Prevalence of minority HIV-1 drug resistant quasi-species in children patients at virologic failure in a rural KwaZulu-Natal cohort

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Author's Declaration

I, Miss Hloniphile Ruth Mthiyane, declare that the work described in this thesis has not been submitted to UKZN or other tertiary institution for the purposes of obtaining an academic qualification, whether by I or any other party. Where each other's work was used, it has been acknowledged in the text. This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (ref. BF052/10) and the Health Research Committee of the KwaZulu-Natal Department of Health (HRKM 176/10).

Signed 

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Publications

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Table of Contents

List of figures

List of Tables

List of acronyms

3TC	Lamivudine
ABC	Abacavir
ART	Antiretroviral therapy
AIDS	Acquired immune deficiency syndrome
ARV	Antiretroviral drug
AZT	Zidovudine
d4T	Lamivudine
ddI	Didanosine
DRM	Drug resistance mutation
EFV	Efavirenz
HIV	Human immunodeficiency virus
GSS	Genetic susceptibility score
HAART	Highly active antiretroviral therapy
IAS	International Aids Society
II	Intergrase inhibitors
IQR	Interquartile range
KZN	Kwa-Zulu Natal
LIMS	Laboratory information management system
MTCT	Mother to child transmission
LPV/r	Lopinavir/ritonavir
NGS	Next generation sequencing
NRTI	Nucleoside reverse transcriptase Inhibitor
NNRTI	Non-nucleoside reverse transcriptase Inhibitor
NVP	Nevirapine
ML	Maximum likelihood
pMTCT	Prevention of mother to child transmission
PCR	Polymerase chain reaction
PHC	Primary Health Care
PI	Protease inhibitors
qPCR	Quantification polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcriptase
RTV	Ritonavir
SA DoH	South African Department of Health
SATuRN	South Africa Treatment Resistance Network
TAM	Thymidine analogue mutation
TDF	Tenofovir
VL	Viral load
WHO	World Health Organisation

ABSTRACT

Background

Missed minority drug resistance mutations (DRMs) may pave a way to therapy failure in a short period of time. Therefore, the use of sensitive assays to monitor the presence of minority DRMs in HIV-1 infected individuals especially children is important and urgently needed for better patient management. Assays have been developed including next generation sequencing (NGS) that are able to identify a larger proportion of quasi-species including those bearing minority DRMs within a patient's viral population. This intervention is crucial particularly among children who would require antiretroviral therapy (ART) for their lifetime. Therefore, the aims of this study was to (1) describe the prevalence of minority HIV quasi-species harbouring DRMs in paediatric patients at virologic failure in a rural KwaZulu-Natal (KZN) paediatric cohort using NGS technology and (2) to compare the genotypes generated using Sanger sequencing with NGS.

Study design

This retrospective study was conducted on archived samples (n= 34) collected from August 2011 to June 2014 from infants and children ≤15 years of age on first-line ART (13 on PI-based regimen and 21 on an NNRTI-based regimen) and experiencing virologic failure (defined as two successive viral load results >1000 copies/ml) from a rural KwaZulu-Natal cohort.

Methods

Thirty four patients were genotyped using both Sanger sequencing and NGS. A 1.3kb region of the Pol gene was genotyped using Sanger sequencing, while the whole 9.7kb HIV genome was sequenced using NGS. All electropherograms were analysed using the Geneious V8.0.5 software system for the presence of drug resistance mutations including minority drug resistance mutations. Sequences were assembled against an HIV-1 subtype C reference sequence from South Africa. For NGS a reference sequence was annotated with known HIV resistance mutations within the protease and RT genes. Drug resistance mutations were identified using the RegaDB which references the Stanford, Rega and ANRS resistance algorithms and analysed in correlation with selected clinical and demographic data in STATA v11.

Results

NGS was able to detect minority DRMs in eleven (32.3%) samples which were missed by Sanger sequencing. NGS also detected an additional three (8.8%) specimens that harboured DRMs but were found to be susceptible by Sanger sequencing. Patients on PI-based regimen had a lower prevalence of mutations compared to those on an NNRTI=based regimen.

Conclusion

The presence of minority DRMs among paediatric patients is likely to obstruct the use of ART and consequently predispose patients to therapy failure. This emphasises the critical importance of using specific and sensitive assays for the detection of minority DRM early in treatment particularly among children. We noted that children on PI-based regimen, while at a lower prevalence still harboured DRMs that remained undetected by conventional Sanger sequencing. Finally, this study emphasised the need to apply more sensitive assays to accurately distinguish patients failing due to the emergence of minority DRMs from those that are non-adherent in order to maximize the efficacy of the limited range of anti-retroviral drugs currently in use in South Africa.

CHAPTER ONE

1.1 Introduction

The human immunodeficiency virus type 1 (HIV-1) subtype C accounts for the majority of HIV-1 infections in the world with the Sub-Saharan African region bearing the highest burden [1]. Globally, it was estimated that in 2014 the number of children (≤15 years) living with HIV-1 was approximately 3.2 million[1, 2], 2.9 million (91%) of which were from Sub-Saharan region alone[3]. The Republic of South Africa has the largest HIV-1 epidemic in the world with approximately 7 million people living with HIV which 240 000 infected individuals are children ≤15 years of age[2, 4]. The KwaZulu-Natal province of South Africa has one of the highest prevalence rates bearing 35.9% of this South African epidemic [2, 4]. Mother to child transmission (MTCT) accounts for the majority of HIV-1 infection in children from resource-limited settings including South Africa[5, 6]**.** However, the use of ART has shown to effectively children[5]. In the absence of ART up to 50% of children will die from infection before they even reach the age of two[7]. It was reported that by the end of 2014, HIV-1 infections among children was reduced by 58% from 520 000 in year 2000 down to 220 000 in 2014 globally[2]. However, the rate of ART coverage in children still lags behind that of adults[2, 8], despite Sub-Saharan region bearing equal HIV-1 disease burdens among children and adults (Fig. 4)[9]. By 2014 only 32% of infected children had access to ART world-wide[2], but the use of the antiretroviral (ARV) prophylaxis nevirapine (NVP) either as a single dose intervention at birth or an extended treatment option into infancy has resulted reduction of HIV-1 infection rates in children and preventing HIV transmission from mothers to children during birth or through breastfeeding[10].

In 2004, South Africa initiated ART rollout to HIV-1 infected individuals including children. According to South African ART recent guidelines all children <1year irrespective of CD4 cell count should start ART immediately based on WHO Clinical Stage 4 or at a CD4 count of <200 cells/µl or 15%. Older children between 5 and 15 years of age should start based on WHO Clinical Stage 3 or 4 or at a CD4 count of <500 cells/µl (Table 1)[6]**.**There has since been a rapid increase in ART access making it the world's largest ART programme yet[4]. Despite these gains, patients might experience treatment failure defined as two successive viral load results >1000 copies/ml following at least one year on ART[4, 11]. Reasons for treatment failure among children include:(1) inadequate dosing, (2) poor adherence, (3) long-term challenges due to the limited number of approved ARTs with paediatric friendly formulations, (4) limited laboratory infrastructure to monitor treatment efficacy and (5) the almost inevitable emergence of drug resistance mutation (DRM)[12]. In a resource-limited setting such challenges severely inhibit the benefits of an effective ART programmes among patients, representing a serious public health problem especially dreadful among children**.** Given the greater overall duration of ART exposure in infants and children, there is an urgent need to minimise these challenges in order to preserve future ART options.

Table 1: Standardised national eligibility criteria for starting ART regimens for Infants and children[6]

Eligible to Start ART
All children < 5 years, irrespective of CD4 Children 5 – 15 years: WHO clinical stage 3 or 4 OR CD4 ≤ 500 cells/µL
Require Fast-Track (i.e. start ART within 7 days of being eligible)
Children less than 1 year of age WHO clinical Stage 4 MDR or XDR-TB CD4 Count < 200 cells/µl Or < 15%

To date, the emergence of HIV-1 DRMs remains the most important factor that contributes to treatment failure in patients on highly active antiretroviral therapy (HAART)[12]. In general ART will prevent the emergence of DRMs and it would be unlikely that a patient will have a virus that is resistant to two or more drugs with different mechanism of action[13], provided the patient is fully adherent to their regimen. Due to the absence or very low proofreading activity of DNA-dependant RNA polymerase, the HIV-1 replication cycle is characterised by a high mutation rate with an average of 10^4 substitution per nucleotide copied[14] resulting in a complex population of diverse particles that contain closely related but not identical genomes termed viral quasi-species or minority variants[14]. As a result, HIV-1 infected patients will have a diverse range of minority variants that may obstruct the effectiveness of ART in cases of sub-optimal ART because a regimen in use will be insufficient to stop viral replication[13, 15]. Among children minority DRMs could occur as a result of mother-to-child transmission (MTCT) during birth or through breastfeeding or they could be acquired spontaneously while on ART[4, 16].

Therefore, WHO recommends that in the process of scaling up ART developing countries should establish a national surveillance program for HIV drug resistance monitoring[4]. This includes genotyping at baseline to detect resistance mutations to be performed for all HIV-1 infected infants who have been exposed to any form of ART taken by the mother or infant for prevention of mother to child transmission (pMTCT) of HIV[4, 17]. In South Africa ARV drug resistance testing guidelines recommend drug resistance genotyping for all patients (children and adults) experiencing virologic failure[4] after adherence and other psycho-social issues have been addressed [17]. In a resource-limited setting such as South Africa where sdNVP was widely employed in pMTCT programmes, nevirapine (NVP) associated DRMs are expected and common[6]. This poses an obstacle in countries where NVP is a major component in first-line ARV regimens[6]; for example, Y181C will persist for longer periods occurring as minority DRMs resulting in therapy failure[15]. However , by 2015 WHO released new guidelines recommending lifelong ART for all pregnant and breastfeeding women living with HIV[6]. Where lifelong ART is provided to all pregnant and breastfeeding women living with HIV regardless of CD4 count or WHO clinical stage, these measures directly impact pMTCT programs[6].

Treatment should be maintained after delivery and completion of breastfeeding for life. Of equal importance is an early identification of patients on a failing regimen due to poor adherence as opposed to drug resistance[18], because once on HAART poor adherence is also strongly related to therapy failure[19]. A standard population sequencing method is currently used for resistance genotyping[4, 15, 20, 21]; however, this method is only sensitive if DRMs are occurring at a frequency of >20% within the total patient's viral population[15, 20, 21]. As a result this assay will miss minority DRMs occurring at frequencies < 20%. However, minority DRMs occurring at < 20% frequency within the patient's viral population are also important to understand[11, 16, 18, 20, 22], whether their presence has any clinical implications remains a point of debate but evidence is emerging that they could result in considerable implications to therapy failure. Therefore, minority DRMs are important to understand[11, 16, 18, 20, 22], studies have shown that the presence of baseline[22], pre-treated minority DRMs acquired during pMTCT[9, 15, 23] or during ART was correlated with the virologic failure on subsequent future NNRTI-based regimen. There is strong evidence of the correlation between pre-existing minorities DRMs to DRMs detected at virologic failure. The question remains could the presence of minority DRMs be used as a marker to predict future virologic failure?

A number of studies have established that DRMs occurring at frequencies of <20% of the total viral population (minority DRMs) will complicate the benefits of current and future ART options[11, 16, 18, 20, 22, 24]. Resource-limited settings such as South Africa follow the public health approach to ART delivery with standardised regimens containing limited first-line and second-line options available for HIV-infected individuals[12]. Two nucleoside reverse transcriptase inhibitors (NRTIs) plus a non-nucleoside reverse transcriptase inhibitor (NNRTI) or two NRTIs plus a protease inhibitor (PI) drug[6]. The emergence of DRMs on first-line therapy compromises second-line therapy success[13]. However, Hosseinipour et al 2013 reported that drug resistance patterns detected early at the time of first-line failure on an NNRTI-based regimen could be predictable and have a low risk of compromising second-line PI-based regimen options if these DRM patterns are identified early after virologic failure or ≤12 months after initiating ART[25]. Given that, resource-limited countries including South Africa have limited ART options available for HIV-1 infected patients[12]. The presence of DRMs including minority DRMs is of the great concern and severely limits ART options as well as future ART successes [7]. ART has proven successful at reducing the burden of the HIV/AIDS epidemic[10], by decreasing the number of AIDS-related deaths by 36.7% by 2014 among children and new HIV infections by 58% among children[2]. A direct result is effectively prolonged lives spans where HIV-infected infants are now surviving to adolescence and even adulthood[17].

Sensitive assays have been developed including next generation sequencing (NGS) and ultra-sensitive real-time polymerase chain reaction (qPCR) that are able to identify a larger proportion of quasi-species including those bearing minority DRMs within patient's viral population[16, 20, 22-26]. This is due to their abilities to detect variants at frequencies as low as ≤1%. Hunt et al 2011 noted from 255 South African infants previously exposed to NVP that Sanger sequencing missed a number of mutations detected using a sensitive allele-specific PCR (ASPCR) assay[15]. While Vignoles et al 2009, demonstrated the importance of using a sensitive assay in a cohort of newly diagnosed vertically infected ART-naive children (n=35) from Argentina. Using population genotyping they found that 63(6%) of the children had M184V mutation; however, when a sensitive qPCR method was used an additional 45.5% of the children harboured DRMs associated with therapy failure[22]. Similarly Rowley et al 2010 conducted a study in patients from Botswana and detected minority variant with K103N and Y181C among 65% patients when a sensitive ASPCR assay was used[22].

Given that, while the use of minority in treatment monitoring and surveillance remains a point of contention given that threshold for clinical revelerance remains unclear. There is nonetheless the possibility that ultra-sensitive DRM detection assay may be used to assess the likelihood of therapy failure in short period of time[14]. The use of sensitive assays to monitor the presence and the prevalence of minority DRMs in HIV-1 infected individuals especially children is crucial and urgently needed. Such cases may result in patients being maintained on a failing regimen for a long period of time (months to years) that leads to a high prevalence of DRMs complex resistance patterns including cross-resistance to multiple drugs within a class or over drug classes and accumulation of thymidine analogue mutations (TAMs)[19]. As a result, long standing virologic failure may compromise the potency of the components within future ART regimens[19]. Equally disturbing is the accumulation of TAMs which results in diminished susceptibility to multiple nucleoside reverse transcriptase inhibitors (NRTI) thus causing broad cross-resistance to the NRTI drug class[19, 26]. This study aims to investigate the prevalence of minority HIV quasi-species harbouring DRMs in paediatric patients at virologic failure in rural KwaZulu-Natal (KZN) cohort. By using NGS platform parallel with standard Sanger sequencing method in children from a decentralized rural public health setting we also contrast the frequency of drug resistance mutations detected by either method.

1.2 Justification

South Africa is one of the countries that is heavily struck by HIV-1/AIDS[2, 4, 27]. By 2014, the use of ART among infected children has shown to effectively reduce new infections by 52% and mortality rates by 50%[10]. However, the emergence of DRMs is inevitable and remains a major setback for ART successes[12]. Among children this is of increasing concern representing a hindrance to the achievement of long-term ART in South Africa[28] and of maximising ART benefits given that South Africa is having limited options. As well as the presence of minority DRMs, since several studies have reported that the presence of these DRMs in infected individuals could result in virologic failure within the first year on ART. All of these studies were conducted from an urban area, no investigation to our knowledge have been conducted in a rural setting (Table 2). However, ART outcomes in children from South African rural areas are poorer than those in urban areas [29].

Table 2: Summary of studies conducted on children at virologic failure harbouring HIV minority drug-resistance mutations

Study	Naïve Patients	Pre-treated Patients	No. of patients with minority DRMs	No. of patients at virologic failure	Clinical outcome	Study setting
Lwembe et al 2007[30]	12	No	5	5	Minority DRMs at baseline became dominant on ART and was associated with virologic failure	Nairobi, Kenya (children)
Vignoles et al 2009[22]	35	No	15	Not specified	Minority M184V DRMs at baseline became dominant on ART	Buenos Aires, Argentina (children<14)
Machado et al 2009[31]	1	No	1	1	Minority DRMs at baseline became dominant on ART	Rio de Jainero, Brazil (child, case study)
McCleod et al 2010[24]	7	33	13	16	Minority DRMs at baseline was associated with virologic failure on ART	Botswana (infants)
Hunt et al 2011[15]	no	255	18	8	Minority DRMs at baseline was associated with virologic failure on ART	Johannesburg, South Africa(infants)
Hauser et al 2012[23]	no	7	3	Not specified	Infants due to pMTCT developed NVP associated minority DRMs without postnatal ingestion of NVP	Tanzania (mothers and infants)

Therefore, the detection and monitoring of HIV drug resistance mutations are important to ensure continuous ART efficacy[4]. In resource-limited settings due to limited ART options, infrastructure and data to guide decision making children may remain in virologic failure for longer periods resulting in high prevalence of DRMs and cross-resistance that severely limit future ART options[13]. A study conducted in infants from Johannesburg, South Africa reported that a decision to switch children from an expensive PI to NNRTI-based regimens was based on the detection and prevalence of DRMs [15]. We found studies conducted in children initiated on PI-based regimen stating that, while on virologic failure the presence of DRMs was very low to none. The identification of DRMs in patients at virologic failure could be a tool for distinguishing patients failing due to resistance mutations from those that are non-adherent. By doing so, unnecessary switches will be avoided, maximising the ART

regimens still available. In general 40% of adults patients will re-suppress HIV without regimen switch[19]. However, given the complexity and severity of paediatric disease, achieving this goal is likely to be challenging in children.

In South Africa drug resistance monitoring is not used routinely to manage patients on ART, probably due to the high cost of genotyping and limited facilities to perform such tests. However, there is a need for interventions that focus on the prevention and monitoring of HIV-1 drug resistance including correct identification of minority variants harbouring DRMs using cost effective sensitive assay. As a result a number of sensitive assays such as NGS have been developed which are able to detect minority variants occurring at frequencies down to <1% within an entire viral population[15]. NGS assays provide accurate and sensitive HIV drug resistance detection to minority variant levels in a high throughput manner. In addition; it provides the capacity to scan the entire HIV genome for the presence of DRMs in a single run. These features provide distinct advantages over Sanger sequencing that detects only anticipated mutations. Therefore, the aim of this study is to investigate the prevalence of minority HIV-1 DRMs in infected infants and children at virologic failure in a rural Kwa-Zulu Natal cohort using both Sanger sequencing and NGS. To the ability of NGS and Sanger sequencing to detect and quantify the prevalence of minority HIV quasi-species harbouring DRMs from this rural KZN cohort.

1.3 Aims of the study

1. To investigate the prevalence of minority HIV quasi-species harbouring DRMs in paediatric patients at virologic failure in rural KwaZulu-Natal (KZN) cohort.

2. To assess how many additional mutations are detected by NGS when compared to Sanger sequencing.

1.4 Objectives

1. To assess the efficacy and application of NGS platform as a sensitive assay for minority DRMs identification thus, distinguishing non-adherent patients from those harboring resistance mutations among infants and children at first-line virologic failure.

2. To determine the prevalence of minority HIV DRMs in children and compare the prevalence and patterns of DRMs detected by Sanger sequencing versus NGS.

3. To compare the prevalence of minority DRMs among patients on PI-based regimens versus those on an NNRTI-based regimens

CHAPTER 2

2. Literature Review

2.1 Human Immunodeficiency Virus-1

HIV-1 (Fig. 1) is an RNA virus that belongs to genus Lentivirus a family of Retroviridae. Lentiviruses are classified as slow viruses which infect many species characterized by long-term illnesses and incubation periods [32]. HIV is divided into two types HIV-1 and HIV-2 with HIV-1 being the most common type[33]. HIV is the cause of the acquired immunodeficiency syndrome (AIDS) which is a slowly progressive and degenerative disease of the human immune system[34]. Globally, in 2013, approximately 35 million were living with HIV-1 virus. Briefly, HIV-1 contains nine open reading frames namely: Gag, Pol, Env, Tat, Rev, Nef, Vif, Vpu and Vpr that produce 15 proteins[35]. These products are divided into three major categories; the Gag polyprotein precursor is proteolytically processed to generate the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins. While Gag-Pol polyprotein contains protease (PR), reverse transcriptase (RT) and intergrase (IN)[36], and lastly env gene that encodes for 30-amino-acid, signal peptide (SP), gp120 and gp41.They are transmitted as a single-stranded, positive-sense, enveloped RNA virus[36].

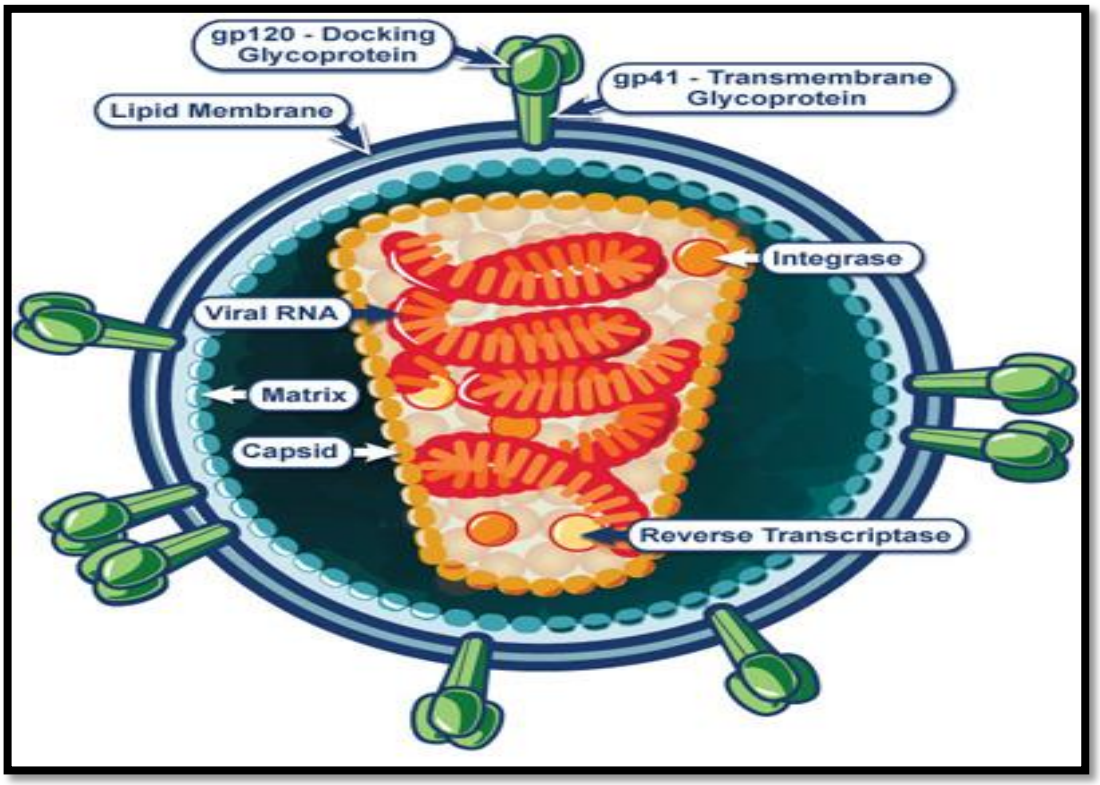


Figure1: The structure of human immunodeficiency virus[32]

2.1.1 Genetic variability

HIV is sub-divided into three distinctive lineages: namely the major group M, the outlier group O and the two new groups N (non-M, non-O) and P[37]. Group M is sub-divided into nine subtypes designated by letters A, B, C, D, F, G, H, J and K [38]. The dominant viral forms are subtypes A and C followed by subtype B; the dominant recombinants are CRF01-AE and CRF02-AG. The dominant viral form in South Africa is subtypes C. The great genetic diversity of HIV-1 has been found in Africa caused by group M viruses with group O and N causing a small minority of infections in central Africa [38].

2.1.2 HIV life cycle

HIV virions enter the human cell by attaching its viral membrane glycoprotein to CD4 receptors and CCR5 or CXCR4 co-receptors of the target cell. Once within the cell, the viral RNA genome is reverse transcribed into a full length double stranded DNA through an error-prone enzyme called reverse transcriptase (Fig. 2). Pro-viral DNA is then integrated into the host chromosome by the integrase enzyme. Whilst in the cell, pro-viral DNA can either enter a latent state or actively replicate to form a large number of virus particles that are then released to infect neighbouring cells[39]. New viral RNA is then translated into precursor protein gp160 which is glycosylated within the endoplasmic reticulum. Gag-pol gene is primarily translated to produce the Gag and Gag-Pol polyprotein; a precursor that is proteolytically processed during the maturation of the virus into six structural proteins which are then rearranged by the protease enzyme to produce mature HIV virions (Fig. 2) [40].

