% [6/92]), 13 children had both major and minor PI mutations, while 10 children only harboured minor PI mutations and 3 children only harboured major PI mutations. Dual class resistance to NRTIs + NNRTIs was the most common (44% [41/92]), followed by NRTIs + PIs (5%) and NNRTIs + PIs (2%). Triple class resistance (NNRTI + NRTI + PI) was detected in 17 children (18%). Interestingly, 40 of the children with detectable resistance (43% [40/92]) were ≤ 3 years of age, with dual class resistance (NNRTI and NRTI) detected in 19 (48%) of these children and triple class resistance detected in 7 children (18%). Among the 52 children aged between 3 and 10 years, dual class resistance was detected in 22 (42%) and 10 children harboured triple class resistance (19%).

Resistance to NNRTIs (87%) among adolescents was the highest, followed by NRTIs (66%) and PIs (30%). Among those with PI mutations (30% [51/167]), 21 of the adolescents harboured both major and minor PI mutations, 26 of the adolescents only harboured minor PI mutations, while 4 of the adolescents only harboured major PI mutations. NNRTI mutations

existed in combination with NRTI mutations in 38% (63/197) of adolescents with resistance, followed by NNRTIs + PIs (4%) and NRTIs + PIs (4%). Triple class resistance (NNRTI + NRTI + PI) was detected in 32 adolescents.

Table 4.2 Frequency of resistance mutations by drug category among children and adolescents included in the study

	Children(n=92)	Adolescents(n=167)
Resistance category	**Frequency of mutations (%)**	**Frequency of mutations (%)**
At least one resistance mutation	92 (100)	167 (100)
At least one NNRTI	69 (75)	146 (87)
At least one NRTI	79 (86)	111 (66)
At least one PI	26 (28)	51 (30)
Dual class resistance		
NNRTI + NRTI	41 (44)	63 (38)
NNRTI + PI	2 (2)	7 (4)
NRTIs + PIs	5 (5)	7 (4)
Triple class resistance		
NNRTI + NRTI + PI	17 (18)	32 (19)

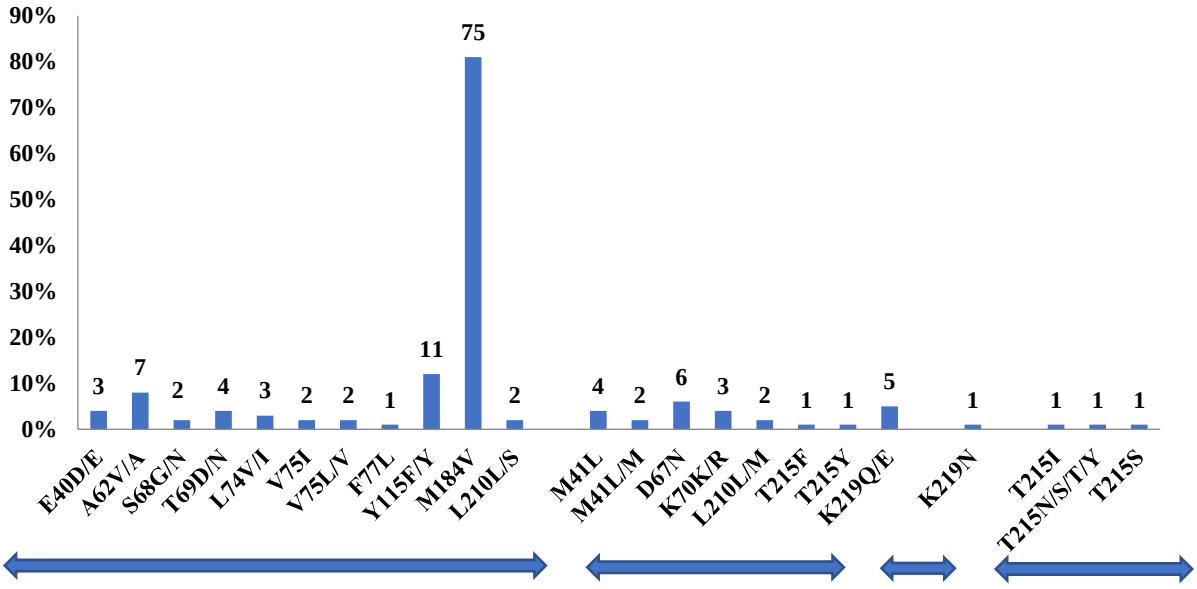
NNRTI Non-nucleoside reverse transcriptase inhibitor NRTI Nucleoside reverse transcriptase inhibitor
PI Protease inhibitor

Figure 4.1 displays the mutations found among the different classes of ARVs from children included in the study population. Among children, the most common NRTI mutation was M184V (81%), which was detected in 75 children. It was followed by Y115F/Y (12%) detected in 11 children and A62V/A (8%) which was found in seven children. Among the thymidine analogue mutations (TAMs), the most common mutations were D67N (6%) and M41L (4%) which were detected in 6 and 4 children, followed by K219Q/E (5%) and K70K/R (3%) which were detected in 5 and 3 children, respectively. Three or more TAMs (D67N, K70R and K219E) were only detected in one child (6 years of age). Additionally, PI resistance was also detected in this child, with major PI resistance mutations, including I54I/V and V82A.

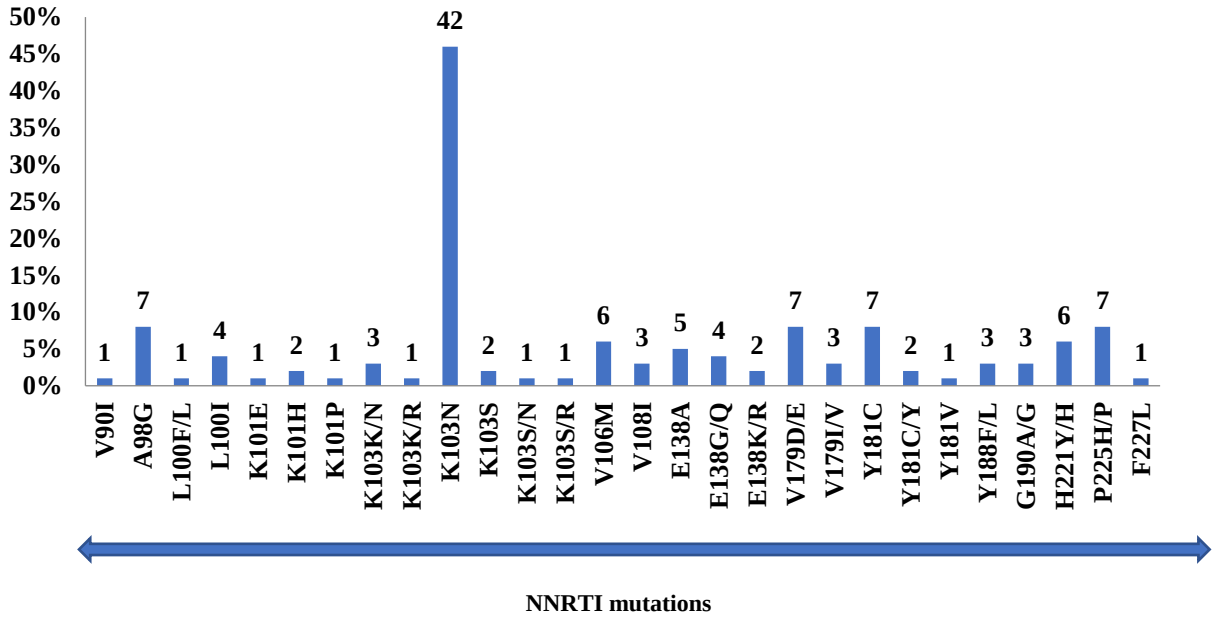
The most common NNRTI mutation among the children was K103N (46%), which was detected in 42 children, followed by A98G, P225H/P, Y181C and V179D/E, which were detected in 7 children each (8%). The less common mutations, such as V106M (6%) and H221Y/H (6%) were detected in 6 children, followed by E138A (5%), which was detected in 5 children.

The most common major PI mutations in the children were M46I (13%) and V82A (13%), which were detected in 12 children, followed by I54V (11%), in 10 children. The less common

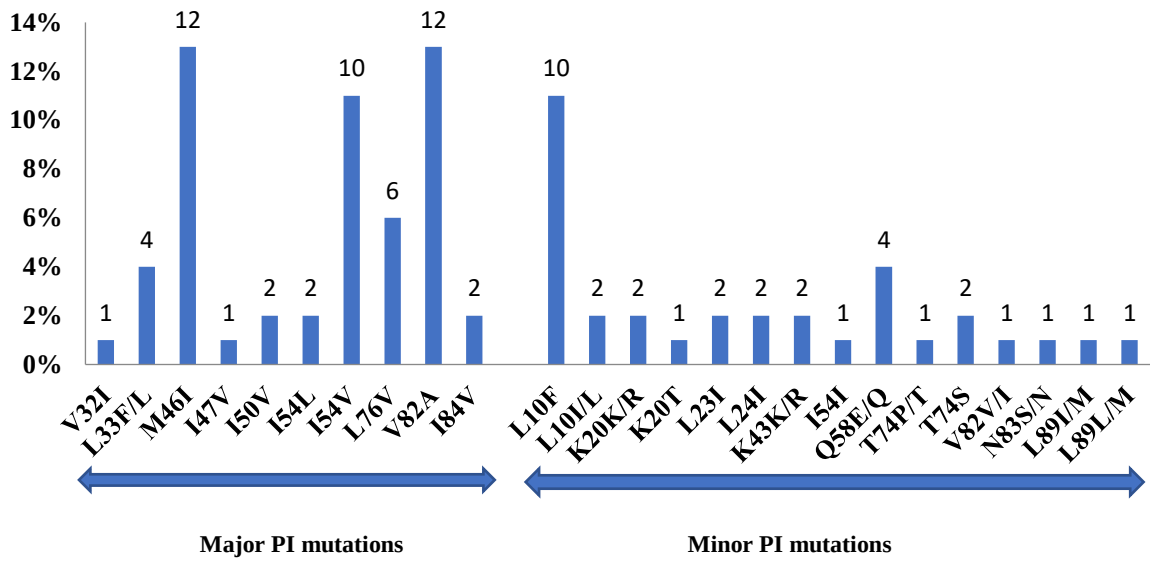
major PI mutations were L76V (6%) in 6 children and L33F/L (4%) in 4 children. The most common minor PI mutation was L10F (11%) which was detected in 10 children.



A



B



C

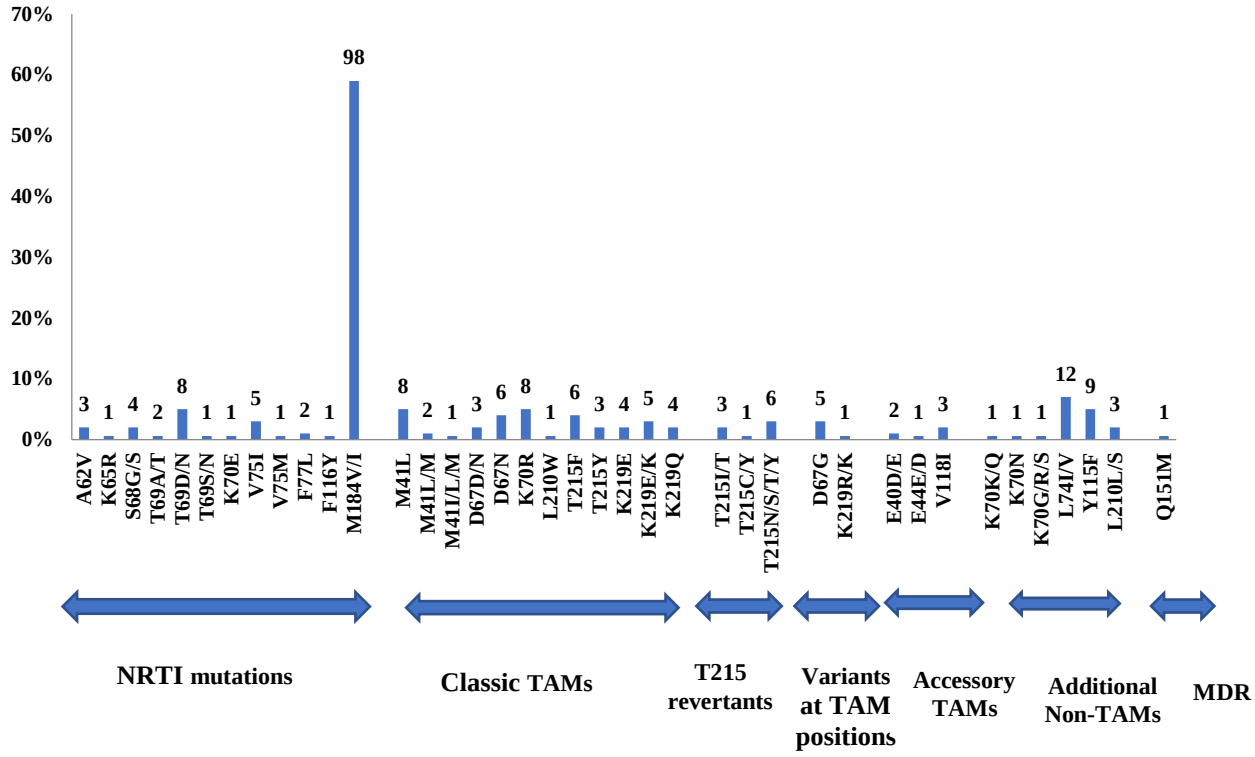
Figure 4.1 **Different mutations from children included in the study population to NRTIs (A), NNRTIs (B) and PIs (C)**

Figure 4.2 displays the mutations found among the different classes of ARVs from adolescents with detectable drug resistance. The most common NRTI mutation was M184V/I (59%), which was detected in 98 adolescents. Among the classic TAMs, the most common mutation was M41L (5%), which was detected in eight adolescents, followed by D67N (4%) which was detected in 6 adolescents. Less common TAMs were K70R (5%) and T215F (4%), which were detected in eight and six adolescents, respectively. A multidrug resistant Q151M complex was also detected in one adolescent. The presence of three or more TAMs was detected in nine adolescents (age range from 11 to 19 years).

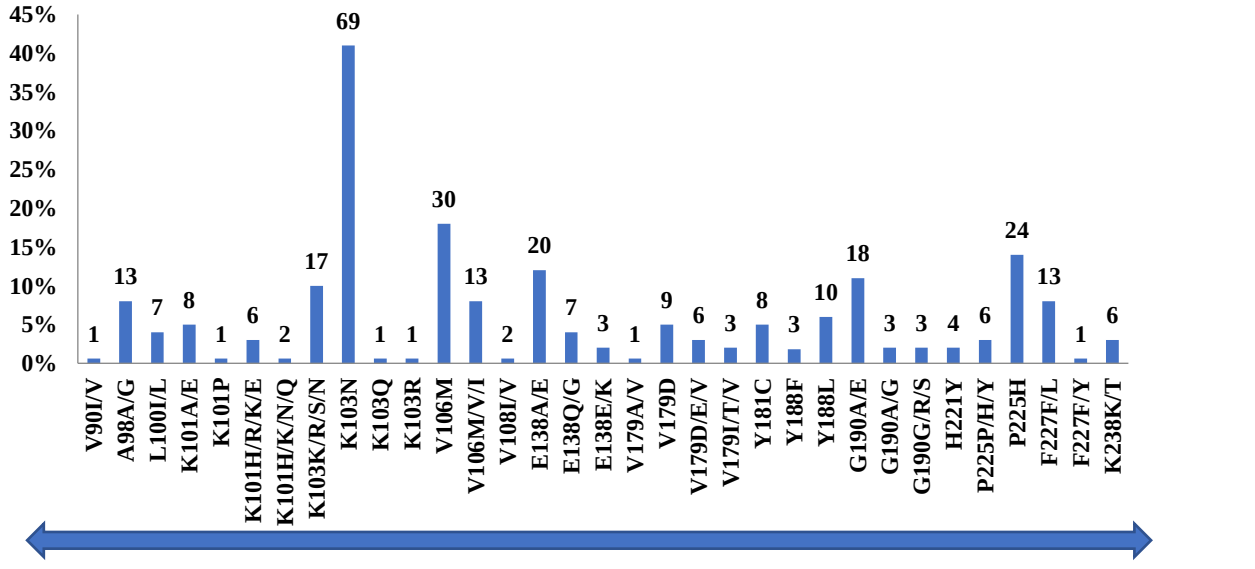
The most common NNRTI mutation among the adolescents was K103N (41%), which was detected in 69 adolescents. It was followed by V106M (18%) and P225H (14%), which were detected in 30 and 24 adolescents, respectively. The less common mutations, such as E138A/E (12%), G190A/E (11%) and K103K/R/S/N (10%) were detected in 20, 18 and 17 adolescents, respectively.

The most common PI mutation was I54I/V (9%), which was detected in 15 adolescents. It was followed by L10F (8%) and V82A (8%), which were detected in 14 adolescents each. The less

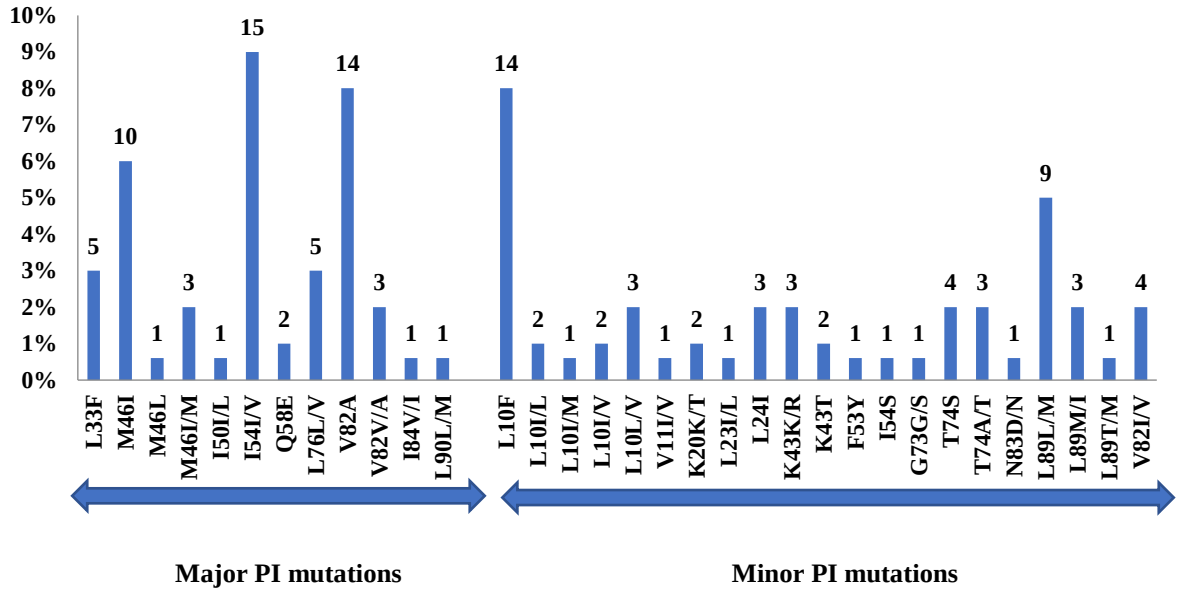
common mutations were M46I (6%) and L89L/M (5%) which were detected in 10 and 9 adolescents, respectively.



A



B



C

Figure 4.2 **Different mutations detected among adolescents included in the study population NRTIs (A), NNRTIs (B) and PIs (C)**

4.5 Discussion

The national roll out of ART has greatly reduced the morbidity and mortality associated with HIV infections (Boyer *et al.,* 2010). However, with the rapid scaling up of ARVs for children and adolescents within sub-Saharan Africa and South Africa in particular, establishing control strategies and surveillance programmes for the prevention of resistance remains a priority (Fokam *et al.,* 2011). Accessibility to these ARVs without adequate monitoring might result in an increase in both transmitted (TDR) and acquired drug resistant (ADR) HIV, thereby decreasing the effectiveness of commonly used ARVs (Fokam *et al.,* 2011). Drug resistance can be divided into ADR in treatment-experienced patients and TDR in treatment-naïve patients, both of which could result in treatment failure (Zou *et al.,* 2020). Treatment failure on ART is a result of sub-optimal treatment and non-adherence leading to increased viral replication in the presence of selected drug pressure resulting in the emergence of drug resistant HIV (Boyer *et al.,* 2010; Fokam *et al.,* 2011). Thus, routine VL monitoring and testing for drug resistance have been identified as essential services to prevent accumulation of drug resistance mutations and preserving HIV susceptibility to ARVs (Inzaule *et al.,* 2016). According to the current NDOH guidelines (2020), routine VL monitoring is recommended for children and adolescents on ART and virological failure on a NNRTI based regimen is

identified as a VL ≥ 1,000 copies/mL on two separate occasions after adherence issues have been addressed. In cases of confirmed virological failure on NNRTI based regimen, resistance testing is not recommended and children and adolescents are switched to PI or INI based regimens after discussion with an expert. PI and INI based regimens demonstrate a high genetic barrier and slow development of resistance. Drug resistance testing is therefore only recommended for children and adolescents on a PI and INI based regimen for at least two years with confirmed virological failure and supported by discussion with an expert.

In the current study, drug resistance mutations were characterised from children and adolescents experiencing virological failure while on ART. Among the samples collected for resistance testing (n=295) from children and adolescents, 3% (9/295) of the children and 9% (27/295) of adolescents were fully susceptible and no resistance was found in these samples. Among these 36 who did not have any detectable resistance to NRTIs, NNRTIs and PIs, 92% (33/36) experienced virological failure while on a PI based regimen. This indicates that non adherence contributed to the virological failure observed. This highlights the need for effective surveillance programmes to monitor adherence and provide adherence counselling, since the presence of virological failure does not necessarily indicate treatment failure or development of resistance among these children and adolescents. A number of systematic reviews analysing different factors associated with non-adherence in children, adolescents and adults have been published (Kim *et al.,* 2014; Hudelson and Cluver, 2015; Shubber *et al.,* 2016). These studies highlight a number of individual, interpersonal, community, environmental and structural factors, which become a barrier towards achieving adherence in children and adolescents. In addition, WHO has recommended the use of adherence counselling and reminder devices to improve adherence in these population groups (World Health Organisation, 2016).

