CHARACTERIZATION OF HIV DRUG RESISTANCE MUTATIONS AND SUBTYPE DIVERSITY OF ISOLATES FROM CHILDREN AND ADOLESCENTS FAILING VIRAL SUPPRESSION IN KENYATTA NATIONAL HOSPITAL

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been submitted or presented for examination in any institution.

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Recommendation

This thesis has been submitted with our approval as supervisors according to Egerton University regulations.

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DEDICATION

I dedicate this work to my parents Mr. Jairus Ombima and Mrs. Rebecca Kamkwi who offered me unwavering support; both financial and moral support. I also dedicate this work to my siblings who believed in my dream and encouraged me to put in hard work and remain patient through my studies.

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ABSTRACT

Human Immunodeficiency Virus (HIV) infection remains a major global public health concern with 36.9 million people infected. The HIV health burden is most felt in Sub-Saharan Africa, where about 70% of the infection occurs. The unprecedented scale-up of access to antiretroviral therapy (ART) has improved the management of HIV and reduced HIV-associated morbidity and mortality. However, long term sustainability of this success requires treatment monitoring and surveillance of emerging HIV drug resistance in patients during combination Antiretroviral Therapy (cART). This study aimed at characterizing HIV-1 drug resistance mutations (HIVDRM) in children and adolescents failing treatment; investigating the relatedness of the circulating viral isolates, and modifying and assessing performance characteristics of the Thermofisher HIV drug resistance genotyping assay. Fifty plasma samples collected from children and adolescents experiencing virologic failure were used to characterize drug resistance mutations and an additional set of 26 plasma samples used to assess the performance of the modified assay. RNA was extracted from 500µl of plasma and subjected to reverse transcriptase (RT) PCR before PCR amplification. The amplicons were purified and sequenced using the ABI 3730 genetic analyzer platform. The modified assay was assessed by testing its accuracy, precision, reproducibility, and amplification sensitivity. Out of the 50 participants tested in this study, 42 harbored at least one major drug resistance mutation. Mutations to nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleotide reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) were present in 34/50, 38/50 and 2/50 participants respectively. HIV-1 subtype A was the most prevalent (73%). The accuracy, precision, and reproducibility of the modified assay were 98.5% (CI, 97.9 – 99.1%); 98.67% (CI, 98.1 – 99.23), and 98.7% (CI, 98.1 – 99.3) respectively. Test for concordance between the two assays showed no difference in mutations detected by both assays ($\chi^2 = 2.358$, df=1, $p<0.05$). The modified assay had an amplification sensitivity of 62.5% for viremia between 200 and 999 copies/ml and 100% for viremia above 1000 copies/ml. Assay modification resulted in a 38.5% reduction in reagent cost per test. The study showed that HIV-1 drug resistance remains to be a major barrier to disease management in children and adolescents. To implement routine HIVDR testing, there is a need to adopt validated cost-effective methods for HIV drug resistance surveillance.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABC	Abacavir
AIDS	Acquired Immunodeficiency Syndrome
ARV	Antiretroviral
ART	Antiretroviral Therapy
ATV/r	Atazanavir
AZT	Azidothymidine
CDC	Center for Disease Control
CRF	Circulating Recombinant Forms
CD4+	Cluster of Differentiation 4
cART	Combination Antiretroviral Therapy
cDNA	Complementary Deoxyribonucleic Acid
CCC	Comprehensive Care Center
CI	Confidence Interval
DEC	Dead End Complex
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide
DTG	Dolutegravir
DBS	Dried Blood Spot
DRT	Drug Resistance Test
EFV	Efavirenz
ELISA	Enzyme-Linked Immunosorbent Assay
ERC	Ethical Review Committee
EDTA	Ethylenediaminetetraacetic acid
FTC	Federal Trade Commission
FDA	Food and Drug Administration
GF	Global Fund
GP	Glycoprotein
HAART	Highly Active Antiretroviral Therapy
HIVDR	HIV Drug Resistance
HIVDRMs	HIV Drug Resistance Mutations
HIV	Human Immunodeficiency Virus
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome