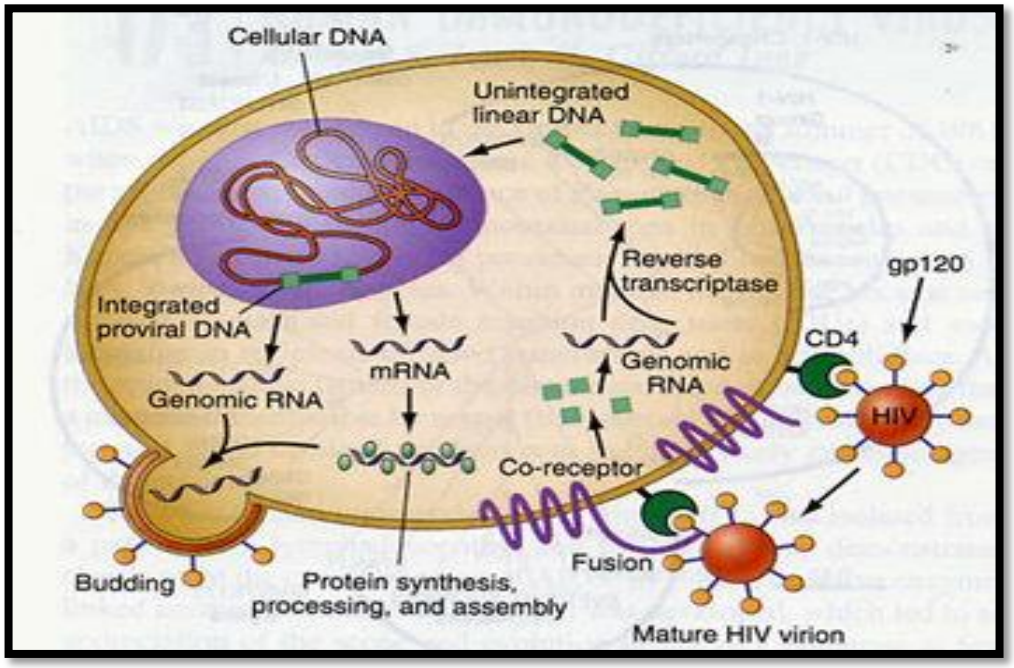


Figure 2: An illustration of the HIV life cycle [40]

2.2 Antiretroviral Drugs (ARVs)

Despite the substantial number of ARVs developed over the past 30 years, since the discovery of HIV. HIV-1 remains incurable and only treatable as a chronic disease[41]. The primary goal of ARVs is to reduce HIV-related morbidity and mortality by hindering HIV replication[42]. ARVs mode of action is by interfering with critical steps of the viral life-cycle namely: adsorption, entry, fusion, un-coating, reverse transcription, integration, transcription and maturation. In recent years newer compounds have been developed including those that target viral entry (CXCR4 and CCR5 antagonists) and virus-cell adsorption/fusion compounds by interacting with either gp120 or gp41 [43]. Consequently, ARVs are classified on the basis of the target with which they interact during HIV-1 replication [44] .

The first HIV-1 specific antiviral drugs were administered as mono-therapy in the early 1990s[45]. However, highly active antiretroviral therapy (HAART) mainly used in South Africa involves a combination of two or three anti-HIV-1 drug classes (NRTI, NNRTI, PI) is effective in suppressing HIV-1 replication[46]. The principle of HAART is to act at the different viral targets to achieve the highest possible benefit, tolerability and compliance and to reduce the risk of resistance development[44].

2.3 Mechanisms action for ARVs

2.3.1 Nucleoside reverses transcriptase inhibitors (NRTIs)

NRTIs were the first class of drugs to be approved by FDA[45], and forms the backbone of ARV regimens in resource-limited settings including South Africa. Currently, there are six FDA-approved NRTIs (nucleoside and nucleotides reverse transcriptase inhibitors) currently available in South Africa for infants and children, namely: Abacavir (ABC), Didanosine (ddI), Lamivudine (3TC), Stavudine (d4T), and Zidovudine (AZT). The nucleotide reverses transcriptase inhibitors Tenofovir (TDF) also forms this [art of this drug class[45].

2.3.2 Non-nucleoside reverses transcriptase inhibitors (NNRTIs)

The NNRTI class of drugs form one of the bases for ARV regimens[47]. NNRTIs block HIV-1 replication by binding distal to the active site of the RT[48] thereby blocking the binding pocket and interfering with the normal functioning of the RT (Fig. 3)[44]. Since 2004, there are four FDA-approved NNRTIs available in South Africa namely: Efavirenz (EFV) and NVP Etravirine (ETR) and Rilpivirine (RPV) [6, 45].

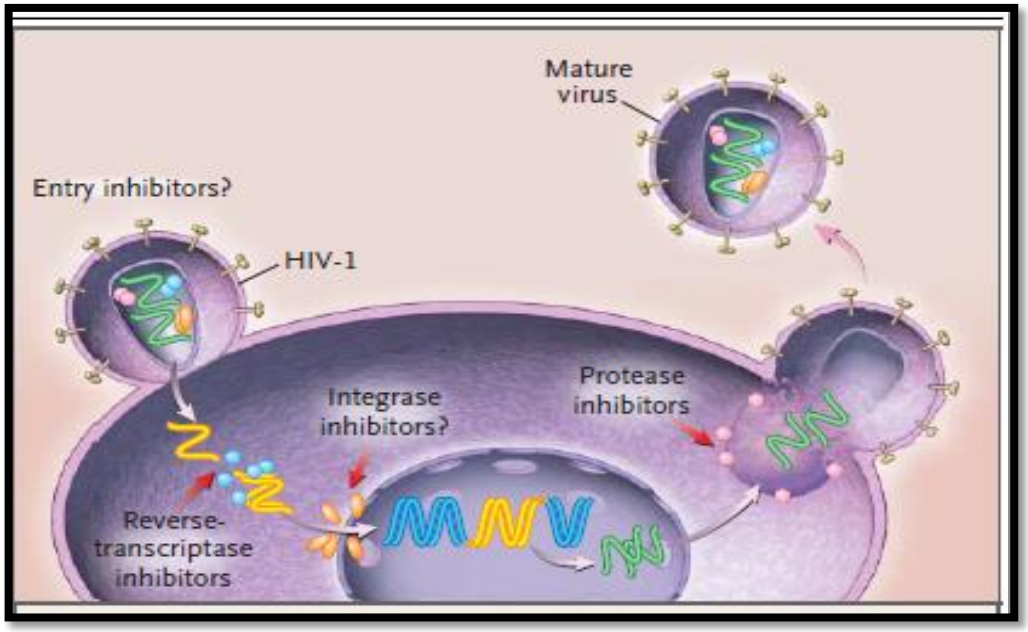


Figure 3: The stages at which different antiretroviral drugs block HIV replication[49]

2.3.3 Protease inhibitors (PIs)

PIs prevent the cleavage of the gag and gag-pol precursor polyproteins to the structural proteins (p17, p24, p7, p6, p2, p1) and functional proteins (protease, RT/RNase-H and integrase), thus arresting maturation and thereby blocking infectivity of the newly synthesised virions (Fig. 3)[43]. PIs presently available for the treatment of HIV infections in South Africa for children include Lopinavir boosted with ritonavir (LPV/r)[45] and Darunavir (DRV)[6].

2.3.4 Entry inhibitors

Entry inhibitors can be subdivided into two distinct classes based on their mode of action namely: fusion inhibitors (FI) and small-molecule CCR5 antagonists[45]**.** FIs form a complex group of ARVs with multiple mechanisms of action depending on the stage of the viral entry process they target. Two entry inhibitors currently approved are enfuvirtide and maraviroc. Enfuvirtide disrupts conformational changes in gp41 that drive membrane fusion,whereas Maraviroc a CCR5 antagonist blocks interactions between the viral envelope proteins and the CCR5 co-receptor [50].

2.3.5 Integrase inhibitors (II)

Retrovirus integration requires at least two viral components: namely the integrase enzyme, and cis-acting sequences at the retroviral DNA termini U3 and U5 ends of the LTRs[43]. IIs interact with the two essential magnesium metal ion cofactors in the integrase active site of the viral DNA[45]. As a result, IIs block the integration step of viral DNA into the host chromosome, preventing the production of the virions. The first IIs licensed for patient's treatment, raltegravir, was approved in 2007[44].

2.4 Antiretroviral Therapy (ART)

The use of ART has changed from mono-therapy in 1990's to combined ART known as HAART in 1996. Since then, HAART has been used to treat HIV-1 infected patients in developed countries[51]. By late 2003 South Africa launched their ART scale-up program which started to run in April 2004. By 2013, 12.9 million people were receiving ART. As a result, the number of people dying of HIV/AIDS has declined by 41% which is 100 000 fewer deaths in 2013[27]. The South African Department of Health (SA DoH) recommends a standard first and second line regimens for children (Table 3)[6].

Despite an extensive rate of ART scale-up program in South Africa, the coverage of children still lags behind that of adults (Fig. 4)[8]. Three of four children living with HIV-1 are not receiving ART.

Table 3: Summary of the standardised South African regimens for infants and children[6]

First-line Regimen	
< 3 years or older children weighing < 10kg	ABC + 3TC + LPV/r
> 3 years and > 10kg	ABC + 3TC + EFV
Currently on d4T-based regimen	Change d4T to ABC if viral load (VL) is undetectable (< 50 copies/mL) If VL > 1000 copies/mL: Manage as possible treatment failure If VL 50 – 1000 copies/mL: Consult with expert or phone the HIV hotline
Adolescents > 15 years AND > 40 kg and CrCl > 80mL/min	TDF + FTC (or 3TC) + EFV Provided as fixed dose combination (FDC)
Currently on ddI containing regimen	Change ddI to ABC, regardless of VL
Second-line Regimen	
Failed first-line Protease Inhibitor (PI) based regimen	
Failed first line PI-based regimen	**Action**
ABC + 3TC + LPV/r d4T + 3TC + LPV/r Unboosted PI-based regimen, while taking rifampicin	Consult with expert for advice and consider resistance testing in patients on LPV/r > 12 months and adherent to treatment
Failed First-line NNRTI-based regimen (discuss with expert before changing)	
Failed first-line NNRTI-based regimen	**Action**
ABC + 3TC + EFV (or NVP)	AZT + 3TC + LPV/r
d4T + 3TC + EFV (or NVP)	AZT + ABC + LPV/r
Third-Line Regimen	
Failing any 2nd line regimen	Should be managed by a Paediatric Infectious Disease Specialist on the basis of genotype resistance testing. Access to third line ART is managed centrally by the National Department of Health

Note: Children ≥ 3 years and exposed to NVP for 6 weeks or longer (PMTCT) should be initiated on ABC + 3TC + LPV/r.

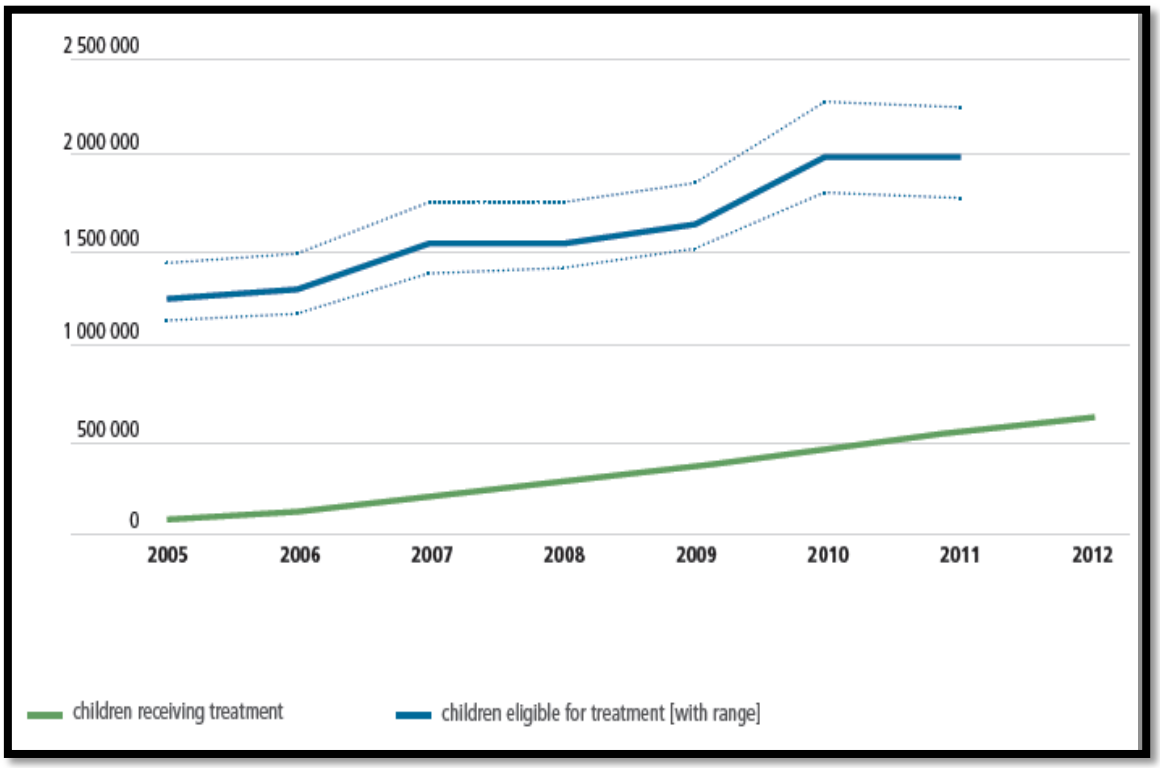


Figure 4: Number of children eligible for, and receiving antiretroviral therapy in low and middle-income countries between 2005 and 2012[3].

2.4.1 ART in children

Access to ART for children has improved over years. By late 2003 South Africa launched their ART scale-up program which started to run in April 2004. Since then the number of children dying from HIV/AIDS has declined by 50% [27]. South African eligibility criteria state that all children less than 5 years irrespective of CD4 should start ART immediately, whereas older children between 5 and 15 years of age should start based on their WHO clinical stage 3 or 4 or CD4 <350 cells/µl[6]. Eleven years later ART options are still limited in South Africa and even fewer options for infants and children [52]. In a South African public sector a number of 9 ARV are available for paediatrics ART offered as paediatric formulation, tablet or capsules [53].

2.5 HIV drug resistance

As the ART coverage continues to grow in Sub-Saharan Africa, some degree of drug resistance development will be inevitable[54]. The development of drug resistance in HIV-1 infected patients is due to the production of genetic variation in the virus due to the selection of drug-resistant pressure during ART. Studies have shown that a major cause of therapy failure in HIV-infected patients is generally associated with the development of HIV DRMs[55]. The Sub-Saharan Africa region has large numbers of HIV-infected individuals in need of life-long ART. However, this region has the limited ART options. Therefore, it is crucial that available regimens are used effectively and to their maximum capacities for patients on ART[54].

The emergence of HIV-1 drug resistance should be distinguished from other causes of therapy failure such as non-adherence, insufficient drug levels and drug regimens with intrinsically weak antiviral activity [56]. To date the ways of making this distinction include adherence counselling, resistance genotyping or measurement of drug levels[17]. These methods may be unreliable, costly and inaccessible, particularly in resource-limited settings. Barriers to adherence namely: regimen complexity, side effects, patient related factors in HAART adherence, psycho-social issues, belief systems and the patient-provider relationship may all result to the development of drug resistance[57]. The emergence of drug resistance should be monitored so that required interventions should be taken to minimise its development[54].

HIV-1 genotypic variants carrying viral protease or RT resistance conferring mutations to one or more ARV drugs can be classified into primary or secondary mutations[56]. Primary (transmitted) drug resistance occurs when a person is infected with a strain of HIV that is resistant to ARV drugs[58]. Secondary (acquired) resistance develops over time[58] and is due to the accumulation of resistance mutations that allow the virus to persist despite the selective pressures exerted by HAART resulting in therapy failure[14]. The mutations in the HIV genome that confers drug resistance can limit the use of subsequent ARV regimens [26].

2.5.1 Drug resistance in children

The development of DRMs in HIV-1 infected children on ART is expected and compromises the benefits of HAART to reduce HIV-related morbidity and mortality[59]. In general, the children develops DRMs that lead to virologic failure within the first year on first line ART regimen[60]. International ART guidelines for infants recommend initiation of ART immediately due to the high risk of rapid disease progression among children[27]. In resource-limited settings infants and children are likely to have been previously exposed to ART during pMTCT programs; therefore, this population is likely to acquire resistance mutations. DRMs will persist as minority variants, thus predisposing these to virologic failure if these individuals require ART later-on in their lives.

Studies have shown that at least 95% of adherence to HAART is required to prevent the development of DRMs which may result to therapy failure, thus compromising long-term benefits of ARV drugs in children [57]. Common causes of poor adherence are usually complex and associated to social issues namely: poor treatment literacy, side-effects, depression and mental illness, poverty, work-related issues, substance use, social problems, denial and pill burden[17]. Children have unique challenges related to ART adherence such as complex dosing regimens, lack of availability of paediatric fixed-dose combinations and poor drug palatability [57]. In addition challenges such as poor socio-economic factors and the literacy levels of caregivers who are usually grandparents may lead to sub-optimal or infrequent administration and ultimately treatment failure[57].

Minimising drug resistance mutations fixed drug combinations and simplified ART strategies are important ways to maintain treatment options as children move through adolescence and possibly reach adulthood [61]. Therefore, in order to provide effective ART for HIV patients, it is essential to understand the mechanisms and the factors that contribute to the development of drug resistance [62]. Among children the development of DRMs is worrying especially with the long term data on children DRMs not available[52]. Furthermore, the development of drug resistance in children is a great concern and should be minimised so that future ART options may be available through adolescence and even to adulthood[63]. Currently, HIV drug resistance testing has proved to be a powerful tool to monitor the development of DRMs [62].

2.6 Detection methods for HIV-1 drug resistance mutations

Since the ART scale-up, the rapid emergence of DRMs has become common[17] . The developing countries including South Africa should focus on maintaining virologic suppression in patients on first line ART[17] by providing simplified first-line regimen for children and adherence support[64]. In order to preserve and maximise the use of ART[17]. The primary goal of drug resistance testing should be to provide necessary information to assist in the selection of ARVs more likely to maintain viral suppression for a long period of time for a better patient's management [58]. Since South African drug resistance testing guidelines recommends that drug resistance genotyping for all patients (children and adults) experiencing virologic failure after adherence and other psycho-social issues addressed [17].

2.6.1 Sanger sequencing method

Until a few years ago the standard method used for sequencing was the Sanger sequencing method first described in 1977[66].This technique involves DNA sequence production carried out with capillary-based semi-automated technologies based on dye-terminator Sanger biochemistry[67]. However, Sanger sequencing is only sensitive to >20%, where genomes are occurring in the majority will be sequenced[68]. Currently, the use of genotypic resistance testing involves DNA sequencing of the pol gene comprising the protease (PR) and reverse transcriptase (RT) genes either in its entirety or a portion thereof containing DRMs relevant to drugs currently in use[69]. The main barrier of genotypic resistance testing to use in resource-limited settings is its cost[70].

Sanger sequencing testing involves DNA sequencing to detect DRMs within the genomic regions relevant to drugs currently in use such as the reverse transcriptase (RT) or protease (PR) genes[69]. Targeted region of the HIV genome is amplified using commercial assay kits *e.g.*TRUGENE and ViroSeq or in-house RT-PCR. Given that the main barrier of genotypic resistance testing to use in resource-limited settings is mainly related to cost[68]. Zhou et al 2011 investigated the cost and sensitivity between commercial assay kits and in-house RT-PCR and found that commercially available kits are not only insensitive to non-B subtypes but they are also more expensive when compared to in-house genotyping[68].

2.6.2 Phenotypic method

The phenotypic method involves cell culture-based assay that measures the concentration of a drug pressure required to reduce replication of the virus (in vitro) [69]. Susceptibility to protease (PR) and reverse transcriptase (RT) inhibitors is measured by using resistance test vectors (RTVs) that contain a luciferase indicator gene and PR and RT sequences derived from HIV-1 in patient plasma[71]. This method is not possible in the resource-limited setting for it requires a specialist laboratory and is very expensive to run. Disadvantages of phenotypic methods are a considerable delay of reporting time due to the wait for culture growth (up to 2 weeks) and its lack of sensitivity to detect minority DRMs[71]. The phenotypic methods can be used when new anti-HIV compounds are being developed to determine their activity against highly drug-resistance patient isolates[72]. However, for clinical purposes this method is difficult to use due to the difficulty of correlating individual mutations with resistance from each of the experimental drug tested[72].

2.6.3 Allele-specific PCR (AS-PCR)

AS-PCR is an allele identification real-time PCR method that is used to detect the genotype of samples by identifying a single mutation in a targeted region. It involves an allele-specific primer set and probes specific to the mutant and wild type variants[73]. AS-PCR is able to detect variants at a frequency as low as ≤1% within a population[74]. Two of the most commonly used probes types are the Taqman and MG probes. These probes have comparable sensitivities and linear ranges of detection. However, the mismatch discrimination of the MGB probe makes it more sensitive when compared to Taqman probe[75, 76].

Although AS-PCR has the limitation of detecting one specific mutation at a time[77] its sensitivity, accuracy, reproducibility and cost-effectiveness make it an effective tool for resistance surveillance of minority DRM s in HIV-1 infected patients[77]. Especially when the key mutations are anticipated for instance Y188C or K103N in nevirapine (NVP) exposed children during pMTCT intervention[15]. Hunt al 2011, compared this method to population sequencing assay in their paediatric cohort (n=255) that was initiated on LPV/r-based ART regimen and were previously NVP-exposed. These children were all screened for the NNRTI key mutations: K103N and Y181C before switching them back to a cheap and tolerant NNRTI-based regimen. From both assays the combined results were as follows: zero - 6 months of age infants harboured 61.9% NNRTIs mutations before starting ART, 6 – 12 months of age children harboured 38.6% mutations, 12-18 months old of age children had 22 % mutations and 18 -24 months of age children 15.5% had persisting mutations occurring as minority variants detected only by AS-PCR. The children with NNRTI mutations were likely to fail when switched back to NNRTI-based regimen[15].

In a resource-limited setting minimising the cost and the emergence of DRMs is important to ensure the effectiveness of a limited number of ART regimens. Therefore, AS-PCR analysis can be used as cost-effective analyses of choice for DRMs as oppose to a costly population sequencing analysis[15]. Given that South African guidelines recommend that all HIV-infected children should start ART immediately. However, that is also the period when DRMs are high and could compromise ART. Therefore, ART regimen should be chosen correctly, AS-PCR can be used to screen for DRMs and detect minority variants that can hinder future ART regimens and monitor patients in an efficient way especially in a resource-limited setting[78].

2.6.4 Next generation sequencing (NGS)

The increase in national and international ART scale-up has necessitated the development of more sensitive, high throughput and cost-effective DRM testing methods. qPCR approach was the first to emerge with AS-PCR demonstrating excellent performance characteristics, detection limits and cost-effectiveness [15]. However, this method is limited to detecting just one DRM per reaction making a high-throughput approach unachievable. Over the past few years NGS platforms have become widely available including Illumina and Ion Torrent and Roche systems[67]. NGS platforms are different from both Sanger sequencing and AS-PCR methods because they use massively parallel sequencing that is high throughput (up to 96 to 384 samples/run) and provides a snapshot of the entire virus population in a single run[67]. Not only can specific genes but the entire genome be interrogated for resistance associated mutations in a single run that can, in the future be helpful for patient management at reduced costs[79].

CHAPTER 3

3. Materials and Methods

3.1 Ethical statement

The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (ref. BF052/10) and the Health Research Committee of the KwaZulu-Natal Department of Health (HRKM 176/10).