Among the 88% (259/295) of samples with evidence of drug resistance, 36% (92/259) of the samples belonged to children, while 64% (167/259) of the samples belonged to adolescents. Among children with resistance, 28% (26/92), displayed PI resistance and 13 of the children had both major and minor PI mutations. The current regimen was a PI based for all children except one child which had no current regimen recorded. Among adolescents, 30% (51/167) of the adolescents displayed PI resistance and 21 of the adolescents had both major and minor PI mutations. The current regimen for these adolescents was PI based for 91% (46/51), four had no current regimen recorded and one was receiving a NNRTI based regimen. This highlights the possibility of acquired PI resistance among these children and adolescents.

However, some children might be infected with a primary drug resistant HIV from their mother and some adolescents might have been infected through sexual transmission, making it difficult to distinguish between primary and acquired drug resistant HIV in the absence of clinical records. However, previous PMTCT surveys have reported that the transmission of PI mutations from mother to child is rare in the current South African landscape (Hunt *et al.,* 2019).

Most of the children displayed resistance to NRTIs (86%), followed by NNRTIs (75%). However, among adolescents, resistance to NNRTIs (87%) was the most common, followed by NRTIs (66%). The high prevalence of NNRTI resistance among young children highlights the possibility of transmitted drug resistance from mother to child or acquiring drug resistance from PMTCT. Among most of the adolescents, the current regimen was PI based. However, the previous regimen for most of these adolescents (n=91) was efavirenz (EFV) or NVP based, indicating previous exposure to NNRTIs. According to the National Department of Health (2015b) guidelines, children between 3 and 10 years of age should be initiated on ABC+3TC+EFV, which was the first-line regimen for most of these adolescents (n=91).
Similarly, studies conducted in sub-Saharan Africa have reported a high prevalence of resistance to NNRTIs (88%), followed by NRTIs (80%) and PIs (54%) among children experiencing first-line virological failure (Sigaloff *et al.*, 2011a).

In the current study triple class resistance, i.e resistance to NNRTIs, NRTIs and PIs was detected in 18% of children and 19% of adolescents. Similarly, a study conducted by Collins *et al.* (2017) reported triple class resistance in 12% of adolescents infected with PHIV. Anderson *et al.* (2020) suggested that the risk of resistance increases with higher duration of ART and the presence of multidrug resistance has been reported from both high-income and low-income countries. However, a higher prevalence of NNRTI mutations among children and adolescents has been reported from high-income countries, which can be explained by the intensive PMTCT measures implemented in these settings.

Among children, the most common NRTI mutation was M184V (81%) detected in 75 children, displaying high level resistance to 3TC/emtricitabine (FTC) and low-level resistance to ABC and didanosine (ddl), while increasing susceptibility to ZDV, stavudine (d4T) and tenofovir (TDF), with impaired viral fitness. TAMs were detected in eleven children, who displayed D67N (6%), K70R (1%), K219Q/E (5%) and T215F (1%) indicating the TAM-2 pathway,

while the TAM-1 pathway was detected in eight of the children, including M41L (4%) and T215Y (1%). One child had three TAMs (D67N, K70R and K219E) detected, which compromises NRTI susceptibility. Similarly, M184V/I (59%) was the most common mutation detected in 98 adolescents. It was mostly selected in adolescents with previous regimen of 3TC + ABC + EFV and current regimen of 3TC + ZDV + LPV/r as guided by NDOH guidelines for adolescents experiencing first-line virological failure (National Department of Health, 2015b). While, M184V/I increases the susceptibility to ZDV, a number of TAMs known to cause reduced susceptibility to ZDV were detected among adolescents, resulting in little residual activity of NRTIs and largely relying on PIs. TAMs were detected in 26 adolescents, who displayed D67N (4%), K70R (5%), K219E (2%), K219Q (2%) and T215F (4%) indicating the TAM-2 pathway, while 19 of the adolescents harboured mutations in the TAM-1 pathway, such as M41L (5%), L210W (1%) and T215Y (2%). The presence of three or more TAMs was detected in 9 adolescents, resulting in compromised affectivity of NRTIs. One of the adolescents displayed the multidrug resistant Q151M (1%) complex. The frequency of K65R was very low in the current study, as none of the children harboured K65R, while only one adolescent displayed the mutation. The most common NNRTI mutation detected among children and adolescents was K103N, which was detected in 42 children and 69 adolescents.

Previous studies conducted in Southern Africa have reported as similar prevalence of M184V/I mutation (range 72% to 81%) compared with findings of the current study of 81% for children, although a lower prevalence [59%] was noted for adolescents (Hosseinipour *et al.,* 2009; Wallis *et al.,* 2010; Sigaloff *et al.,* 2012b).

The accumulation of multiple TAMs and complex resistance pathways among individuals experiencing virological failure causes reduced activity of NRTIs (Van Vaerenbergh *et al.,* 2000; Whitcomb *et al.,* 2003). Once cross resistance among NRTIs has developed, the PI based second-line regimens will offer the activity of the boosted PI with little effect of the NRTI backbone (Sigaloff *et al.,* 2012b). Furthermore, recent studies including the EARNEST trial have reported that the NRTI backbone has limited activity in the PI based second-line regimens and the presence of cross resistance to NRTIs does not clinically affect the outcome of the PI based regimen as long as the NRTIs are combined with a boosted PI (Paton *et al.,* 2017). High levels of PI resistance were detected among children (28% [26/92]) and adolescents (30%

[51/167]) in the current study, which might be due to the intermediate adherence frequently encountered in children and adolescents.

Interestingly, to reduce the development of resistance and increase the duration on ART for children, certain preventive measures have been undertaken to identify children that experience early virological failure as compared to children experiencing late virological failure (Huibers *et al.,* 2019). Early virological failure is most commonly detected among young children ≤ 3 years of age, with the presence of pre-treatment drug resistant (PDR) mutations and a high pre-ART viral load (Boerma *et al.,* 2017a, Boerma *et al.,* 2017b; Huibers *et al.,* 2019). Late virological failure is detected among older children > 3 years of age, with advanced WHO stage and a history of previous treatment switches (Dalhatu *et al.,* 2016; Huibers *et al.,* 2019). Poor adherence has been associated with both early and late virological failure but plays a more crucial role in young children experiencing early virological failure (Huibers *et al.,* 2019). In the current study, forty (43% [40/92]) of the children were ≤ 3 years of age and dual class resistance (NNRTI and NRTI) was detected in 19 (48% [19/40]) of these children, while triple class resistance was detected in seven children. These findings have implications for the clinical management of HIV infection among children and adolescents experiencing early or late virological failure.

The major limitation of the study was the unavailability of the composition and duration of current and previous treatment and PMTCT regimens, the maternal treatment history and the rout of infection, in the majority of study participants. In addition, pre-treatment drug resistance might have been underestimated since transmitted mutations or those acquired during PMTCT are archived in older children and cannot be distinguished from acquired mutations in the absence of clinical data.

In conclusion, limited information is available regarding the long-term treatment outcomes of perinatally and behaviourally acquired HIV among adolescents. Future studies are required to fill the knowledge gap and to provide better clinical management options for children and adolescents. Optimising adherence counselling, resistance surveillance studies and continued monitoring of children and adolescents are crucial to prevent the emergence of drug resistance mutations and limit the transmission of acquired resistance.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Conclusion

The use of antiretroviral therapy (ART) has significantly reduced the morbidity and mortality caused by the human immunodeficiency virus (HIV) epidemic in South Africa (Brumme and Poon, 2017). However, a major barrier to successful treatment is the emergence of drug resistant variants. These variants are a result of the lack of proof reading ability of the enzyme reverse transcriptase (RT) coupled with a high error rate of reverse transcription, resulting in the diverse population of HIV quasispecies within the first few weeks following infection. As a result of this rapid expansion of quasispecies, the infected individual may harbour variants that contain mutations which can confer resistance to antiretrovirals (ARVs) and may be present before the initiation of treatment. With the administration of ARVs, the drug pressure results in the selection of resistant variants in the presence of ongoing viral replication (Coffin, 1995). Previous studies have reported only partial inhibition of viral replication when exposed to mono or dual therapy with nucleoside or non-nucleoside reverse transcriptase inhibitors (NRTIs/NNRTIs) due to the presence of resistant variants (Rooke *et al.*, 1989; Shirasaka *et al.*, 1995). In contrast, the use of modern ART results in the complete inhibition of viral replication leading to undetectable viraemia (Brumme and Poon, 2017). The major barrier to successful therapy is partial adherence to ARVs.

Drug resistant HIV can be transmitted to drug naïve individuals, known as transmitted drug resistance (TDR), or can be acquired (ADR) due to the presence of selective drug pressure (Brumme and Poon, 2017). Both TDR and ADR have been associated with poorer virological response leading to treatment failure and earlier mortality. HIV drug resistance testing has therefore been recommended to select appropriate treatment regimens in individuals experiencing treatment failure. The aim of this study was therefore to determine and characterise drug resistance mutations in newly diagnosed HIV-infected children and in children and adolescents with virological failure on ART.

To detect drug resistance mutations in children and adolescents, the study developed and validated a Sanger assay for HIV drug resistance testing using dried blood spot (DBS) samples.

Plasma has been the specimen of choice for performing drug resistance testing but the use of plasma in not feasible in resource limited areas. Therefore, the use of DBS cards has been recommended for detecting resistance mutations in infants experiencing treatment failure. In addition, light weight of the cards, small quantity of blood required for preparation, ease of sample collection without phlebotomy and easy transport and storage at ambient temperature are some of the added advantages for performing DBS testing.

In this study, the DBS assay was validated against different parameters to ensure that the assay produce reliable results and was compared with an in-house plasma Sanger sequencing assay. These parameters included amplification sensitivity, linearity, accuracy, precision and reproducibility. The results indicated that similar major mutations were detected with both the DBS and plasma assays, although additional minor mutations were detected using the DBS assay. Since all the additional mutations found were minor with the majority present as mixtures, the interpretation of the resistance profiles were found to be identical using plasma and DBS samples. Hence, the discordant mutations had no clinical impact on the selection of treatment regimens for these patients. These validation results using samples from adults on ART are promising since previous studies have reported the presence of proviral deoxyribonucleic acid (DNA) in DBS samples, which causes discrepancies due to amplification and sequencing of HIV DNA (Ziemniak *et al.,* 2006; Masciotra *et al.,* 2007; Bertagnolio *et al.,* 2007; McNulty *et al.,* 2007; Hearps *et al.,* 2010). The presence of proviral DNA results in the detection of additional mutations from the archived virus and these mutations would be enhanced in an HIV-infected individual with advanced treatment history and treatment interruptions and may account for the additional minor mutations detected using the DBS assay in the current study.

Another major challenge associated with the use of DBS specimens is the small blood volume present on the card (Singh *et al.,* 2017). The lower volume of whole blood sample leads to a smaller quantity of ribonucleic acid (RNA) present in the sample, which could affect the sensitivity of the assay. However, in the present study high amplification sensitivity ($\geq$ 90%) was observed in samples with VL between 5,000 and $\geq$ 10,000 copies/mL. Hence, the use of DBS technology may revolutionise drug resistance genotyping among the paediatric population in resource limited settings.

The rate of transmission of HIV infection from mother to child (MTCT) has greatly decreased over the years due to the introduction of prevention of mother to child transmission (PMTCT) and early infant diagnosis (EID) services (Salou *et al.,* 2016). However, the prevalence of drug resistance among children has greatly increased particularly to the class of NNRTIs. Most of these children are known to have pre-treatment drug resistant (PDR) HIV, which was either acquired through MTCT, prophylaxis with nevirapine (NVP) as part of PMTCT or though breastfeeding. The present study aimed to determine the prevalence of PDR in HIV positive samples collected from the routine EID platform. Overall, PDR was detected among 73% of the children, with the majority of the children harbouring resistance to NNRTIs (72%). The findings were similar to national and international PMTCT surveys conducted previously (Goga *et al.,* 2012; Penazzato, 2013; Goga *et al.,* 2015; Goga *et al.,* 2016; Salou *et al.,* 2016; Fokam *et al.,* 2018). The national surveys indicated the prevalence of PDR among infants has increased from 37% to 68.7% between 2010 and 2014 (Goga *et al.,* 2012; Jordan *et al.,* 2017). This change has coincided with the change in the PMTCT policy from single dose of NVP to 6 weeks of NVP prophylaxis for HIV-exposed infants, which resulted in the increase in the prevalence of NNRTI resistance observed over the years.

The nationally representative South African PMTCT surveys reported that MTCT has decreased over the years, from 3.5% in 2010, to 2.7% in 2011-2012, 2.6% in 2012-2013, 1.1% in 2015-2016 and 0.9% in 2016-2017 measured as the *in utero* paediatric HIV infection rate per 100,000 live births (Goga *et al.,* 2012; Goga *et al.,* 2015; Goga *et al.,* 2016; Goga *et al.,* 2018; Hunt *et al.,* 2019). Due to PMTCT the risk of vertical infections has dramatically decreased, but the infants who do become infected and harbour drug resistance to ARVs being used for PMTCT prophylaxis (i.e NNRTIs) is increasing. This is relevant in terms of children which are being infected with a resistant strain (TDR) is reducing overall but the infants harbouring PDR to NNRTIs is increasing. Among the entire cohort, NVP resistance (66%) was the highest and almost all infants with resistance displayed resistance to NVP (91% 203/224). This was similar to other studies, which reported increased resistance to NVP among infants given extended exposure of NVP prophylaxis (60%) as compared to single dose of NVP (27%) (Hunt *et al.,* 2011; Salou *et al.,* 2016). In addition, overall dual class resistance i.e resistance to both NNRTIs and NRTIs (11% [33/308]) was the most prevalent, followed by NNRTIs + PIs (0.6% [2/308]). Triple class resistance (NNRTI+NRTI+PI) was very rare and was only detected in two infants. Among the NRTIs, the most common mutation was M184V/I (6%), which was detected in 18 infants and was followed by K65R (5%), which was detected

in 11 infants. Protease resistance was very rare among the infants and none of the infants displayed high-level resistance against any PI, which indicates the probability that infants would respond adequately to the current first line regimen recommended for HIV exposed infants within the paediatric management guidelines. The present study was limited due to the unavailability of maternal and infant treatment history and prophylaxis for the majority of samples, which makes it difficult to determine the source of drug resistance among infants i.e either through MTCT or through PMTCT given in the form of extended NVP prophylaxis.

In addition to TDR, the rate of ADR among infants and adolescents is increasing (Steegen *et al.,* 2014). This is worrisome, since it limits the future treatment options for infants who already have a history of resistance to NNRTIs (Steegen *et al.,* 2014). These infants with high PDR to NNRTIs often have a high pre-ART VL, which makes it difficult to achieve viral suppression resulting in high prevalence of early virological failure (Steegen *et al.,* 2014). In the present study, Sanger sequencing data were characterised from children and adolescents experiencing virological failure while on ART. The study population was divided into children (0 to 9) and adolescents (10 to 19) and their respective resistance profiles were reported. The results indicated that most of the children were resistant to NRTIs (86% [79/92]), followed by NNRTIs (75% [69/92]) and PIs (28% [26/92]). Among adolescents, prevalence of resistance to NNRTIs (87% [146/167]) was the highest, followed by NRTIs (66% [111/167]) and PIs (30% [51/167]). The high prevalence of NNRTI resistance among children can be explained by previous exposure to PMTCT prophylaxis; however, since the history of exposure to neonatal ART as PMTCT was not available, this could not be confirmed. This also highlights the possibility of children primarily being infected with NNRTI resistant HIV due to transmission of NNRTI resistant HIV from mother to child. However, since maternal records were not available, the maternal treatment history could not be confirmed.

M184V and K103N were the most common NRTI and NNRTI mutations detected among children (n=75, M184V and n=42, K103N) and adolescents (n=98, M184V and n=69, K103N). Most of the TAMs which were detected in children (n=11) and adolescents (n=26) indicated the TAM-2 pathway. This corresponded with previous studies conducted in Southern Africa, which highlighted the presence of multiple TAMs and the M184V mutation among patients failing first-line regimens (Novitsky *et al.,* 2007; Hosseinipour *et al.,* 2009; Wallis *et al.,* 2010; Sigaloff *et al.,* 2012b).

In conclusion, the prevalence of children and adolescents experiencing treatment failure due to the presence of transmitted and acquired resistance is increasing. Although the implementation of drug resistance testing plays a huge role in monitoring drug resistance prevalence, access to this essential service is limited in many resource limited settings. The lack of adherence to ARVs in many children and adolescents leads to higher mortality reported among these age groups. Furthermore, awareness is required for strengthening adherence strategies tailored to these particular vulnerable populations, which can result in the increased survival of HIV-infected infants, children and adolescents in South Africa.

5.2 Future perspectives

The world has embarked on a fast-track strategy to achieve the HIV/AIDS 90-90-90 treatment target set by the Joint United Nations Programme on HIV/AIDS (UNAIDS) to curb the spread of new HIV infections (Clutter *et al.,* 2016). The programme aims to diagnose 90% of people with HIV infection, with 90% of those with a positive diagnosis receiving antiretroviral therapy (ART) and 90% of people on ART achieving suppressed viral loads (UNAIDS, 2014). South Africa has been one of the countries with the largest epidemic of HIV and the country has adopted the UNAIDS treatment target to eliminate the burden of HIV infections (UNAIDS, 2014). In support of the treatment targets set by UNAIDS, the World Health Organisation (WHO) has recommended that ART must be initiated immediately after the diagnosis as part of the "treat all" option and pre-exposure prophylaxis (PrEP) must be considered in individuals with a high risk of acquiring HIV infection (World Health Organisation, 2016). While the adoption of these recommendations will reduce the number of new HIV infections, they will however most likely increase the prevalence of ADR in treated individuals and TDR in newly diagnosed individuals (Phillips *et al.,* 2014). Effective implementation of the programme would likely also control the spread of new HIV infections in the most vulnerable populations, such as infants, children and adolescents.

The fact that newly-diagnosed infants are becoming infected with HIV due to failing PMTCT is problematic, since these infections are preventable and it increases the risk of acquiring drug resistant variants due to continuous selective pressure of ARVs (Inzaule *et al.,* 2018a). Due to PMTCT the risk of vertical infections has dramatically decreased, but the infants who do become infected are more likely to harbour PDR to NNRTIs. This is particularly worrisome in infants, since infants need treatment for a longer period of time as compared to adults and

the presence of PDR can limit the available treatment options, which can lead to treatment and virological failure and eventually increased mortality. This requires the implementation of routine and robust nationally representative surveys to continuously assess the rate of PDR in HIV-infected individuals starting ART, irrespective of their previous exposure to ARVs (Gupta *et al.,* 2018). The PDR surveys should be conducted among all provinces with special reference to infants to highlight the current rate of PDR in this specific population. Furthermore, the introduction of dolutegravir (DTG) will likely have a huge impact on the prevention of MTCT of HIV and further preventing PDR (infants infected primarily with NNRTI drug resistant HIV) in infants where the mother is on a DTG based regimen. The use of DTG in pregnant women will provide rapid viral suppression and a high genetic barrier to resistance, resulting in increased viral suppression. However, DTG may increase the risk of neural tube defects (NTD) in infants and therefore is not recommended preconception and in the first 6 weeks of pregnancy (National Department of Health, 2020). A stable pregnant woman on ART is only switched from EFV to DTG if 1) VL < 50 copies/mL and 2) no longer in first 6 weeks of pregnancy (National Department of Health, 2020).

Management of HIV in paediatric patients is evolving rapidly with the introduction of new ARVs to better manage treatment and virological failure among this vulnerable population (Penazzato *et al.*, 2018). However most of these ARVs are not approved for use in infants due to lack of clinical trials to determine the long term side effects of using ART. Adherence is difficult to achieve in the paediatric population (Boucher *et al.,* 2018). Research should be conducted in developing new ARVs, which have 1) low-pill burden and available paediatric formulations, 2) lower toxicity, 3) high genetic barrier and 4) higher half-life for specific use in the paediatric population. Clinical trials should be conducted to determine the optimal dosing of such ARVs for their usage in children of different ages (and weight) to ensure the appropriate use in this population (Hazra *et al.,* 2010). These infants should be followed up as they transition into adolescence to determine the effect of treatment of HIV on a developing child (Hazra *et al.,* 2010). ARVs, such as DTG have been proven to have a higher genetic barrier to resistance with a longer half life and are currently being investigated for usage in young children.

Drug resistance is an inevitable consequence of the use of ARVs to reduce morbidity and mortality in the HIV-infected population (Clutter *et al.,* 2016). While concern for ARV drug resistance has been rising, it should never limit the access to ARVs in HIV-infected individuals

(Clutter *et al.*, 2016). However, earlier initiation of ART, continuous adherence and the mechanisms and pathways for drug resistance mutations to cause resistance to a specific ARV and cross resistance to other ARVs of the same class needs to be monitored closely. In high-income countries, drug resistance testing has been implemented as part of the routine care to select a better treatment regimen for initiation of ART in HIV-infected individuals (Clutter *et al.*, 2016). However, in resource limited settings, such as South Africa, drug resistance testing has only been implemented after treatment failure has been reported, to guide the response for second or third line regimens due to feasibility, logistics and cost constraints (Clutter *et al.*, 2016). Drug resistance testing is therefore only recommended for children and adolescents on a PI and INI based regimen over a course of two years, with confirmed virological failure and accompanied by discussion with an expert (National Department of Health, 2020).