3.2 The setting

This study was conducted within the Hlabisa area, a sub-division of uMkhanyakude district situated in predominantly rural northern KwaZulu-Natal which is one of the epicentres for HIV-1 subtype C burden in the world (Fig. 5), with a prevalence of 5% in children aged 0-14 years. Details of the programme have been previously described[80]**.** Briefly, the programme employs a public health approach to ART delivery for the patients from this area facilitating a rapid scale-up of HIV treatment services at the 17 primary health care clinics and one district hospital[80]. The treatment and care is provided free of charge; however, the infrastructure is typical of many other rural health districts in South Africa having a limited-resource setting. The programme adheres to the South African eligibility criteria stating that all children less than 5 years irrespective of CD4 should start ART immediately, older children between 5 and 15 years of age should start based on their WHO clinical stage 3 or 4 or CD4 <350 cells/ul (Table 2)[6]**.** At the time of the study, children eligible for ART aged 0–3 years (or weight under 10 kg) were initiated on a protease inhibitor (PI) based regimen while children older than 3 years were initiated on a non-nucleoside reverse-transcriptase inhibitor (NNRTI)-based regimen[6]. All patients were seen weekly, fortnightly or monthly by a nurse and counsellor for the ART collections and the patients counselling session is conducted prior to receiving ART medication. Basic clinical and demographic data is collected on a standardised clinical form in parallel to the records in the Africa Centre's ART evaluation and monitoring System (ARTemis) that is an operational database holding treatment and laboratory monitoring information[18, 81]. Some of the steps followed on the patient visit:

- The counsellor informed the caregivers accompanying the child, as well as the child about the resistance study and gave them information sheet (Appendix 3).
- The caregivers provided written informed consent (Appendices 4 and 5) and the children older than 12 years of age also provided their own written consent.
- A virologic failure clinical history sheet (appendix 6) including details of ART drugs, a record of each viral load and CD4 count and adherence (clinical and social) and history on Mycobacterium tuberculosis treatment and pMTCT was completed for each child recruited into the study.

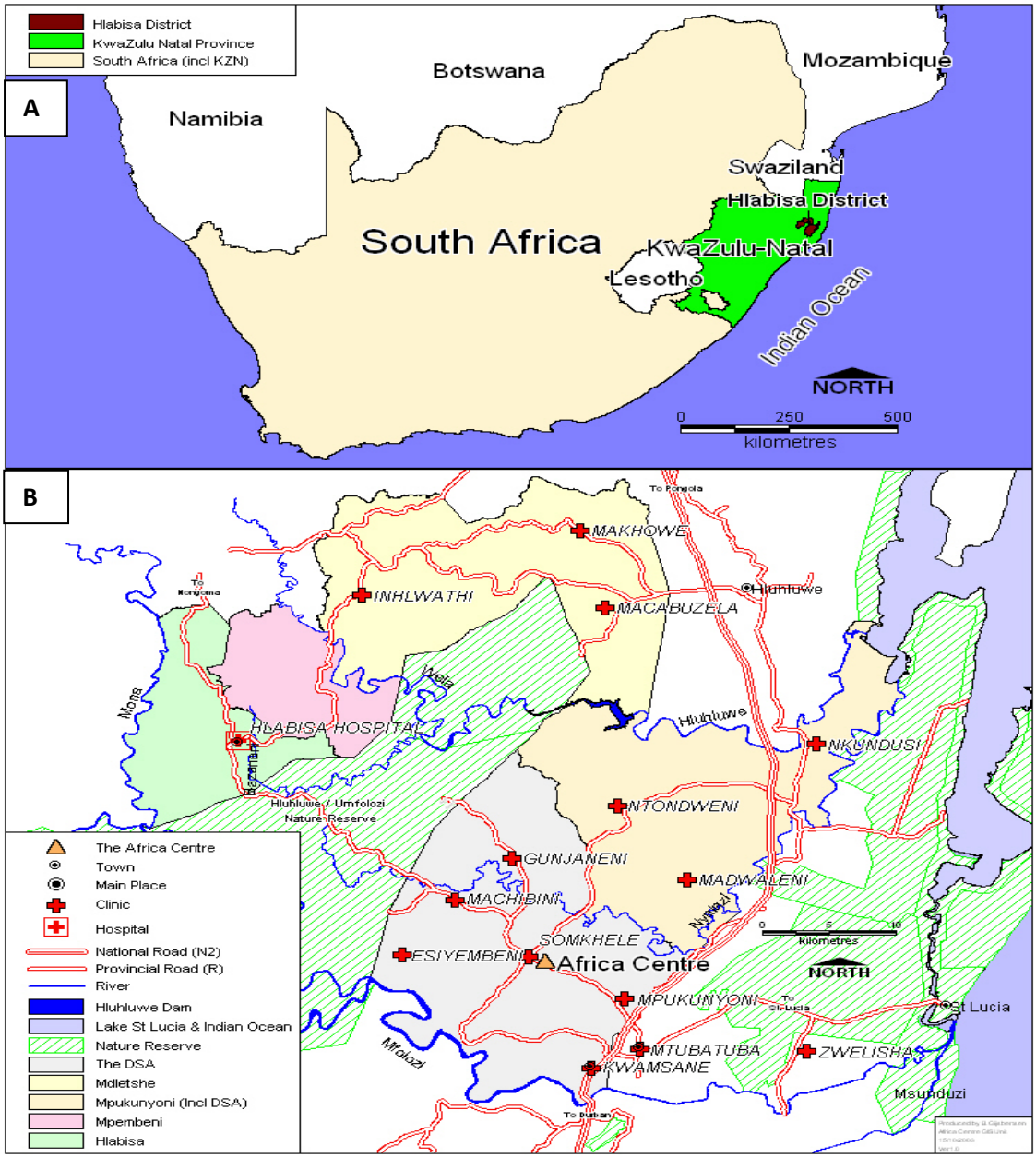


Figure 5: The locations within Hlabisa demographic surveillance area, where patient recruitment and sample collection was conducted[82] In panel A: green indicates KwaZulu Natal province, red indicates Hlabisa demographic surveillance area. In panel B: red crosses indicates 17 clinics within the Hlabisa sub-district where sample collection was conducted and yellow triangle indicates Africa Centre's location within an area

3.3 Study design and sample size

This was a retrospective study conducted on HIV-1 infected infants and children ≤ 15 years of age with virologic failure (defined as two consecutive viral loads >1000 copies/ml) who had been receiving ART for at least a year at the time of HIV drug resistance genotyping. The study was conducted from August 2011 until the end of June 2014[81]. The children from 17 clinics within the uMkhanyakude sub-district, a total number of 119 patients were recruited and included into the Africa Centre paediatric resistance cohort. Of the 119 children, 38 children were included in this study. We selected (Fig. 6) all children that were initiated on PI first-line regimen (n=17) and never had experienced an NNRTI-based regimen, with an exception of those who had received NVP during pMTCT. Plus (n=21) initiated on NNRTI first-line based regimen were also selected for comparison. Children 16 years and older were excluded from the study and referred to the adult resistance cohort if experiencing virologic failure also managed by the Africa Centre. Details of patient's recruitment have been reported previously[81]. For any child at virologic failure 5ml EDTA whole blood sample for HIV drug resistance genotyping collected during the clinical evaluation and sent to the Africa Centre laboratory in Durban, South Africa for testing.

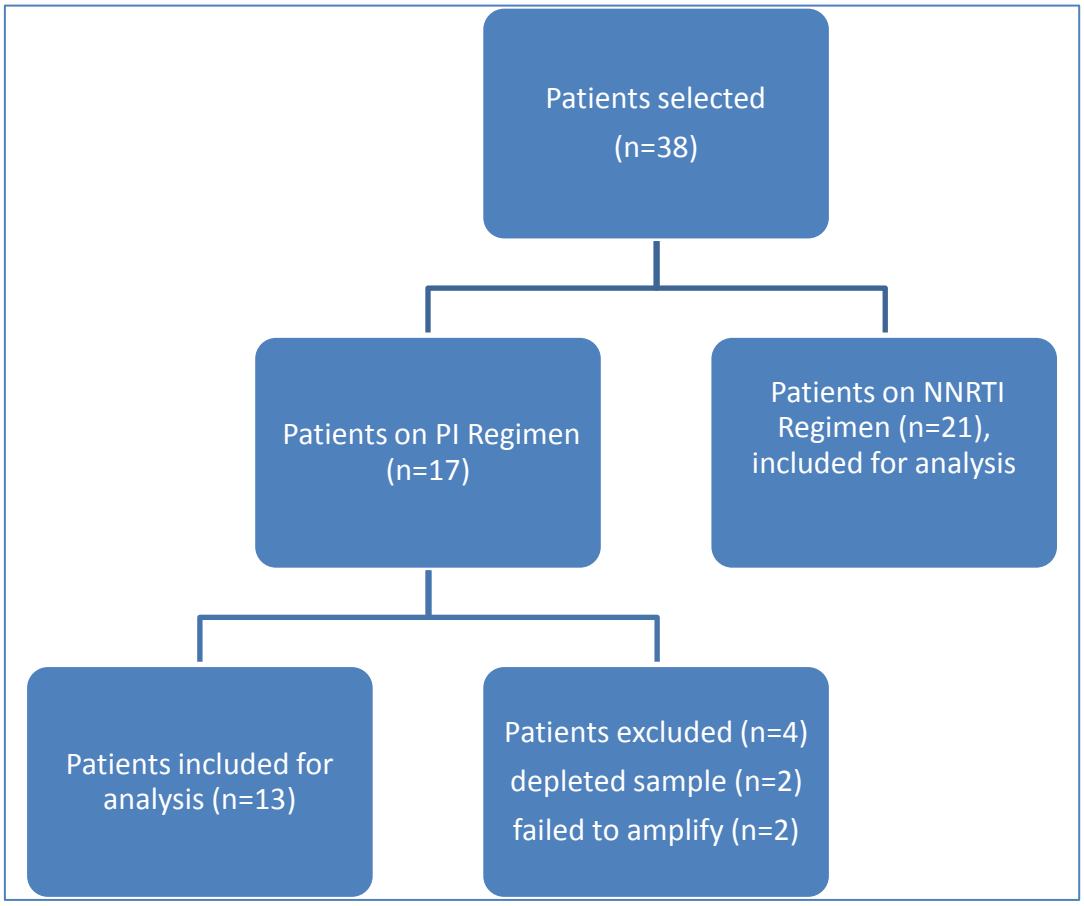


Figure 6: Flow chart showing the patients included and excluded from the analysis
Note. Of the excluded samples, two samples were depleted and the other two samples failed to amplify

3.4 Africa Centre Laboratory

All laboratory methods were conducted at the Africa Centre Laboratory (ACL), a Welcome Trust funded research institute situated approximately 220 kilometers south of Mtubatuba at the UKZ medical school, Durban. The laboratory conducts HIV serology, HIV viral loads (qPCR), in-house polymerase chain reaction (PCR), genotyping using Sanger sequencing and NGS using Illumina, Miseq.

3.5 Laboratory methods

3.5.1 Sample collection

Approximately 5ml EDTA whole blood sample was collected during the clinical evaluation of the patient from one of the 17 sites and sent to Africa Centre laboratory for HIV drug resistance testing. The samples were collected as the part of paediatric ART failure cohort through Hlabisa HIV treatment and Care programme (PHC). EDTA blood samples were received at the laboratory on ice within 8 hours of collection. The basic clinical data was recorded in the laboratory information management system (LIMS). The plasma was harvested within 18 to 24 hours of sample collection and stored immediately at -80◦C until further use.

3.5.2 HIV RNA extraction

HIV RNA was extracted from the plasma using the manual QiAmp Viral RNA mini kit (Qiagen). This protocol is modified to extract RNA from 200µl plasma spun for an hour at maximum speed to concentrate the viral RNA for better amplification rates instead of 140µl, this protocol is previously described[18]. HIV RNA was eluted using 60µl of the elution buffer and then HIV RNA extract was stored immediately at -80◦C to prevent the RNA from degrading until further use.

3.5.3 Resistance genotyping using Sanger capillary sequencing method

3.5.3.1 In-house PCR

An affordable and open access Southern African Treatment Resistance Network (SATuRN) drug resistance method previously described [18], was used. HIV RNA was reverse transcribed into cDNA using the Superscript III First-Strand Synthesis kit (Life Technologies, Carlsbad, CA) and RT21 gene specific primer (CTGTATTTCAGCTATCAAGTCCTTTGATGGG), this protocol is summarised in Table 4. Amplicons (Fig. 7) were generated from 3µl of the cDNA in a total reaction volume of 25µl followed by a nested PCR. The list of primers is listed in Table 5 and amplification protocol outlined in Table 6.

Table 4: Master Mix for cDNA synthesis using superscript III polymerase

MM1		
Reagent	Volume/Sample(µl)	Final concentration
Sterile Water	0.0	0
RT21 (20mM)	0.5	0.2 m.M
dNTP Mix (10 mM)	0.5	0.4 m.M
Volume/Sample	1.0	
Add 6ul of RNA to the MM1 for each of the samples. Prepare MM2 as per table below, do not aliquot this mix.		
MM2		
Reagent	Volume/Sample(µl)	Final concentration
10 x Buffer	1.0	1X
MgCl (25mM)	2.0	4mM
DTT (0.1M)	1.0	0.008M
RNAse Out (40U/µl)	0.5	1.6U/µl
SuperScript III	0.5	
Volume/Sample	5	
Cycling conditions for reverse transcription using superscript III polymerase		
Temperature (°C)	Time (minutes)	
65	5	
4	2	
Pause to add 5µl MM2 for the synthesis of cDNA (total volume of 12µl)		
50	60	
85	5	
Pause to add 1ul of RNaseH		
37	20	
4	∞	

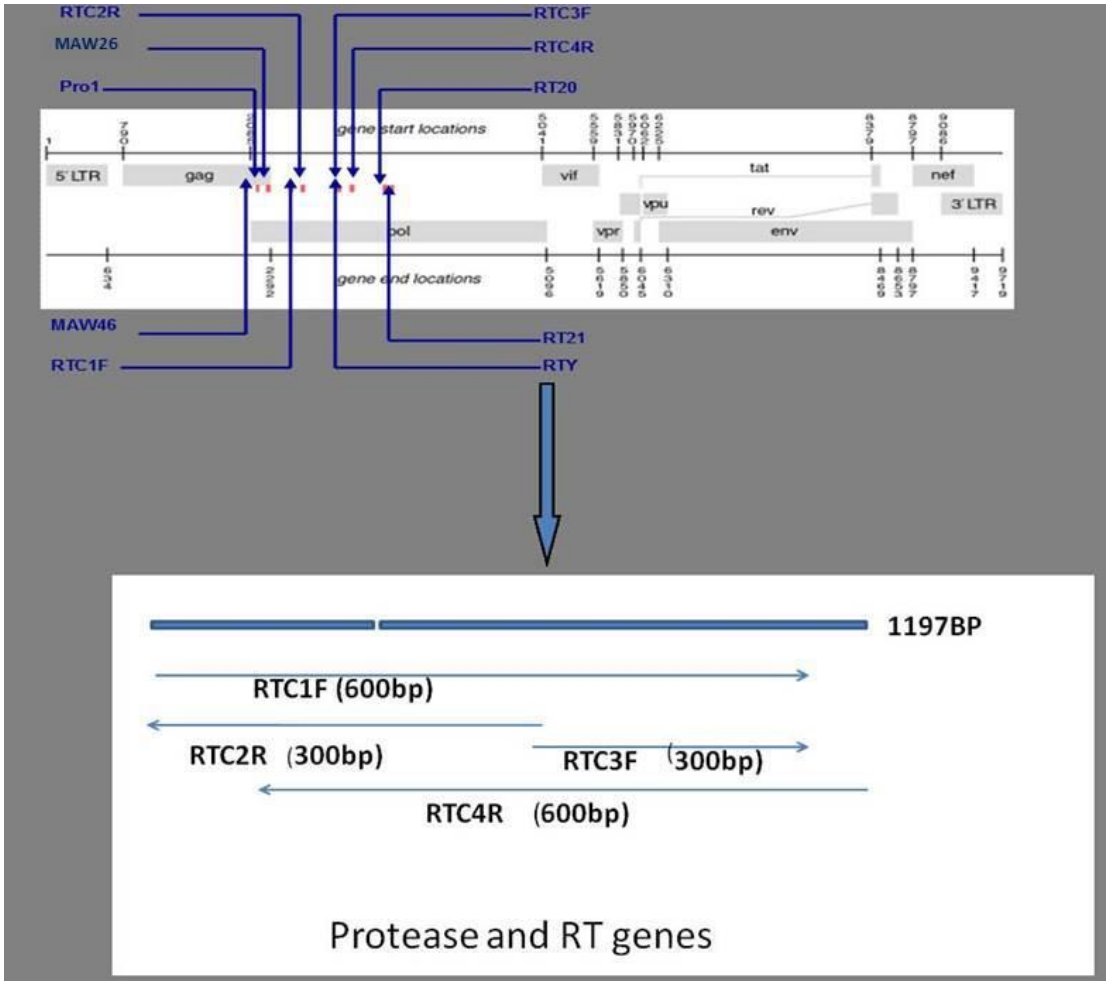


Figure 7: A diagram showing the primers and their positions covering protease and reverse transcriptase regions and the amplicon size generated employed in genotyping using Sanger sequencing

Table 5: Summary of primer sequences used for the first and second round nested in-house PCR for pol gene amplification

Stage	Primer name	Nucleotide Sequence	Length	Direction	HXB2 position
1st round	MAW - 26	TTGGAAATGTGGAAAGGAAGGAC	23	Forward	2028-2050
	RT-21	CTGTATTTCAGCTATCAAGTCCTTTGATGGG	31	Reverse	3539-3509
2nd round	Pro-1	TAGAGCCAACAGCCCCACCA	20	Forward	2147-2166
	RT-20	CTGCCAATTCTAATTCTGCTTC	22	Reverse	*3462*-3441

Table 6: First round and second round Master Mix for nested in-house PCR

First round Master Mix		
Reagent	Volume/Sample(µl)	Final concentration
Sterile Water	18.4	0
10 x Buffer	2.5	4
MgCl (50mM)	1.0	2
dNTP (10mM)	0.5	0.2
MAW26 (5 pmol/ul)	0.25	0.1
RT21 (5 pmol/ul)	0.25	0.1
Platinum Taq	0.1	0.02
Volume/Sample	23	
Template (cDNA)	2.0	
Total Reaction Volume	25	
Second round Master Mix		
Reagent	Volume/Sample(µl)	Final concentration
Sterile Water	18.4	0
10 x Buffer	2.5	4
MgCl (50mM)	1.0	2
dNTP (10mM)	0.5	0.2
PRO1 (5 pmol/ul)	0.25	0.1
RT20 (5 pmol/ul)	0.25	0.1
Platinum Taq	0.1	0.02
Volume/Sample	23	
Sample	2.0	
Total Reaction Volume	25	
The cycling conditions for the nested in-house PCR		
Temperature (°C)	Time	Number of cycles
94	2 minutes	Hold
95	30 seconds	30 cycles
58	20 seconds	
72	2 minutes	
72	10 minutes	Hold

3.5.3.2 Agarose gel

To assess the success of the PCR reaction, second round PCR products were stained with a fluorescent dye called novel juice (GeneDireX, Taipei Taiwan) and visualised using 1% agarose gel electrophoresis (45 min at 70V and 400 mA) under ultra-violet light. A 200-bp DNA ladder from (Fermentas, Maryland, USA) was used as a reference for the desired positive 1.3 kb PCR products. Successfully amplified PCR products were purified using the PureLink PCR purification kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

3.5.3.3 HIV genotyping using Big Dye terminator chemistry

Sequencing reaction was done on purified PCR products using Big Dye ® terminator V3.1 (Applied bio-systems Inc., Foster City, CA) protocol outlined in Table 7 and a summary of gene specific primers listed in Table 8. Following the sequencing reaction, sequencing products were purified to remove any excess primers, unincorporated dye terminators, salts or enzymes. The plate clean-up was done using sodium acetate precipitation reaction method with ethanol washes. DNA pellet was reconstituted in 10µl of formamide and denatured at 95ºC for 2 minutes before running on a 3130xl Genetic Analyser (Applied Biosystems Inc, Foster City, CA).

Table 7: Master Mix for Big Dye terminator chemistry sequencing reaction

Label four sets of tubes as follows for each of the 4 primers		
MM-1 RTC1F		
MM-2 RTC2R		
MM-3 RTC3F		
MM-4 RTC4R		
Prepare the master mixes as follows:		
Reagent	Volume/Sample(µl)	
Sterile Water	6.10	
Big Dye Ready Reaction mix	0.40	
Primer (3.20pmol/µl)	0.50	
5X sequencing buffer	2	
Volume per sample	9	
Template (DNA)	1	
Total Reaction Volume	10	
The cycling conditions for the big dye sequencing reaction		
Temperature (°C)	Time	Number of cycles
94	2 minutes	Hold
95	30 seconds	35 cycles
58	20 seconds	
72	2 minutes	
72	10 minutes	Hold

Table 8: The summary of primers used in sequencing reaction of the pol gene

Primer name and direction	Sequence	Size	HXB2 (p)
RTC1_Forward	ACCTACACCTGTCAACATAATTG	23	2486-2508
RTC2_Reverse	TGTCAATGGCCATTGTTTAACCTTTGG	27	2630-2604
RTC3_Forward	CACCAGGGATTAGATATCAATATAATGTGC	30	2956-2994
RTC4_Reverse	CTAAATCAGATCCTACATACAAGTCATCC	29	3129-3101
RTy _Reverse	GTGTCTCATTGTTTATACTAGG	22	2967-2946
MAW 46_Forward	TCCCTCAGATCACTCTTTGGCAACGAC	27	2251-2277

3.5.3.4 Sequence assembly and quality analysis

Electropherograms generated from the Sanger sequencing were imported into Geneious V8.0.5 software (www.geneious.com), the quality of the reads for each of the four primers was manually assessed and poor quality bases at the 5' and 3' ends were trimmed to improve the quality of each sequence. The sequences were manually edited and deemed high quality if the quality score was higher than 80% after trimming. A consensus sequence covering 300 amino acids of RT and 99 amino acids of the protease gene was generated following assembly of the four fragments. The first 240 codons of the RT gene cover all currently recognised RT mutations associated with resistance to available RT inhibitors.

Once trimmed and manually edited the sequences were aligned to a subtype C reference (Genebank, accession # JN665021.1) sequence to generate a contig. The contig sequence generated from an assembly was manually edited by reading through each sequence for possible base mixtures, deletions and insertions at different sites. Thereafter, the quality assessment and HIV subtyping of these sequences were performed using the HIV-1 Quality Analysis Tool and REGA HIV-1 Subtyping Tool v. 2.0, respectively. Firstly, the quality of the sequences was assessed using the HIV quality analysis tool hosted on BioAfrica.net. The sequences were analysed using the Stanford HIVDB programme accessed on the mirror of the Stanford database also hosted on BioAfrica.net. Prior to the detection of DRM using bioinformatics software applications, we submitted each consensus to the Calibrated Population Resistance Tool (CPR) (http://hivdb.stanford.edu) for a final quality check.