Future research should be conducted to design a better coordinated and cost-effective response to implement the use of drug resistance testing before the initiation of ART in HIV-infected individuals with special reference to newly-diagnosed infants. Scaling up of drug resistance testing will lead to better treatment outcomes and will be useful in developing appropriate treatment regimens, better treatment algorithms and managing of particularly complex resistance mechanisms in infected individuals (Hamers *et al.*, 2018).

Since the preparation of DBS cards require a small volume of whole blood sample and provide for easier transportation and storage, the cards have been considered as a better alternative to perform drug resistance testing in infants failing treatment regimens (Zhang *et al.*, 2018). However, some studies have reported poor concordance between plasma and DBS cards due to the presence of provirus and lack of RNA integrity (Dickover *et al.*, 1998; Parry *et al.*, 2014). Future research should be directed into developing pre-extraction steps for processing DBS samples to improve the sensitivity of the assays. Additionally, there is a need for developing, standardizing and validating future sensitive assays for performing drug resistance genotyping using DBS.

Sanger sequencing has provided essential insights into drug resistance testing which has been used in resource limited settings. However, the disadvantage of this sequencing technology is its inability to detect minority variants which are present at below 20-30% of the viral population (Halvas *et al.*, 2006). Next generation sequencing technology has therefore been developed to detect these minority variants to as low as 1% but the clinical significance of these

low frequency variants is still unclear (Shao *et al.,* 2013; Clutter *et al.,* 2016). In addition, the technology offers great advantages, such as automated sample processing, advanced sequencing technology, and generation of large amounts of information. The substantial information generated from NGS enables better understanding of mutational patterns present under a selective ART combination (Eriksson *et al.,* 2008; Poon *et al.,* 2010; Willerth *et al.,* 2010). Future research should therefore investigate the usage of NGS in infants failing second or third line treatment regimens to better understand the mutation populations present. This will make it easier to develop better treatment regimens and algorithms to treat these infants with an extensive previous history of ART.

To conclude, the drug resistance genotyping laboratories need to develop a longitudinal centralised database to record the treatment regimens and their associated resistance profiles in HIV-infected individuals experiencing treatment failure. These drug resistance laboratories should be well co-ordinated to provide input on the relevant and recent resistance patterns and mutational profiles obtained from patients failing therapy. The database should be kept up to date with the demographics, patient history, previous treatment regimens, current treatment regimens, viral load profile, CD4 count and presence of all the resistance mutations and associated resistance profiles detected in an individual. The information generated from the database can be used to monitor HIV-infected individuals with a previous history of drug resistance and treatment failure. Finally, the information generated can be used for routine surveillance of population level HIV drug resistance genotyping combined with better approaches to prevent the development of additional resistance in the population which will be essential in the attainment of the goal to eliminate AIDS as a public threat.

References

Abram, M.E., Ferris, A.L., Shao, W., Alvord, W.G. and Hughes, S.H. (2010). Nature, position, and frequency of mutations made in a single cycle of HIV-1 replication. *Journal of Virology*, *84*(19), pp.9864-9878.

Abrams, E.J., Weedon, J., Steketee, R.W., Lambert, G., Bamji, M., Brown, T., Kalish, M.L., Schoenbaum, E.E., Thomas, P.A., Thea, D.M. and New York City Perinatal HIV Transmission Collaborative Study Group (1998). Association of human immunodeficiency virus (HIV) load early in life with disease progression among HIV-infected infants. *Journal of Infectious Diseases*, *178*(1), pp.101-108.

Aghokeng, A.F., Monleau, M., Eymard-Duvernay, S., Dagnra, A., Kania, D., Ngo-Giang-Huong, N., Toni, T.D., Touré-Kane, C., Truong, L.X., Delaporte, E. and Chaix, M.L. (2013). Extraordinary heterogeneity of virological outcomes in patients receiving highly antiretroviral therapy and monitored with the World Health Organization public health approach in sub-saharan Africa and southeast Asia. *Clinical Infectious Diseases*, *58*(1), pp.99-109.

Amellal, B., Murphy, R., Maiga, A., Brucker, G., Katlama, C., Calvez, V. and Marcelin, A.G. (2008). Stability of HIV RNA in plasma specimens stored at different temperatures. *HIV Medicine*, *9*(9), pp.790-793.

Anderson, K., Muloiwa, R. and Davies, M.A. (2020). Long-term outcomes in perinatally HIV-infected adolescents and young adults on antiretroviral therapy: a review of South African and global literature. *African Journal of AIDS Research*, *19*(1), pp.1-12.

Ayouba, A., Souquières, S., Njinku, B., Martin, P.M., Müller-Trutwin, M.C., Roques, P., Barré-Sinoussi, F., Mauclère, P., Simon, F. and Nerrienet, E. (2000). HIV-1 group N among HIV-1-seropositive individuals in Cameroon. *AIDS*, *14*(16), pp.2623-2625.

Barth, R.E., Van Der Loeff, M.F.S., Schuurman, R., Hoepelman, A.I. and Wensing, A.M. (2010). Virological follow-up of adult patients in antiretroviral treatment programmes in sub-Saharan Africa: a systematic review. *The Lancet Infectious Diseases*, *10*(3), pp.155-166.

Barth, R.E., Tempelman, H.A., Smelt, E., Wensing, A.M., Hoepelman, A.I. and Geelen, S.P. (2011). Long-term outcome of children receiving antiretroviral treatment in rural South Africa: substantial virologic failure on first-line treatment. *The Pediatric Infectious Disease Journal*, *30*(1), pp.52-56.

Battini, L. and Bollini, M. (2019). Challenges and approaches in the discovery of human immunodeficiency virus type-1 non-nucleoside reverse transcriptase inhibitors. *Medicinal Research Reviews*, *39*(4), pp.1235-1273.

Baxter, J.D., Mayers, D.L., Wentworth, D.N., Neaton, J.D., Hoover, M.L., Winters, M.A., Mannheimer, S.B., Thompson, M.A., Abrams, D.I., Brizz, B.J. and Ioannidis, J.P. (2000). A randomized study of antiretroviral management based on plasma genotypic antiretroviral resistance testing in patients failing therapy. *AIDS*, *14*(9), pp.83-93.

Bbosa, N., Kaleebu, P. and Ssemwanga, D. (2019). HIV subtype diversity worldwide. *Current Opinion in HIV and AIDS*, *14*(3), pp.153-160.

Bennett, D.E. (2006). The requirement for surveillance of HIV drug resistance within antiretroviral rollout in the developing world. *Current Opinion in Infectious Diseases*, *19*(6), pp.607-614.

Bertagnolio, S., Soto-Ramirez, L., Pilon, R., Rodriguez, R., Viveros, M., Fuentes, L., Harrigan, P.R., Mo, T., Sutherland, D. and Sandstrom, P. (2007). HIV-1 drug resistance surveillance using dried whole blood spots. *Antiviral Therapy*, *12*(1), pp.107-113.

Bertagnolio, S., Parkin, N.T., Jordan, M., Brooks, J. and García-Lerma, J.G. (2010). Dried blood spots for HIV-1 drug resistance and viral load testing: A review of current knowledge and WHO efforts for global HIVDR surveillance. *AIDS Reviews*, *12*(4), pp.195-208.

Bester, P.A., De Vries, A., Riekert, S., Steegen, K., Van Zyl, G. and Goedhals, D. (2019). PhyloPi: an affordable, purpose built phylogenetic pipeline for the HIV drug resistance testing facility. *PloS One*, *14*(3), pp.e0213241. DOI: https://doi.org/10.1371/journal.pone. 0213241 (Accessed April 2019).

Beyrer, C., Sullivan, P., Sanchez, J., Baral, S.D., Collins, C., Wirtz, A.L., Altman, D., Trapence, G. and Mayer, K. (2013). The global HIV epidemics in men who have sex with men (MSM): time to act. *AIDS*, *27*(1), pp.2665-2678.

Birrell, P.J., Gill, O.N., Delpech, V.C., Brown, A.E., Desai, S., Chadborn, T.R., Rice, B.D. and De Angelis, D. (2013). HIV incidence in men who have sex with men in England and Wales 2001–10: a nationwide population study. *The Lancet Infectious Diseases*, *13*(4), pp.313-318.

Bock, P., Boulle, A., White, C., Osler, M. and Eley, B. (2008). Provision of antiretroviral therapy to children within the public sector of South Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *102*(9), pp.905-911.

Boender, T.S., Hoenderboom, B.M., Sigaloff, K.C., Hamers, R.L., Wellington, M., Shamu, T., Siwale, M., Labib Maksimos, E.E., Nankya, I., Kityo, C.M. and Adeyemo, T.A. (2015). Pretreatment HIV drug resistance increases regimen switches in sub-Saharan Africa. *Clinical Infectious Diseases*, *61*(11), pp.1749-1758.

Boerma, R.S., Sigaloff, K.C., Akanmu, A.S., Inzaule, S., Boele van Hensbroek, M., Rinke de Wit, T.F. and Calis, J.C. (2016). Alarming increase in pretreatment HIVDR in children living in sub-Saharan Africa: a systematic review and meta-analysis. *Journal of Antimicrobial Chemotherapy*, *72*(2), pp.365-371.

Boerma, R.S., Bunupuradah, T., Dow, D., Fokam, J., Kariminia, A., Lehman, D., Kityo, C., Musiime, V., Palumbo, P., Schoffelen, A. and Sophan, S. (2017a). Multicentre analysis of second-line antiretroviral treatment in HIV-infected children: adolescents at high risk of failure. *Journal of the International AIDS Society*, *20*(1). DOI: https://doi.org/10.7448/IAS.20.1.21930 (Accessed August 2020).

Boerma, R.S., Kityo, C., Boender, T.S., Kaudha, E., Kayiwa, J., Musiime, V., Mukuye, A., Kiconco, M., Nankya, I., Nakatudde, L. and Mugyenyi, P.N. (2017b). Second-line HIV treatment in Ugandan children: favorable outcomes and no protease inhibitor resistance. *Journal of Tropical Pediatrics*, *63*(2), pp.135-143.

Booth, C.L. and Geretti, A.M. (2007). Prevalence and determinants of transmitted antiretroviral drug resistance in HIV-1 infection. *Journal of Antimicrobial Chemotherapy*, *59*(6), pp.1047-1056.

Boucher, C.A., Bobkova, M.R., Geretti, A.M., Hung, C.C., Kaiser, R., Marcelin, A.G., Streinu-Cercel, A., Dorr, P. and Vandamme, A.M. (2018). State of the antiretroviral therapy in human immunodeficiency virus drug resistance: science and technology knowledge gap. *AIDS Reviews*, *20*(1), pp.26-41.

Boyer, S., Eboko, F., Camara, M., Abé, C., Nguini, M.E.O., Koulla-Shiro, S. and Moatti, J.P. (2010). Scaling up access to antiretroviral treatment for HIV infection: the impact of decentralization of healthcare delivery in Cameroon. *AIDS*, *24*, pp.5-15. DOI: 10.1097/01.aids.0000366078.45451.46 (Accessed August 2020).

Bratholm, C., Johannessen, A., Naman, E., Gundersen, S.G., Kivuyo, S.L., Holberg-Petersen, M., Ormaasen, V. and Bruun, J.N. (2010). Drug resistance is widespread among children who receive long-term antiretroviral treatment at a rural Tanzanian hospital. *Journal of Antimicrobial Chemotherapy*, *65*(9), pp.1996-2000.

Brenner, B., Turner, D., Oliveira, M., Moisi, D., Detorio, M., Carobene, M., Marlink, R.G., Schapiro, J., Roger, M. and Wainberg, M.A. (2003). A V106M mutation in HIV-1 clade C viruses exposed to efavirenz confers cross-resistance to non-nucleoside reverse transcriptase inhibitors. *AIDS*, *17*(1), pp.F1-F5.

Brown, K., Williams, D.B., Kinchen, S., Saito, S., Radin, E., Patel, H., Low, A., Delgado, S., Mugurungi, O., Musuka, G. and Barr, B.A.T. (2018). Status of HIV epidemic control among adolescent girls and young women aged 15–24 years-seven African countries, 2015-2017. *Morbidity and Mortality Weekly Report*, *67*(1), pp.29-32.

Bruisten, S.M., Reiss, P., Loeliger, A.E., Van Swieten, P.E.T.E.R., Schuurman, R., Boucher, C.A., Weverling, G.J. and Huisman, J.G. (1998). Cellular proviral HIV type 1 DNA load persists after long-term RT-inhibitor therapy in HIV type 1 infected persons. *AIDS Research and Human Retroviruses*, *14*(12), pp.1053-1058.

Brumme, C.J. and Poon, A.F. (2017). Promises and pitfalls of Illumina sequencing for HIV resistance genotyping. *Virus Research, 239,* pp.97-105. DOI: https://doi.org/10.1016/j .virusres.2016.12.008 (Accessed April 2019).

Bunupuradah, T., Sricharoenchai, S., Hansudewechakul, R., Klinbuayaem, V., Teeraananchai, S., Wittawatmongkol, O., Akarathum, N., Prasithsirikul, W., Ananworanich, J. and Pediatric PROGRESS Study Team (2015). Risk of first-line antiretroviral therapy failure in HIV-infected Thai children and adolescents. *The Pediatric Infectious Disease Journal*, *34*(3), pp.58-62.

Buonaguro, L., Tornesello, M.L. and Buonaguro, F.M. (2007). Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications. *Journal of Virology*, *81*(19), pp.10209-10219.

Bygrave, H., Mtangirwa, J., Ncube, K., Ford, N., Kranzer, K. and Munyaradzi, D. (2012). Antiretroviral therapy outcomes among adolescents and youth in rural Zimbabwe. *PloS One*, *7*(12), pp.52856. DOI: 10.1371/journal.pone.0052856 (Accessed May 2020).

Calmy, A., Ford, N., Hirschel, B., Reynolds, S.J., Lynen, L., Goemaere, E., De La Vega, F.G., Perrin, L. and Rodriguez, W. (2007). HIV viral load monitoring in resource-limited regions: optional or necessary? *Clinical Infectious Diseases*, *44*(1), pp.128-134.

Carr, J.K., Foley, B.T., Leitner, T., Salminen, M., Korber, B. and McCutchan, F. (1998). Reference sequences representing the principal genetic diversity of HIV-1 in the pandemic. *Human Retroviruses and AIDS*, *111*(10). Available from: https://www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/1998/III/Carr.pdf (Accessed November, 2020).

Chakrabarti, L., Guyader, M., Alizon, M., Daniel, M.D., Desrosiers, R.C., Tiollais, P. and Sonigo, P. (1987). Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. *Nature*, *328*(6130), pp.543-547.

Chen, T.K. and Aldrovandi, G.M. (2008). Review of HIV antiretroviral drug resistance. *The Pediatric Infectious Disease Journal*, *27*(8), pp.749-752.

Chiappini, E., Galli, L., Tovo, P.A., Gabiano, C., Lisi, C., Giacomet, V., Bernardi, S., Esposito, S., Rosso, R., Giaquinto, C. and Badolato, R. (2012). Antiretroviral use in Italian children with perinatal HIV infection over a 14-year period. *Acta Paediatrica*, *101*(7), pp.287-295.

Chimukangara, B., Lessells, R.J., Rhee, S.Y., Giandhari, J., Kharsany, A.B., Naidoo, K., Lewis, L., Cawood, C., Khanyile, D., Ayalew, K.A. and Diallo, K. (2019). Trends in pretreatment HIV-1 drug resistance in antiretroviral therapy-naive adults in South Africa, 2000–2016: a pooled sequence analysis. *EClinicalMedicine*, *9*, pp.26-34. DOI: https://doi.org/10.1016/j.eclinm.2019.03.006 (Accessed November 2019).

Chun, T.W., Carruth, L., Finzi, D., Shen, X., DiGiuseppe, J.A., Taylor, H., Hermankova, M., Chadwick, K., Margolick, J., Quinn, T.C. and Kuo, Y.H. (1997). Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature*, *387*(1), pp.183-188.

Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M.A., Santos-Ferreira, M.O., Laurent, A.G., Dauguet, C., Katlama, C. and Rouzioux, C. (1986). Isolation of a new human retrovirus from West African patients with AIDS. *Science*, *233*(4761), pp.343-346.

Clavel, F. and Hance, A.J. (2004). HIV drug resistance. *New England Journal of Medicine*, *350*(10), pp.1023-1035.

Clevenbergh, P., Durant, J., Halfon, P., Del Giudice, P., Mondain, V., Montagne, N., Schapiro, J.M., Boucher, C.A. and Dellamonica, P. (2000). Persisting long-term benefit of genotype-guided treatment for HIV-infected patients failing HAART. The Viradapt Study: week 48 follow-up. *Antiviral Therapy*, *5*(1), pp.65-70.

Clutter, D.S., Jordan, M.R., Bertagnolio, S. and Shafer, R.W. (2016). HIV-1 drug resistance and resistance testing. *Infection, Genetics and Evolution*, *46*, pp.292-307. DOI: https://doi.org /10.1016/j.meegid.2016.08.031 (Accessed May 2020).

Cockerill, F.R. (1999). Genetic methods for assessing antimicrobial resistance. *Antimicrobial Agents and Chemotherapy*, *43*(2), pp.199-212.

Coetzee, D., Hilderbrand, K., Boulle, A., Draper, B., Abdullah, F. and Goemaere, E. (2005). Effectiveness of the first district-wide programme for the prevention of mother-to-child transmission of HIV in South Africa. *Bulletin of the World Health Organization*, *83*(7), pp.489-494.

Coffin, J.M. (1995). HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. *Science*, *267*(5197), pp.483-489.

Collins, I.J., Foster, C., Tostevin, A., Tookey, P., Riordan, A., Dunn, D., Gibb, D.M., Judd, A. and Collaborative HIV Paediatric Study (CHIPS) Steering Committee (2017). Clinical status of adolescents with perinatal HIV at transfer to adult care in the UK/Ireland. *Clinical Infectious Diseases*, *64*(8), pp.1105-1112.

Costin, J.M. (2007). Cytopathic mechanisms of HIV-1. *Virology Journal*, *4*(100). DOI: 10.1186/1743-422X-4-100. (Accessed June 2017).

Coutsoudis, A., Pillay, K., Spooner, E., Kuhn, L., Coovadia, H.M. and South African Vitamin A Study Group. (1999). Influence of infant-feeding patterns on early mother-to-child transmission of HIV-1 in Durban, South Africa: a prospective cohort study. *The Lancet*, *354*(9177), pp.471-476.

Crowley, T., van der Merwe, A., Kidd, M. and Skinner, D. (2020). Adolescent human immunodeficiency virus self-management: associations with treatment adherence, viral suppression, sexual risk behaviours and health-related quality of life. *Southern African Journal of HIV Medicine*, *21*(1), pp.1054. DOI: 10.4102/sajhivmed.v21i1.1054 (Accessed November 2020).

Dabis, F., Bequet, L., Ekouevi, D.K., Viho, I., Rouet, F., Horo, A., Sakarovitch, C., Becquet, R., Fassinou, P., Dequae-Merchadou, L. and Welffens-Ekra, C. (2005). Field efficacy of zidovudine, lamivudine and single-dose nevirapine to prevent peripartum HIV transmission. *AIDS*, *19*(3), pp.309-318.

Dalhatu, I., Onotu, D., Odafe, S., Abiri, O., Debem, H., Agolory, S., Shiraishi, R.W., Auld, A.F., Swaminathan, M., Dokubo, K. and Ngige, E. (2016). Outcomes of Nigeria's HIV/AIDS

treatment program for patients initiated on antiretroviral treatment between 2004-2012. *PloS One*, *11*(11), pp.e0165528. DOI: https://doi.org/10.1371/journal.pone.0165528 (Accessed August 2020).

Davies, M.A., Moultrie, H., Eley, B., Rabie, H., Van Cutsem, G., Giddy, J., Wood, R., Technau, K., Keiser, O., Egger, M., Boulle, A. and for the International epidemiologic Databases to Evaluate Aids Southern Africa (IeDEA-SA) collaboration (2011). Virologic failure and second-line antiretroviral therapy in children in South Africa-The IeDEA Southern Africa Collaboration. *Journal of Acquired Immune Deficiency Syndromes*, *56*(3), pp.270-278.

DeGruttola, V., Dix, L., D'Aquila, R., Holder, D., Phillips, A., Ait-Khaled, M., Baxter, J., Clevenbergh, P., Hammer, S., Harrigan, R. and Katzenstein, D. (2000). The relation between baseline HIV drug resistance and response to antiretroviral therapy: re-analysis of retrospective and prospective studies using a standardized data analysis plan. *Antiviral Therapy*, *5*(1), pp.41-48.

Delaugerre, C., Chaix, M.L., Blanche, S., Warszawski, J., Cornet, D., Dollfus, C., Schneider, V., Burgard, M., Faye, A., Mandelbrot, L. and Tubiana, R. (2009). Perinatal acquisition of drug-resistant HIV-1 infection: mechanisms and long-term outcome. *Retrovirology*, *6*(1), pp.85. DOI: 10.1186/1742-4690-6-85 (Accessed November 2019).

Derache, A., Shin, H.S., Balamane, M., White, E., Israelski, D., Klausner, J.D., Freeman, A.H. and Katzenstein, D. (2015). HIV drug resistance mutations in proviral DNA from a community treatment program. *PLoS One*, *10*(1), p.e0117430. DOI: https://doi.org /10.1371/journal.pone. 0117430 (Accessed April 2019).

Dickover, R.E., Herman, S.A., Saddiq, K., Wafer, D., Dillon, M. and Bryson, Y.J. (1998). Optimization of specimen-handling procedures for accurate quantitation of levels of human immunodeficiency virus RNA in plasma by reverse transcriptase PCR. *Journal of Clinical Microbiology*, *36*(4), pp.1070-1073.

Durant, J., Clevenbergh, P., Halfon, P., Delgiudice, P., Porsin, S., Simonet, P., Montagne, N., Boucher, C.A.B., Schapiro, J.M. and Dellamonica, P. (1999). Drug-resistance genotyping in

HIV-1 therapy: the VIRADAPT randomised controlled trial. *The Lancet*, *353*(9171), pp.2195-2199.

Ekici, H., Rao, S.D., Sönnerborg, A., Ramprasad, V.L., Gupta, R. and Neogi, U. (2014). Cost-efficient HIV-1 drug resistance surveillance using multiplexed high-throughput amplicon sequencing: implications for use in low-and middle-income countries. *Journal of Antimicrobial Chemotherapy*, *69*(12), pp.3349-3355.

Emerman, M. and Malim, M.H. (1998). HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. *Science*, *280*(5371), pp.1880-1884.

Eriksson, N., Pachter, L., Mitsuya, Y., Rhee, S.Y., Wang, C., Gharizadeh, B., Ronaghi, M., Shafer, R.W. and Beerenwinkel, N. (2008). Viral population estimation using pyrosequencing. *PLoS Computational Biology*, *4*(5), pp.e1000074. DOI: 10.1371/journal .pcbi.1000074. (Accessed February 2020).

Escaich, S., Ritter, J., Rougier, P., Lepot, D., Lamelin, J.P., Sepetjan, M. and Trepo, C. (1992). Relevance of the quantitative detection of HIV proviral sequences in PBMC of infected individuals. *AIDS Research and Human Retroviruses*, *8*(10), pp.1833-1837.

Etta, E.M., Mavhandu, L., Manhaeve, C., McGonigle, K., Jackson, P., Rekosh, D., Hammarskjold, M.L., Bessong, P.O. and Tebit, D.M. (2017). High level of HIV-1 drug resistance mutations in patients with unsuppressed viral loads in rural northern South Africa. *AIDS Research and Therapy*, *14*(1), pp.36. DOI: 10.1186/s12981-017-0161-z (Accessed November, 2020).

European AIDS Clinical Society. (2019). Guidelines. Available from: https://www.eacsociety.org/files/2019_guidelines-10.0_final.pdf (Accessed November 2020).

European Centre for Disease Prevention and Control. (2019). Developing a reporting system for the surveillance of HIV drug resistance in Europe. Available from: https://www.ecdc.europa.eu/sites/portal/files/documents/HIV-drug-resistance-surveillance-system-web.pdf (Accessed December 2020).

Fanales-Belasio, E., Raimondo, M., Suligoi, B. and Buttò, S. (2010). HIV virology and pathogenetic mechanisms of infection: a brief overview. *Annali Dell'istituto Superiore di Sanita*, *46*(1), pp.5-14.

Fitzgerald, F., Penazzato, M. and Gibb, D. (2013). Development of antiretroviral resistance in children with HIV in low-and middle-income countries. *The Journal of Infectious Diseases*, *207*(2), pp.85-92.

Fokam, J., Salpini, R., Santoro, M.M., Cento, V., Perno, C.F., Colizzi, V., Ndumbe, P.M., Ntungen, C.F., Tetang, S.M.N., Nanfack, A.J. and Komego, D.A.T. (2011). Drug resistance among drug-naive and first-line antiretroviral treatment-failing children in Cameroon. *The Pediatric Infectious Disease Journal*, *30*(12), pp.1062-1068.

Fokam, J., Billong, S.C., ZK, B.A.C., Kembou, E., Milenge, P., Abessouguie, I., Nkwescheu, A.S., Tsomo, Z., Aghokeng, A.F., Ngute, G.D. and Ndumbe, P.M. (2013). Declining trends in early warning indicators for HIVDR in Cameroon from 2008–2010: lessons and challenges for low-resource settings. *BMC Public Health*, *13*(1), pp.308. DOI: 10.1186/1471-2458-13-308 (Accessed November 2019).

Fokam, J., Bellocchi, M.C., Armenia, D., Nanfack, A.J., Carioti, L., Continenza, F., Takou, D., Temgoua, E.S., Tangimpundu, C., Torimiro, J.N. and Koki, P.N. (2018). Next-generation sequencing provides an added value in determining drug resistance and viral tropism in Cameroonian HIV-1 vertically infected children. *Medicine*, *97*(13), pp.0176. DOI: 10.1097/MD.0000000000010176 (Accessed November 2019).

Fokam, J., Sosso, S.M., Yagai, B., Billong, S.C., Mbadie, R.E.D., Simo, R.K., Edimo, S.V., Nka, A.D., Ayissi, A.T., Yimga, J.F. and Takou, D. (2019). Viral suppression in adults, adolescents and children receiving antiretroviral therapy in Cameroon: adolescents at high risk of virological failure in the era of "test and treat". *AIDS Research and Therapy*, *16*(1), pp.1-8.

Gao, F., Bailes, E., Robertson, D.L., Chen, Y., Rodenburg, C.M., Michael, S.F., Cummins, L.B., Arthur, L.O., Peeters, M., Shaw, G.M. and Sharp, P.M. (1999). Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. *Nature*, *397*(6718), pp.436-441.

Garcia, J.A. and Gaynor, R.B. (1994). Regulatory mechanisms involved in the control of HIV-1 gene expression. *AIDS*, *8*(1), pp.3-17.

Garcia, P.M., Kalish, L.A., Pitt, J., Minkoff, H., Quinn, T.C., Burchett, S.K., Kornegay, J., Jackson, B., Moye, J., Hanson, C. and Zorrilla, C. (1999). Maternal levels of plasma human immunodeficiency virus type 1 RNA and the risk of perinatal transmission. *New England Journal of Medicine*, *341*(6), pp.394-402.

Gardner, E.M., Burman, W.J., Steiner, J.F., Anderson, P.L. and Bangsberg, D.R. (2009). Antiretroviral medication adherence and the development of class-specific antiretroviral resistance. *AIDS*, *23*(9), pp.1035-1046.

Goga, A., Dinh, T., Jackson, D. and for the SAPMTCTE study group (2012). Evaluation of the effectiveness of the national prevention of mother-to-child transmission (PMTCT) programme measured at six weeks postpartum in South Africa, 2010. South African Medical Research Council, National Department of Health of South Africa and PEPFAR/US Centers for Disease Control and Prevention. Available from: http://www.mrc.ac.za/sites/default /files/files/2016-07-12/SAPMTCTE2010.pdf (Accessed January 2020).

Goga, A.E., Dinh, T.H., Jackson, D.J., Lombard, C., Delaney, K.P., Puren, A., Sherman, G., Woldesenbet, S., Ramokolo, V., Crowley, S. and Doherty, T. (2015). First population-level effectiveness evaluation of a national programme to prevent HIV transmission from mother to child, South Africa. *Journal of Epidemiology and Community Health*, *69*(3), pp.240-248.

Goga, A.E., Dinh, T.H., Jackson, D.J., Lombard, C.J., Puren, A., Sherman, G., Ramokolo, V., Woldesenbet, S., Doherty, T., Noveve, N., Magasana, V., Singh, Y., Ramraj, T., Bhardwaj, S., Pillay, Y. and for the South Africa PMTCT Evaluation (SAPMCTE) Team (2016). Population–level effectiveness of PMTCT Option A on early mother–to–child (MTCT) transmission of HIV in South Africa: implications for eliminating MTCT. *Journal of Global Health*, *6*(2), pp.020405. DOI: 10.7189/jogh.6.020405 (Accessed January 2020).

Goga, A., Chirinda, W., Ngandu, N.K., Ngoma, K., Bhardwaj, S., Feucht, U., Davies, N., Ntloana, M., Mhlongo, O., Silere-Maqetseba, T. and Moyo, F. (2018). Closing the gaps to eliminate mother-to-child transmission of HIV (MTCT) in South Africa: Understanding MTCT

case rates, factors that hinder the monitoring and attainment of targets, and potential game changers. *South African Medical Journal*, *108*(3), pp.17-24.

Gonzalez-Serna, A., Min, J.E., Woods, C., Chan, D., Lima, V.D., Montaner, J.S.G., Harrigan, P.R. and Swenson, L.C. (2014). Performance of HIV-1 drug resistance testing at low-level viremia and its ability to predict future virologic outcomes and viral evolution in treatment-naive individuals. *Clinical Infectious Diseases*, *58*(8), pp.1165-1173.

Govindasamy, D., Ferrand, R.A., Wilmore, S.M., Ford, N., Ahmed, S., Afnan-Holmes, H. and Kranzer, K. (2015). Uptake and yield of HIV testing and counselling among children and adolescents in sub-Saharan Africa: a systematic review. *Journal of the International AIDS Society*, *18*(1), pp.20182. DOI: https://doi.org/10.7448/IAS.18.1.20182 (Accessed May 2020).

Guay, L.A., Musoke, P., Fleming, T., Bagenda, D., Allen, M., Nakabiito, C., Sherman, J., Bakaki, P., Ducar, C., Deseyve, M. and Emel, L. (1999). Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. *The Lancet*, *354*(9181), pp.795-802.

Gupta, R.K., Gibb, D. and Pillay, D. (2009). Management of paediatric HIV-1 resistance. *Current Opinion in Infectious Diseases*, *22*(3), pp.256-263.

Gupta, R.K., Jordan, M.R., Sultan, B.J., Hill, A., Davis, D.H., Gregson, J., Sawyer, A.W., Hamers, R.L., Ndembi, N., Pillay, D. and Bertagnolio, S. (2012). Global trends in antiretroviral resistance in treatment-naive individuals with HIV after rollout of antiretroviral treatment in resource-limited settings: a global collaborative study and meta-regression analysis. *The Lancet*, *380*(9849), pp.1250-1258.

Gupta, R.K., Gregson, J., Parkin, N., Haile-Selassie, H., Tanuri, A., Forero, L.A., Kaleebu, P., Watera, C., Aghokeng, A., Mutenda, N. and Dzangare, J. (2018). HIV-1 drug resistance before initiation or re-initiation of first-line antiretroviral therapy in low-income and middle-income countries: a systematic review and meta-regression analysis. *The Lancet Infectious Diseases*, *18*(3), pp.346-355.

Guyader, M., Emerman, M., Sonigo, P., Clavel, F., Montagnier, L. and Alizon, M. (1987). Genome organization and transactivation of the human immunodeficiency virus type 2. *Nature*, *326*(6114), pp.662-669.

Hallack, R., Doherty, L.E., Wethers, J.A. and Parker, M.M. (2008). Evaluation of dried blood spot specimens for HIV-1 drug-resistance testing using the Trugene® HIV-1 genotyping assay. *Journal of Clinical Virology*, *41*(4), pp.283-287.

Halvas, E.K., Aldrovandi, G.M., Balfe, P., Beck, I.A., Boltz, V.F., Coffin, J.M., Frenkel, L.M., Hazelwood, J.D., Johnson, V.A., Kearney, M. and Kovacs, A. (2006). Blinded, multicenter comparison of methods to detect a drug-resistant mutant of human immunodeficiency virus type 1 at low frequency. *Journal of Clinical Microbiology*, *44*(7), pp.2612-2614.

Hamers, R.L., Wallis, C.L., Kityo, C., Siwale, M., Mandaliya, K., Conradie, F., Botes, M.E., Wellington, M., Osibogun, A., Sigaloff, K.C. and Nankya, I. (2011). HIV-1 drug resistance in antiretroviral-naive individuals in sub-Saharan Africa after rollout of antiretroviral therapy: a multicentre observational study. *The Lancet Infectious Diseases*, *11*(10), pp.750-759.

Hamers, R.L., Schuurman, R., Sigaloff, K.C., Wallis, C.L., Kityo, C., Siwale, M., Mandaliya, K., Ive, P., Botes, M.E., Wellington, M. and Osibogun, A. (2012). Effect of pretreatment HIV-1 drug resistance on immunological, virological, and drug-resistance outcomes of first-line antiretroviral treatment in sub-Saharan Africa: a multicentre cohort study. *The Lancet Infectious Diseases*, *12*(4), pp.307-317.

Hamers, R.L., De Wit, T.F.R. and Holmes, C.B. (2018). HIV drug resistance in low-income and middle-income countries. *The Lancet HIV*, *5*(10), pp.e588-e596.

Hammer, S.M., Eron, J.J., Reiss, P., Schooley, R.T., Thompson, M.A., Walmsley, S., Cahn, P., Fischl, M.A., Gatell, J.M., Hirsch, M.S. and Jacobsen, D.M. (2008). Antiretroviral treatment of adult HIV infection: 2008 recommendations of the International AIDS Society–USA panel. *Journal of the American Medical Association*, *300*(5), pp.555-570.

Hanna, G.J. and Richard, T.D. (1999). Antiretroviral drug resistance in HIV-1. *Current Infectious Disease Reports*, *1*(3), pp.289-297.

Hazra, R., Siberry, G.K. and Mofenson, L.M. (2010). Growing up with HIV: children, adolescents, and young adults with perinatally acquired HIV infection. *Annual Review of Medicine*, *61*, pp.169-185. DOI: https://doi.org/10.1146/annurev.med.050108.151127. (Accessed June 2020).

Hearps, A.C., Ryan, C.E., Morris, L.M., Plate, M.M., Greengrass, V. and Crowe, S.M. (2010). Stability of dried blood spots for HIV-1 drug resistance analysis. *Current HIV Research*, *8*(2), pp.134-140.

Herrera-Carrillo, E. and Berkhout, B. (2015). Bone marrow gene therapy for HIV/AIDS. *Viruses*, *7*(7), pp.3910-3936.

Hertogs, K., de Béthune, M.P., Miller, V., Ivens, T., Schel, P., Van Cauwenberge, A., Van den Eynde, C., Van Gerwen, V., Azijn, H., Van Houtte, M. and Peeters, F. (1998). A rapid method for simultaneous detection of phenotypic resistance to inhibitors of protease and reverse transcriptase in recombinant human immunodeficiency virus type 1 isolates from patients treated with antiretroviral drugs. *Antimicrobial Agents and Chemotherapy*, *42*(2), pp.269-276.

HIV sequence database. Los Alamos National Laboratory. Available from: https://www.hiv.lanl.gov/content/sequence/HIV/CRFs /CRFs.html (Accessed November 2020).

Ho, D.D., Neumann, A.U., Perelson, A.S., Chen, W., Leonard, J.M. and Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature*, *373*(6510), pp.123-126.

Hogg, R.S., Bangsberg, D.R., Lima, V.D., Alexander, C., Bonner, S., Yip, B., Wood, E., Dong, W.W., Montaner, J.S. and Harrigan, P.R. (2006). Emergence of drug resistance is associated with an increased risk of death among patients first starting HAART. *PLoS Medicine*, *3*(9), pp.e356. DOI: https://doi.org/10.1371/journal.pmed.0030356 (Accessed April 2019).

Hosseinipour, M.C., van Oosterhout, J.J., Weigel, R., Phiri, S., Kamwendo, D., Parkin, N., Fiscus, S.A., Nelson, J.A., Eron, J.J. and Kumwenda, J. (2009). The public health approach to

identify antiretroviral therapy failure: high-level nucleoside reverse transcriptase inhibitor resistance among Malawians failing first-line antiretroviral therapy. *AIDS*, *23*(9), pp.1127-1134.

Hudelson, C. and Cluver, L. (2015). Factors associated with adherence to antiretroviral therapy among adolescents living with HIV/AIDS in low-and middle-income countries: a systematic review. *AIDS Care*, *27*(7), pp.805-816.

Huibers, M.H., Kityo, C., Boerma, R.S., Kaudha, E., Sigaloff, K.C., Balinda, S.N., Bertagnolio, S., Nakanjako, R., Mugyenyi, P., Calis, J.C. and Boele van Hensbroek, M. (2019). Long-term virological outcomes, failure and acquired resistance in a large cohort of Ugandan children. *Journal of Antimicrobial Chemotherapy*, *74*(10), pp.3035-3043.

Human Sciences Research Council. (2018). The fifth South African national HIV prevalence, incidence, behaviour and communication survey. Available from: http://www.hsrc.ac.za/uploads/pageContent/9234/SABSSMV_Impact_Assessment_Summary_ZA_ADS_cleared_PDFA4.pdf (Accessed May 2020).

Hunt, G.M., Coovadia, A., Abrams, E.J., Sherman, G., Meyers, T., Morris, L. and Kuhn, L. (2011). HIV-1 drug resistance at antiretroviral treatment initiation in children previously exposed to single-dose nevirapine. *AIDS*, *25*(12), pp.1461-1469.

Hunt, G., Venter, F., Morris, L. and Maartens, G. (2015). Monitoring, surveillance and control of HIVDR: the experience of a high burden country. *Overcoming Global Antimicrobial resistance, AMR Control*, pp. 94-97. Available from: http://resistancecontrol .info/wp-content/uploads/2017/07/14_hunt-venter-morris-maartens.pdf (Accessed November 2019).

Hunt, G.M., Ledwaba, J., Salimo, A., Kalimashe, M., Dinh, T.H., Jackson, D., Sherman, G., Puren, A., Ngandu, N.K., Lombard, C. and Morris, L. (2019). Prevalence of HIV-1 drug resistance amongst newly diagnosed HIV-infected infants age 4-8 weeks, enrolled in three nationally representative PMTCT effectiveness surveys, South Africa: 2010, 2011–12 and 2012–13. *BMC Infectious Diseases*, *19*(1), pp.787. DOI: https://doi.org/10.1186/s12879-019-4339-y (Accessed January 2020).

Ibeto, M., Giddy, J. and Cox, V. (2014). Closing the gaps: steps towards elimination of mother-to-child transmission of HIV. *Southern African Journal of HIV Medicine, 15*(3), pp.107-109.

Iliff, P.J., Piwoz, E.G., Tavengwa, N.V., Zunguza, C.D., Marinda, E.T., Nathoo, K.J., Moulton, L.H., Ward, B.J., Humphrey, J.H. and ZVITAMBO Study Group. (2005). Early exclusive breastfeeding reduces the risk of postnatal HIV-1 transmission and increases HIV-free survival. *AIDS, 19*(7), pp.699-708.

Inzaule, S.C., Ondoa, P., Peter, T., Mugyenyi, P.N., Stevens, W.S., De Wit, T.F.R. and Hamers, R.L. (2016). Affordable HIV drug-resistance testing for monitoring of antiretroviral therapy in sub-Saharan Africa. *The Lancet Infectious Diseases, 16*(11), pp.267-275.

Inzaule, S.C., Hamers, R.L., Calis, J., Boerma, R., Sigaloff, K., Zeh, C., Mugyenyi, P., Akanmu, S. and De Wit, T.F.R. (2018a). When prevention of mother-to-child HIV transmission fails: preventing pretreatment drug resistance in African children. *AIDS, 32*(2), pp.143-147.

Inzaule, S.C., Osi, S.J., Akinbiyi, G., Emeka, A., Khamofu, H., Mpazanje, R., Ilesanmi, O., Ndembi, N., Odafe, S., Sigaloff, K.C. and De Wit, T.F.R. (2018b). High prevalence of HIVDR among newly diagnosed infants aged< 18 months: results from a nationwide surveillance in Nigeria. *Journal of Acquired Immune Deficiency Syndromes*, *77*(1), pp.1-7. DOI: 10.1097/QAI.0000000000001553 (Accessed January 2020).

Johannessen, A., Holberg-Petersen, M., Lovgaarden, G., Naman, E., Ormaasen, V., Matee, M.I., Gundersen, S.G. and Bruun, J.N. (2010). HIV type-1 drug resistance testing on dried blood spots is feasible and reliable in patients who fail antiretroviral therapy in rural Tanzania. *Antiviral Therapy, 15*(7), pp.1003-1009.

Johnson, L.F., Davies, M.A., Moultrie, H., Sherman, G.G., Bland, R.M., Rehle, T.M., Dorrington, R.E. and Newell, M.L. (2012). The effect of early initiation of antiretroviral treatment in infants on pediatric AIDS mortality in South Africa: a model-based analysis. *The Pediatric Infectious Disease Journal, 31*(5), pp.474-480.

Johnson, V.A., Calvez, V., Gunthard, H.F., Paredes, R., Pillay, D., Shafer, R.W., Wensing, A.M. and Richman, D.D. (2013). Update of the drug resistance mutations in HIV-1: March 2013. *Topics in Antiviral Medicine*, *21*(1), pp.6-7.

Jordan, M.R., Bennett, D.E., Wainberg, M.A., Havlir, D., Hammer, S., Yang, C., Morris, L., Peeters, M., Wensing, A.M., Parkin, N. and Nachega, J.B. (2012). Update on World Health Organization HIV drug resistance prevention and assessment strategy: 2004-2011. *Clinical Infectious Diseases*, *54*(4), pp.245-249.

Jordan, M.R., Penazzato, M., Cournil, A., Vubil, A., Jani, I., Hunt, G., Carmona, S., Maphalala, G., Mthethwa, N., Watera, C. and Kaleebu, P. (2017). Human immunodeficiency virus (HIV) drug resistance in African infants and young children newly diagnosed with HIV: a multicountry analysis. *Clinical Infectious Diseases*, *65*(12), pp.2018-2025.

Judd, A., Lodwick, R., Noguera-Julian, A., Gibb, D.M., Butler, K., Costagliola, D., Sabin, C., Van Sighem, A., Ledergerber, B., Torti, C. and Mocroft, A. (2017a). Higher rates of triple-class virological failure in perinatally HIV-infected teenagers compared with heterosexually infected young adults in Europe. *HIV Medicine*, *18*(3), pp.171-180.

Judd, A., Collins, I.J., Parrott, F., Hill, T., Jose, S., Ford, D., Asad, H., Gibb, D.M., Sabin, C. and Collaborative HIV Paediatric Study (CHIPS) Steering Committee and the UK Collaborative HIV Cohort Study (UK CHIC) (2017b). Growing up with perinatal HIV: changes in clinical outcomes before and after transfer to adult care in the UK. *Journal of the International AIDS Society*, *20*(3), pp.71-80.