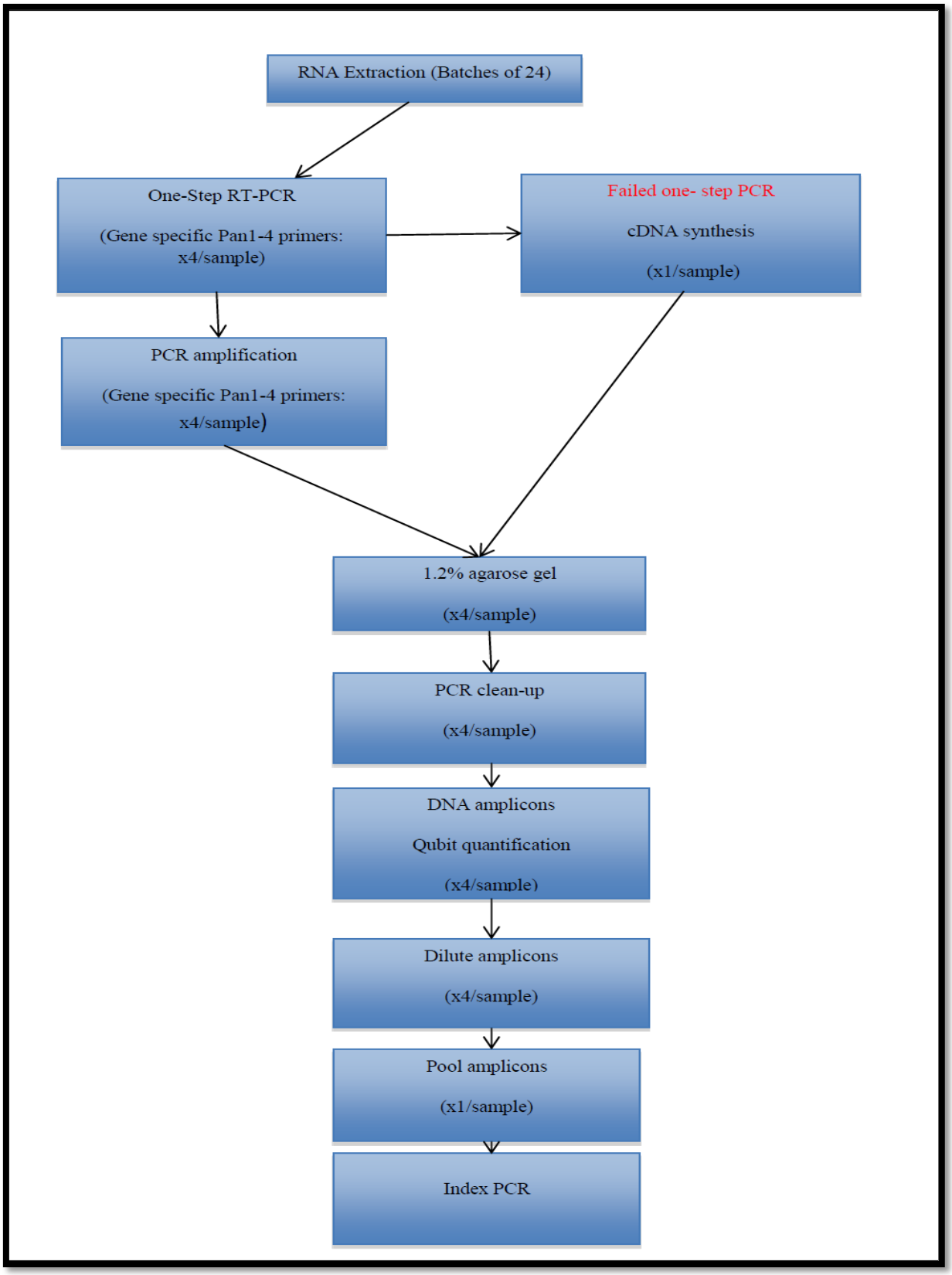
To test for contamination the sequences were blasted against the public dataset using NCBI blast (http://www.ncbi.nlm.nih.gov/ blast) as well as a local database using a Blast Server application. Sequences were deemed not a contaminant if the identity to previously genotyped samples was lower than 98%. Post quality assessment the sequences were loaded onto the SATuRN database. This database uses the online drug resistance algorithms (ANRS 2009.07, HIVDB6.0.5 and REGAv8.0.2) to interpret the drug resistance data from the submitted sequence.

3.6 Next generation sequencing

3.6.1 Amplicon generation

Fig. 8 depicts HIV-1 whole genome sequencing sample preparation from extraction to sequencing for Miseq, Illumina. Briefly, HIV RNA was used to synthesise cDNA using the SuperScript III one-step RT-PCR with platinum Taq high fidelity previously described at a temperature of 50°C for 30 minutes[83]. Amplification was performed in the same tube (one-step) using SuperScript III-Platinum Taq High fidelity enzyme mix and 5µl of RNA to synthesise four overlapping amplicons that span the near full 9.7kb HIV genome[83] (Table 9) and gene specific primers listed in Table 12. The amplicons generated Pan 1 of 1.9kb, Pan 2 of 3.6kb, Pan 3 of 3.0kb and Pan 4 of 3.5kb respectively including all 9 open reading frames as well as the U5 and partial R region of 5'-LTR and the partial U3 of the 3'-LTR shown in (fig. 9)[83] .

In the event of 1 to 2 fragments failing to amplify, we used a separate RT and PCR reaction using the reverse gene specific primer and SuperScript III First-Strand Synthesis Kit (Invitrogen) for reverse transcriptase and then Platinum Taq High Fidelity DNA Polymerase (Invitrogen) for amplicons generation. For cDNA synthesis 5µl of RNA was used in a 12.5µl reaction volume and final primer concentration of 1.6µM (Table 10). Amplicons were generated using Platinum Taq High Fidelity DNA Polymerase (Invitrogen) (Table 11) and gene specific primers listed in Table 12. All positive amplicons were visualised on a 1% agarose gel and then purified using the QiaQuick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.


RNA Extraction (Batches of 24)
One-Step RT-PCR
(Gene specific Pan1-4 primers: x4/sample)
Failed one- step PCR
cDNA synthesis
(x1/sample)
PCR amplification
(Gene specific Pan1-4 primers: x4/sample)
1.2% agarose gel
(x4/sample)
PCR clean-up
(x4/sample)
DNA amplicons
Qubit quantification
(x4/sample)
Dilute amplicons
(x4/sample)
Pool amplicons
(x1/sample)
Index PCR

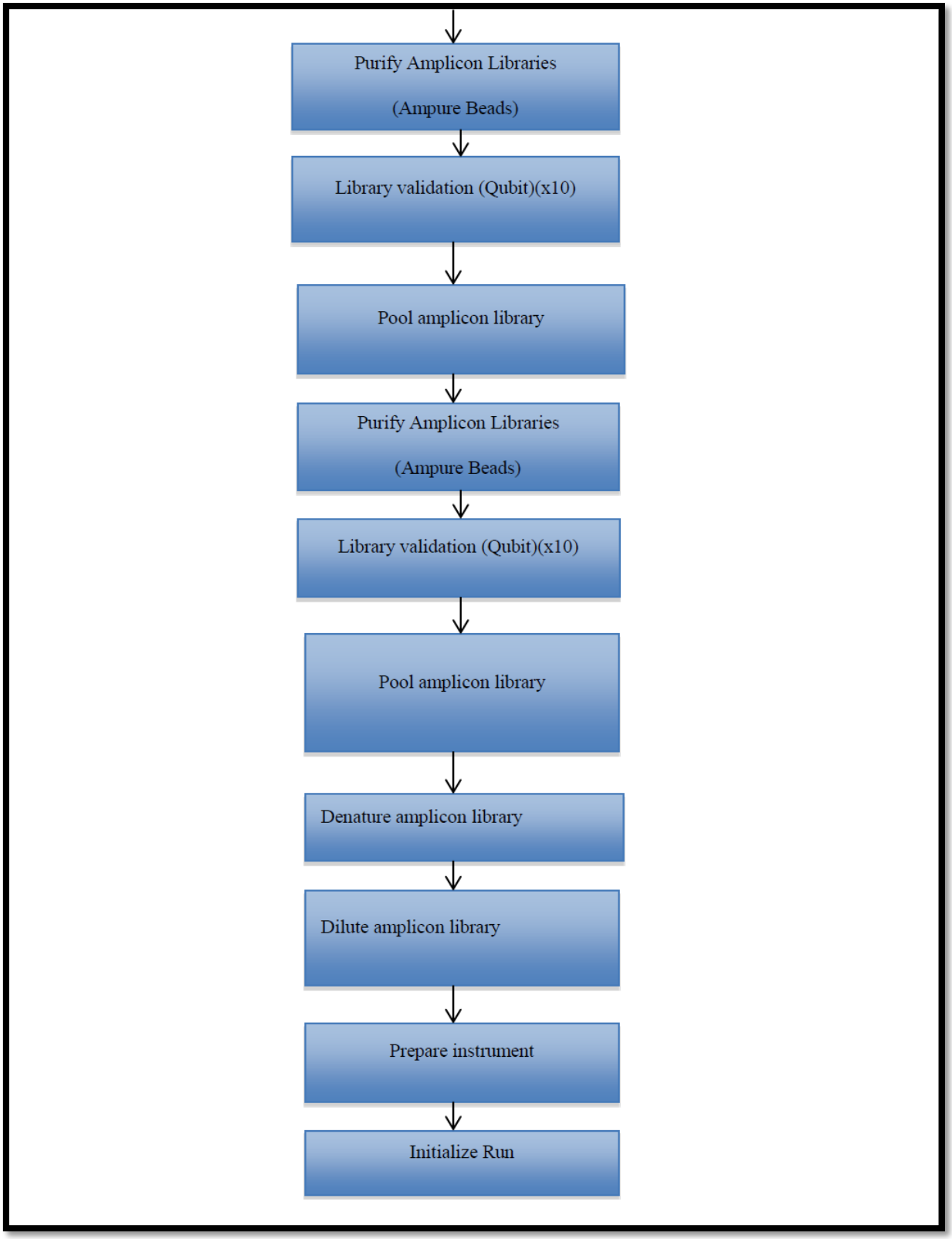


Figure 8: Flow diagram for HIV-1 whole genome sequencing sample preparation using Illumina, Miseq

Table 9: Master Mix for SuperScript III one-step RT-PCR using platinum Taq high fidelity enzyme

Reagent	Vol/sample (µl)	Vol in MM (µl)
Sterile water	4.5	72
2x Reaction Buffer	12.5	200
Pan1-4 primer mix (10pmol/µl each)	2.0	32
SSIII/Platinum Taq polymerase (5U/µl)	1.0	16
Volume/sample	20.0	
Template (HIV RNA)	5	
Total reaction volume	25	
The cycling conditions for superscript III one-step RT-PCR		
Temperature (°C)	Time	Number of cycles
50	30minutes	Hold
94	2minutes	Hold
94	15seconds	
60	30seconds	
68	4.5 minutes	40 cycles
68	10minutes	Hold
4	∞	Hold

Note. Label four sets of tubes for each gene-specific pan primer

Table 10: Master Mix for cDNA synthesis using superscript III polymerase

MM1		
Reagent	Volume per sample(µl)	Final concentration
Sterile Water	0.0	0
Primer-R(20µM)	1.0	0.5 m.M
dNTP Mix (10 mM)	1.0	2.5 m.M
Volume/Sample	2.0	
Template(HIV RNA)	5.0	
Total Reaction volume	7.0	
Prepare MM2 as per table below, do not aliquot this mix.		
MM2		
Reagent	Volume/Sample(µl)	Final concentration
5xBuffer	2.5	1X
MgCl	1.0	
DTT (0.1M)	0.75	0.01M
RNAse Out (40U/µl)	0.75	2U/µl
SuperScriptIII (200U/µl)	0.75	10U/µl
Volume/Sample	5.75	
The cycling conditions for reverse transcription using superscript III polymerase		
Temperature (°C)	Time (minutes)	Hold
65	5	Hold
4	2	Hold
Pause to add 5.75µl MM2 for the synthesis of cDNA (total volume of 12µl)		
50	60	Hold
55	60	Hold
Pause to add 1ul of RNaseH		Hold
70	15	Hold
4	∞	Hold

Table 11: Master Mix for the RT-PCR using platinum Taq high fidelity polymerase

Reagent	Volume per sample (µl)	Final concentration
Sterile water	16.20	0
10x buffer	2.50	1X
$MgSO_4$ (50mM)	1.00	1X
dNTP (10mM)	0.60	2.5mM
Primer-F (20uM)	1.00	0.2mM
Primer-R (20uM)	1.00	0.2µM
Platinum HF(5U/µl)	0.20	0.025U/µl
Volume/sample	22.50	
Template(cDNA)	2.50	
Total reaction volume	25.00	
The cycling conditions for the RT-PCR using platinum Taq high fidelity		
Temperature	Time	Number of cycles
94	4min	hold
94	15sec	40 cycles
60	30sec	
68	4min30sec	
68	10min	hold
4	∞	hold

Table 12: Summary of primers covering protease, reverse transcriptase and integrase regions used for amplification

Set and primer	**Sequence (5'-3')**	**Position(nt)**	**Product size (bp)**
Pan-HIV-1_1F	AGC CYG GGA GCT CTG TG	26-42	1928
Pan-HIV-1_1R	CCT CCA ATT CCY ATC ATT TT	1953-1931	
Pan-HIV-1_2F	GGG AAG TGA YAT AGC WGG AAC	1031-1051	3574
Pan-HIV-1_2R	CTG CCA TCT GTT TTC CAT ARTC	4604-4583	
Pan-HIV-1_3F	TTA AAA GAA AGG GGG GGA TTG GG	4329-4351	3066
Pan-HIV-1_3R	TGG CYT GTA CCG TCA GCG	7394-7377	
Pan-HIV-1_4F	CCT ARG GCA GGA AGA AGC G	5513-5531	3551
Pan-HIV-1_4R	CTT WTA TGC AGC WTC TGA GGG	9063-9043	

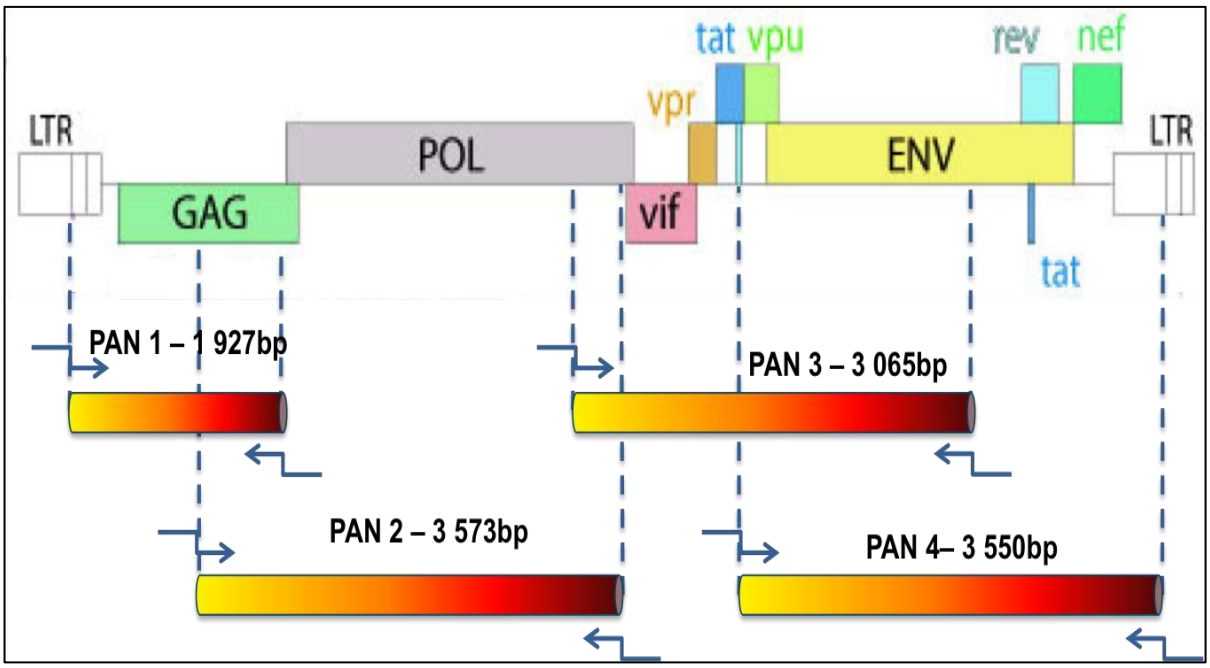


Figure 9: An illustration showing the location of Pan 1-4 primers and amplicon size generated by each

3.6.2 Genotyping using Miseq, Illumina

The amplicons were quantified using the Qubit sdDNA HS Assay Kit (Thermo/Life Technologies, Oregon; USA). Each amplicon was diluted to a concentration of 0.3ng/µl before pooling in a 1:3:3:3 ratio of Pan1 to Pan 4. Pooled amplicons were prepared for sequencing using the Nextera XT DNA sample preparation kit (Illumina) and the Nextera XT DNA sample preparation index kit (Illumina), following the manufacturers protocol. Normalisation of the libraries was bead-based, also as per the manufacturer's protocol and using the Nextera XT DNA sample preparation kit (Illumina). Libraries were pooled and sequenced on the MiSeq, Illumina using 300bp paired-end technology and the MiSeq Reagent Kit V3 (Illumina). The run comprised pools of 96 samples that included 3 controls (one negative sample, one inter-run and one intra-run control).

3.6.3 Assembly and consensus generation

The generated reads were assembled using Geneious V8.0.5 software package to map and align all reads. The quality of the reads was manually assessed and the ends trimmed to improve the quality, before mapping and aligning to a subtype C reference sequence (Genebank, accession # AF411967) from South Africa. Reads <100bp were excluded and poor quality reads were trimmed up to 50bp from 5' and 3' ends. We imposed these inclusion and exclusion criteria in order to decrease the probability of ambiguous read mapping which occurs when shorter reads of lower accuracy are included in assemblies.

3.6.4. Phylogeny

The pol gene was extracted from 34 consensus sequences generated using NGS and aligned with consensus sequences generated using Sanger sequencing together with reference sequences representative of all major subtypes currently circulating. We included a greater number of subtype C sequences from Southern Africa, Brazil and India in order to verify the subtype of our sequences given that South Africa has a predominantly subtype C epidemic. Alignments were generated in ClustalW [84] and then manually edited in Geneious V 8.1 (www.geneious.com). Trees were generated using RAxML[85] and a general time reversible model with an estimated gamma heterogeneity alpha parameter and 1000 bootstrap replicates to estimate the reliability of internal nodes. We used FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) to visualise and annotate the resultant tree.

3.6.5. Detection for drug resistance mutations

A set of 34 sequence reads >301 base pairs were obtained and uploaded onto the Geneious V8.0.5 software system. The sequences were trimmed between 100bp and 301bp, mapped and assembled against full-length HXB2 subtype C (Genebank, accession # AF411967) reference sequence. The reference sequence used was annotated with HIV DRMs of interest at the pol gene as outlined in the Stanford HIV drug resistance 2013 database (http://hivdb.stanford.edu). Too short sequences and sequences that were of the poor quality according to the statistical scoring requirements were excluded from the analysis.

Contig from each sample was mapped against a reference sequence to find minority DRMs with a 5% variant call cut-off to exclude the impact of polymerase introduced errors. We identified polymorphisms with a 5% variant call cut-off to exclude the impact of polymerase introduced errors. The table list of motifs and polymorphism was extracted and exported as a working excel table. To analyse minor DRMs: read depth and frequencies of mutations associated with drug resistance, as outlined in the Stanford HIV drug resistance database were analysed. The use of an annotated reference sequence allowed us to confirm the presence of minority resistance calls at DRMs located at the contig. DRMs present at a proportion ≥20% were regarded to be wild type and those present at a proportion <20% were considered to be minority DRMs

3.7 Statistical analysis

In order to investigate the DRMs in this cohort, the drug resistance mutations, clinical measurements and demographic data were exported for all patients genotyped from RegaDB for further statistical analysis using STATA version 10 (StataCorp LP, Texas, USA). Descriptive statistics were used to summarize the demographic and clinical characteristics in this cohort**.** For analysis of drug resistance mutations, frequency distributions of the major DRMs were derived and 5% level of significance to investigate minority DRMs was selected. Medians and the interquartile range (IQR) were calculated for continuous data.

3.8 Interpretation of resistance results and report generation

Sanger genotyping results for this cohort including drug resistance profile for each sequence together with the clinical data and treatment history (Appendix 6) of the patient's was used to generate a drug resistance report (Appendix 8). The report contained DRMs identified from the patient virus and drug resistance interpretation based on the HIVDB version 6.0 algorithms was generated and sent to an Infectious Disease Specialist who evaluated the report together with the patient's clinical data and provided treatment recommendations based on the current South African guidelines (Table 1). The report also contained genotypic susceptibility scores (GSS) where a GSS of 1.0 indicates drugs that are still active against the virus, 0.5 indicates drugs that have intermediate resistance and 0.0 is indicative of high-level resistance (Figure 10). However, NGS analysis was done retrospectively only for research purposes on the patients from the same cohort and the results were not sent back to the Specialist.

CHAPTER 4

4. Results

4.1 Study Population

The demographic and clinical details of this cohort are summarised in Table 13. Briefly, a total number of 34 patients were included in the analysis with 20 (58.8%) males with a median age of 1.4 (IQR 0.6-4 years). Twenty-one children (61.7%) were enrolled on an NNRTI-based regimen while the remaining 13 were initiated on a PI regimen. Children on a PI-regimen were significantly (p=0.0029) younger in age at initiation with a median age of 0.7 (IQR 0.6-1.4 years) compared with those on an NNRTI regimen with a median age of 7.1(IQR 3.4-10 years). Patients were genotyped at virologic failure following two successive viral load measurements >1000cp/ml.The median age at genotype was 6.6 years (IQR 4.5-11.7), (Table 13). The median time between the date of genotype and the last viral load was 3.4 months (IQR 1.5-8.3 months), (Table 13).

For CD4 measurements this time period was 6.2 months (IQR 3-9 months). Children from NNRTI-based regimen group had been on ART for longer period than those on PI-based regimen group. Interestingly, among children on PI-based regimen only 15.3% had ever achieved virologic suppression (a confirmed HIV RNA cp/ml level below the limit of detection) compared to 53.2% of those on NNRTI-base regimen. Of the 34 patients in our cohort, twenty patients had at least one drug substitution in their regimen with 13 patients having drug substitution prior to genotyping and seven patients who had a drug substitution after genotyping. Due to drug toxicity in 2013, the South African Department of Health recommended replacing d4T with ABC if the VL is undetectable[6]. In this cohort only six children were subject to this recommendation and had a drug substitution of ABC replacing d4T in their regimen and among other fourteen drugs substitution was due to virologic failure. The remaining 14 patients in the cohort were maintained on the same regimen that they had been on prior to genotyping. More patients on PI-regimens (n=5) were exposed to single-dose nevirapine (sdNVP) at birth compared with patients on NNRTI-based regimens.