Kahana, S.Y., Fernandez, M.I., Wilson, P.A., Bauermeister, J.A., Lee, S., Wilson, C.M. and Hightow-Weidman, L.B. (2015). Rates and correlates of antiretroviral therapy use and virologic suppression among perinatally and behaviorally infected HIV+ youth linked to care in the United States. *Journal of Acquired Immune Deficiency Syndromes*, *68*(2), pp.169-177.

Kamya, M.R., Mayanja-Kizza, H., Kambugu, A., Bakeera-Kitaka, S., Semitala, F., Mwebaze-Songa, P., Castelnuovo, B., Schaefer, P., Spacek, L.A., Gasasira, A.F. and Katabira, E. (2007). Predictors of long-term viral failure among ugandan children and adults treated with antiretroviral therapy. *Journal of Acquired Immune Deficiency Syndromes*, *46*(2), pp.187-193.

Kaneda, T., Shiraki, K., Hirano, K. and Nagata, I. (1997). Detection of maternofetal transfusion by placental alkaline phosphatase levels. *The Journal of Pediatrics*, *130*(5), pp.730-735.

Kanthula, R., Rossouw, T.M., Feucht, U.D., Van Dyk, G., Beck, I.A., Silverman, R., Olson, S., Salyer, C., Cassol, S. and Frenkel, L.M. (2017). Persistence of HIVDR among South African children given nevirapine to prevent mother-to-child-transmission. *AIDS*, *31*(8), pp.1143-1148.

Kantor, R., Diero, L., DeLong, A., Kamle, L., Muyonga, S., Mambo, F., Walumbe, E., Emonyi, W., Chan, P., Carter, E.J. and Hogan, J. (2009). Misclassification of first-line antiretroviral treatment failure based on immunological monitoring of HIV infection in resource-limited settings. *Clinical Infectious Diseases*, *49*(3), pp.454-462.

Kantor, R., Smeaton, L., Vardhanabhuti, S., Hudelson, S.E., Wallis, C.L., Tripathy, S., Morgado, M.G., Saravanan, S., Balakrishnan, P., Reitsma, M. and Hart, S. (2015). Pretreatment HIV drug resistance and HIV-1 subtype C are independently associated with virologic failure: results from the multinational PEARLS (ACTG A5175) clinical trial. *Clinical Infectious Diseases*, *60*(10), pp.1541-1549.

Karger, B.L. and Guttman, A. (2009). DNA sequencing by CE. *Electrophoresis*, *30*(1), pp.196-202.

Kaufmann, G.R., Perrin, L., Pantaleo, G., Opravil, M., Furrer, H., Telenti, A., Hirschel, B., Ledergerber, B., Vernazza, P., Bernasconi, E. and Rickenbach, M. (2003). CD4 T-lymphocyte recovery in individuals with advanced HIV-1 infection receiving potent antiretroviral therapy for 4 years: the Swiss HIV cohort study. *Archives of Internal Medicine*, *163*(18), pp.2187-2195.

Kempf, D.J., King, M.S., Bernstein, B., Cernohous, P., Bauer, E., Moseley, J., Gu, K., Hsu, A., Brun, S. and Sun, E. (2004). Incidence of resistance in a double-blind study comparing lopinavir/ritonavir plus stavudine and lamivudine to nelfinavir plus stavudine and lamivudine. *The Journal of Infectious Diseases*, *189*(1), pp.51-60.

Kim, S.H., Gerver, S.M., Fidler, S. and Ward, H. (2014). Adherence to antiretroviral therapy in adolescents living with HIV: systematic review and meta-analysis. *AIDS*, *28*(13), pp.1945-1956.

Kourtis, A.P., Lee, F.K., Abrams, E.J., Jamieson, D.J. and Bulterys, M. (2006). Mother-to-child transmission of HIV-1: timing and implications for prevention. *The Lancet Infectious Diseases*, *6*(11), pp.726-732.

Lallemant, M., Jourdain, G., Le Coeur, S., Mary, J.Y., Ngo-Giang-Huong, N., Koetsawang, S., Kanshana, S., McIntosh, K. and Thaineua, V. (2004). Single-dose perinatal nevirapine plus standard zidovudine to prevent mother-to-child transmission of HIV-1 in Thailand. *New England Journal of Medicine*, *351*(3), pp.217-228.

Lam, P.K., Fidler, S. and Foster, C. (2017). A review of transition experiences in perinatally and behaviourally acquired HIV-1 infection; same, same but different? *Journal of the International AIDS Society*, *20*(3), pp.91-99.

Lapointe, H.R., Dong, W., Lee, G.Q., Bangsberg, D.R., Martin, J.N., Mocello, A.R., Boum, Y., Karakas, A., Kirkby, D., Poon, A.F.Y., Harrigan, P.R. and Brumme, C.J. (2015). HIV drug resistance testing by high-multiplex "wide" sequencing on the Illumina MiSeq. *Antimicrobial Agents and Chemotherapy*, *59*(11), pp.6824-6833.

Ledergerber, B., Egger, M., Erard, V., Weber, R., Hirschel, B., Furrer, H., Battegay, M., Vernazza, P., Bernasconi, E., Opravil, M. and Kaufmann, D. (1999). AIDS-related opportunistic illnesses occurring after initiation of potent antiretroviral therapy: the Swiss HIV cohort study. *Journal of the American Medical Association*, *282*(23), pp.2220-2226.

Lehman, D.A. and Farquhar, C. (2007). Biological mechanisms of vertical human immunodeficiency virus (HIV-1) transmission. *Reviews in Medical Virology*, *17*(6), pp.381-403.

Leroy, V., Sakarovitch, C., Cortina-Borja, M., McIntyre, J., Coovadia, H., Dabis, F. and Newell, M.L. (2005). Is there a difference in the efficacy of peripartum antiretroviral regimens in reducing mother-to-child transmission of HIV in Africa?. *AIDS*, *19*(16), pp.1865-1875.

Levy, D.N., Aldrovandi, G.M., Kutsch, O. and Shaw, G.M. (2004). Dynamics of HIV-1 recombination in its natural target cells. *Proceedings of the National Academy of Sciences*, *101*(12), pp.4204-4209.

Lew, J., Reichelderfer, P., Fowler, M., Bremer, J., Carrol, R., Cassol, S., Chernoff, D., Coombs, R., Cronin, M., Dickover, R. and Fiscus, S. (1998). Determinations of levels of human immunodeficiency virus type 1 RNA in plasma: reassessment of parameters affecting assay outcome. *Journal of Clinical Microbiology*, *36*(6), pp.1471-1479.

Lewis, P., Nduati, R., Kreiss, J.K., John, G.C., Richardson, B.A., Mbori-Ngacha, D., Ndinya-Achola, J. and Overbaugh, J. (1998). Cell-free human immunodeficiency virus type 1 in breast milk. *The Journal of Infectious Diseases*, *177*(1), pp.34-39.

Lewis, S.H., Fox, H.E., Reynolds-Kohler, C. and Nelson, J.A. (1990). HIV-1 in trophoblastic and villous Hofbauer cells and haematological precursors in eight-week fetuses. *The Lancet*, *335*(8689), pp.565-568.

Maartens, G., Celum, C. and Lewin, S.R. (2014). HIV infection: epidemiology, pathogenesis, treatment, and prevention. *The Lancet*, *384*(9939), pp.258-271.

Mansky, L.M. (1996). Forward mutation rate of human immunodeficiency virus type 1 in a T lymphoid cell line. *AIDS Research and Human Retroviruses*, *12*(4), pp.307-314.

Marais, B.J., Kangwana, B.B., Rabie, H. and Cotton, M.F. (2007). Caring for our children. *South African Family Practice*, *49*(4), pp.18. DOI: https://doi.org/10.108020786204 .2007.10873537 (Accessed May 2020).

Marino-Merlo, F., Macchi, B., Armenia, D., Bellocchi, M.C., Ceccherini-Silberstein, F., Mastino, A. and Grelli, S. (2018). Focus on recently developed assays for detection of resistance/sensitivity to reverse transcriptase inhibitors. *Applied Microbiology and Biotechnology*, *102*(23), pp.9925-9936.

Masciotra, S., Garrido, C., Youngpairoj, A.S., McNulty, A., Zahonero, N., Corral, A., Heneine, W., De Mendoza, C. and García-Lerma, J.G. (2007). High concordance between HIV-1 drug resistance genotypes generated from plasma and dried blood spots in antiretroviral-experienced patients. *AIDS*, *21*(18), pp.2503-2511.

Maskew, M., Bor, J., MacLeod, W., Carmona, S., Sherman, G.G. and Fox, M.P. (2019). Adolescent HIV treatment in South Africa's national HIV programme: a retrospective cohort study. *The Lancet HIV*, *6*(11), pp.760-768.

Mayer, K.H., Hanna, G.J. and Richard, T.D. (2001). Clinical use of genotypic and phenotypic drug resistance testing to monitor antiretroviral chemotherapy. *Clinical Infectious Diseases*, *32*(5), pp.774-782.

Mazanderani, A.H., Moyo, F., Kufa, T., Maritz, J. and Sherman, G.G. (2018). Differentiating clearly positive from indeterminate results: A review of irreproducible HIV-1 PCR positive samples from South Africa's Early Infant Diagnosis Program, 2010–2015. *Diagnostic Microbiology and Infectious Disease*, *91*(3), pp.248-255.

McMahon, J.H., Elliott, J.H., Bertagnolio, S., Kubiak, R. and Jordan, M.R. (2013). Viral suppression after 12 months of antiretroviral therapy in low-and middle-income countries: a systematic review. *Bulletin of the World Health Organization*, *91*, pp.377-385. DOI: http://dx.doi.org/10.2471/BLT.12.112946 (Accessed April 2019).

McNulty, A., Jennings, C., Bennett, D., Fitzgibbon, J., Bremer, J.W., Ussery, M., Kalish, M.L., Heneine, W. and García-Lerma, J.G. (2007). Evaluation of dried blood spots for human immunodeficiency virus type 1 drug resistance testing. *Journal of Clinical Microbiology*, *45*(2), pp.517-521.

Menson, E.N., Walker, A.S., Sharland, M., Wells, C., Tudor-Williams, G., Riordan, F.A.I., Lyall, E.H. and Gibb, D.M. (2006). Underdosing of antiretrovirals in UK and Irish children with HIV as an example of problems in prescribing medicines to children, 1997-2005: cohort study. *The British Medical Journal*, *332*(7551), pp.1183-1187.

Meyers, T., Moultrie, H., Naidoo, K., Cotton, M., Eley, B. and Sherman, G. (2007). Challenges to pediatric HIV care and treatment in South Africa. *The Journal of Infectious Diseases*, *196*(3), pp.474-481.

Meynard, J.L., Vray, M., Morand-Joubert, L., Race, E., Descamps, D., Peytavin, G., Matheron, S., Lamotte, C., Guiramand, S., Costagliola, D. and Brun-Vézinet, F. (2002). Phenotypic or genotypic resistance testing for choosing antiretroviral therapy after treatment failure: a randomized trial. *AIDS*, *16*(5), pp.727-736.

Milligan, C. and Overbaugh, J. (2014). The role of cell-associated virus in mother-to-child HIV transmission. *TheJournal of Infectious Diseases*, *210*(3), pp.631-640.

Mitsuya, Y., Varghese, V., Wang, C., Liu, T.F., Holmes, S.P., Jayakumar, P., Gharizadeh, B., Ronaghi, M., Klein, D., Fessel, W.J. and Shafer, R.W. (2008). Minority human immunodeficiency virus type 1 variants in antiretroviral-naive persons with reverse transcriptase codon 215 revertant mutations. *Journal of Virology*, *82*(21), pp.10747-10755.

Mocroft, A., Phillips, A.N., Gatell, J., Ledergerber, B., Fisher, M., Clumeck, N., Losso, M., Lazzarin, A., Fatkenheuer, G., Lundgren, J.D. and EuroSIDA study group (2007). Normalisation of CD4 counts in patients with HIV-1 infection and maximum virological suppression who are taking combination antiretroviral therapy: an observational cohort study. *The Lancet*, *370*(9585), pp.407-413.

Monleau M, Aghokeng AF, Eymard-Duvernay S, Dagnra A, Kania D, Ngo-Giang-Huong N, Toure-Kane C, Truong LX, Chaix ML, Delaporte E, Ayouba A, Peeters M. and for the ANRS 12235 study group. (2014). Field evaluation of dried blood spots for routine HIV-1 viral load and drug resistance monitoring in patients receiving antiretroviral therapy in Africa and Asia. *Journal of Clinical Microbiology*, 52(2), pp.578–586.

Moorthy, A., Gupta, A., Bhosale, R., Tripathy, S., Sastry, J., Kulkarni, S., Thakar, M., Bharadwaj, R., Kagal, A., Bhore, A.V. and Patil, S. (2009). Nevirapine resistance and breast-milk HIV transmission: effects of single and extended-dose nevirapine prophylaxis in subtype C HIV-infected infants. *PloS One*, *4*(1), pp.e4096. DOI: 10.1371/journal.pone .0004096 (Accessed May 2020).

Muri, L., Gamell, A., Ntamatungiro, A.J., Glass, T.R., Luwanda, L.B., Battegay, M., Furrer, H., Hatz, C., Tanner, M., Felger, I. and Klimkait, T. (2017). Development of HIV drug resistance and therapeutic failure in children and adolescents in rural Tanzania: an emerging public health concern. *AIDS*, *31*(1), pp.61-70.

Musiime, V., Kaudha, E., Kayiwa, J., Mirembe, G., Odera, M., Kizito, H., Nankya, I., Ssali, F., Kityo, C., Colebunders, R. and Mugyenyi, P. (2013). Antiretroviral drug resistance profiles and response to second-line therapy among HIV type 1-infected Ugandan children. *AIDS Research and Human Retroviruses*, *29*(3), pp.449-455.

Mutwa, P.R., Boer, K.R., Rusine, J., Muganga, N., Tuyishimire, D., Schuurman, R., Reiss, P., Lange, J.M. and Geelen, S.P. (2014). Long-term effectiveness of combination antiretroviral therapy and prevalence of HIV drug resistance in HIV-1-infected children and adolescents in Rwanda. *The Pediatric Infectious Disease Journal*, *33*(1), pp.63-69.

Nachega, J.B., Hislop, M., Nguyen, H., Dowdy, D.W., Chaisson, R.E., Regensberg, L., Cotton, M. and Maartens, G. (2009). Antiretroviral therapy adherence, virologic and immunologic outcomes in adolescents compared with adults in southern Africa. *Journal of Acquired Immune Deficiency Syndromes*, *51*(1), pp.65-71.

Nachega, J.B., Uthman, O.A., Anderson, J., Peltzer, K., Wampold, S., Cotton, M.F., Mills, E.J., Ho, Y.S., Stringer, J.S., McIntyre, J.A. and Mofenson, L.M. (2012). Adherence to antiretroviral therapy during and after pregnancy in low-income, middle-income, and high-income countries: a systematic review and meta-analysis. *AIDS*, *26*(16), pp.2039-2052.

Nasir, I.A., Emeribe, A.U., Ojeamiren, I. and Aderinsayo Adekola, H. (2017). Human immunodeficiency virus resistance testing technologies and their applicability in resource-limited settings of Africa. *Infectious Diseases: Research and Treatment*, *10*. DOI: 10.1177/1178633717749597 (Accessed November 2020).

National Department of Health. (2008). Policy and guidelines for the implementation of the PMTCT programme. Available from: http://www.doh.gov.za/docs/policy/pmtct-f.html (Accessed November 2019).

National Department of Health. (2010a). Clinical guidelines: PMTCT (prevention of mother-to child transmission). Available from: https://sahivsoc.org/Files/NDOH_PMTCT% 20Apr%202008.pdf (Accessed November 2019).

National Department of Health. (2010b). National antenatal sentinel HIV and syphilis prevalence survey in South Africa. Available from: http://www.hst.org.za/publications/2010-national-antenatal-sentinel-hiv-and-syphilis-prevalence-survey-south-africa. (Accessed June 2016).

National Department of Health. (2013). The South African antiretroviral treatment guidelines Available from: https://sahivsoc.org/Files/2013%20ART%20Treatment%20Guidelines%20 Final% 2025%20March%202013%20corrected.pdf (Accessed November 2019).

National Department of Health. (2015a). National antenatal sentinel HIV prevalence survey South Africa. Available from: https://www.health-e.org.za/wp-content/uploads/2016/03/ Dept-Health-HIV-High-Res-7102015.pdf. (Accessed March 2017).

National Department of Health. (2015b). National consolidated guidelines for the prevention of mother-to-child transmission of HIV (PMTCT) and the management of HIV in children, adolescents and adults. Available from: http://www.health.gov.za/index.php/2014-03-17-09-09-38/policies-and-guidelines/category/230-2015p?download=937:national-art-guidelines-2015final (Accessed November 2019).

National Department of Health. (2017). The 2017 national antenatal sentinel HIV survey key findings, South Africa. Available from: https://www.nicd.ac.za/wp-content/uploads/2019/07/Antenatal_survey-report_24July19.pdf (Accessed December 2020).

National Department of Health. (2019). ART clinical guidelines for the management of HIV in adults, pregnancy, adolescents, children, infants and neonates. Available from: https://sahivsoc.org/Files/2019%20Abridged%20ART%20Guidelines%2010%20October%202019.pdf (Accessed June 2020).

National Department of Health. (2020). 2019 ART clinical guidelines for the management of HIV in adults, pregnancy, adolescents, children, infants and neonates. Available from: https://sahivsoc.org/Files/2019%20ART%20Guideline%2028042020%20pdf.pdf (Accessed January 2020).

Nglazi, M.D., Kranzer, K., Holele, P., Kaplan, R., Mark, D., Jaspan, H., Lawn, S.D., Wood, R. and Bekker, L.G. (2012). Treatment outcomes in HIV-infected adolescents attending a community-based antiretroviral therapy clinic in South Africa. *BMC Infectious Diseases*, *12*(1), pp.21. DOI: http://www.biomedcentral.com/1471-2334/12/21 (Accessed May 2020).

Novitsky, V., Wester, C.W., DeGruttola, V., Bussmann, H., Gaseitsiwe, S., Thomas, A., Moyo, S., Musonda, R., Van Widenfelt, E., Marlink, R.G. and Essex, M. (2007). The reverse transcriptase 67N 70R 215Y genotype is the predominant TAM pathway associated with virologic failure among HIV type 1C-infected adults treated with ZDV/ddI-containing HAART in Southern Africa. *AIDS Research and Human Retroviruses*, *23*(7), pp.868-878.

Orrell, C., Levison, J., Ciaranello, A., Bekker, L.G., Kuritzkes, D.R., Freedberg, K.A. and Wood, R. (2013). Resistance in pediatric patients experiencing virologic failure with first-and second-line antiretroviral therapy. *The Pediatric Infectious Disease Journal*, *32*(6). DOI: 10.1097/INF.0b013e3182829092 (Accessed May 2020).

Palella Jr, F.J., Delaney, K.M., Moorman, A.C., Loveless, M.O., Fuhrer, J., Satten, G.A., Aschman, D.J. and Holmberg, S.D. (1998). Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. *New England Journal of Medicine*, *338*(13), pp.853-860.

Palmer, S., Boltz, V., Martinson, N., Maldarelli, F., Gray, G., McIntyre, J., Mellors, J., Morris, L. and Coffin, J. (2006). Persistence of nevirapine-resistant HIV-1 in women after single-dose nevirapine therapy for prevention of maternal-to-fetal HIV-1 transmission. *Proceedings of the National Academy of Sciences*, *103*(18), pp.7094-7099.

Paredes, R., Marconi, V.C., Lockman, S., Abrams, E.J. and Kuhn, L. (2013). Impact of antiretroviral drugs in pregnant women and their children in Africa: HIV resistance and treatment outcomes. *The Journal of Infectious Diseases*, *207*(2), pp.93-100.

Parry, C.M., Parkin, N., Diallo, K., Mwebaza, S., Batamwita, R., DeVos, J., Bbosa, N., Lyagoba, F., Magambo, B., Jordan, M.R. and Downing, R. (2014). Field study of dried blood spot specimens for HIV-1 drug resistance genotyping. *Journal of Clinical Microbiology*, *52*(8), pp.2868-2875.

Paton, N.I., Kityo, C., Thompson, J., Nankya, I., Bagenda, L., Hoppe, A., Hakim, J., Kambugu, A., Van Oosterhout, J.J., Kiconco, M. and Bertagnolio, S. (2017). Nucleoside reverse-transcriptase inhibitor cross-resistance and outcomes from second-line antiretroviral therapy in the public health approach: an observational analysis within the randomised, open-label, EARNEST trial. *The Lancet HIV*, *4*(8), pp.e341-e348.

Pau, A.K. and George, J.M. (2014). Antiretroviral therapy: current drugs. *Infectious Disease Clinics*, *28*(3), pp.371-402.

Peeters, M., Gueye, A., Mboup, S., Bibollet-Ruche, F., Ekaza, E., Mulanga, C., Ouedrago, R., Gandji, R., Mpele, P., Dibanga, G. and Koumare, B. (1997). Geographical distribution of HIV-1 group O viruses in Africa. *AIDS*, *11*(4), pp.493-498.

Penazzato, M. (2013). WHO HIV drug resistance surveillance in children less than 18 months old newly diagnosed with HIV: results from Swaziland and Zimbabwe. 5th International Workshop on HIV Pediatrics; Kuala Lumpur, Malaysia. 28–29 June, 2013; (Abstract O_2012AB).

Penazzato, M., Sugandhi, N., Essajee, S., Doherty, M., Ficht, A. and Phelps, B.R. (2018). Getting treatment and care services right for children and adolescents to reach high viral suppression. *Journal of Acquired Immune Deficiency Syndromes*, *78*, pp.128-133. DOI: 10.1097/QAI.0000000000001738 (Accessed December 2020).

Perelson, A.S., Neumann, A.U., Markowitz, M., Leonard, J.M. and Ho, D.D. (1996). HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. *Science*, *271*(5255), pp.1582-1586.