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Table 13: Demographic and clinical data of the 34 children on non-nucleoside reverse transcriptase and protease inhibitors based regimen groups at ART initiation and at the time of genotype

Characteristic	**All patients (n = 34)**	**PI-based (n = 13)**	**NNRTI-based (n = 21)**
Gender, male, *n* (%)	20 (58.8)	7 (53.8)	13 (61.9)
Age at ART initiation, years, median (IQR)	**3 (1-8.3)**	**0.7 (0.6 - 1.3)**	**7.1(3.3-10)**
Number of patients in age categories (%):			
0-3 years	17 (50.0)	13(100)*	4 (19.1)*
3-5 years	3 (8.8)	0	3 (14,3)
5-10 years	9 (26.5)	0	9 (42.9)
10-15 years	5 (14.7)	0	5 (23.8)
Age at genotype, years**,** median (IQR)	6.6 (4.7-11.7)	4.1 (3.3-5.8)*	10.5 (6.6-12.6)*
Number of patients in age categories (%):			
0-3 years	2 (5.9)	2 (15.3)	0
3-5 years	4 (11.8)	3 (23.1)	1 (4.8)
5-10 years	16 (47.1)	8 (61.5)	8 (38.1)
10-15 years	12 (35.3)	0	12 (57.1)
CD4 at ART initiation, cells/µl median (IQR)	469 (176 - 865)	865 (661-1305)*	306 (112-461)
Number of patients by CD4 categories: (%)			
<50 cells/µl	2 (5.9)	0	2 (9.5)
50 – 200 cells/µl	7 (20.6)	0	7 (33.3)
201 – 500 cells/µl	9 (26.5)	1 (7.7)	8 (38.1)
501 – 1 000 cells/µl	9 (26.5)	7 (53.9)	2 (9.5)
>1 000 cells/µl	7 (20.6)	5 (38.5)	2 (9.5)
CD4 prior to genotyping, cells/µl median (IQR)	706 (488.5-1108.5)	1105(732-1369)	574(382-797.5)
Number of patients by CD4 count categories: (%)			
<50 cells/µl	1(2.9)		1(4.7)
50 - 100 cells/µl	1(2.9)	0	1(4.7)
101 - 200 cells/µl	-	0	-
201 – 500 cells/µl	6(17.6)	-	5(23.8)
>500 cells/µl	26(76.4)	1(7.6)	14(66.6)
		12(92.3)	

Viral load at ART initiation, log_{10} copies/ml median (IQR)	4.2 (3.51 – 5.23)	4.4 (3.51-4.98)	4.2 (3.67-5.38)
Number of patients by viral load categories: (%)			
≤ 2 log_{10} copies/ml	3 (8.8)	1 (7.7)	2 (9.5)
2 - 3 log_{10} copies/ml	4 (11.8)	1 (7.7)	3 (14.3)
3 - 4 log_{10} copies/ml	6 (17.7)	3 (23.1)	3 (14.3)
4 - 5 log_{10} copies/ml	10 (29.4)	5 (38.5)	5 (23.8)
> 5 log_{10} copies/ml	11(32.4)	3 (23.1)	8 (38.1)
[1]**Viral load prior to genotype,** log_{10} copies/ml median (IQR)	3.89 (1.78 – 4.43)	4.39 (2.35-5.11)	3.89 (1.61-4.21)
Number of patients by viral load categories: (%)			
<2 log_{10} copies/ml	1(2.9)		0
2-3 log_{10} copies/ml	1(2.9)	1(7.6)	1(4.7)
3-4 log_{10} copies/ml	16(50)	0	9(42.8)
4-5 log_{10} copies/ml	10(29.4)	7(53.8)	8(38)
> 5 log_{10} copies/ml	6 (17.6)	2(15.3)	3(14.2)
		3(23)	
Time between last CD4 and genotype Months, median (IQR)	6.2 (3-9.3)	6.7 (2.1-10.6)	5.9 (3-8.8)
Time between last VL and genotype Months, median (IQR)	3.4 (1.5-8.3)	4.6 (0.9-8.6)	3.1 (1.6-7.2)
Ever achieved virological suppression, n (%)			
Yes	13 (38.2)	2 (15.3)	11 (52.3)
No	21 (61.7)	11 (84.6)	10 (47.6)
Duration of antiretroviral therapy since ART initiation Months, median (IQR)	36.7 (24.7-48)	37.4 (27.5-44)	35.9 (18.2-51.6)
[2]**Duration of antiretroviral failure,** Months ,median (IQR)	21.4 (11-32.2)	14.2 (9.9-31) *	23.2 (14.9-32.8) *
Number of patients within ART regimens, n (%)			
3TC+D4T+EFV	20 (58.8)	0	20 (95.2)
3TC+D4T+LPV/r	9 (26.5)	9 (69.2)	0
3TC+ABC+LPV/r	4 (11.8)	3 (23.1)	1 (4.8)
3TC+D4T+RTV	1 (2.9)	1 (7.7)	0
Number of patients by ART regimen at the time of genotyping, *n* (%)			
3TC+AZT+LPV/r	8 (23.5)	2 (15.4)	6 (28.6)
3TC+D4T+LPV/r	7 (20.6)	7 (53.9)	0
3TC+ABC+LPV/r	7 (20.6)	4 (30.8)	3 (14.3)
3TC+D4T+EFV	5 (14.7)	0	5 (23.8)
3TC+ABC+EFV	1 (2.9)	0	1 (4.8)

AZT+DDI+LPV/r 3TC+TDF+LPV/r	2 (5.9) 4 (11.8)	0 0	2 (9.5) 4 (19.1)
[3]Number of patients with history of drug substitution n (%) Before genotype After genotype Not changed	 20 (58.8) 13 (38.2) 7 (20.5) 14 (41.1)	 4 (30.7) 4 (30.7) 0 9 (69.2)	 16 (76) 9 (42.8) 7 (33.3) 5 (23.8)
Number of patients with Single-dose nevirapine (sdNVP) exposure n (%) **None** **Yes** **Unknown**	 16 (47.1) 7 (20.5) 11 (32.4)	 4 (30.8) 5 (38.4) 4 (30.8)	 12 (57.1) 2 (9.5) 7 (33.3)

Key:

IQR, interquartile range, ART, antiretroviral therapy, PI, Protease inhibitor, NNRTI, non-nucleoside reverse-transcriptase inhibitor, 3TC, lamivudine, d4T, stavudine, ABC, abacavir, AZT, zidovudine, TDF, tenofovir, DDI, Didanosine, EFV, efavirenz, NVP, nevirapine, LPVr, lopinavir/ rotinavir, 1 = Refers to the last viral load measurement documented prior to date of genotype, 2 = Duration of antiretroviral failure was calculated from the date of the first viral load >1000 copies/ml, If there was no viral load <1000 copies/ml then time was calculated from date of ART initiation, 3= Substitution refers to changing one or two drugs due to antiretroviral failure or guidelines modification. * = $p<0.01$

4.2 Clinical characteristics of children

The median age at the time of ART initiation was 3 years (IQR 1-8.3). Children on PI-based regimen group were initiated at 0.7 years (IQR 0.6-1.3), significantly (p=0.0029) younger in age compared with those on an NNRTI regimen. We noted that four children younger than 3 years of age were incorrectly initiated on an NNRTI-based regimen (3TC+D4T+ EFV)[6] such that only one patient in this age group was correctly initiated on PI-based regimen. Seven of the 34 children were exposed to sdNVP for pMTCT, five from PI-based regimen group and two from NNRTI-based regimen group.

The median VL at ART initiation was 4.2 $\log_{10}$ copies/ml (IQR 3.5-5.2) with comparable viral loads between the NNRTI group (median = 4.2 $\log_{10}$ copies/ml, IQR 3.6-5.4) and PI groups (4.4 $\log_{10}$ copies/ml, IQR 3.5-4.9). However, the median CD4 at the time of ART initiation was significantly higher among patients initiated on a PI-based regimen (p>0.05). At the time of genotype children from NNRTI-based regimen group were significantly older than those on PI-based regimen (p=0.0500). The median duration of 23.2 months (IQR 14.9-32.8) on ART failure was longer for children on NNRTI-based regimen group than those on the PI-based regimen group with 14.2 months (IQR 9.9-31)**.** Children on the NNRTI group remained on a failing regimen for significantly (p<0.01) longer than those in the PI group (Table 13). At genotype, we noted no significant differences in viral loads (p>0.05) when comparing both groups (Table 13). Similarly, CD4 counts prior to genotyping were not significantly different (p>0.05, Table 13).

4.3 Drug resistance mutations detected by Sanger sequencing

We generated population amplicons of a 1.3kb region of the pol gene and sequenced this amplicon using the Sanger sequencing platform. Of the 34 patients in the cohort we failed to generate a sequence from one patient using the Sanger sequencing platform. For the remaining 33 patients sequences of >75% quality were used in the assembly and generation of a consensus sequence.

Among these 33 sequences, we detected a mixture of major (79.5%) and accessory (23.8%) mutations in the pol gene that are associated with resistance to ARVs currently in use (Table 14,16, Fig. 10). Twenty-four (72.7%) children harboured at least one DRM, while 9 (27.2%) were completely susceptible to their regimens. NRTI-associated mutations were the most commonly occurring and were detected in 23 (69.6%) children with M184 being the most frequent NRTI mutation (n=22 of 33, 66.6%). We detected at least one TAM in 7 (21.2%) children comprising four children with one TAM, two in six and multiple of ≥3 TAMs in one child. D67N TAM pathway II mutation predominated among these children.

We noted that children from NNRTI-based regimen group, the M184 was the most commonly occurring mutation detected in 15(71.4%) of children on an NNRTI –based regimen (Table14, Fig.10). In comparison, the most common NNRTI-associated DRM was K103 which was present in 8 (38 %) children. TAMs were detected in 6 (28.5%) children. Six children had a complex mixture of cross-resistance and TAMs suggesting an extended period of time on a failing therapy.

Among children on PI-based regimens, we noted that 3(25%) had PI-associated drug resistance mutations. Included among the PI-associated mutations detected were the major V82A and I54V mutations, as well as the L10I/F and L24I accessory mutations (Table 14, Fig.10). NRTI-associated drug resistance mutations were the most prevalent mutations within this group with M184V the most frequently occurring NRTI mutation (n=7, 58.3%). One patient had a single TAM, the M41L mutation. No NNRTI-associated mutations were detected in this group where approximately 42% remained susceptible to their regimens (Table 14, Fig.10).

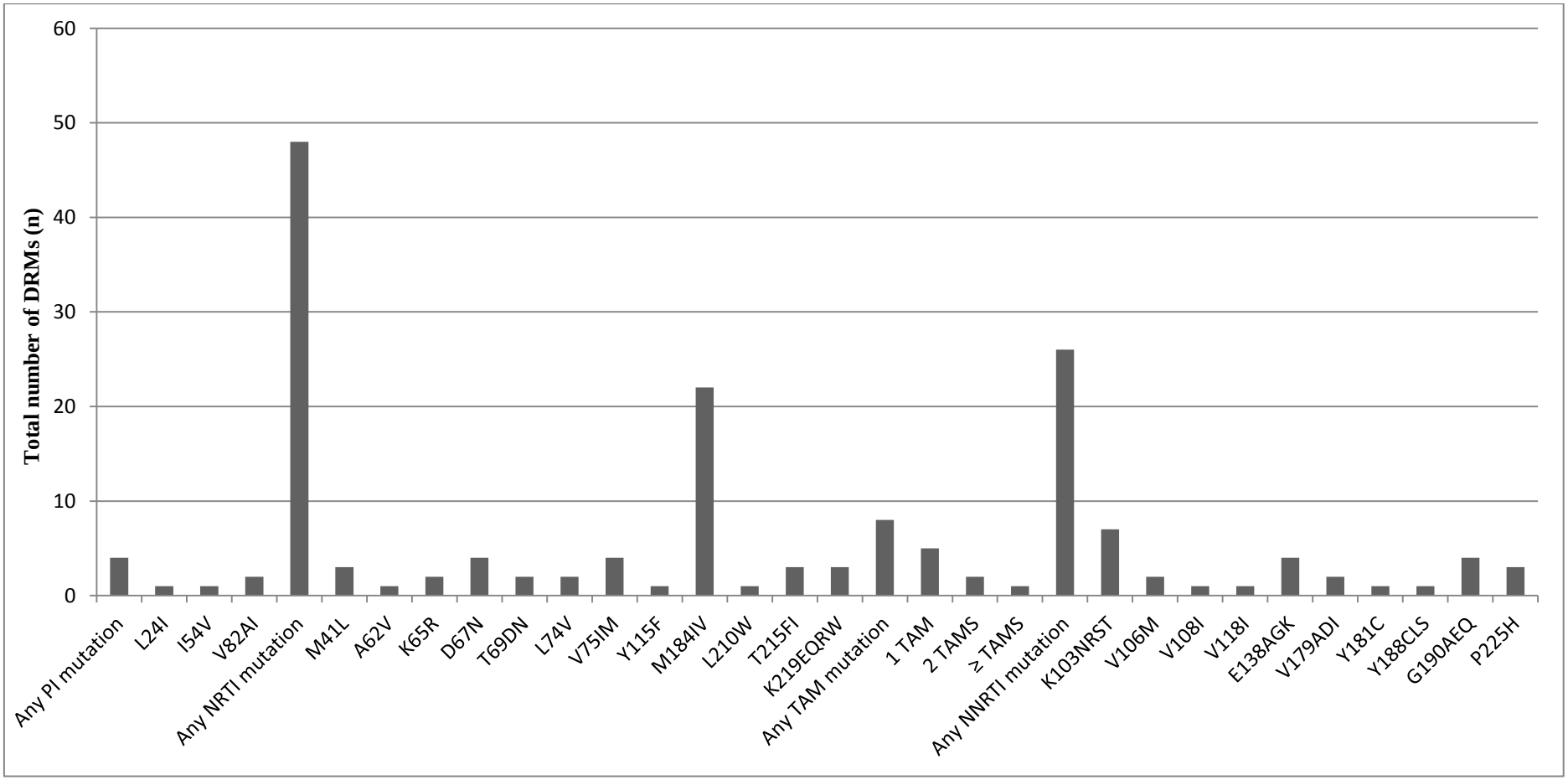


Figure 10: A summary of all HIV-1 drug resistance mutations identified in the pol genome including thymidine analogue mutations (TAMs) from 33 patients that were successfully genotyped using Sanger sequencing platform

Table 14: Frequency of major and minor drug resistance mutations associated with protease inhibitors, nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase regimens in 33 children genotyped using Sanger sequencing

DRM[i]	Overall patients (n=33) Number (%)	PI-based regimen (n=12)	NNRTI-based regimen (n=21)
PI mutations			
Any mutation	3(9.0)	3(25.0)	
L10FI	**2(6.0)**	**2(16.6)**	
L24I	**1(3.0)**	**1(8.3)**	
I54V	1(3.0)	1(8.3)	
V82A	2(6.0)	2(16.6)	
NRTI mutations			
Any mutation	23(69.6)	7(58.3)	16(76.1)
M41L	3(9.0)	1(8.3)	2(9.5)
A62V	**1(3.0)**		**1(4.7)**
K65R	2(6.0)		2(9.5)
D67N	4(12.1)		4(19)
T69D	**2(6.0)**	**1(8.3)**	**1(4.7)**
L74V	2(6.0)	1(8.3)	1(4.7)
V75IM	**4(12.1)**		**4(19.0)**
Y115F	1(3.0)	1(8.3)	
M184V	22(66.7)	7(58.3)	15(71.4)
L210W	1(3.0)		1(4.7)
T215F	2(6.0)		2(9.5)
K219QRW	**3(9.0)**		**2(9.5)**
TAMs			
Any TAMs	7(21.2)	1(8.3)	6(28.5)
1 TAM	4(12.1)	1(8.3)	3(14.2)
2 TAMs	2(6.0)		2(9.5)
≥ 3 TAMs	1(3.0)		1(4.7)
NNRTI mutations			
Any mutation	17(51.5)	17(80.9)	17(80.9)
K101EIHN	4(12.1)	4(19.0)	4(19.0)
K103NRST	8(24.2)	8(38.0)	8(38.0)
V106M	1(3.0)	1(4.7)	1(4.7)
V108I	**1(3.0)**	**1(4.7)**	**1(4.7)**
V118I	1(3.0)	1(4.7)	1(4.7)
E138A	2(6.0)	2(9.5)	2(9.5)
V179D	**1(3.0)**	**1(4.7)**	**1(4.7)**

Table 14: Frequency of major and minor drug resistance mutations associated with protease inhibitors, nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase regimens in 33 children genotyped using Sanger sequencing (continued)

Y181C	1(3.0)		1(4.7)
Y188CL	2(6)		2(9.5)
G190AEQ	5(15.1)		5(23.8)
P225H	**3(9.0)**		**3(14.2)**
Susceptible	9(27.2)	5(41.6)	4(19.0)

- Bold font indicates accessory mutations, regular font indicates major mutations
- All identified drug resistance mutations were defined using IAS-USA mutation list _2013 (appendices 1 and 2)

4.4 NGS

4.4.1 Data quality

In order to examine the patterns and prevalence of DRMs at minority frequencies, we performed ultra-deep next generation sequencing using the Illumina MiSeq platform. Each sample generated a total number of sequence reads that averaged >300 000 but following quality control of the data (trim 5' and 3' end, discard <Q25 and reads <100bp) approximately half that number were used in the final assembly (mean = 179 731 reads). We achieved a mean depth of coverage of the assembly of >4 600x; however, this varied over the entire genome. We noted that the average coverage over positions relevant to drug resistance codons varied considerably with the highest depth of coverage noted at the M184V position while the V32E/L, K103T, Q151M, E138A Y181C, Y188C/S, T215I and M230L codons did not have notable depth of coverage (Table 15). We used 3 different thresholds to investigate the impact of minority DRMs namely: 1%, 5% and 10% and will present the findings of this comparison subsequently.

4.4.2 Drug resistance mutations detected by NGS

Among 34 near complete HIV genomes generated (34 included children) using NGS, 29 (85%) harboured at least one DRM while five remained susceptible. At least one accessory DRMs was detected in 25 (73.5%) children. We noted that seven children harboured a mixture of NRTI, NNRTI and TAMs DRMs. While NRTI mutations were the most commonly occurring DRMs detected in 24 (76.1%) children with M184 being the most prevalent (n= 22, 64.7%) of these. NNRTI-associated mutations were less frequent occurring in 19 of 34 (55.8%) children. At least one TAM was detected in 9 (26.4%) patients, among those nine patients and five had at least three or more TAMs within the total viral population and four had at least one TAM each detected within their total viral population. We noted a child with no prior exposure to integrase inhibitors (IN) having major N155R intergrase-associated mutation detected. Eleven children (32.3%) were found to be harbouring at least one minority drug-resistance mutation that was missed by Sanger sequencing (Table 15, 16, Fig. 11).

Among patients on an NNRTI-based regimen group, we noted that NRTI mutations were the most frequent detected in 24(70.5%). M184 mutation was the most common NRTI mutation detected in 16 (76.1%) children. NNRTI-associated mutations were detected in 15(71.4%) with K103 being the most common mutation detected in 9 (42.8 %) children. Six children (28.5%) had a mixture of NRTI, NNRTI and TAMs mutations. While at least one TAM was detected in 8 (38 %) children with D67N being the most common detected in six children. Of the 34 children, eight children (38%) were found to be harbouring minority drug-resistance mutations that were totally missed by Sanger sequencing (Table 15, 16).

Even within the group of patients PI-based regimen group NRTI-associated mutations were the most frequently occurring mutations (Table 15), despite that these children were on PI-based regimen detected from 61.5% children. However, that was expected given that NRTIs forms a backbone for ARVs. Only 4 (30.7%) children had PI-associated mutations. One child had TAMs occurring as multiple TAMs, while four (30.7%) children were found to be susceptible. We noted a child who had never been on NNRTI-based regimen nor had experienced sdNVP during pMTCT to be harbouring NNRTI-associated V179D mutation likely due to transmitted resistance.

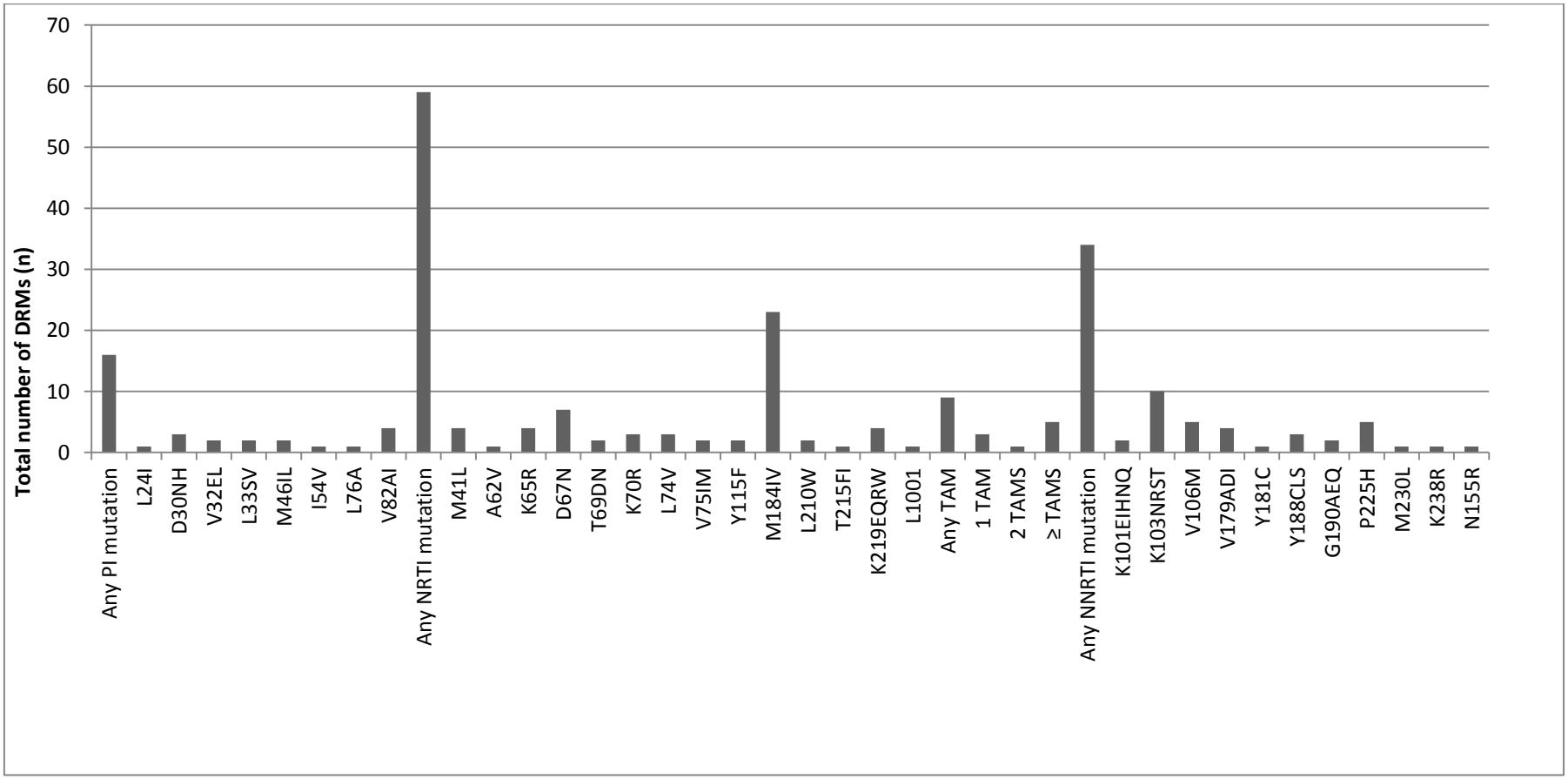


Figure 11: A summary of all HIV-1 drug resistance mutation identified from the whole HIV genome including thymidine analogue mutations (TAMs) outside the pol gene among 34 patients using NGS platform

Table 15: Frequencies of major and accessory drug resistance mutations associated with protease inhibitors, nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors in 34 genotyped children using next generation sequencing

DRM°	**Overall patients(n=34)**	**PI-based regimen (n=13)**	**NNRTI-based regimen (n=21)**
PI mutations	**n (%)**	**n (%)**	**n (%)**
Any mutation	6 (17.6)	4(30.7)	2 (9.5)
L24I	**1 (2.9)**	**1(7.6)**	
D30NH	2 (5.8)	1(7.6)	1 (4.7)
V32EL	**1 (2.9)**	**1(7.6)**	
L33V	1 (2.9)	1(7.6)	
M46IL	2 (5.8)	1(7.6)	1(4.7)
I54V	1 (2.9)	1(7.6)	
L76AI	**2 (5.8)**	**2(15.3)**	
V82AI	3 (8.8)	3(23)	
NRTI mutations			
Any mutation	24(70.5)	8(61.5)	16(76.1)
M41L	4(11.7)		4(19)
A62V	**1(2.9)**		**1(4.7)**
K65R	4(11.7)	1(7.6)	3(14.2)
D67N	7(20.5)		7(33.3)
T69DN	**2(5.8)**	1(7.6)	**1(4.7)**
K70R	3(8.8)		3(14.2)
L74V	3(8.8)	1(7.6)	2(9.5)
V75IM	**2(5.8)**		**2(9.5)**
Y115F	2(5.8)	1(7.6)	2(9.5)
M184IV	22(64.7)	7(53.8)	16(76.1)
L210W	2(5.8)		2(9.5)
T215I	**1(2.9)**		**1(4.7)**
K219EQ	**4(11.7)**		**4(19)**
TAMs			
Any TAM	9(26.4)	1(7.6)	8(38)
1 TAM	3(8.8)		3(14.2)
2 TAMs	1(2.9)		1(4.7)
≥ 3 TAMs	5(14.7)	1(7.6)	4(19)

Table 15: Frequencies of major and accessory drug resistance mutations associated with protease inhibitors, nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors in 34 genotyped children using next generation sequencing (continued)

NNRTI mutations			
Any mutation	19(55.8)	4(30.7)	15(71.4)
L100I	1(2.9)		1(4.7)
K101EHN	2(5.8)		2(9.5)
KI03NRST	9(26.4)		9(42.8)
V106M	5(14.7)	1(7.6)	4(19)
V179ADI	**4(11.7)**	**3(23)**	**1(4.7)**
Y181C	1(2.9)		
Y188CLS	3(8.8)	1(7.6)	2(9.5)
G190AEQ	2(5.8)		2(9.5)
P225H	**5(14.7)**		**5(23.8)**
M230L	1(2.9)		1(4.7)
K238R	**1(2.9)**		**1(4.7)**
Integrase mutation			
N155R	1(2.9)		1(4.7)
Susceptible	5(14.7)	4(30)	1(4.7)

- Bold font indicates accessory mutations
- Regular font indicate major mutations
- All identified drug resistance mutations were defined using IAS-USA mutation list _2013 (appendices 1 and 2)

4.5 Comparison between Sanger sequencing and NGS

4.5.1Phylogeny

All sequences were subtype C and clustered within the subtype C clade with high bootstrap support for each. Sequences for each patient derived from Sanger sequencing and those generated by NGS clustered together indicating that there was no cross-contamination between patient samples.