Persaud, D., Palumbo, P., Ziemniak, C., Chen, J., Ray, S.C., Hughes, M., Havens, P., Purswani, M., Gaur, A.H., Chadwick, E.G. and Pediatric AIDS clinical trials group P1030 team. (2007). Early archiving and predominance of nonnucleoside reverse transcriptase inhibitor-resistant HIV-1 among recently infected infants born in the United States. *The Journal of Infectious Diseases*, *195*(10), pp.1402-1410.

Petropoulos, C.J., Parkin, N.T., Limoli, K.L., Lie, Y.S., Wrin, T., Huang, W., Tian, H., Smith, D., Winslow, G.A., Capon, D.J. and Whitcomb, J.M. (2000). A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. *Antimicrobial Agents and Chemotherapy*, *44*(4), pp.920-928.

Phanuphak, P., Sirivichayakul, S., Jiamsakul, A., Sungkanuparph, S., Kumarasamy, N., Lee, M.P., Sirisanthana, T., Kantipong, P., Lee, C., Kamarulzaman, A. and Mustafa, M. (2014). Transmitted drug resistance and antiretroviral treatment outcomes in non-subtype B HIV1-infected patients in South East Asia. *Journal of Acquired Immune Deficiency Syndromes*, *66*(1), pp.74-79.

Phillips, A.N., Cambiano, V., Miners, A., Revill, P., Pillay, D., Lundgren, J.D., Bennett, D., Raizes, E., Nakagawa, F., De Luca, A. and Vitoria, M. (2014). Effectiveness and cost-effectiveness of potential responses to future high levels of transmitted HIV drug resistance in antiretroviral drug-naive populations beginning treatment: modelling study and economic analysis. *The Lancet HIV*, *1*(2), pp.85-93.

Pingen, M., van der Ende, M.E., Wensing, A.M., el Barzouhi, A., Simen, B.B., Schutten, M. and Boucher, C.A.B. (2013). Deep sequencing does not reveal additional transmitted mutations in patients diagnosed with HIV-1 variants with single nucleoside reverse transcriptase inhibitor resistance mutations. *HIV Medicine*, *14*(3), pp.176-181.

Plantier, J.C., Leoz, M., Dickerson, J.E., De Oliveira, F., Cordonnier, F., Lemée, V., Damond, F., Robertson, D.L. and Simon, F. (2009). A new human immunodeficiency virus derived from gorillas. *Nature Medicine*, *15*(8), pp.871-872.

Poon, A.F., Swenson, L.C., Dong, W.W., Deng, W., Kosakovsky Pond, S.L., Brumme, Z.L., Mullins, J.I., Richman, D.D., Harrigan, P.R. and Frost, S.D. (2010). Phylogenetic analysis of population-based and deep sequencing data to identify coevolving sites in the *nef* gene of HIV-1. *Molecular Biology and Evolution*, *27*(4), pp.819-832.

Poppe, L.K., Chunda-Liyoka, C., Kwon, E.H., Gondwe, C., West, J.T., Kankasa, C., Ndongmo, C.B. and Wood, C. (2017). HIVDR in infants increases with changing prevention of mother-to-child transmission regimens. *AIDS*, *31*(13), pp.1885-1889.

Prosperi, M.C., Mackie, N., Di Giambenedetto, S., Zazzi, M., Camacho, R., Fanti, I., Torti, C., Sönnerborg, A., Kaiser, R., Codoñer, F.M. and Van Laethem, K. (2011). Detection of drug resistance mutations at low plasma HIV-1 RNA load in a European multicentre cohort study. *Journal of Antimicrobial Chemotherapy*, *66*(8), pp.1886-1896.

Puren, A., Gerlach, J.L., Weigl, B.H., Kelso, D.M. and Domingo, G.J. (2010). Laboratory operations, specimen processing, and handling for viral load testing and surveillance. *Journal of Infectious Diseases*, *201*(1), pp.27-36.

Rasoolinejad, M., Sarraf, M., Najafi, Z., SeyedAlinaghi, S., Moradmand Badie, B., Salehi, M. and Azevedo Voltarelli, F. (2019). Virologic failure in different antiretroviral regimens among pediatric patients with HIV referring to a voluntary counseling and testing (VCT) center in Tehran, Iran (2004-2017). *Archives of Pediatric Infectious Diseases*, *7*(4), pp.e80318. DOI: 10.5812/pedinfect.80318 (Accessed November 2020).

Rhee, S.Y., Gonzales, M.J., Kantor, R., Betts, B.J., Ravela, J. and Shafer, R.W. (2003). Human immunodeficiency virus reverse transcriptase and protease sequence database. *Nucleic Acids Research*, *31*(1), pp.298-303.

Rhee, S.Y., Blanco, J.L., Jordan, M.R., Taylor, J., Lemey, P., Varghese, V., Hamers, R.L., Bertagnolio, S., De Wit, T.F.R., Aghokeng, A.F. and Albert, J. (2015a). Geographic and

temporal trends in the molecular epidemiology and genetic mechanisms of transmitted HIV-1 drug resistance: an individual-patient and sequence-level meta-analysis. *PLoS Medicine*, *12*(6), pp.e1001810. DOI: https://doi.org/10.1371/journal.pmed.1001810 (Accessed April 2019).

Rhee, S.Y., Jordan, M.R., Raizes, E., Chua, A., Parkin, N., Kantor, R., Van Zyl, G.U., Mukui, I., Hosseinipour, M.C., Frenkel, L.M. and Ndembi, N. (2015b). HIV-1 drug resistance mutations: potential applications for point-of-care genotypic resistance testing. *PLoS One*, *10*(12), pp.0145772. DOI: https://doi.org/10.1371/journal.pone.0145772 (Accessed April 2019).

Rhee, S.Y., Kassaye, S.G., Barrow, G., Sundaramurthi, J.C., Jordan, M.R. and Shafer, R.W. (2020). HIV-1 transmitted drug resistance surveillance: shifting trends in study design and prevalence estimates. *Journal of the International AIDS Society*, *23*(9), pp.e25611. DOI: https://doi.org/10.1002/jia2.25611 (Accessed November 2020).

Rooke, R., Tremblay, M., Soudeyns, H., DeStephano, L., Yao, X.J., Fanning, M., Montaner, J.S., O'Shaughnessy, M., Gelmon, K., Tsoukas, C. and Gill, J. (1989). Isolation of drug-resistant variants of HIV-1 from patients on long-term zidovudine therapy. *AIDS*, *3*(7), pp.411-416.

Rottinghaus, E.K., Ugbena, R., Diallo, K., Bassey, O., Azeez, A., DeVos, J., Zhang, G., Aberle-Grasse, J., Nkengasong, J. and Yang, C. (2012). Dried blood spot specimens are a suitable alternative sample type for HIV-1 viral load measurement and drug resistance genotyping in patients receiving first-line antiretroviral therapy. *Clinical Infectious Diseases*, *54*(8), pp.1187-1195.

Salimo, A.T., Ledwaba, J., Coovadia, A., Abrams, E.J., Technau, K.G., Kuhn, L., Morris, L. and Hunt, G.M. (2015). The use of dried blood spot specimens for HIV-1 drug resistance genotyping in young children initiating antiretroviral therapy. *Journal of Virological Methods*, *223*, pp.30-32. DOI: https://doi.org/10.1016/j.jviromet.2015.07.005 (Accessed January 2020).

Salou, M., Butel, C., Konou, A.A., Ekouevi, D.K., Vidal, N., Dossim, S., Lawson-Evi, K., Nyasenu, Y.T., Singo-Tokofaï, A., d'Almeida, S. and Tchama, R. (2016). High rates of drug

resistance among newly diagnosed HIV-infected children in the national prevention of mother-to-child transmission program in Togo. *The Pediatric Infectious Disease Journal*, *35*(8), pp.879-885.

Sánchez, P.R. and Holguín, A. (2014). Drug resistance in the HIV-1-infected paediatric population worldwide: a systematic review. *Journal of Antimicrobial Chemotherapy*, *69*(8), pp.2032-2042.

Santoro, M.M., Fabeni, L., Armenia, D., Alteri, C., Di Pinto, D., Forbici, F., Bertoli, A., Di Carlo, D., Gori, C., Carta, S. and Fedele, V. (2014). Reliability and clinical relevance of the HIV-1 drug resistance test in patients with low viremia levels. *Clinical Infectious Diseases*, *58*(8), pp.1156-1164.

Scarlata, S. and Carter, C. (2003). Role of HIV-1 Gag domains in viral assembly. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1614*(1), pp.62-72.

Schlatter, A.F., Deathe, A.R. and Vreeman, R.C. (2016). The need for pediatric formulations to treat children with HIV. *AIDS Research and Treatment*. DOI: http://dx.doi.org/10.1155/2016/1654938. (Accessed November 2020).

Sebire, K., McGavin, K., Land, S., Middleton, T. and Birch, C. (1998). Stability of human immunodeficiency virus RNA in blood specimens as measured by a commercial PCR-based assay. *Journal of Clinical Microbiology*, *36*(2), pp.493-498.

Shafer, R.W., Jung, D.R. and Betts, B.J. (2000). Human immunodeficiency virus type 1 reverse transcriptase and protease mutation search engine for queries. *Nature Medicine*, *6*(11), pp.1290-1292.

Shafer, R.W. (2006). Rationale and uses of a public HIV drug-resistance database. *The Journal of Infectious Diseases*, *194*(1), pp.51-58.

Shao, W., Boltz, V.F., Spindler, J.E., Kearney, M.F., Maldarelli, F., Mellors, J.W., Stewart, C., Volfovsky, N., Levitsky, A., Stephens, R.M. and Coffin, J.M. (2013). Analysis of 454 sequencing error rate, error sources, and artifact recombination for detection of low-frequency

drug resistance mutations in HIV-1 DNA. *Retrovirology*, *10*(1), pp.18. DOI: https://doi.org/10.1186/1742-4690-10-18. (Accessed February 2020).

Sharp, P.M. and Hahn, B.H. (2011). Origins of HIV and the AIDS pandemic. *Cold Spring Harbor Perspectives in Medicine*, *1*(1), pp.a006841. DOI: 10.1101/cshperspect.a006841. (Accessed November 2020).

Sherman, G.G., Jones, S.A., Coovadia, A.H., Urban, M.F. and Bolton, K.D. (2004). PMTCT from research to reality-results from a routine service. *South African Medical Journal*, *94*(4), pp.289-292.

Shirasaka, T., Kavlick, M.F., Ueno, T., Gao, W.Y., Kojima, E., Alcaide, M.L., Chokekijchai, S., Roy, B.M., Arnold, E. and Yarchoan, R. (1995). Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. *Proceedings of the National Academy of Sciences*, *92*(6), pp.2398-2402.

Shubber, Z., Mills, E.J., Nachega, J.B., Vreeman, R., Freitas, M., Bock, P., Nsanzimana, S., Penazzato, M., Appolo, T., Doherty, M. and Ford, N. (2016). Patient-reported barriers to adherence to antiretroviral therapy: a systematic review and meta-analysis. *PLoS Medicine*, *13*(11), pp.e1002183. DOI: 10.1371/journal.pmed.1002183 (Accessed November 2020).

Sierra, S., Kupfer, B. and Kaiser, R. (2005). Basics of the virology of HIV-1 and its replication. *Journal of Clinical Virology*, *34*(4), pp.233-244.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J. and Thompson, J.D. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, *7*(1). DOI: 10.1038/msb.2011.75 (Accessed April 2019).

Sievers, F. and Higgins, D.G. (2018). Clustal Omega for making accurate alignments of many protein sequences. *Protein Science*, *27*(1), pp.135-145.

Sigaloff, K.C., Calis, J.C., Geelen, S.P., Van Vugt, M. and De Wit, T.F.R. (2011a). HIV-1-resistance-associated mutations after failure of first-line antiretroviral treatment among children in resource-poor regions: a systematic review. *The Lancet Infectious Diseases*, *11*(10), pp.769-779.

Sigaloff, K.C., Hamers, R.L., Wallis, C.L., Kityo, C., Siwale, M., Ive, P., Botes, M.E., Mandaliya, K., Wellington, M., Osibogun, A. and Stevens, W.S. (2011b). Unnecessary antiretroviral treatment switches and accumulation of HIV resistance mutations; two arguments for viral load monitoring in Africa. *Journal of Acquired Immune Deficiency Syndromes*, *58*(1), pp.23-31.

Sigaloff, K.C., Hamers, R.L., Menke, J., Labib, M., Siwale, M., Ive, P., Botes, M.E., Kityo, C., Mandaliya, K., Wellington, M. and Osibogun, A. (2012a). Early warning indicators for population-based monitoring of HIVDR in 6 African countries. *Clinical Infectious Diseases*, *54*(4), pp.294-299.

Sigaloff, K.C., Ramatsebe, T., Viana, R., De Wit, T.F.R., Wallis, C.L. and Stevens, W.S. (2012b). Accumulation of HIV drug resistance mutations in patients failing first-line antiretroviral treatment in South Africa. *AIDS Research and Human Retroviruses*, *28*(2), pp.171-175.

Simoni, J.M., Montgomery, A., Martin, E., New, M., Demas, P.A. and Rana, S. (2007). Adherence to antiretroviral therapy for pediatric HIV infection: a qualitative systematic review with recommendations for research and clinical management. *Pediatrics*, *119*(6), pp.1371-1383.

Singh, D., Dhummakupt, A., Siems, L. and Persaud, D. (2017). Alternative sample types for HIV-1 antiretroviral drug resistance testing. *The Journal of Infectious Diseases*, *216*(9), pp.834-837.

Smit, P.W., Sollis, K.A., Fiscus, S., Ford, N., Vitoria, M., Essajee, S., Barnett, D., Cheng, B., Crowe, S.M., Denny, T. and Landay, A. (2014). Systematic review of the use of dried blood spots for monitoring HIV viral load and for early infant diagnosis. *PloS One*, *9*(3), pp.e86461. DOI: https://doi.org/10.1371/journal.pone.0086461.

Snijdewind, I.J., Van Kampen, J.J., Fraaij, P.L., Van Der Ende, M.E., Osterhaus, A.D. and Gruters, R.A. (2012). Current and future applications of dried blood spots in viral disease management. *Antiviral Research*, *93*(3), pp.309-321.

Sohn, A.H. and Hazra, R. (2013). The changing epidemiology of the global paediatric HIV epidemic: keeping track of perinatally HIV-infected adolescents. *Journal of the International AIDS Society*, *16*(1), pp.18555. DOI: https://doi.org/10.7448/IAS.16.1.18555 (Accessed May 2020).

Statistics South Africa. (2018). Statistical Release, mid-year population estimates. Available from: https://www.statssa.gov.za/publications/P0302/P03022018.pdf (Accessed November 2019).

Statistics South Africa. (2019). Statistical Release, mid-year population estimates. Available from: https://www.statssa.gov.za/publications/P0302/P03022019.pdf (Accessed May 2020).

Statistics South Africa. (2020). Statistical Release, mid-year population estimates. Available from: http://www.statssa.gov.za/publications/P0302/P03022020.pdf (Accessed December 2020).

Steegen, K., Levin, L., Ketseoglou, I., Bronze, M., Papathanasopoulos, M.A., Carmona, S. and Stevens, W. (2014). High-level cross-resistance to didanosine observed in South African children failing an abacavir-or stavudine-based 1st-line regimen. *PloS One*, *9*(5), pp.97067. DOI: 10.1371/journal.pone.0097067 (Accessed May 2020).

Sutcliffe, C.G., Van Dijk, J.H., Bolton, C., Persaud, D. and Moss, W.J. (2008). Effectiveness of antiretroviral therapy among HIV-infected children in sub-Saharan Africa. *The Lancet Infectious Diseases*, *8*(8), pp.477-489.

Tang, M.W. and Shafer, R.W. (2012). HIV-1 antiretroviral resistance. *Drugs*, *72*(9), pp.1-25.

Taylor, B.S., Hunt, G., Abrams, E.J., Coovadia, A., Meyers, T., Sherman, G., Strehlau, R., Morris, L. and Kuhn, L. (2011). Rapid development of antiretroviral drug resistance mutations

in HIV-infected children less than two years of age initiating protease inhibitor-based therapy in South Africa. *AIDS Research and Human Retroviruses*, *27*(9), pp.945-956.

Tebit, D.M. and Arts, E.J. (2011). Tracking a century of global expansion and evolution of HIV to drive understanding and to combat disease. *The Lancet Infectious Diseases*, *11*(1), pp.45-56.

Tuboi, S.H., Brinkhof, M.W., Egger, M., Stone, R.A., Braitstein, P., Nash, D., Sprinz, E., Dabis, F., Harrison, L.H. and Schechter, M. (2007). Discordant responses to potent antiretroviral treatment in previously naive HIV-1-infected adults initiating treatment in resource-constrained countries: the antiretroviral therapy in low-income countries (ART-LINC) collaboration. *Journal of Acquired Immune Deficiency Syndromes*, *45*(1), pp.52-59.

Tural, C., Ruiz, L., Holtzer, C., Schapiro, J., Viciana, P., González, J., Domingo, P., Boucher, C., Rey-Joly, C., Clotet, B. and Havana study group. (2002). Clinical utility of HIV-1 genotyping and expert advice: the Havana trial. *AIDS*, *16*(2), pp.209-218.

UNAIDS. (2013). UNAIDS report on the global AIDS epidemic. Available from: http://www.unaids.org/sites/default/files/media_asset/UNAIDS_Global_Report_2013_en_1.pdf. (Accessed April 2016).

UNAIDS. (2014). 90-90-90: An ambitious treatment target to help end the AIDS. Available from: https://www.unaids.org/sites/default/files/media_asset/90-90-90_en.pdf (Accessed May 2020).

UNAIDS. (2016a). South Africa: HIV and AIDS estimates. Available from: https://www.unaids.org/en/regionscountries/countries/southafrica (Accessed May 2020).

UNAIDS. (2016b). WHO validates elimination of mother-to-child transmission of HIV and syphilis in Armenia, Belarus and the Republic of Moldova. Available from: https://www.unaids.org/en/resources/presscentre/pressreleaseandstatementarchive/2016/june/20160607_PR_EMTCT_Europe#:~:text=The%20World%20Health%20Organization%20(WHO,child%20transmission%20of%20HIV%20and (Accessed December 2020).

UNAIDS. (2017). Ending AIDS: Progress towards 90-90-90 targets. Available from: https://www.unaids.org/sites/default/files/media_asset/Global_AIDS_update_2017_en.pdf (Accessed August 2019).

UNAIDS. (2018). South Africa. Available from: https://www.unaids.org/en/regions countries/countries/southafrica (Accessed May 2020).

UNAIDS. (2019a). Global AIDS update. Communities at the centre, defending rights, breaking barriers, reaching people with HIV services. Available from: https://www.unaids.org/sites/default/files/media_asset/2019-global-AIDS-update_en.pdf (Accessed November 2020).

UNAIDS. (2019b). AIDS info: Number of people living with HIV in South Africa. Available from: http://aidsinfo.unaids.org/ (Accessed May 2020).

UNAIDS. (2019c). South Africa. Available from: https://www.unaids.org/en/regions countries/countries/southafrica (Accessed December 2020).

UNAIDS. (2020). Global HIV and AIDS statistics- 2020 fact sheet. Available from: https://www.unaids.org/en/resources/fact-sheet (Accessed November 2020).

Van Laethem, K., De Munter, P., Schrooten, Y., Verbesselt, R., Van Ranst, M., Van Wijngaerden, E. and Vandamme, A.M. (2007). No response to first-line tenofovir + lamivudine + efavirenz despite optimization according to baseline resistance testing: impact of resistant minority variants on efficacy of low genetic barrier drugs. *Journal of Clinical Virology*, *39*(1), pp.43-47.

Van Oosterhout, J.J., Brown, L., Weigel, R., Kumwenda, J.J., Mzinganjira, D., Saukila, N., Mhango, B., Hartung, T., Phiri, S. and Hosseinipour, M.C. (2009). Diagnosis of antiretroviral therapy failure in Malawi: poor performance of clinical and immunological WHO criteria. *Tropical Medicine and International Health*, *14*(8), pp.856-861.

Van Vaerenbergh, K., Van Laethem, K., Albert, J., Boucher, C.A., Clotet, B., Floridia, M., Gerstoft, J., Hejdeman, B., Nielsen, C., Pannecouque, C. and Perrin, L. (2000). Prevalence and characteristics of multinucleoside-resistant human immunodeficiency virus type 1 among European patients receiving combinations of nucleoside analogues. *Antimicrobial Agents and Chemotherapy*, *44*(8), pp.2109-2117.

Van Zyl, G.U., Van der Merwe, L., Claassen, M., Cotton, M.F., Rabie, H., Prozesky, H.W. and Preiser, W. (2009). Protease inhibitor resistance in South African children with virologic failure. *The Pediatric Infectious Disease Journal*, *28*(12), pp.1125-1127.

Vergne, L., Peeters, M., Mpoudi-Ngole, E., Bourgeois, A., Liegeois, F., Toure-Kane, C., Mboup, S., Mulanga-Kabeya, C., Saman, E., Jourdan, J. and Reynes, J. (2000). Genetic diversity of protease and reverse transcriptase sequences in non-subtype-B human immunodeficiency virus type 1 strains: evidence of many minor drug resistance mutations in treatment-naive patients. *Journal of Clinical Microbiology*, *38*(11), pp.3919-3925.

Violari, A., Cotton, M.F., Gibb, D.M., Babiker, A.G., Steyn, J., Madhi, S.A., Jean-Philippe, P. and McIntyre, J.A. (2008). Early antiretroviral therapy and mortality among HIV-infected infants. *New England Journal of Medicine*, *359*(21), pp.2233-2244.