4.5.2 Detection of minority drug resistance mutations

A total of 108 DRMs were detected by NGS platform, 32 from PI based regimen group and 76 from NNRTI-based regimen group. While 88 DRMs were detected using Sanger sequencing; NGS detected an additional 24 DRMs, 22% more DRMs from what was detected using Sanger sequencing. Of those additional mutations 15 were minority DRMs present in 11 (32.3%) children (Table 17, Fig. 12). The minority DRMs percentage frequency ranged between 10.2% and 20.9% (Table 17). Minority DRMs detected includes: PI-associated mutations L76A (n=1), V82I (n=1), M46L (n=1) and D30H (n=1). NNRTI-associated minority mutations: Y188C (n=1), P225H (n=1), V179A (n=1). NRTI-associated minority variants detected were M41L (n=2), K65R (n=1), T69D (n=1), K70R (n=1), Y115F (n=1), M184I (n=1) and L210W (n=1) (Table 17, Fig. 12).We noted that among four children (three from PI and one from NNRTI-based regimen groups) when NGS was used, DRMs not associated with the ART regimen in use were detected. Of the three children from the PI-based regimen; two had a history of sdNVP. The other two children (including one from NNRTI-based regimen groups) the reason for these DRMs was unknown. We hypothesise that these DRMs could have been transmitted vertically or during breastfeeding, since both children had no history of sdNVP.

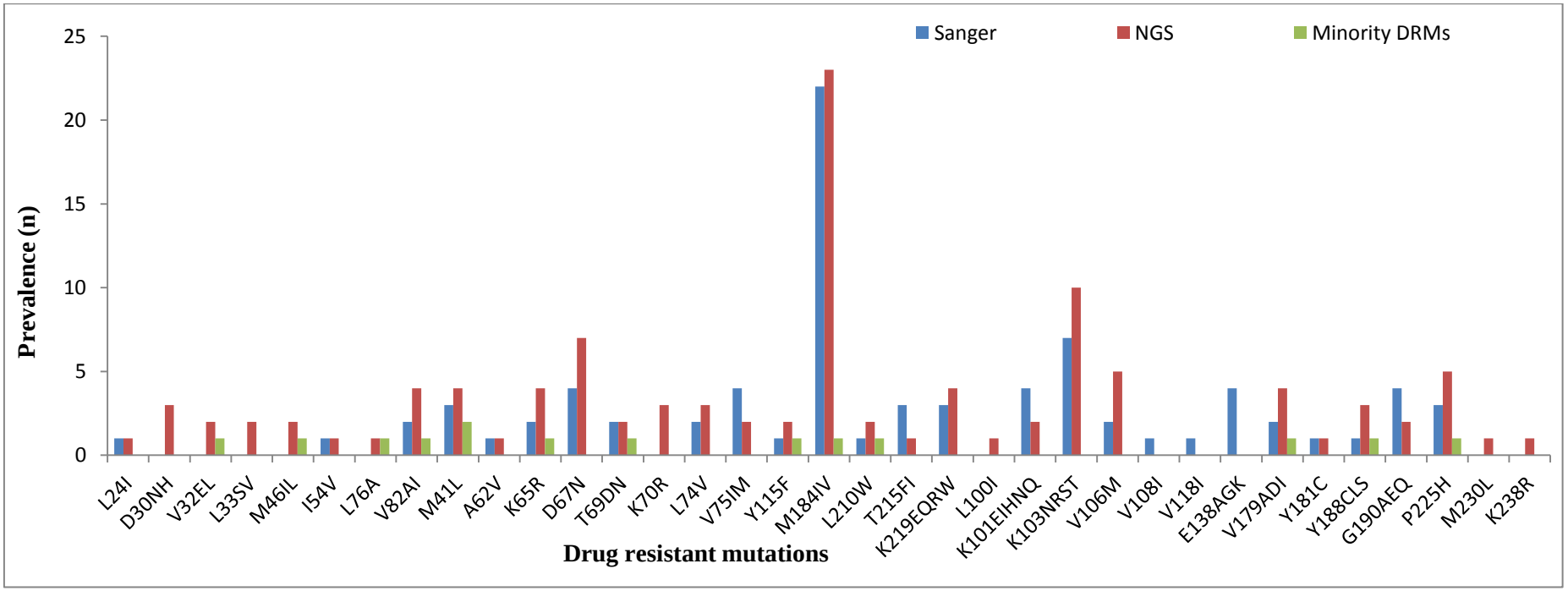


Figure 12: A summary of the prevalence of HIV-1 drug-resistance mutations detected by Sanger and next generation sequencing. DRMs detected by Sanger sequencing are indicated in blue, DRMs detected by NGS are indicated in red and minority drug resistance mutations indicated in green which were detected from eleven children using only NGS

Table 16: Average sequencing read coverage and frequencies for all HIV-1 drug resistance mutations identified by Sanger sequencing and NGS

Gene	DRM	Sanger sequencing	NGS at 10% mutation frequency			NGS at 1- 5% mutation frequency		
		Frequency of DRMs	Frequency of DRMs	Average coverage	Allele frequency (%)	Frequency of DRMs	Average coverage	Allele frequency (%)
PR	L24I	1	1	958	99	1	1045	96.3
	D30N					1	28	10.7
	D30H		2	8294	59.75	2	516	20.5
	V32E		1	12	16.7	1	26	23.1
	V32L		1	12	16.7	1	26	23.1
	L33V		1	11	18.2	1	26	15.4
	M46I		1	1125	98.6	1	1168	98.2
	M46L		1	785	13.9	1	791	13.8
	I54V	1	1	1088	98.7	1	1145	97.3
	L76A		1	3327	98.9	1	4333	21.9
	V82A	2	2	3615	87.2	3	2750	90.5

Table 16: Average sequencing read coverage and frequencies for all HIV-1 drug resistance mutations identified by Sanger sequencing and NGS (Continued)

Gene	DRM	Frequency of DRMs	Frequency of DRMs	Average coverage	Allele frequency (%)	Frequency of DRMs	Average coverage	Allele frequency (%)
	V82I					1	3546	21.3
RT/NRTI	M41L	3	3	9374	51.4	4	2082.2	40.8
	A62V	1				1	6202	84.2
	K65R	2	3	8567	86	4	3794.5	68
	D67N	4	6	11849	87.8	7	2977	69.6
	T69D	2				1	2123	89.1
	T69N		1	4889	47.1	2	5550.5	32.1
	K70R		3	1314	70	3	3297	61.3
	L74V	2	3	14648	57.2	3	5179.6	56.3
	V75M	2						
	V75I	2	1	2568	99.3	2	2312	98.3
	Y115F	1	1	4377	98.9	2	4601	54
	M184I					1	2139	12.8
	M184V	22	17	56348	89	21	4312	82
	L210W	1				2	6927.5	56
	T215I	3	1	8	75	1	8	65.5
	K219E	1	3	9382	99.8	3	3563.6	71.7
	K219Q		1	3	100	1	1021	47.4

Table 16: Average sequencing read coverage and frequencies for all HIV-1 drug resistance mutations identified by Sanger sequencing and NGS (Continued)

RT/NNRTI	DRM	Frequency of DRMs	Frequency of DRMs	Average coverage	Allele frequency (%)	Frequency of DRMs	Average coverage	Allele frequency (%)
	L100I					1	6033	96.9
	K101E	1	1	1035	99.5	1	1096	97.9
	K101Q					1	486	80.9
	K103N	4	7	20849	91.6	7	3817.8	88.4
	K103R	2	2	8737	90.6	2	3448	88.3
	K103T		1	17	55.9	1	17	52.9
	V106M		4	2904	97	5	3848.6	96.4
	V179A					1	4527	19.8
	V179D	1				2	1211.5	96.3
	V179I		1	4475	68.5	1	4624	68.3
	Y181C		1	6	33.3	1	819	18.3
	Y188C	1	2	80	100	2	4779	38.9
	Y188S		1	6	100	1	685	31.5
	G190A	3	2	8126	86	2	4045.5	88.6
	G190E		1	8695	99.3	1	8949	98.7
	P225H	3	4	16439	98.6	5	4890.4	71.5
	M230L		1	3	100	1	719	51.3
	K238R					1	586	36.2

- All identified drug resistance mutations were defined using IAS-USA mutation list _2013 (appendices 1 and 2)

Table 17: Summary of minority drug-resistance mutations detected from eleven children using next generation sequencing

Patient #	**Sanger sequencing DRMs**	**NGS DRMs including minority variants[a]**	**Regimen[b]**	**sdNVP (y/n)**
#5	V82A, M184V	L76A (20.9)*, V82A (76.9%), V82I (20.3%)*, M184V (31.5%)	3TC+AZT+ LPV/r	n
#14	M184V, G190A	M41L (10.4%)*, D67N (35.6%), V106M (98.7%), M184V (97.8%), G190A (97.3%), L210W (17.3%)*	3TC+ABC+ LPV/r[c]	n
#19	M184V	M46L (13.8)*, M184V (98.4%)	3TC+DDI+ LPV/r[c]	n
#28	susceptible	D67N (98.2%), K70R (98.2%), V106M (96%), M184V (42.2%), Y188C (17.9%)*, K219E (39.6%)	3TC+ABC+LPV/r	y
#29	V75M, K101E, M184V	M41L (15.9%)*, D67N (21.3%)*, K70R (20.8%)*, K101E (97.9%), M184V (98.0%)	3TC+AZT+ LPV/r[c]	n
#45	K65R, L101I, K103N	K65R (98.2%), L100I (96.9%), K103N (96.6%), P225H (14.5%)*	3TC+AZT+ LPV/r[c]	n
#56	M184V	T69D (16.7%)*, L74V (30.7%), Y115F (10.2%)*, M184V (99%)	3TC+ABC+ LPV/r[c]	n
#57	susceptible	D30H(10.5)*	3TC+D4T+ EFV	n
#85	M41L, K65R, V75I, K103R, V106M, M184V	M41L(75.3%), K65R(97.2%), V75I(97.9%), K103R(78.8%), V106M(98.3%),M184V(92.7%), M184I(12.8)*	3TC+AZT+ LPV/r[c]	n
#309	A62V, D67N, V75I, K103R, M184V, G109E, K219E	A62V(84.2%),K65R(16.7%)*,D67N(98.6%),V75I(98.8%),K103R(97.8%),M184V(98.3%), G190E(98.7%), K219E(98.5)	3TC+TDF+ LPV/r[c]	n
#318	M184V	L76I(98.8%), V82A(98%),V179A(19.8%)*, M184V(98.7%)	3TC+ABC+ LPV/r[c]	y

[a] DRMs with asterisk were found to be occurring as minority variants in these children and only detected by NGS

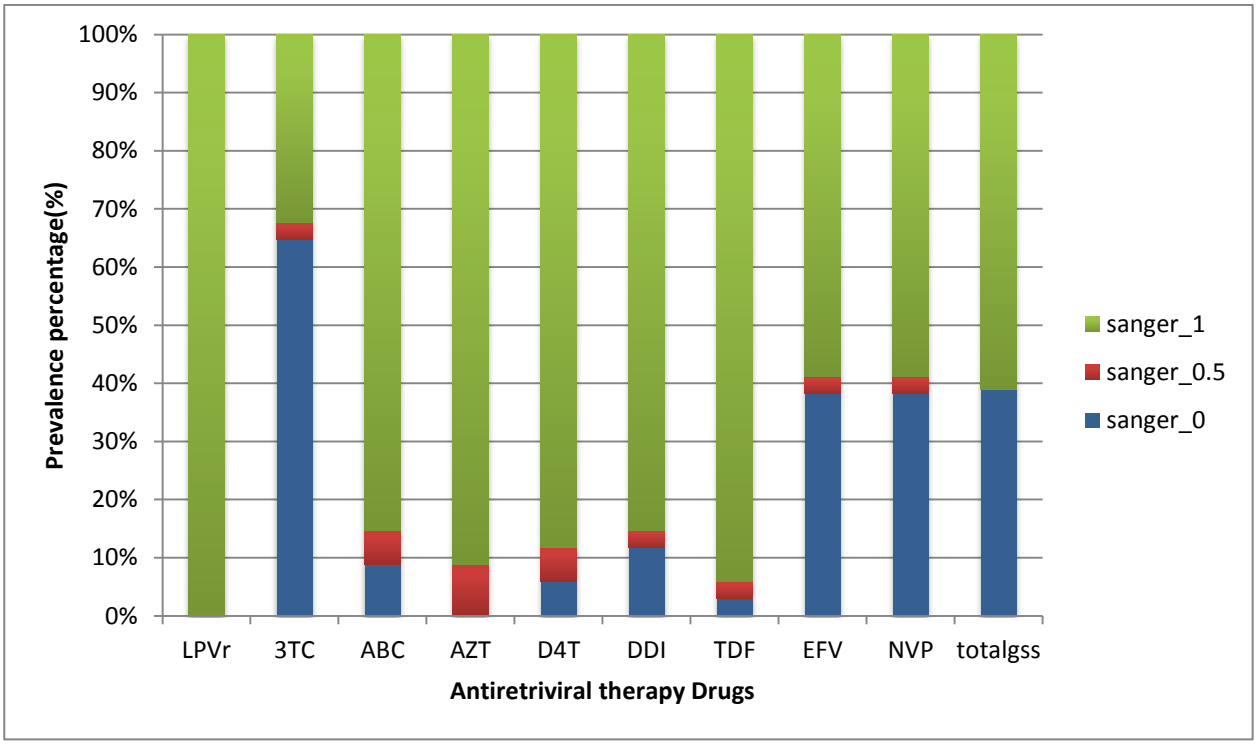
[b] 3TC (lamivudine), AZT (zidovudine), ABC (abacavir), DDI (didanosine), TDF (tenofovir), LPV/r (lopinavir boosted with rotinavir)

[c] switched from NNRTI-based regimen to PI-based regimen

4.5.3 Genotypic susceptibility score (GSS) correlations between drug resistance mutations detected and drug regimens

We interpreted genotypic results using Rega V 9.1.0 genotypic susceptibility score (GSS) as a predictor of virologic failure (Fig. 13). Rega V 9.1.0 system reports three levels of resistance: susceptible (≤1), intermediate (≤0.5) and resistant (0). We noted that some of these patients had developed resistance to some ARV in use (NRTIs and NNRTIs) with an exception of PI drugs. A total number of (24, 70.5%) children had developed resistance mutations to NRTIs, predominant 3TC. GSS scoring system showed that M184 mutation resulted in a high resistance (~65%) to 3TC, intermediate resistance (20%) to ABC and DDI, and low resistance (10%) to AZT and d4T. These finding were consisted with a high prevalence of M184 mutation in out cohort; therefore, high resistance to 3TC. However, high resistance to NRTI is of the great concern, given that NRTIs make up the backbone of ARVs in South Africa and other limited-resources countries. However, the majority of children were still susceptible to other NRTIs including ABC, AZT, d4T, DDI and TDF allowing these children to switch to these drugs if needed (Fig.13). We also detected the low level of resistance to NNRTI-associated ARVs. K103 mutation was the most common, occurred in approximately 26.4% children resulting in resistance to EFV and NVP. Though our cohort had no child on NVP, except for seven who were sdNVP exposed, resistance to NVP could have been due to the fact that EFV and NVP drugs from the same class. The majority of the children were still susceptible to PI-based drug, LPV/r, in spite of experiencing virologic failure.

A



B

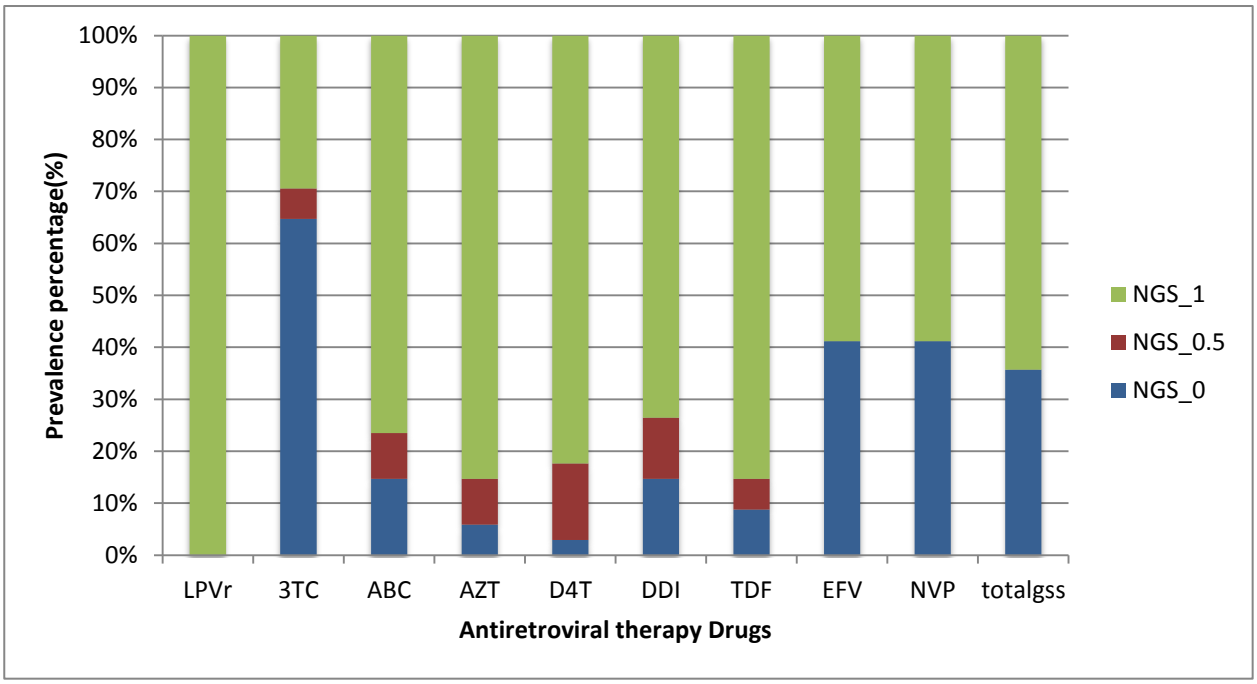


Figure 13: Overall Genotypic susceptibility scores stratified by drugs in use at the time of therapy failure where 0 indicates drug resistance (blue), 0.5 indicates intermediate drug resistance (red), and 1 indicates susceptible (green). Panel A: represents GSS scores inferred from Sanger sequencing while Panel B: illustrates GSS scores calculated from NGS data

CHAPTER 5

5. Discussion

5.1 General comments

Transmission of HIV among children has been significantly reduced with pMTCT intervention yet nearly 200 000 HIV-infected children are currently receiving ART in South Africa[3]. Since 2004 when South Africa effectively initiated ART rollout to HIV-infected individuals, ART scale-up has increased rapidly and this intervention has greatly reduced HIV-1 morbidity and mortality among adults and children alike[6]. However, the development of DRMs including minority drug resistance mutations in HIV-1 infected patients is inevitable and will compromise the benefits of ART to reduce HIV-1 related morbidity and mortality[59]. This is particularly devastating for children who have to remain on ART for significantly longer than adults. Therefore, WHO recommends that in the process of ART scale-up developing countries should establish a national surveillance program for HIV drug resistance monitoring[4]. The Southern African HIV Clinicians Society guidelines recommend drug resistance genotyping for all patients experiencing virologic failure, following attempts to improve adherence and other psycho-social challenges[17]. Resistance genotyping at ART initiation and during therapy can guide the selection of optimal drug regimens to suppress viral replication and promote successful management of HIV-1 infection[86].

The Sanger sequencing is currently used for resistance genotyping[4] ; however, studies have shown that this method is only sensitive when DRMs are occurring at a frequency of >20% within the total patient's viral population[15]. With advancement in technology there has been a development of a number of sensitive assays which can detect DRMs occurring at <20% of the viral population termed minority DRMs[15]. The presence of minority drug resistance mutations has been shown to compromise long-term treatment success as early as within the first year of ART initiation[14, 15, 20-22, 28, 55, 87-95]. It is for this reason that a deeper understanding of the impact of minority drug resistance mutations on ART failure particularly in resource-limited setting needs to be gained. We sought to contribute to this understanding by profiling and investigating the prevalence of minority DRMs occurring at <20% frequencies in children failing ART in a rural KwaZulu-Natal setting.Our findings highlights that deep sequencing does provide useful information regarding drug resistance that can be used in ART management programs particularly with its abilities to detect minority DRMs which were not detected by traditional Sanger sequencing.

5.2 Results discussion

This retrospective study that included 34 children ≤15 years of age on ART for at least a year and experiencing virologic failure was to the best of our knowledge, the first to be conducted in a rural KwaZulu-Natal cohort in South Africa. The prevalence of DRMs was measured by both Sanger sequencing with its ability to detect DRMS present in >20% of the total viral population and NGS able to detect DRMs present at frequencies down to <1% of the viral population. Among these children, 21 were initiated on an NNRTI-based regimen (classified as NNRTI-based regimen group) and 13 were initiated on PI regimen (classified as PI-based regimen group). Although our sample size was small, this study is unique because it is the first study to our knowledge (1) to compare patients failing first line NNRTI and PI-based regimens at minority levels, (2) use two high technology platforms to interrogate DRMs, (3) investigate the prevalence of minority DRMs at virologic failure among children in a rural setting, the depth of information detected would indicate that this technology may be a useful tool in patient management. All patients in this cohort were from the Hlabisa sub-district, a resource-limited setting where health services are decentralized into 17 clinics in the Hlabisa Treatment and Care Programme[96].

Among 34 patients, 29 (85.2%) had at least one DRM. This suggests that these children were failing due to the emergence of resistance, and a high prevalence of resistance has been previously reported by Pillay et al[81]. While five patients were susceptible, the detection of DRMs could be a tool to distinguish children failing therapy due to non-adherence as opposed to those failing due to resistance in a timely manner such that patients do not remain on failing regimen for extended periods of time. M184V was the most prevalent mutation detected in our cohort. Mutations at the M184 location are usually the first to appear when patients are exposed to 3TC[19, 97]. NNRTI-associated K103N was the second most prevalent mutation detected and is associated with nevirapine and efavirenz use. We detected 14 (41%) children having a mixture of NNRTI/NRTI mutations. The prevalence of these two mutations occurring simultaneously results in complex resistance patterns such as cross-resistance to multiple drugs within the same class or over other drug classes[81]. In resource-limited settings such as South Africa, first and second line regimens consist of 2 NRTI (as a backbone) with either an NNRTI or a PI [6]. The emergence of mutations to these classes is of the great concern especially in cases of children who are failing therapy as this will compromise future ART options and consequently long-term ART success[19].

A total of 9 (26.4%) children harboured at least one TAM , five of whom had multiple (>3) TAMs. Only two children from the PI-based regimen group had TAMs compared to eight from the NNRTI-based regimen group. Children in the NNRTI-based group remained on a failing regimen for significantly ($p<0.01$) longer than patients on a PI-based regimen (median = 8.6 years versus 3.5 years respectively). Given that prolonged failure on first-line regimens leads to the accumulation of drug resistance mutations and subsequently therapy failure[18]. However, it is concerning to know that even after viral load monitoring, patients remained on failing regimens for prolonged periods without any intervention ultimately resulting in the accumulation of DRMs. Others have noted similar patterns of complex DRMs in children in both urban [18, 21, 27, 28, 38] and rural settings [81]. Unfortunately, the presence of TAMs is associated with the high-level and cross-resistance to ddI and TDF [18, 30, 81, 88], while multiple TAMs results in high-level resistance to NRTIs (e.g. AZT and d4T) as well as intermediate resistance to ddI, ABC and TDF [18, 30, 81, 88]. Equally disturbing is that the accumulation of TAMs results in diminished susceptibility to multiple NRTI thus causing broad cross-resistance to the NRTI drug class and compromising future ART options[98].