Violin, M., Cozzi-Lepri, A., Velleca, R., Vincenti, A., D'Elia, S., Chiodo, F., Ghinelli, F., Bertoli, A., Monforte, A.D.A., Perno, C.F. and Moroni, M. (2004). Risk of failure in patients with 215 HIV-1 revertants starting their first thymidine analog-containing highly active antiretroviral therapy. *AIDS*, *18*(2), pp.227-235.

Wainberg, M.A. and Brenner, B.G. (2012). The impact of HIV genetic polymorphisms and subtype differences on the occurrence of resistance to antiretroviral drugs. *Molecular Biology International*, *2012*. DOI: 10.1155/2012/256982 (Accessed November, 2020).

Walker, B.R., Colledge, N.R., Ralston, S.H. and Penman, I. (2014). Davidson's principles and practice of medicine, 22nd edition. Churchill Livingstone, London, UK. ISBN: 9780702052248. pp.1-1392.

Walker, S.E. and Lorsch, J. (2013). Sanger dideoxy sequencing of DNA. *Methods in Enzymology*, 529, pp.171-184. DOI: https://doi.org/10.1016/B978-0-12-418687-3.00014-8. (Accessed June 2016).

Wallis, C.L., Erasmus, L., Varughese, S., Ndiweni, D. and Stevens, W.S. (2009). Emergence of drug resistance in HIV-1 subtype C infected children failing the South African national antiretroviral roll-out program. *The Pediatric Infectious Disease Journal*, *28*(12), pp.1123-1125.

Wallis, C.L., Mellors, J.W., Venter, W.D., Sanne, I. and Stevens, W. (2010). Varied patterns of HIV-1 drug resistance on failing first-line antiretroviral therapy in South Africa. *Journal of Acquired Immune Deficiency Syndromes*, *53*(4), pp.480-484.

Wang, J., Bambara, R.A., Demeter, L.M. and Dykes, C. (2010). Reduced fitness in cell culture of HIV-1 with nonnucleoside reverse transcriptase inhibitor-resistant mutations correlates with relative levels of reverse transcriptase content and RNase H activity in virions. *Journal of Virology*, *84*(18), pp.9377-9389.

Wei, X., Ghosh, S.K., Taylor, M.E., Johnson, V.A., Emini, E.A., Deutsch, P., Lifson, J.D., Bonhoeffer, S., Nowak, M.A., Hahn, B.H. and Saag, M.S. (1995). Viral dynamics in human immunodeficiency virus type 1 infection. *Nature*, 373(6510), pp.117-122.

Wensing, A.M., Calvez, V., Ceccherini-Silberstein, F., Charpentier, C., Günthard, H.F., Paredes, R., Shafer, R.W. and Richman, D.D. (2019). 2019 update of the drug resistance mutations in HIV-1. *Topics in Antiviral Medicine*, *27*(3). Available from: https://www.iasusa.org/wp-content/uploads/2019/07/2019-drug-resistance-mutations-figures.pdf (Accessed December 2020).

Wettstein, C., Mugglin, C., Egger, M., Blaser, N., Salazar, L., Estill, J., Bender, N., Davies, M.A., Wandeler, G. and Keiser, O. (2012). Missed opportunities to prevent mother-to-child-transmission in sub-Saharan Africa: systematic review and meta-analysis. *AIDS*, *26*(18), pp.2361-2373.

Whitcomb, J.M., Parkin, N.T., Chappey, C., Hellmann, N.S. and Petropoulos, C.J. (2003). Broad nucleoside reverse-transcriptase inhibitor cross-resistance in human immunodeficiency virus type 1 clinical isolates. *The Journal of Infectious Diseases*, *188*(7), pp.992-1000.

Willerth, S.M., Pedro, H.A., Pachter, L., Humeau, L.M., Arkin, A.P. and Schaffer, D.V. (2010). Development of a low bias method for characterizing viral populations using next generation sequencing technology. *PloS One*, *5*(10), pp.e13564. DOI: 10.1371/journal.pone .0013564. (Accessed February 2020).

Wirden, M., Soulie, C., Valantin, M.A., Fourati, S., Simon, A., Lambert-Niclot, S., Bonmarchand, M., Clavel-Osorio, C., Marcelin, A.G., Katlama, C. and Calvez, V. (2011). Historical HIV-RNA resistance test results are more informative than proviral DNA genotyping in cases of suppressed or residual viraemia. *Journal of Antimicrobial Chemotherapy*, *66*(4), pp.709-712.

Wittkop, L., Günthard, H.F., De Wolf, F., Dunn, D., Cozzi-Lepri, A., De Luca, A., Kücherer, C., Obel, N., Von Wyl, V., Masquelier, B. and Stephan, C. (2011). Effect of transmitted drug resistance on virological and immunological response to initial combination antiretroviral therapy for HIV (EuroCoord-CHAIN joint project): a European multicohort study. *The Lancet Infectious Diseases*, *11*(5), pp.363-371.

Woods, C.K., Brumme, C.J., Liu, T.F., Chui, C.K., Chu, A.L., Wynhoven, B., Hall, T.A., Trevino, C., Shafer, R.W. and Harrigan, P.R. (2012). Automating HIV drug resistance genotyping with RECall, a freely accessible sequence analysis tool. *Journal of Clinical Microbiology*, *50*(6), pp.1936-1942. DOI: 10.1128/JCM.06689-11.

World Health Organization. (2005). Antiretroviral drugs and the prevention of mother-to-child transmission of HIV infection in resource limited settings. Recommendations for a public health approach. Available from: http://www.who.int/hiv/pub/mtct/en/arvd drugsmeeting report.pdf. (Accessed October 2016).

World Health Organisation. (2006). Antiretroviral drugs for treating pregnant women and preventing HIV infection in infants: towards universal access. Recommendations for a public

health approach. Available from: http://www.who.int/hiv/pub/mtct/arv_guidelines_mtct.pdf. (Accessed October 2016).

World Health Organisation. (2009). Surveillance of transmitted HIV drug resistance. Available from: https://www.who.int/hiv/topics/drugresistance/surveillance/en/ (Accessed December 2020).

World Health Organisation. (2010). Antiretroviral drugs for treating pregnant women and preventing HIV infections in infants. Recommendations for a public health approach. Available from: http://apps.who.int/iris/bitstream/10665/75236/1/9789241599818_eng.pdf. (Accessed October 2016).

World Health Organisation. (2012). WHO manual for HIV drug resistance testing using dried blood spot specimens. Available from: https://www.who.int/hiv/pub/drugresistance/dried_blood_spots/en/ (Accessed October 2020).

World Health Organisation. (2013). Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: Recommendations for a public health approach. Available from: http://www.who.int/hiv/pub/guidelines/arv2013/en/ (Accessed November 2019).

World Health Organisation. (2016). Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV Infection: recommendations for a public health approach-second edition. Available from: http://www.who.int/hiv/pub/arv/ arv-2016/en/ (Accessed June 2020).

World Health Organisation. (2017a). Guidelines on the public health response to pretreatment HIV drug resistance. Available from: https://www.who.int/hiv/pub/guidelines/hivdr-guidelines-2017/en/. (Accessed February 2020).

World Health Organisation. (2017b). HIV drug resistance report. Available from: https://www.who.int/hiv/pub/drugresistance/hivdr-report-2017/en/. (Accessed March 2019).

World Health Organisation. (2018). Global action plan on HIVDR 2017-2021. Available from: https://apps.who.int/iris/bitstream/handle/10665/273049/WHO-CDS-HIV-18.12-eng .pdf?ua=1 (Accessed November 2019).

World Health Organization. (2019a). Policy brief: Update of recommendations on first- and second-line antiretroviral regimens, HIV treatment. Available from: https://www.who.int/hiv/pub/arv/arv-update-2019-policy/en/ (Accessed January, 2020).

World Health Organization. (2019b). HIVDR report. Available from: https://www .who.int/hiv/pub/drugresistance/hivdr-report-2019/en/ (Accessed November, 2019).

World Health Organization. (2020). HIV/AIDS. Available from: https://www.who.int/news-room/fact-sheets/detail/hivaids (Accessed November, 2020).

Youngpairoj, A.S., Masciotra, S., Garrido, C., Zahonero, N., De Mendoza, C. and García-Lerma, J.G. (2008). HIV-1 drug resistance genotyping from dried blood spots stored for 1 year at 4°C. *Journal of Antimicrobial Chemotherapy*, *61*(6), pp.1217-1220.

Zaidi, J., Grapsa, E., Tanser, F., Newell, M.L. and Bärnighausen, T. (2013). Dramatic increases in HIV prevalence after scale-up of antiretroviral treatment: a longitudinal population-based HIV surveillance study in rural Kwazulu-Natal. *AIDS*, *27*(14), pp.2301-2305.

Zhang, G., DeVos, J., Medina-Moreno, S., Wagar, N., Diallo, K., Beard, R.S., Zheng, D.P., Mwachari, C., Riwa, C., Jullu, B. and Wangari, N.E. (2018). Utilization of dried blood spot specimens can expedite nationwide surveillance of HIV drug resistance in resource-limited settings. *PloS One*, *13*(9), pp.e0203296. DOI: https://doi.org/10.1371/journal.pone.0203296 (Accessed November, 2020).

Zhao, Y., Mu, W., Harwell, J., Zhou, H., Sun, X., Cheng, Y., Li, C. and Zhang, F. (2011). Drug resistance profiles among HIV-1-infected children experiencing delayed switch and 12-month efficacy after using second-line antiretroviral therapy: an observational cohort study in rural China. *Journal of Acquired Immune Deficiency Syndromes*, *58*(1), pp.47-53.

Zhou, Z., Wagar, N., DeVos, J.R., Rottinghaus, E., Diallo, K., Nguyen, D.B., Bassey, O., Ugbena, R., Wadonda-Kabondo, N., McConnell, M.S. and Zulu, I. (2011). Optimization of a low cost and broadly sensitive genotyping assay for HIV-1 drug resistance surveillance and monitoring in resource-limited settings. *PloS One*, *6*(11), pp.e28184. DOI: https://doi.org /10.1371/journal.pone.0028184 (Accessed April 2019).

Ziemniak, C., George-Agwu, A., Moss, W.J., Ray, S.C. and Persaud, D. (2006). A sensitive genotyping assay for detection of drug resistance mutations in reverse transcriptase of HIV-1 subtypes B and C in samples stored as dried blood spots or frozen RNA extracts. *Journal of Virological Methods*, *136*(1-2), pp.238-247.

Zou, X., He, J., Zheng, J., Malmgren, R., Li, W., Wei, X., Zhang, G. and Chen, X. (2020). Prevalence of acquired drug resistance mutations in antiretroviral-experiencing subjects from 2012 to 2017 in Hunan Province of central South China. *Virology Journal*, *17*(1), pp.1-9.

Zoufaly, A., Fillekes, Q., Hammerl, R., Nassimi, N., Jochum, J., Drexler, J.F., Awasom, C.N., Sunjoh, F., Burchard, G.D., Burger, D.M. and Van Lunzen, J. (2013). Prevalence and determinants of virological failure in HIV-infected children on antiretroviral therapy in rural Cameroon: a cross-sectional study. *Antiviral Therapy*, *18*(5), pp.681-690.

Appendix 1a Protease and RT mutations identified in matched plasma and DBS sequences for accuracy parameter

	Protease		RT	
ID	**Plasma**	**DBS**	**Plasma**	**DBS**
1	T12S, I15V, L19I, N37S, R41K, L63P, H69K, T74TA, V77I, L89M, I93L	T12S, I15V, L19I, N37S, R41K, L63P, H69K, T74TA, V77I, L89M, I93L	**M184V**, **K103N, V1061,** V35K, T39E, S48T, V106I, K122E, D123G, K173A, D177E, T200A, Q207A, R211K, F214FL, E224D	**M184V, K103N**, **V106I,**V35K, T39E, S48T, V106I, K122E, D123G, K173A, D177E, T200A, Q207A, R211K, F214FL, E224D
2	**M46I, 154V, L76V, V82VA, I84IV**, **L10F**, I13V, L19I, K20R, M36I, N37D, R41K, D60E,Q61DEKN,I62IV, L63P, H69K, A71AV, L89IM, I93IL	**M46I, 154V, L76V, V82VA, I84IV**, **L10F** ,I13V, L19I, K20R, M36I, N37D, R41K, D60E,Q61DEKN,I62IV, L63P, H69K, A71AV, L89IM, I93IL	**M41ML, D67N, K70R, M184V, T215F, K219Q**, **K103N**, V35T, T39E, S48T, E53D, D121Y, K122E, I35V, I142V, K173AV, Q174K, D177E, I178L, T200A, E203D, Q207E, R211K	**M41ML, D67N, K70R, M184V, T215F, K219Q**, **K103N**, V35T, T39E, S48T, E53D, D121Y, K122E, I35V, I142V, K173AV, Q174K, D177E, I178L, T200A, E203D, Q207E, R211K
3	**V82VA**, T12S, I15V, G16E, L19E, K20M, E35N, M36I, R41K, R57K, D60E, L63A, H69K, I93L	**V82A**, T12S, I15V, G16E, L19E, K20M, E35N, M36I, R41K, R57K, D60E, L63A, H69K, I93L	**D67N, K70R, M184V, K219Q**, **K103N, V106M, E138Q**, V21I, T27S, V35T, T39D, T69N, R83K, V90I, I94L, A98AS, L109I, K122E, D123N, S162A, K173T, D177E, I178L, V179I, T200A, E204Q, Q207E	**D67N, K70R, M184V, K219Q**, **K103N, V106M, E138Q**, V21I, T27S, V35T, T39D, T69N, R83K, V90I, I94L, L109I, K122E, D123N, T139I, S162A, K173T, D177E, I178L, V179I, T200A, E204Q, Q207E
4	T12S, I15V, G16E, L19I, K20R, E35D, M36I, P39T, R41K, K45KR, D60E, L63P, H69K, L89M, I93L	T12S, I15V, G16E, L19I, K20R, E35D, M36I, P39T, R41K, D60E, L63P, H69K, L89M, I93L	**D67DG, K70KE, T215TNSY, K219KR**, **K101KE, Y188YFL, G190GA**, V35T, T39TK, P55PS, V60VI, S68SR, A98S, K122E, D123S, S162SC, K173A, Q174K, D177E, T200A, Q207DE, R211K, F214FL	**D67DG, K70KE, T215TNSY, K219KR, K101KE, Y188YFL, G190GA**, V35T, T39TK, P55PS, V60VI, S68SR, A98S, K122E, D123S, S162SC, K173A, Q174K, D177E, T200A, Q207DE, R211K, F214FL
5	T12S, I15V, L19I, M36I, R41K, H69K, I93L	T12S, I15V, L19I, M36I, R41K, H69K, I93L	**M184V**, **K103N, V106VIM**, V35T, E36A, T39E, S48T, W88C, I94L, K122E, K173A, T200A, Q207E, R211K, F214L,	**M184V**, **K103N, V106VIM**, V35T, E36A, T39E, S48T, W88C, I94L, K122E, K173A, T200A, Q207E, R211K, F214L
6	T12S, I15V, L19I, M36I, L63P, H69K, L89M, I93L	T12S, I15V, L19I, M36I, L63P, H69K, L89M, I93L	V35T, T39N, V106I, K122E, D123DGNS, I142V, S162HY, K173A, D177E, T200A, Q207E, R211K	V35T, T39N, V106I, K122E, D123DGNS, I142V, S162HY, K173A, D177E, T200A, Q207E, R211K
7	K14R, I15V, L19I, K20KR, R41K, L63P, H69K, V77I, L89M, I93L	K14R, I15V, L19I, K20R, M36MI, R41K, L63P, H69K, V77I, L89M, I93L	**E138A**, V35T, T39E, S48T, K122E, D123DN, K173A, Q174K, D177E, I78M, Q197S, T200A, I202V, Q207D, R211K	**E138A**, V35T, T39E, S48T, V106VG, K122E, D123DN, Q145QR, P157PS, K173A, Q174K, D177E, I78M, Q182QP, Q197S, T200A, I202V, Q207D, L209LP, L210LP, R211K
8	T12S, I15V, L19I, K20KR, E35D, M36I, N37ND, R41RK, L63LP, H69K, T74TS, L89M, I93L	T12S, I15V, L19I, K20KR, E35D, M36I, N37ND, R41RK, L63LP, H69K, T74TS, L89M, I93L	V35T, E36A, T39E, S48T, V60I, K173A, Q174K, D177E, T200A, E203D, Q207A	V35T, E36A, T39E, S48T, V60I, K173A, Q174K, D177E, T200A, E203ED, Q207A

9	T12S, L19E, K20R, M36I, R41K, I62IV, L63P, H69K, L89IM, I93L	T12S, L19E, K20R, M36I, R41K, I62IV, L63P, H69K, L89IM, I93L	V35T, E36A, T39E, S48T, K122E, D123E, I135T, S162A, K173M, Q174R, T200A, Q207E, R211K	V35T, E36EA, T39E, S48T, K122E, D123E, I135T, S162A, K173M, Q174R, T200A, Q207E, R211K
10	**M46I, 154V, L76V, V82VA, L10F** T4S, I15V, G16A, L19I, K20R, E35D, M36I, R41K, R57K, Q61N, I62V, L63V, E65D, H69K, T74S, L89M	**M46I, 154V, L76V, V82VA**, **L10F**, T4S, I15V, G16A, L19I, K20R, E35D, M36I, R41K, R57K, Q61N, I62V, L63V, E65D, H69K, T74S, L89M	**M41L, E44D, D67N, K70E, M184V, L210W, T215Y**, **A98G**, V35T, E36K, T39E, S48T, K122E, D123N, K173A, Q174K, D177E, T200A, E203D, Q207E, R211K	**M41L, E44D, D67N, K70E, M184V, L210W, T215Y**, **A98G**, V35T, E36K, T39E, S48T, G93GE, K122E, D123N, K173A, Q174K, D177E, T200A, I202V, E203D, Q207E, R211K
11	T12S, I15V, G16E, L19I, E35D, M36I, N37Q, P39S, R41K, D60E, Q61N, H69K, I72IV, L89M, I93L	T12S, I15V, G16E, L19I, E35D, M36I, N37Q, P39S, R41K, D60E, Q61N, H69K, I72V, L89M, I93L	**M184V** V35T, E36A, T39E, S48T, K122E, D123S, I135T, K173A, Q174K, D177E, T200A, E203EK, Q207DN, L210LF, R211K, F214L	**M184V**, V35T, E36A, T39E, S48T, V60VI, K122E, D123S, I135T, K173A, Q174K, D177E, T200A, E203EK, Q207DN, L210LF, R211K, F214L

DBS dried blood spots RT reverse transcriptase

Major protease and reverse transcriptase inhibitor mutations are shown in bold. Discordances are underlined.

Appendix 1b Interpretation of Protease and RT mutations identified in plasma and DBS sequences

ID	Protease Inhibitors		NRTIs		NNRTIs	
1	Atazanavir Darunavir Lopinavir	Susceptible Susceptible Susceptible	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	Low-Level Resistance Susceptible High-Level Resistance High-Level Resistance Susceptible	Efavirenz Etravirine Nevirapine Rilpivirine	High-Level Resistance Potential Low Level Resistance High-Level Resistance Potential Low Level Resistance
2	Atazanavir Darunavir Lopinavir	High-Level Resistance Intermediate Resistance High-Level Resistance	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	High-Level Resistance High-Level Resistance High-Level Resistance High-Level Resistance Intermediate Resistance	Efavirenz Etravirine Nevirapine Rilpivirine	High-Level Resistance Susceptible High-Level Resistance Susceptible
3	Atazanavir Darunavir Lopinavir	Low-Level Resistance Susceptible Intermediate Resistance	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	High-Level Resistance Intermediate Resistance High-Level Resistance High-Level Resistance Low-Level Resistance	Efavirenz Etravirine Nevirapine Rilpivirine	High-Level Resistance Potential Low-Level Resistance High-Level Resistance Low-Level Resistance
4	Atazanavir Darunavir Lopinavir	Susceptible Susceptible Susceptible	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	Intermediate Resistance Intermediate Resistance Potential Low-Level Resistance Potential Low-Level Resistance Intermediate Resistance	Efavirenz Etravirine Nevirapine Rilpivirine	High-Level Resistance Intermediate Resistance High-Level Resistance High-Level Resistance
5	Atazanavir Darunavir Lopinavir	Susceptible Susceptible Susceptible	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	Low-Level Resistance Susceptible High-Level Resistance High-Level Resistance Susceptible	Efavirenz Etravirine Nevirapine Rilpivirine	High-Level Resistance Susceptible High-Level Resistance Susceptible
6	Atazanavir Darunavir Lopinavir	Susceptible Susceptible Susceptible	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	Susceptible Susceptible Susceptible Susceptible Susceptible	Efavirenz Etravirine Nevirapine Rilpivirine	Susceptible Susceptible Susceptible Susceptible
7	Atazanavir Darunavir Lopinavir	Susceptible Susceptible Susceptible	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	Susceptible Susceptible Susceptible Susceptible Susceptible	Efavirenz Etravirine Nevirapine Rilpivirine	Susceptible Potential Low-Level Resistance Susceptible Low-Level Resistance

8	Atazanavir Darunavir Lopinavir	Susceptible Susceptible Susceptible	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	Susceptible Susceptible Susceptible Susceptible Susceptible	Efavirenz Etravirine Nevirapine Rilpivirine	Susceptible Susceptible Susceptible Susceptible
9	Atazanavir Darunavir Lopinavir	Susceptible Susceptible Susceptible	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	Susceptible Susceptible Susceptible Susceptible Susceptible	Efavirenz Etravirine Nevirapine Rilpivirine	Susceptible Susceptible Susceptible Susceptible
10	Atazanavir Darunavir Lopinavir	High-Level Resistance Low-Level Resistance High-Level Resistance	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	High-Level Resistance High-Level Resistance High-Level Resistance High-Level Resistance High-Level Resistance	Efavirenz Etravirine Nevirapine Rilpivirine	Low-Level Resistance Potential Low-Level Resistance Intermediate Resistance Low-Level Resistance
11	Atazanavir Darunavir Lopinavir	Susceptible Susceptible Susceptible	Abacavir Zidovudine Emtricitabine Lamivudine Tenofovir	Low-Level Resistance Susceptible High-Level Resistance High-Level Resistance Susceptible	Efavirenz Etravirine Nevirapine Rilpivirine	Susceptible Susceptible Susceptible Susceptible

DBS dried blood spots NRTIs nucleoside reverse transcriptase inhibitors NNRTIs non-nucleoside reverse transcriptase inhibitors RT reverse transcriptase

Appendix 2a Protease and RT mutations and the interpretation of resistance profiles identified in five replicates of three samples amplified together in a single run to determine the precision of the assay

	Resistance mutations		Interpretation of resistance profiles		
ID	**Protease**	**RT**	**Protease**	**NRTIs**	**NNRTIs**
1a	T12S, I15V, L19I, M36I, R41K, L63P, H69K, L89M, I93L	**K70E**, **M184V, K103N**,**P225H,** P4S, K20R, K32N, V35K, E36A, T39E, S48T, A98S, K122E, D123S, S162C, K173A, D177E, I178M, T200A, Q207E, L228R	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-IR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-LLR	Doravirine-IR Efavirenz-HLR Etravirine-S Nevirapine-HLR Rilpivirine-S
1b	T12S, I13IL, I15V, L19I, M36I, R41K, L63P, H69K, L89M, I93L	**K70E**, **M184V**, **K219KR, K103N**, **P225H,** P4S, I5IV, K20R, K32N, V35K, E36A, T39E, S48T, A98S, K122E, D123S, S162C, K173A, D177E, I178M, T200A, Q207E, L228R	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-IR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-LLR	Doravirine-IR Efavirenz-HLR Etravirine-S Nevirapine-HLR Rilpivirine-S
1c	T12S, I15V, L19I, M36I, R41K, L63P, H69K, L89M, I93L	**K70E**, **M184V, K103N**, **P225H,** P4S, K20R, K32N, V35K, E36A, T39E, S48T, A98S, K122E, D123S, S162C, K173A, D177E, I178M, T200A, Q207E, L228R	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-IR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-LLR	Doravirine-IR Efavirenz-HLR Etravirine-S Nevirapine-HLR Rilpivirine-S
1d	T12S, I15V, L19I, M36I, R41K, L63P, H69K, L89M, I93L	**K70E**, **M184V**, **K219KR, K103N**, **P225H,** P4S, K20R, K32N, V35K, E36A, T39E, S48T, A98S, K122E, D123S, S162C, K173A, D177E, I178M, T200A, Q207E, L228R	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-IR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-LLR	Doravirine-IR Efavirenz-HLR Etravirine-S Nevirapine-HLR Rilpivirine-S
1e	T12S, I15V, L19I, M36I, R41K, G49K, L63P, H69K, L89M, I93L	**K70E**, **M184V**, **K219R, K103N**, **P225H,** P4S, K20R, K32N, V35K, E36A, T39E, S48T, A98S, K122E, D123S, S162C, K173A, D177E, I178M, T200A, Q207E, L228R	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-IR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-LLR	Doravirine-IR Efavirenz-HLR Etravirine-S Nevirapine-HLR Rilpivirine-S
2a	T12AS, I15V, L19T, M36I, R41K, L63P, H69K, L89M, I93L	**A62AV**, **K65R**, **V75VIM**, **M184V, L100I**, **K103N**, **P225H,** E28R, K32E, V35T, E36A, T39E, S68SN, K122E, D123S, I142Q, K173A, I178L, T200A, Q207K, R211Q, F214L	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-HLR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
2b	T12S, I15V, L19T, M36I, R41K, L63P, H69K, L89M, I93L	**K65R**, **V75VIM**, **Y115YF**, **M184V, L100I**, **K103N**, **P225H,** E28R, K32E, V35T, E36A, T39E, S68SN, K122E, D123S, I142Q, K173A, I178L, T200A, Q207K, R211Q, F214L	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-HLR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR

2c	T12AS, I15V, L19T, M36I, R41K, L63P, H69K, L89M, I93L	**A62AV**, **K65R**, **V75VIM**, **Y115YF**, **M184V, L100I**, **K103N**, **P225H,** E28R, K32E, V35T, E36A, T39E, S68SN, K122E, D123S, I142Q, K173A, I178L, T200A, Q207K, R211Q, F214L	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-HLR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
2d	T12AS, I15V, L19T, M36I, R41K, L63P, H69K, L89M, I93L	**A62AV**, **K65R**, **Y115YF**, **M184V, L100I**, **K103N**, **P225H,** E28R, K32E, V35T, E36A, T39E, K122E, D123S, I142Q, K173A, I178L, T200A, Q207K, R211Q, F214L	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-HLR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
2e	T12S, I15V, L19T, M36I, R41K, L 63P, H69K, L89M, I93L	**K65R**, **Y115YF**, **M184V, L100I**, **K103N**, **P225H,** E28R, K32E, V35T, E36A, T39E, S68SN, K122E, D123S, I142Q, K173A, I178L, T200A, Q207K, R211Q, F214L	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-HLR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
3a	I15V, L19I, M36I, R41K, H69K, T 74S, L89M, I93L	**K65R**, **M184I, K103N**, **M230L,** V35T, T39E, V60I, K122E, D123S, I135LV, S162C, T165 I, K173A, D177E, Q197K, T200E, Q207A, R211K	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-IR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
3b	T12TS, I15V, L19I, M36I, R41K, H69K, T74S, L89M, I93L	**K65R**, **M184MI, K103N**, **M230L,** V35T, T39E, V60I, K122E, D123S, I135LV, S162C, T165 I, K173A, D177E, Q197K, T200E, Q207A, R211K	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-IR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
3c	I15V, L19I, M36I, R41K, H69K, T 74S, L89M, I93L	**K65R**, **M184I, K103N**, **M230L,** V35T, T39E, V60I, K122E, D123S, I135LV, S162C, T165 I, K173A, D177E, Q197K, T200E, Q207A, R211K	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-IR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
3d	I15V, L19I, M36I, R41K, H69K, T 74S, L89M, I93L	**K65R**, **M184I, K103N**, **M230L,** V35T, T39E, V60I, K122E, D123S, I135L, S162C, T165I, K173A, D177E, Q197K, T200E, Q207A, R211K	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-IR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
3e	I15V, L19I, M36I, R41K, H69K, T 74S, L89M, I93L	**K65R**, **M184I, K103N**, **M230L,** V35T, T39E, V60I, K122E, D123S, I135L, S162C, T165I, K173A, D177E, Q197K, T200E, Q207A, R211K	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-IR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR

HLR high level resistance IR intermediate resistance LLR low level resistance NRTIs nucleoside reverse transcriptase inhibitors NNRTIs non-nucleoside reverse transcriptase inhibitors RT reverse transcriptase S Susceptible

Major protease and reverse transcriptase inhibitor mutations are shown in bold

Appendix 2b Protease and RT mutations and the interpretation of resistance profiles identified in five replicates of three samples to determine the reproducibility of the assay

	Resistance mutations		Interpretation of resistance profiles		
ID	**Protease**	**RT**	**Protease**	**NRTIs**	**NNRTIs**
1a	T12S, I15V, L19I, M36I, R41K, L63P, H69K, L89M, I93L	**K70E**, **M184V**, **K219R, K103N**, **P225H,** P4S, K20R, K32N, V35K, E36A, T39E, S48T, A98S, K122E, D123S, S162C, K173A, D177E, I178M, T200A, Q207E, L228R	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-IR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-LLR	Doravirine-IR Efavirenz-HLR Etravirine-S Nevirapine-HLR Rilpivirine-S
1b	T12AS, I15V, L19I, M36I, R41K, L63P, H69K, L89M, I93L	**K70E**, **M184V**, **K219KR, K103N**, **P225H,** P4S, K20R, K32N, V35K, E36A, T39E, S48T, A98S, K122E, D123S, S162C, K173A, D177E, I178M, T200A, Q207E, L228R	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-IR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-LLR	Doravirine-IR Efavirenz-HLR Etravirine-S Nevirapine-HLR Rilpivirine-S
1c	T12S, I15V, L19V, M36I, R41K, L63P, H69K, L89M, I93L	**K70E**, **M184V, K103N**, **P225H,** P4S, K20R, K32N, V35K, E36A, T39E, S48T, A98S, K122E, D123S, S162C, K173A, D177E, I178M, T200A, Q207E, L228R	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-IR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-LLR	Doravirine-IR Efavirenz-HLR Etravirine-S Nevirapine-HLR Rilpivirine-S
1d	T12S, I15V, L19I, M36I, R41K, L63P, H69K, L89M, I93L	**K70E**, **M184V, K103N**, **P225H,** P4S, K20R, K32N, V35K, E36A, T39E, S48T, A98S, K122E, D123S, S162C, I167X, K173A, D177E, I178M, T200A, Q207E, L228R	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-IR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-LLR	Doravirine-IR Efavirenz-HLR Etravirine-S Nevirapine-HLR Rilpivirine-S
1e	T12S, I15V, L19I, M36I, R41K, L63P, H69K, L89M, I93L	**K70E**, **M184V, K103N**, **P225H,** P4S, K20R, K32N, V35K, E36A, T39E, S48T, A98S, K122E, D123S, S162C, K173A, D177E, I178M, T200A, Q207E, L228R	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-IR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-LLR	Doravirine-IR Efavirenz-HLR Etravirine-S Nevirapine-HLR Rilpivirine-S
2a	T12S, I15V, L19T, M36I, R41K, L63P, H69K, L89M, I93L	**A62AV**, **K65R**, **V75VIM**, **Y115YF**, **M184V, L100I**, **K103N**, **P225H,** E28R, K32E, V35T, E36A, T39E, S68SN, K122E, D123S, I142Q, K173A, I178L, T200A, Q207K, R211Q, F214L	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-HLR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
2b	T12S, I15V, L19T, M36I, R41K, L63P, H69K, L89M, I93L	**K65R**, **V75VIM**, **Y115YF**, **M184V, L100I**, **K103N**, **P225H,** K20KR, E28R, K32E, V35T, E36A, T39E, S68SN, K122E, D123S, I142Q, K173A, I178L, T200A, Q207K, R211Q, F214L	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-HLR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR

2c	T12AS, I15V, L19T, M36I, R41K, L63P, H69K, L89M, I93L	**K65R**, **V75VIM**, **Y115YF**, **M184V,** **L100I**, **K103N**, **P225H,** E28R, K32E, V35T, E36A, T39E, S68SN, K122E, D123S, I142Q, K173A, I178L, T200A, Q207K, R211Q, F214L	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-HLR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
2d	T12S, I15V, L19T, M36I, R41K, L 63P, H69K, L89M, I93L	**K65R**, **V75VIM**, **Y115YF**, **M184V,** **L100I**, **K103N**, **P225H,** E28R, K32E, V35T, E36A, T39E, S68SN, K122KE, D123 S, I142Q, K173A, I178L, T200A, Q207K, R211Q, F214L	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-HLR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
2e	T12AS, I15V, L19T, M36I, R41K, L63P, H69K, L89M, I93L	**A62AV**, **K65R**, **Y115YF**, **M184V,** **L100I**, **K103N**, **P225H,** E28R, K32E, V35T, E36A, T39E, K122E, D123S, I142Q, K173A, I178L, T200A, Q207K, R211Q, F214L	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-HLR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
3a	I15V, L19I, M36I, R41K, H69K, T 74S, L89M, I93L	**K65R**, **M184I, K103N**, **M230L,** V35T, T39E, V60I, K122E, D123S, I135L, S162C, T165I, K173A, D177E, Q197K, T200E, Q207A, R211K	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-IR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
3b	I15V, L19I, M36I, R41K, H69K, T 74S, L89M, I93L	**K65R**, **M184I, K103N**, **M230L,** V35T, T39E, V60I, K122E, D123S, I135LV, S162C, T165 I, K173A, D177E, Q197K, T200E, Q207A, R211K	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-IR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
3c	I15V, L19I, M36I, R41K, H69K, T 74S, L89M, I93L	**K65R**, **M184MI, K103N**, **V108VI**, **M230L,** V35T, T39E, V60I, K122E, D123S, I135LV, S162C, T165 I, K173A, D177E, Q197K, T200E, Q207A, R211K	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-IR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
3d	I15V, L19I, M36I, R41K, H69K, T 74S, L89M, I93L	**K65R**, **M184MI, K103N**, **V108VI**, **M230L,** V35T, T39E, V60I, K122E, D123S, I135LV, S162C, T165 I, K173A, D177E, Q197K, T200E, Q207A, R211K	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-IR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR
3e	I15V, L19I, M36I, R41K, H69K, T 74S, L89M, I93L	**K65R**, **M184I, K103N**, **M230L,** V35T, T39E, V60I, K122E, D123S, I135L, S162C, T165I, K173A, D177E, Q197K, T200E, Q207A, R211K	Atazanavir-S Darunavir-S Lopinavir-S	Abacavir-HLR Zidovudine-S Emtricitabine-HLR Lamivudine-HLR Tenofovir-IR	Doravirine-HLR Efavirenz-HLR Etravirine-IR Nevirapine-HLR Rilpivirine-HLR

HLR high level resistance IR intermediate resistance LLR low level resistance NRTIs nucleoside reverse transcriptase inhibitors NNRTIs non-nucleoside reverse transcriptase inhibitors RT reverse transcriptase Susceptible S

Major protease and reverse transcriptase inhibitor mutations are shown in bold

Appendix 3: Ethics committee letter, project approval UFS-HSD2017/0633

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

26-Mar-2020

Dear **Miss Iqra Barakzai**

Ethics Number: UFS-HSD2017/0633

Ethics Clearance: **Drug resistance mutations in newly diagnosed HIV-infected infants and in children and adolescents with virological failure on ART**

Principal Investigator: **Miss Iqra Barakzai**

Department: **Medical Microbiology Department (Bloemfontein Campus)**

SUBSEQUENT SUBMISSION APPROVED

With reference to your recent submission for ethical clearance from the Health Sciences Research Ethics Committee. I am pleased to inform you on behalf of the HSREC that you have been granted ethical clearance for your request as stipulated below:

Major amendment:

* Title change from "HIV drug resistance and viral dynamics in HIV-infected paediatric patients in the Free State province, South Africa" to "Drug resistance mutations in newly diagnosed HIV-infected infants and in children and adolescents with virological failure on ART"
* Change to Objective 3 affecting sample size
* Delete Objective 4
* Additional Objective incorporated

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this request for ethical clearance and we wish you continued success with your research.

Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00011992; REC 230408-011; IORG 0010096; FWA 00027947
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

Appendix 4: Ethics committee letter, project approval UFS-HSD2017/0612

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

08 November 2017

DR D GOEDHALS
DEPT OF MEDICAL MICROBIOLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Dr D Goedhals

HSREC 69/2017 (UFS-HSD2017/0612)
PRINCIPAL INVESTIGATOR: DR D GOEDHALS
PROJECT TITLE: EARLY INFANT DIAGNOSIS AND DRUG RESISTANCE IN HIV-INFECTED PAEDIATRIC PATIENTS

APPROVED

1. You are hereby kindly informed that the Health Sciences Research Ethics Committee (HSREC) approved this protocol after all conditions were met. This decision will be ratified at the next meeting to be held on 05 Desember 2017.
2. The Committee must be informed of any serious adverse event and/or termination of the study.
3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.
4. A progress report should be submitted within one year of approval and annually for long term studies.
5. A final report should be submitted at the completion of the study.
6. Kindly use the **HSREC NR** as reference in correspondence to the HSREC Secretariat.
7. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services-(HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

Yours faithfully

MS MGE MARAIS
HEAD: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE ADMINISTRATION

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

Appendix 5: National Department of Health approval

health
Department of Health
FREE STATE PROVINCE

09 October 2017

Dr D Goedhals
Dept. of Medical Microbiology
Faculty of Health Science
UFS

Dear Dr D Goedhals

Subject: Early infant diagnosis and drug resistance in HIV-infected paediatric patients.

- Please ensure that you read the whole document, Permission is hereby granted for the above – mentioned research on the following conditions:
- Serious Adverse events to be reported to the Free State department of health and/ or termination of the study
- Ascertain that your data collection exercise neither interferes with the day to day running of the facilities nor the performance of duties by the respondents or health care workers.
- Confidentiality of information will be ensured and please do not obtain information regarding the identity of the participants.
- Research results and a complete report should be made available to the Free State Department of Health on completion of the study (a hard copy plus a soft copy).
- Progress report must be presented not later than one year after approval of the project to the Ethics Committee of the University of Free State and to Free State Department of Health.
- Any amendments, extension or other modifications to the protocol or investigators must be submitted to the Ethics Committee of the University of Free State and to Free State Department of Health.
- **Conditions stated in your Ethical Approval letter should be adhered to and a final copy of the Ethics Clearance Certificate should be submitted to** sebeelats@fshealth.gov.za **before you commence with the study**
- No financial liability will be placed on the Free State Department of Health
- Please discuss your study with the institution manager/CEOs on commencement for logistical arrangements
- Department of Health to be fully indemnified from any harm that participants and staff experiences in the study
- Researchers will be required to enter in to a formal agreement with the Free State department of health regulating and formalizing the research relationship (document will follow)
- You are encouraged to present your study findings/results at the Free State Provincial health research day
- Future research will only be granted permission if correct procedures are followed see http://nhrd.hst.org.za

Trust you find the above in order.

Kind Regards

Dr D Moeti
HEAD: HEALTH
Date: 10/10/2017

Head : Health
PO Box 227, Bloemfotein, 9300
4th Floor, Executive Suite, Bophelo House, cnr Maitland and, Harvey Road, Bloemfotein
Tel: (051) 408 1646 Fax: (051) 408 1556 e-mail:khusemj@fshealth.gov.za@fshealth.gov.za/chikobvup@fshealth.gov.za
www.fs.gov.za

Appendix 6: National Health Laboratory Services approval

NATIONAL HEALTH LABORATORY SERVICE

Office of the Pathology Representative
Faculty of Health Science
Francois Retief Building, Block C, Ground Floor, Room 210
Tel: 051 405 2807
Email: magda.theron@nhls.ac.za / TheronM1@ufs.ac.za
Prof Magda Theron

9 May 2017

Dear Dr D Goedhals

Department of Medical Microbiology and Virology
Project title: *Early infant diagnosis and drug resistance in HIV-infected paediatric patients.*
Project leader: Dr D Goedhals
Co-investigators: Prof J Frater, Nuffield Department of Medicine, University of Oxford,
Dr P Matthews, Nuffield Department of Medicine, University of Oxford
Dr R van Zyl, Dept Paediatrics and Child Health, UFS
Dr MD Morobadi, Dept Medical Microbiology and Virology, UFS/NHLS
Mr PA Bester, Dept Medical Microbiology and Virology, UFS/NHLS
Ms I Barakzai, Dept Medical Microbiology and Virology, NHLS

Application: Permission to perform study in NHLS laboratories.

- Permission is hereby grated to perform the study entitled: *"Early infant diagnosis and drug resistance in HIV-infected paediatric patients"* within the laboratories of Department of Medical Microbiology and Virology on the following condition:
  - Dual authorship for both the University of the Free State and National Health Laboratory Service on all outputs.

1. Approval of project proposal, research funding and approval by head of department are noted.
2. Your multi-facility approach has been noted.
3. We appreciate your constant contribution towards the university and the NHLS and we wish you a successful study.

Yours faithfully

Prof Magda Theron Ph.D.

Appendix 7: Elsevier permission obtained for figure 1.1

RE: Obtain permission request - Journal (975940)

ELSEVIER

Dear Mrs Iqra Barakzai

We hereby grant you permission to reprint the material below at no charge **in your thesis** subject to the following conditions:

RE: DAVIDSON'S PRINCIPLES AND PRACTICE OF MEDICINE 22ND EDITION, 2014, ISBN: 9780702052248, Walker et al, 1 Figure only

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"This article was published in Publication title, Vol number, Author(s), Title of article, Page Nos, Copyright Elsevier (or appropriate Society name) (Year)."

3. Your thesis may be submitted to your institution in either print or electronic form.

4. Reproduction of this material is confined to the purpose for which permission is hereby given

5. This permission is granted for non-exclusive world **English** rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form other than submission. Should you have a specific electronic project in mind please reapply for permission.

6. This includes permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

7. Posting of the full article/ chapter online is not permitted. You may post an abstract with a link to the Elsevier website www.elsevier.com, or to the article on ScienceDirect if it is available on that platform.

Thanks & Regards,
Roopa Lingayath
Sr Copyrights Coordinator - Copyrights Team
ELSEVIER | Health Content Operations

Appendix 8: Elsevier permission obtained for figure 1.3

RE: Obtain permission request - Journal (975940)

ELSEVIER

Dear Mrs Iqra Barakzai

We hereby grant you permission to reprint the material below at no charge **in your thesis** subject to the following conditions:

RE: DAVIDSON'S PRINCIPLES AND PRACTICE OF MEDICINE 22ND EDITION, 2014, ISBN: 9780702052248, Walker et al, 1 Figure only

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"This article was published in Publication title, Vol number, Author(s), Title of article, Page Nos, Copyright Elsevier (or appropriate Society name) (Year)."

3. Your thesis may be submitted to your institution in either print or electronic form.

4. Reproduction of this material is confined to the purpose for which permission is hereby given

5. This permission is granted for non-exclusive world **English** rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form other than submission. Should you have a specific electronic project in mind please reapply for permission.

6. This includes permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

7. Posting of the full article/ chapter online is not permitted. You may post an abstract with a link to the Elsevier website www.elsevier.com, or to the article on ScienceDirect if it is available on that platform.

Thanks & Regards,

Roopa Lingayath

Sr Copyrights Coordinator - Copyrights Team

ELSEVIER | Health Content Operations