Mutations detected by NGS were reliable if the coverage was above 100, which increased the confidence of calling mutations occurring at <20% frequency, given that studies have reported that NGS is prone to homopolymer reading error resulting in false positive detection of different DRMs especially minority DRMs[99]. We chose NGS because of its abilities to provide a snapshot of the entire spectrum of resistance DRMs within the viral population in a single run[67], provides a high throughput of data and at a comparable; if not, reduced cost[79]. In our cohort Sanger sequencing detected the majority of anticipated mutations associated with ARTs currently in use. However, we noted that the use of NGS platform revealed a number of DRMs missed by Sanger sequencing. Furthermore, we identified three children who were found to be susceptible by Sanger sequencing to be harbouring at least one DRM when NGS was used. This confirms the higher sensitivity of NGS over Sanger sequencing and it is consistent with the findings from a number of studies.

NGS was able to detect at least one minority DRMs in eleven children (32.3%) that were not detected by Sanger sequencing. Minority DRMs were detected at frequencies between 10.2% and 20.9%. Whether these minority DRMs have any clinical impact on patients is still a point of debate[15]. Through studies conducted both on adults[20] and children [14, 15, 20, 22, 55, 87-95], we now know that minority DRMs predispose patients to virologic failure. Studies conducted on children have shown that minority DRMs detected at baseline regardless of the history of sdNVP predisposed those children to therapy failure[15]. Indeed, minority mutations detected at baseline were shown to increase into major mutations over time on ART resulting in therapy failure[15]. This demonstrates the critical role of accurate DRM identification as a clinical guide for treatment switch and choice of appropriate ART regimen[15].

We noticed the lesser prevalence of DRMs in PI-based regimen group, in agreement with other studies [39, 40]. In our study children were still susceptible on a PI regimen compared to those on an NNRTI-based regimen who had a greater number of both major and accessory mutations as well as TAMs. The lack of PI mutations may be a result of the high genetic barrier of LPV/r or the presence of drug resistance mutations outside of the protease region e.g. the HIV gag or env genes. Studies are emerging showing that the presence of mutations in Gag or the cytoplasmic tail of Env could affect drug resistance to PIs[87, 100], even in the absence of major DRMs within the pol gene. PI-based regimens due to their robust genetic barrier appear to be ideal as a first-line option in children given that they will have to remain on ART for longer periods compared to adults. Whole genome sequencing of HIV using NGS will; therefore, allow researchers and clinicians to identify patients that are in virologic failure due to mutation in genes other than the presence of DRMs in the pol gene.

We found a study done in the UK[64] on when to switch to second-line regimen after elevated VL results in children enrolled on either NNRTI or PI- based regimens. They noted that if these children were kept on failing regimens, there was an additional 10% NRTI DRMs (predominantly TAMs and M184V) in the delayed switching of NNRTI-based regimen group that was not seen in the PI-based regimen group[87]. Therefore, in our cohort this scenario is further explaining low prevalence of DRM among PI-based regimen group though on virologic failure when compared to a high prevalence of resistance detected among NNRTI based-regimen group. Using NGS, we found that 25(73.5 %) children harboured accessory mutations, of these 11.7 % did not have any major DRMs. Accessory mutations are polymorphisms that alone do not result in drug resistance and only contribute in the presence of major mutations to resistance[56]. The role of these mutations at a minority level to treatment failure requires further investigation.

The use of NVP in mothers and infants as part of pMTCT strategies could select for DRMs resulting in treatment failure when on first-line regimens containing NNRTI. Of the seven children who were previously exposed to sdNVP, three from PI group had at least one NNRTI-associated mutation. YI88C and V179D were observed occurring as minority variants in two children, while another child harboured only the V179D mutation. We speculate that these DRMs were persisted as minority variants following sdNVP exposure in two of the three children. We noted that children previously exposed to NVP reacted better to PI-based first line regimens. In contrast to other studies, we noted that the presence of Y181C was not common in our cohort, although a number of studies from the Sub-Saharan Africa region conducted on children reported predominantly Y181C and K103N in their cohorts[15, 94, 101-103]. Three studies from South Africa[15, 101, 102] and one from Tanzania[103] and multi-centre study[94] investigated the occurrence of the major mutations K103N and Y181C among children previously exposed to sdNVP, and found that the presence of these mutations was associated with virologic failure. In resource-limited settings such as these countries, this poses a serious problem, since NVP forms a major part of the first-line regimens and pMTCT protocols. Moreover, the emergence of NVP resistance can result in cross-resistance to EFV a key component of first-line regimens.

The emergence of drug resistance progresses fast when drugs are not maintained within an optimal therapeutic range [86]. There are several challenges in paediatric management that complicate adherence to their regimens, among them include medication being administered by grandparents and the inherent problems associated with an older caregiver, the palatability of liquid formulations and inability to swallow ARV drugs and storage conditions of medications that do not heat stable[86]. It is; therefore, critical that patients adhere to their ART regimens for adequate suppression of the HIV-1.

5.3 Limitations

Our study has some strength and limitations, strengths include that this study was conducted in a rural KwaZulu-Natal cohort having one of the highest burdens in South Africa[18, 81]. Previous investigations of resistance patterns in children have primarily focused on urban setting and none to our knowledge; was done using NGS technology on patients from a rural setting. Unravelling the complex patterns of drug-resistance mutations occurring at majority and minority frequencies in variable settings will add a critical depth to successful, long-term management of children on ART. A limitation such as since this was a retrospective study patients who could have been included were not due to that sample was depleted. Limitations of our study included a small sample size and a single time point which made it difficult to well establish a good understanding on persistence of minority DRMs among these children. Despite a small sample size, we were able to establish that minority mutations are prevalent among patients at virologic failure and that DRMs are missed using traditional Sanger sequencing where patients may be incorrectly identified as susceptible to their regimens. Further studies to look at minority variants in larger cohorts at different time points are required for the better understanding of the clinical impact by minority variants in order to inform national policies. We propose that our study be expanded to a larger cohort and include multiple time points in order to fully interrogate the impact of the patterns of minority drug resistance mutation and their persistence on treatment failure among children on NRTI- and PI-based ART regimens. It was noted that very few patients had PI-associated DRMs, we detected three using Sanger sequencing and six using NGS platform. However, these patients were experiencing virologic failure; therefore, it is likely that we could have missed mutations outside the pol region. We also propose that further studies should be done to interrogate gag and gag-pol cleavage sites that could influence the virologic outcome among patients on a PI-based regimen.

5.4 Conclusion

When faced with a need of ART for a lifetime among children it is crucial that the use of ART should be maximized due to the limited ART options in South Africa. Given that the emergence of DRMs leads directly to therapy failure among patients on ART. Accurate identification of DRMs is pivotal and we found that NGS appears to be more specific, sensitive and efficient as compared with Sanger sequencing[79]. Its abilities could present a management tool in patient's harbouring DRMs at an affordable cost[79]. In contrast, we have shown as others have, that traditional Sanger sequencing will miss a number of resistance mutations occurring at minority frequencies[15].

In addition we detected a high prevalence of resistance mutations by both technologies suggesting that patients remained in virologic failure for extended periods of time. This highlights the need for timely identification of patients failing ART and the implementation of early interventions, be it drug switches or effective, reinforced, adherence counselling with appropriate follow-up[19, 99]. The PI-based regimens were more effective than NNRTI-based regiments most likely due to PI's higher genetic barrier. However, PIs are sometimes not well-tolerated by children and there remains a lack of syrup formulations for children. It is; therefore, likely that these children were failing due to reasons other than the presence of DRMs as shown by patients with susceptible virus determined by both methods.

Although we had a small sample size, it was noted that children previously exposed to sdNVP responded well on ART particularly if they were initiated on a PI-based regimen. In agreement with a number of previous studies[14, 15, 20, 22, 26, 56, 88-95]. We noted that Sanger sequencing missed a number of mutations and even classified children (n=3) as susceptible but who harboured mutations detected using NGS. The prevalence of minority DRMs was high (32.3%) in our cohort; however, it must be noted that this is a snapshot of a single time-point. A larger study which would interrogate the clinical impact of minority DRMs at baseline, therapy failure and a follow-up sample will inform us on a deeper level regarding minority DRMs.

6. APPENDICES

Appendix 1

MUTATIONS IN THE REVERSE TRANSCRIPTASE GENE ASSOCIATED WITH RESISTANCE TO REVERSE TRANSCRIPTASE INHIBITORS

Nucleoside and Nucleotide Analogue Reverse Transcriptase Inhibitors (nRTIs)[a]

Multi-nRTI Resistance: 69 Insertion Complex[b] (affects all nRTIs currently approved by the US FDA)

M	A	▼	K	L	T	K
41	**62**	**69**	**70**	**210**	**215**	**219**
L	V	Insert	R	W	Y F	Q E

Multi-nRTI Resistance: 151 Complex[c] (affects all nRTIs currently approved by the US FDA except tenofovir)

A	V	F	F	Q
62	**75**	**77**	**116**	**151**
V	I	L	Y	M

Multi-nRTI Resistance: Thymidine Analogue-Associated Mutations[d,e] (TAMs; affect all nRTIs currently approved by the US FDA)

M	D	K	L	T	K
41	**67**	**70**	**210**	**215**	**219**
L	N	R	W	Y F	Q E

Abacavir[f,g]

K	L	Y	M
65	**74**	**115**	**184**
R	V	F	V

Didanosine[g,h]

K	L
65	**74**
R	V

Emtricitabine

K	M
65	**184**
R	V I

Lamivudine

K	M
65	**184**
R	V I

Stavudine[d,e,g,i,j,k]

M	K	D	K	L	T	K
41	**65**	**67**	**70**	**210**	**215**	**219**
L	R	N	R	W	Y F	Q E

Tenofovir[l]

K	K
65	**70**
R	E

Zidovudine[d,e,j,k]

M	D	K	L	T	K
41	**67**	**70**	**210**	**215**	**219**
L	N	R	W	Y F	Q E

Nonnucleoside Analogue Reverse Transcriptase Inhibitors (NNRTIs)[a,m]

Efavirenz

L	K	K	V	V	Y	Y	G	P
100	**101**	**103**	**106**	**108**	**181**	**188**	**190**	**225**
I	P	N	M	I	C I	L	S A	H

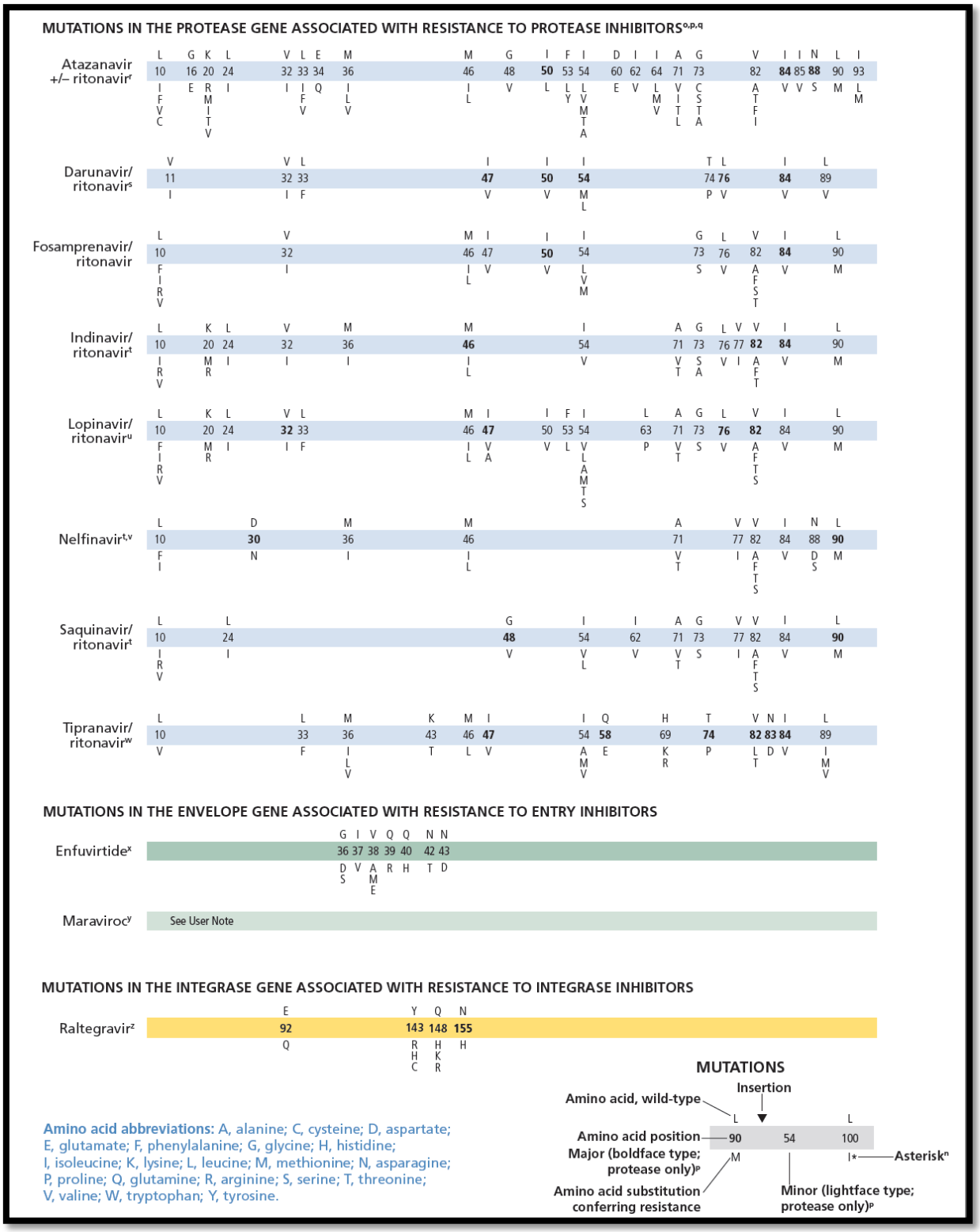
Etravirine[n]

V	A	L	K	V	E	V	Y	G	M
90	98	100	101	106	138	179	181	190	230
I	G	I*	E H P*	I	A G K	D F T	C* I* V*	S A	L

Nevirapine

L	K	K	V	V	Y	Y	G
100	**101**	**103**	**106**	**108**	**181**	**188**	**190**
I	P	N	A M	I	C I	C L H	A

Appendix 2


MUTATIONS IN THE PROTEASE GENE ASSOCIATED WITH RESISTANCE TO PROTEASE INHIBITORS[o,p,q]
Atazanavir +/– ritonavir[r]
Darunavir/ ritonavir[s]
Fosamprenavir/ ritonavir
Indinavir/ ritonavir[t]
Lopinavir/ ritonavir[u]
Nelfinavir[t,v]
Saquinavir/ ritonavir[t]
Tipranavir/ ritonavir[w]
MUTATIONS IN THE ENVELOPE GENE ASSOCIATED WITH RESISTANCE TO ENTRY INHIBITORS
Enfuvirtide[x]
Maraviroc[y]
See User Note
MUTATIONS IN THE INTEGRASE GENE ASSOCIATED WITH RESISTANCE TO INTEGRASE INHIBITORS
Raltegravir[z]
Amino acid abbreviations: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
MUTATIONS
Insertion
Amino acid, wild-type
Amino acid position
Major (boldface type; protease only)[p]
Amino acid substitution conferring resistance
Asterisk[n]
Minor (lightface type; protease only)[p]

Appendix 3

HIV drug resistance study **Information sheet (Children)**

Isethulo

Simema umntwana wakho ukuba abambe iqhaza ocwaningeni oluhlola ukungazweli kwemishanguzo yesandulela ngculazi.

Le ncwajana ikunikeza ulwazi ngocwaningo okuzoxoxiswana ngalo nawe. Uma ngabe uluqonda ucwaningo, nokuthi uma uvuma ukuba umntwana wakho abambe iqhaza, uyocelwa ukuba usayine ifomu, noma wenze uphawu efomini ngaphambi kukafakazi.

Incazelo ngalokho esizama ukukwenza

Ukudla amaphilisi okudambisa isandulela ngculazi (ARVs) kuyinto yaphakade. Kunokwenzeka ukuthi ngesikhathi udla ama-ARVs, isandulela ngculazi singafika esigabeni esibizwa ngokuthi 'ngesingezweli'. Lokhu kuchaza ngesimo soshintsho lwegciwane kangangoba ezinye izinhlobo zama ARV's azisakwazi ukumelana naso. Asazi ukuthi lokhu kwande kangakanani ohlelweni lwethu lokwelapha nokuthi imiphi imithelela okungenzeka ihambisane nokwanda kokungezweli kwemithi. Ucwaningo luyithuba lokukhulisa ulwazi lwethu ngalokhu kanye nokusebenzisa ulwazi ekuthuthukiseni uhlelo lwezokwelapha.

Isimemo sokubamba iqhaza

Sicela ukuba umntwana wakho abambe iqhaza kulolu cwaningo ngoba ebedla ama-ARV's isikhathi esingaphezu konyaka kanti imiphumela yakhe iveza ukuthi inani legciwane egazini alehlile ngokwanele.

Kuchaza ukuthini ukuzibandakanya kulolu cwaningo?

Uma evuma ukubamba iqhaza kulolu cwaningo, udokotela noma umhlengikazi wocwaningo uyomubuza imibuzo ngesikhathi elande imithi ngezikhathi ezijwayelekile. Uyomubuza imibuzo ngokwelashwa kwakhe engeke ithathe ngaphezu kwemizuzu engaphezu kwama 20 bese ethatha igazi eliyothunyelwa elabhorethri eThekwini. Inqubo yasemtholampilo iyokwenzeka kuphela kanye nje kuphela. Igazi lakhe liyohlolwa elabhorethri ukuthi alinalo yini igciwane kanye nokungadodobali kwesandulela ngculazi. Ngemvume yakho, ulwazi olutholakale kulolu cwaningo luyongahlanganiswa nolwazi oselukhona kwisilondoloza lwazi sase-Africa Centre. Uyothola ithuba lokuhlangana nomhlengikazi kanye nodokotela

Appendix 4

HIV drug resistance study **Consent form**

Mina……ngiyavuma ukuba umntwana wami abe yingxenye yocwaningo lokuhlola ukungazweli kwemishanguza yesandulela ngculazi. Sengichazeliwe ngocwaningo ngaliqondisisa nephepha lolwazi.

Ngiyayiqonda imithelela yokungenela komntwana wami kulolu cwaningo nokuthi kunokwenzeka kucelwe olunye ulwazi mayelana nempilo kanye nokwelashwa kwakhe ngesikhathi socwaningo.

Ngiyabagunyaza abasebenzi bocwaningo ukuba babheke efayelini kanye nasekhadini lakhe nokuthi ulwazi olutholakala kulolu cwaningo lungahlanganiswa nolwazi oselukhona kwisilondoloza lwazi sase-Africa Centre. Ngiyaqonda nokuthi kuzothathwa elinye isampula legazi kulolucwaningo.

Ngiyaqonda ukuthi ngiyolithola ithuba lokubonisana ngemiphumela yomntwana wami nomhlengikazi noma nodokotela.

Ngiyaqonda ukuthi umntwana wami angashiya noma nini ocwaningweni futhi ngeke abandlululwe ngokwenze njalo. Siyoqhubeka nokusebenzisa imitholampilo ye-ART futhi ngithole ukunakekelwa ngokujwayelekile.

Isishicilelo sobambe iqhaza Usuku……/……/

Isishicilelo sikafakazi Usuku……/……/

Appendix 5

HIV drug resistance study **Consent form**

I..... agree to be part of the **HIV drug resistance study**. The study has been explained to me and I fully understand the information in the study information sheet.

I understand the implications of me / my child/ward joining the study and that I / my child/ward may be asked additional information regarding my / his/her health and my / his/her treatment during the study visit.

I give permission to the research staff to look at my / my child's/ward's clinic file and clinic card and that information from this study may be linked to information already held on the clinical and demographic databases at the Africa Centre. I understand that an extra blood sample will be taken as part of this study

I understand that I will have the opportunity to discuss the results with a nurse or doctor

I understand that I / my child/ward may leave the study at any time and I / he/she will not be discriminated for doing so. I will continue to use the ART clinic and be given appropriate care as usual.

Signature of the study participant:..... date:...../...../.....

Witness signature :.....date:...../...../.....

Appendix 6

Virological Failure Clinical History Sheet: Used by (circle): DOCTOR/NURSE

Dates	CD4 Count	Viral Load result	Identified Reason for Detectable VL (e.g. Non-adherence, Treatment break, Pharmacological...)	(KG)
[illegible]	69	[illegible]	[illegible]	[illegible]
[illegible]	246	49	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	442	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	625	[illegible]	[illegible]

2. *Nitrotroviral Therapy:* (Continue overleaf if more regimens than space...)

Dates	Regimen/Drugs		Reasons for changing/Toxicities
FROM:	i. D4T	iv. TDF	vii. LPVr
TO:	ii. DDI	v. 3TC	viii. NVP
	iii. AZT	vi. ABC	ix. EFV
	x. RTV		
FROM:	i. D4T	iv. TDF	vii. LPVr
TO:	ii. DDI	v. 3TC	viii. NVP
	iii. AZT	vi. ABC	ix. EFV
	x. RTV		

3) *Perceived Adherence:*

- **Patient estimate of adherence in last 3 months:**
  GOOD (>90% doses taken) **LESS GOOD** (<90%) **POOR** (<50%) NON-(adherence?)
- Details of **any treatment breaks?** Dates: FROM: TO:
  REASON:
- ASK: Do you sometimes find it difficult to remember to give your child medicine? YES/NO
- ASK: When your child feels better do you sometimes stop giving the medications? YES/NO
- ASK: Thinking back over the past 4 days, has your child missed any doses? YES/NO
- ASK: Sometimes if your child feels worse when they take the medicine, do you stop giving it? YES/NO
- Do they know names of medications? YES/NO

Appendix 6 (Continued)

- Have you disclosed to anyone at home about your child? YES/NO
- Do you have a treatment buddy? YES/NO
- Have you been to adherence counseling? YES/NO

4) *Other current medications: (Anti-epileptic, Steroids, Warfarin, Statins particularly important)*

1.	2.	3.

5) *TB therapy:* Nil

Start Date	Regimen/Drugs		
	i. REG 1	ii. REG 2	iii. REG 3 □
	vi. MDR		
	i. REG 1	ii. REG 2	iii. REG 3 □
	vi. MDR		
	i. REG 1	ii. REG 2	iii. REG 3 □
	vi. MDR		

6) *Most Recent Blood Results:*

Dates	Hb	ALT
[illegible]	11.5	20.

7) Do you give your child traditional remedies/ immune boosters? YES/NO/PREVIOUSLY___ DATES:

8a) Alcohol consumption by caregiver: NONE/AVERAGE/HEAVY (>3 drinks most days of week)

8b) Alcohol consumption by child: NONE/AVERAGE/HEAVY (>3 drinks most days of week)

9) Hepatitis B Status: POS/NEG/NOT TESTED: *(Order test)*

10) Currently Pregnant? YES/NO/NA

11) Has there been any exposure to Single Dose NVP at delivery? YES/NO If YES: Year:_____

12) Is the caregiver on ARV's? YES/NO/Unknown

13) Other Co-morbidities:_____

Is the patient on or likely to start TB treatment soon? YES/NO

Is there significant diarrhea or vomiting causing malabsorption? YES/NO

Have you identified any reasons for virological failure? Use the summary below to explain

Poor Adherence	□	Drug Interactions	□	Social issues Impacting Rx □	Transmitted Resistance □
Treatment Breaks	□	Traditional Remedies	□	Financial □	Single Dose NVP □
Drug toxicity	□	Malabsorption	□	Non-Disclosure □	

14) Summary: [illegible]

15) Current action: **HBC/Treatment buddy/Adherence/ Other** _____

Appendix 7

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

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Westville Campus, Govan Mbeki Building
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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

13 July 2016

Dr Tulio de Oliveria
Africa Centre for Health and Population Studies
Mtubatuba
KwaZulu- Natal
3935

Dear Dr de Oliveria

PROTOCOL: High drug resistance assessment in a rural area in South Africa. REF: BF052/010

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	07 July 2016
Expiration of Ethical Approval:	06 July 2017

I wish to advise you that your application for Recertification received 06 July 2016 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The approval will be **ratified** by a full Committee at a meeting to be held on **16 August 2016.**

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

Appendix 8

bioafrica.net/saturn/ Genotypic Resistance Interpretation Algorithm

Durban, 06/09/2011

Dear Clinician,

I enclose the report of the genotyping that you requested

Patient ID on the SATuRN Rega database*: PRES
**Please notice that no patient personal identification information should be stored in this database, please use an sequential study number as patientID.*

Sample ID / Sample Date: PRES - 26/08/2011
Antiretroviral experience: [D4T, 3TC, EFV]
Subtype: HIV-1 Subtype C

Resistance interpretations: HIVDB 6.0.5

HIVDB 6.0.5

Drug	Mutations	Description	Level	GSS
zidovudine	67N 70R 118I 184V 219Q	Intermediate resistance	4	0.5
zalcitabine	N/A	N/A	N/A	N/A
didanosine	67N 118I 184V	Low-level resistance	3	0.5
lamivudine	118I 184V	High-level resistance	5	0.0
stavudine	67N 70R 118I 184V 219Q	Low-level resistance	3	0.5
abacavir	67N 118I 184V	Low-level resistance	3	0.5
emtricitabine	118I 184V	High-level resistance	5	0.0
tenofovir	67N 70R 118I 184V	Susceptible	1	1.0
nevirapine	103N 106M 138A	High-level resistance	5	0.0
delavirdine	103N 106M 138A	High-level resistance	5	0.0
efavirenz	103N 106M 138A	High-level resistance	5	0.0
etravirine	103N 106M 138A	Low-level resistance	3	0.5
saquinavir	N/A	N/A	N/A	N/A
saquinavir/r		Susceptible	1	1.0
ritonavir	N/A	N/A	N/A	N/A
indinavir	N/A	N/A	N/A	N/A
indinavir/r		Susceptible	1	1.0
nelfinavir		Susceptible	1	1.0
fosamprenavir	N/A	N/A	N/A	N/A
fosamprenavir/r		Susceptible	1	1.0
lopinavir/r		Susceptible	1	1.0
atazanavir	N/A	N/A	N/A	N/A
atazanavir/r		Susceptible	1	1.0
tipranavir/r		Susceptible	1	1.0
darunavir/r		Susceptible	1	1.0

Advice:
- Antiretrovirals for which the virus showed a reduced sensitivity, may still be partially active in a combination therapy. Antiretroviral agents against resistant virus are not recommended but may still exhibit a temporary activity when on HAART (> 3 Antiretrovirals).
- The interpretations of enfuvirtide (Envelope entry inhibitor) and tipranavir/r (boosted PI) are based on limited clinical information. These interpretations should be take with care.

List of all amino acid mutations observed in:
Protease: V3I T12S I15V L19I M36I S37N R41K D60E H69K L89M I93L
Reverse transcriptase: E6K K20R V35T T39E S48T V60I D67N K70R K103N V106M V118I D123N E138A T165L K173A Q174K M184V T200A Q207E K219Q V245Q K275R R277K T286A E291D V292I I293V

7. REFERENCES

1. WHO.UNAIDS.UNICEF. Global HIV/AIDS Response: Epidemic Update and Health Sector Progress Toward Universal Access. 2011.
2. UNAIDS. THE GAP REPORT. 2014.
3. WHO. Global Update on HIV Treatment 2013 : Results, Impact and Opportunities. 2013.
4. Africa DoHROS. Final HIV Drug Resistance Strategy. *NATIONAL HIV DRUG RESISTANCE STRATEGY* 2015.
5. Marconi. VC, Sunpath. H, Lu. Z, et al. Prevalence of HIV-1 Drug Resistance after Failure of a First Highly Active Antiretroviral Therapy Regimen in KwaZulu Natal, South Africa. *HIV/AIDS* 2008, **46**(10): 1589-97.
6. Africa DoHROS. The South African Antiretroviral Treatment Guidelines

2015.
7. Collins IJ, Judd A, Gibb DM. Immediate antiretroviral therapy in young HIV-infected children: benefits and risks. *Current opinion in HIV and AIDS* 2014, **9**(1): 87-94.
8. UNICEF. Towards an AIDS-Free Generation: Sixth Stocktalking Report. *Children and AIDS* 2013.
9. Hauser. A, Mugenyi. K, Kabasinguzi. R, et al. Detection and Quantification of Minor Human Immunodeficiency Virus Type 1 Variants Harboring K103N and Y181C Resistance Mutations in Subtype A and D Isolates by Allele-Specific Real-Time PCR. *Antimicrobial Agents and Chemotherapy* 2009, **53**(7): 2965-73.
10. Sa´nchez. PR, Holguı´n. A. Drug resistance in the HIV-1-infected paediatric population worldwide: a systematic review. *Journal of Antimicrobial Chemotherapy* 2014, **69**(8): 2032-42.
11. Johannessen A, Naman E, Kivuyo SL, et al. Virological efficacy and emergence of drug resistance in adults on antiretroviral treatment in rural Tanzania. *BMC Infect Dis* 2009, **9**: 108.
12. Holguín Af, Karen Erazo GE, Mulder Md, et al. Drug resistance prevalence in Human Immunodeficiency Virus Type 1 infected pediatric populations in Honduras and El Salvador during 1989–2009. *The pediatrian infectious disease journal* 2011, **30**(5): e82-e7.
13. Gupta RK, Pillay D. HIV resistance and the developing world. *Int J Antimicrob Agents* 2007, **29**(5): 510-7.
14. Briones. C, Domingo. E. Minority Report:Hidden Memory Genomes in HIV-1 Quasispecies and Possible Clinical Implications. *AIDS REVIEW* 2008, **2008**(10): 93-109.
15. Hunt. GM, Coovadia. A, Abrams. EJ, et al. HIV-1 drug resistance at antiretroviral treatment initiation in children previously exposed to single-dose nevirapine. *AIDS* 2011, **25**(12): 1461-9.
16. Li JZ. HIV-1 drug-resistant minority variants: sweating the small stuff. *J Infect Dis* 2014, **209**(5): 639-41.
17. Conradie. F, Wilson. D, Basson. A, et al. Guidelines:The 2012 southern African ARV drug resistance testing guidelines by the Southern African HIV Clinicians Society. *SAJHIVMED* 2012, **11**(4): 162-7.
18. Manasa J, Lessells RJ, Skingsley A, et al. High-levels of acquired drug resistance in adult patients failing first-line antiretroviral therapy in a rural HIV treatment programme in KwaZulu-Natal, South Africa. *PLoS One* 2013, **8**(8): e72152.
19. Hoffmann CJ, Charalambous S, Sim J, et al. Viremia, resuppression, and the time to resistance in HIV subtype C during first-line antiretroviral therapy in South Africa. *Clinical Infectious Disease* 2009, **49**(12): 1928-35.
20. Coovadia. A, Hunt. G, Abrams. EJ, et al. Persistent Minority K103N Mutations among Women Exposed to Single-Dose Nevirapine and Virologic Response to Nonnucleoside Reverse-Transcriptase Inhibitor–Based Therapy. *Clinical infectious deases* 2009, **48**(4): 462-72.

21. Hunt GM, Morris L, Moorthy A, et al. Concordance between allele-specific PCR and ultra-deep pyrosequencing for the detection of HIV-1 non-nucleoside reverse transcriptase inhibitor resistance mutations. *J Virol Methods* 2014, **207**: 182-7.
22. Vignoles. M, Barboni. G, Agosti. MR, et al. Evaluation of minority populations of HIV type-1 with K103N and M184V drug resistance mutations among children in Argentina

Antiviral Therapy 2009, **14**(8): 1175-81.
23. Hauser A, Sewangi J, Mbezi P, et al. Emergence of minor drug-resistant HIV-1 variants after triple antiretroviral prophylaxis for prevention of vertical HIV-1 transmission. *PLoS ONE* 2012, **7**(2): e32055.
24. MacLeod. IJ, Rowley. CF, Thior. I, et al. Minor resistant variants in nevirapine-exposed infants may predict virologic failure on nevirapine-containing ART. *Clincal Virology* 2010, **48**(3): 162-7.
25. Hosseinipour MC, Gupta RK, Van Zyl G, Eron JJ, Nachega JB. Emergence of HIV drug resistance during first- and second-line antiretroviral therapy in resource-limited settings. *J Infect Dis* 2013, **207 Suppl 2**: S49-56.
26. Hamers. RL, Wallis. CL, Kityo. C, et al. HIV-1 drug resistance in antiretroviral-naive individuals in sub-Saharan Africa after rollout of antiretroviral therapy:a multicentre observational study. *Lancet Infectious desease* 2011, **11**(10): 750-9.
27. UNAIDS. UNAIDS: Report on the Global AIDS Epidemic. 2012.
28. WHO. Scaling up antiteroviral therapy in resource-limited settings: Treatment guideline for a public health approach. 2004.
29. Omole OB, Semenya M-AML. Treatment outcomes in a rural HIV clinic in South Africa: Implications for health care. *Southern African Journal of HIV Medicine* 2016, **17**(1): 6.
30. Lwembe. R, Ochieng. W, Panikulam. A, et al. Anti-retroviral drug resistance-associated mutations among non-subtype B HIV-1-infected Kenyan children with treatment failure. *Journal of Medical Virology* 2007, **79**(7): 865-72.
31. Machado. ES, Afonso. AA, Nissley. DV, et al. Emergency of Primary NNRTI Resistance Mutations without Antiretroviral Selective Pressure in a HAART Treated Child. *PLoS ONE* 2009, **4**(3): e4806.
32. Levy JA. HIV pathogenesis and long term survival. *AIDS* 1993, **7**(11): 1401-10.
33. Hakim JG. HIV/AIDS: Update on Epidemiology, Prevention and Treatment- including Available South Sudan Literature. *Southern Sudan Medical Bulletin* 2009, **2**(3): 1-11.
34. Nielsen. MH, Pedersen. FS, Kjems. J. Molecular strategies to inhibit HIV-1 replication. *Retrovirology* 2005, **2**(10): 1-20.
35. Watts. JM, Dang. KK, Gorelick. RJ, et al. Architecture and secondary structure of an entire HIV-1 RNA genome. *NATURE* 2009, **460**(6): 711-9.
36. Doherty. RS, Oliveira. TD, Seebregts. C, Danaviah. S, Gordon. M, Cassol. S. BioAfrica's HIV-1 Proteomics Resource: Combining protein data with bioinformatics tools. *Retrovirology* 2005, **2**(18): 1-14.
37. Peeters. M. The genetic variability of HIV-1 and its implications. *Transfussion Clinical Biology* 2001, **2001**(8): 222-5.
38. Hemelaara. J, Gouws. E, Ghys. PD, Osmanov. S. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS* 2006, (20): W13-W23.
39. E.O F. HIV-1 Replication. *Somatic Cell and Molecular Genetics* 2001, **26**(1-6): 13-33.
40. Sierra. S, Kupfer. B, Kaiser. R. Basics of the virology of HIV-1 and its replication. *Journal of clinical virology* 2005, **34**(2005): 233-44.
41. Coffin. JM. HIV Population Dynamics in Vivo: mplications for Genetic Variation, Pathogenesis, and Therapy. *SCIENCE* 1995, **267**(27 January 1995): 483-9.
42. Africa NDoHS. National Antiretroviral Treatment Guidelines. *Department of Health* 2004.

43. Clercq. ED. New developments in anti-HIV chemotherapy. *Biochimica et Biophysica Acta* 2002, **1587**(2000): 258-75.
44. Clercq. ED. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. *International Journal of Antimicrobial Agents* 2009, **33**(2009): 307-20.
45. Arts. EJ, Hazuda. DJ. HIV-1 Antiretroviral Drug Therapy. *Cold Spring Harbor Perspectives in Medicine* 2012, **2**(a007161): 1-23.
46. XF. L, ZW. C. The development of anti-HIV-1 drugs. *Acta pharmaceutica Sinica* 2010, **45**(2): 165-76.
47. Zhan. P, Liu. X. Novel HIV-1 non-nucleoside reverse transcriptase inhibitors: a patent review (2005 -- 2010). *Expert Opinion in Therapeutic Patents* 2011, **21**(5): 717-96.
48. Béthune M-Pd. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: A review of the last 20 years (1989–2009). *Antiviral research* 2009, **85**(2010): 75-90.
49. Kilby. JM, Eron. JJ. Novel therapies based on mechanisms of HIV-1 cell entry. *The New England Journal of Medicine* 2003, **2003**(348): 2228-38.
50. Haqqani. AA, Tilton. JC. Entry inhibitors and their use in the treatment of HIV-1 infection. *Antiviral Research* 2013, **98**(2): 158-70.
51. Eley B, Davies M-A, Apolles P, et al. Antiretroviral treatment for children. *South African Medical Journal* 2006, **96**(9): 988-93.
52. Cotton. MF, Violari. A, Otwombe. K, et al. Early time-limited antiretroviral therapy versus deferred therapy in South African infants infected with HIV: results from the children with HIV early antiretroviral (CHER) randomised trial. 2013: 1-9.
53. Maro´n. G, Gaur. AH, Flynn. PM. Antiretroviral Therapy in HIV-Infected Infants and Children. *The Pediatric Infectious Disease Journal* 2010, **29**(4): 360-3.
54. WHO. HIV/AIDS Programm for HIV Drug Resistance Report. 2012.
55. Gianella. S, Richman. DD. Minority variants of drug-resistant HIV. *The journal of Infectious diseases* 2010, **2002**(5): 657-66.
56. Shafer. RW. Genotypic Testing for HIV-1 Drug Resistance. *HIV InSite Knowledge Base Chapter* 2004.
57. Chesney. M. Adherence to HAART Regimens. *AIDS PATIENT CARE and STDs* 2003, **17**(4): 169-77.
58. Stott. K, Michel. J, Oliveira. Td. Principles of HIV drug resistance for clinical management in South Africa. 2012.
59. Vaz. P, Augusto. O, Bila. D, et al. Surveillance of HIV Drug Resistance in Children Receiving Antiretroviral Therapy: A Pilot Study of the World Health Organization's Generic Protocol in Maputo, Mozambique. *Clinical Infectious Diseases* 2012, **54**(S4): S369-S74.
60. Barth. RE, Tempelman. HA, Smelt. E, Wensing. AMJ, Hoepelman. AI, Geelen. SP. Long-term Outcome of Children Receiving Antiretroviral Treatment in Rural South Africa. *The Pediatric Infectious Disease Journal* 2011, **30**(1): 52-6.
61. Johnston. V, Fielding. KL, Charalambous. S, Churchyard. G, Phillips. A, Grant. AD. Outcomes Following Virological Failure and Predictors of Switching to Second-line Antiretroviral Therapy in a South African Treatment Program. *AIDS* 2012, **61**(3): 370-80.
62. Zdanowicz. MM. The Pharmacology of HIV Drug Resistance. *American Journal of Pharmaceutical Education* 2006, **70**(5): 1-9.
63. (COHERE) TpltoIPIptftCoOHERE. Risk of triple-class virological failure in children with HIV:a retrospective cohort study. *The Lancet* 2011, **377**(9777): 1580-7.
64. Fitzgerald F, Penazzato M, Gibb D. Development of antiretroviral resistance in children with HIV in low- and middle-income countries. *J Infect Dis* 2013, **207 Suppl 2**: S85-92.

65. Lessells RJ, Avalos A, de Oliveira T. Implementing HIV-1 genotypic resistance testing in antiretroviral therapy programs in Africa: needs, opportunities, and challenges. *AIDS reviews* 2013, **15**(4): 221-9.
66. Ansorge WJ. Next-generation DNA sequencing techniques. *New Biotechnology* 2009, **25**(4): 195-203.
67. Jay Shendure HJ. Next-generation DNA sequencing. *Nature Biotechnology* 2008, **26**(10): 1135-45.
68. Zhou Z, Wagar N, DeVos JR, et al. Optimization of a low cost and broadly sensitive genotyping assay for HIV-1 drug resistance surveillance and monitoring in resource-limited settings. *PLoS One* 2011, **6**(11): e28184.
69. Dunne AL, Mitchell FM, Coberly SK, et al. Comparison of genotyping and phenotyping methods for determining susceptibility of HIV-1 to antiretroviral drugs. *AIDS* 2001, **15**(12): 1471-5.
70. Dudley. DM, Chin. EN, Bimber. BN, et al. Low-Cost Ultra-Wide Genotyping Using Roche/454 Pyrosequencing for Surveillance of HIV Drug Resistance. *PLoS ONE* 2012, **7**(5): 1-11.
71. Petropoulous. CJ, Parkin. NT, Limoli. KL, et al. A Novel Phenotypic Drug Susceptibility Assay for Human Immunodeficiency Virus Type 1. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY* 2000, **44**(4): 920-8.
72. Palmer. S, Shafer. RW, Merigan. TC. Highly drug-resistant HIV-1 clinical isolates are cross-resistant to many antiretroviral compounds in current clinical development. *AIDS* 1999, **13**(6): 661.
73. technologies. L. TaqMan® Mutation Detection Assay:Competitive Allele-Specific TaqMan® PCR. *Applied biosytems* 2012, **4467011** (Rev. B).
74. Roger Paredes VCM, Thomas B. Campbell , Daniel R. Kuritzkes Systematic evaluation of allele-specific real-time PCR for the detection of minor HIV-1 variants with pol and env resistance mutations. *Journal of Virological Methods* 2007, **146**(2007): 136-46.
75. Yao. Y, ker. CN, Karlsson. Hk. Evaluation of minor groove binding probe and Taqman probe PCR assays: Influence of mismatches and template complexity on quantification. *Molecular and Cellular Probes* 2006, **20**(2006): 311-6.
76. Van Dorst B, Cremers A, Jans K, et al. Integration of clinical point-of-care requirements in a DNA microarray genotyping test. *Biosensors & bioelectronics* 2014, **61**: 605-11.
77. Gega A, Kozal MJ. New Technology to Detect Low-level Drug-resistant HIV Variants. *Future virology* 2011, **6**(1): 17-26.
78. Hauser A, Mugenyi K, Kabasinguzi R, Kuecherer C, Harms G, Kunz A. Emergence and persistence of minor drug-resistant HIV-1 variants in Ugandan women after nevirapine single-dose prophylaxis. *PLoS ONE* 2011, **6**(5): e20357.
79. Lin Liu YL, Siliang Li, Ni Hu, Yimin He, Ray Pong, Danni Lin, Lihua Lu, Maggie Law. Comparison of Next-Generation Sequencing Systems. *Journal of Biomedicine and Biotechnology* 2012, **2012**: 1-11.
80. Mutevedzi PC, Lessells RJ, Heller T, Barnighausen T, Cooke GS, Newell ML. Scale-up of a decentralized HIV treatment programme in rural KwaZulu-Natal, South Africa: does rapid expansion affect patient outcomes? *Bulletin of the World Health Organization* 2010, **88**(8): 593-600.
81. Pillay S, Bland RM, Lessells RJ, Manasa J, de Oliveira T, Danaviah S. Drug resistance in children at virological failure in a rural KwaZulu-Natal, South Africa, cohort. *AIDS research and therapy* 2014, **11**(1): 3.
82. Muhwava W, Nyirenda M, Mutevedzi T, Herbst K, Hosegood V. Operational and Methodological Procedures of the Africa Centre Demographic Information System. *Monograph Series* 2008, (1).
83. Gall A, Ferns B, Morris C, et al. Universal amplification, next-generation sequencing, and assembly of HIV-1 genomes. *J Clin Microbiol* 2012, **50**(12): 3838-44.
84. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids research* 1994, **22**(22): 4673-80.

85. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014, **30**(9): 1312-3.
86. Allison L. Agwu JYC, Ryan E. Wiegand, John T. Wheeling,Beverly A. Bohannon,Kenneth L. Dominguez. Prevalence and Outcomes of Recycling NNRTIs Despite Documented NNRTI Resistance in HIV-Infected Children and Youth. *AIDS PATIENT CARE and STDs* 2014, **28**(1): 10-4.
87. F. Nicot AS, , S. Raymonda, K. Saunéa, M. Dubois, P. Massip, B. Marchou, P. Delobel, J. Izopet. Minority variants associated with resistance to HIV-1 nonnucleoside reverse transcriptase inhibitors during primary infection. *Journal of Clinical Virology* 2012, **55**(2012): 107 - 13.
88. Nicot F, Saliou A, Raymond S, et al. Minority variants associated with resistance to HIV-1 nonnucleoside reverse transcriptase inhibitors during primary infection. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2012, **55**(2): 107-13.
89. Delobel. P, Saliou. A, Nicot. F, et al. Minor HIV-1 Variants with the K103N Resistance Mutation during Intermittent Efavirenz-Containing Antiretroviral Therapy and Virological Failure. *PLoS ONE* 2011, **6**(6): e21655-e215.
90. KJ. M, P. R, V. vW, et al. Efficient suppression of minority drug-resistant HIV type 1 (HIV-1) variants present at primary HIV-1 infection by ritonavir-boosted protease inhibitor-containing antiretroviral therapy. *The journal of Infectious Deseases* 2010, **201**(17): 1063-71.

91. Li JZ, Kuritzkes DR. Clinical implications of HIV-1 minority variants. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2013, **56**(11): 1667-74.
92. Metzner. KJ, Leemann. C, Giallonardo. FD, et al. Reappearance of Minority K103N HIV-1 Variants after Interruption of ART Initiated during Primary HIV-1 Infection. *PLoS ONE* 2011, **6**(7): e21734-e.
93. Pilger D, Hauser A, Kuecherer C, et al. Minor drug-resistant HIV type-1 variants in breast milk and plasma of HIV type-1-infected Ugandan women after nevirapine single-dose prophylaxis. *Antivir Ther* 2011, **16**(1): 109-13.
94. Rowley CF, Boutwell CL, Lee EJ, et al. Ultrasensitive detection of minor drug-resistant variants for HIV after nevirapine exposure using allele-specific PCR: clinical significance. *AIDS Res Hum Retroviruses* 2010, **26**(3): 293-300.
95. Rowley. CF, Boutwell. CL, Lee. EJ, et al. Ultrasensitive Detection of Minor Drug-Resistant Variants for HIV After Nevirapine Exposure Using Allele-Specific PCR: Clinical Significance. *AIDS RESEARCH AND HUMAN RETROVIRUSES* 2010, **26**(3): 293-300.
96. Muhwava W NM, Mutevedzi T, Herbst K, Hosegood V. . Operational and Methodological Procedures of the Africa Centre Demographic Information System. *Monograph Series* 2008, **1**.
97. Aitken SC, Bronze M, Wallis CL, et al. A pragmatic approach to HIV-1 drug resistance determination in resource-limited settings by use of a novel genotyping assay targeting the reverse transcriptase-encoding region only. *J Clin Microbiol* 2013, **51**(6): 1757-61.
98. WHO. Antiretroviral therapy of HIV infection in infants and children:Towards unirvesal access 2010.
99. Fisher RG, Smith DM, Murrell B, et al. Next generation sequencing improves detection of drug resistance mutations in infants after PMTCT failure. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2015, **62**: 48-53.
100. Dawn M Dudley ALB, Shruti H Mehta, Austin L Hughes, Gregory D Kirk, Ryan P Westergaard and David H O'Connor. Cross-clade simultaneous HIV drug resistance genotyping for reverse transcriptase, protease, and integrase inhibitor mutations by Illumina MiSeq. *Retrovirology* 2014.
101. Orrell C, Levison J, Ciaranello A, et al. Resistance in pediatric patients experiencing virologic failure with first-line and second-line antiretroviral therapy. *Pediatr Infect Dis J* 2013, **32**(6): 644-7.
102. Wallis CL, Papathanasopolous MA, Fox M, et al. Low rates of nucleoside reverse transcriptase inhibitor resistance in a well-monitored cohort in South Africa on antiretroviral therapy. *Antivir Ther* 2012, **17**(2): 313-20.

103. Shao ER, Kifaro EG, Chilumba IB, et al. HIV-1 drug mutations in children from northern Tanzania. *The Journal of antimicrobial chemotherapy* 2014, **69**(7): 1928-32.