HTLV	Human T-Lymphocyte Retrovirus
OH	Hydroxyl
IQR	Interquartile Range
JPHMM	Jumping Profile Hidden Markov Model
KNH	Kenyatta National Hospital
3TC	(2R,cis)-4-amino-1(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one.
LPV/r	Lopinavir
LMICs	Low and Middle-Income Countries
MOH	Ministry of Health
MEGA	Molecular Evolutionary Genetic Analysis
MTCT	Mother to Child Transmission
MCR	Multiclass Resistance
NC	Negative Control
NVP	Nevirapine
NGS	Next Generation Sequencing
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
PLHIV	People Living with HIV
PHIV	Perinatal HIV
PBMCs	Peripheral Blood Mononuclear Cells
PVL	Plasma Viral Load
PAMs	Point Mutation Assays
POC	Point of Care
PCR	Polymerase Chain Reaction
Pol Gene	Polymerase Gene
PC	Positive Control
PEPFAR	President's Emergency Plan for AIDS Relief
PMTCT	Prevention of Mother to Child Transmission
PIs	Protease Inhibitors
RLS	Resource-Limited Settings
RT	Reverse Transcriptase

RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic Acid
STIs	Sexually Transmitted Infections
SIV	Simian Immunodeficiency Virus
SD	Standard Deviation
d4T	Stavudine
SSA	Sub-Saharan Africa
TDF	Tenofovir
TAM	Thymidine Analog Mutations
TDR	Transmitted Drug Resistance
UNAIDS	United Nations Programme on HIV and AIDS
USA	United States of America
UoN-MID-R	University of Nairobi Molecular and Infectious Diseases Research
VL	Viral Load
VF	Virologic Failure
WHO	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 Background information

The unprecedented scaled-up access to antiretroviral therapy (ART) has improved the management of Human Immunodeficiency Virus (HIV) patients, and consequently, it is now perceived as a chronic weakening of immune system rather than a fatal illness. Both in resource-rich and resource-limited settings, the use of the Highly Active Antiretroviral Therapy (HAART) has tremendously lowered HIV-associated mortality and morbidity (Wang *et al.,* 2015). However, the virus remains a formidable health burden in resource-limited settings (Chandra-Mouli *et al.,* 2013). As part of the post-2015 global goals for HIV control initiative by United Nations Programme on HIV/AIDS (UNAIDS) (United Nations Programme on HIV/AIDS, 2018), majority of low and middle-income countries (LMIC) including Kenya have increased access to ART (Chandra-mouli *et al*., 2013). In the past decade, the efforts towards the prevention, care, and treatment of HIV have been intensified by support for HIV treatment and prevention programmes. The number of patients receiving ART has significantly increased as a result of multilateral efforts by AIDS control programmes including U.S. President's Emergency Plan for AIDS Relief (PEPFAR) and the Global Fund to Fight AIDS, Tuberculosis, and Malaria (United Nations Programme on HIV and AIDS, 2008; Hanefeld, 2014). These efforts have led to a significant increase in the number of HIV-diagnosed patients initiated on antiretroviral (ARV) treatment. ARV coverage increased from 8 million patients in 2012 to 19.5 million worldwide in 2016 (World Health Organization, 2017). In Kenya, an estimated 1,136,000 patients are currently enrolled in treatment (Ministry of Health, 2016). Nonetheless, to attain the targets set for vision 2030, an additional 17.2 million people living with HIV (PLHIV) globally must be initiated on treatment (United Nations Programme on HIV and AIDS, 2017).

While the efforts of AIDS control programmes to increase access to ARV have resulted in a significant reduction in the reported morbidity and mortality, a new challenge of emerging and spreading of HIV-1 drug-resistant strains threatens this progress (Bennett *et al.,* 2008). HIV drug resistance compromises treatment success by lowering the rate at which viral suppression is achieved. Furthermore, drug resistance limits treatment options available depleting the already limited ARVs especially in resource-limited settings (RLS) (Bennett *et al.,* 2008). Thus, the problem of HIV drug resistance raises a major concern in RLS where treatment options are limited (Petrella *et al.,* 2004).

According to the United Nations Programme on HIV and AIDS, close to 1.8 million children are infected with HIV globally; the majority (90%) of reported cases occurred in sub-Saharan Africa (United Nations Programme on HIV and AIDS & World Health Organization, 2019). Despite the scale-up of access to ART for adults, the number of HIV-infected children who were receiving ART treatment in 2016 were only 49% of children living with HIV (Gamell *et al.*, 2016). A combination of factors defines long-term viral suppression in this sub-population. Firstly, young children show poor virologic response compared to adults due to the high viral loads prior to treatment initiation (Szubert *et al.*, 2017). Secondly, the risk of sub-therapeutic drug concentration as a result of poor adherence to treatment and inaccurate dosage hinder HIV treatment in children (Bortich, 2016). Thirdly, the more frequently changing body weight in actively growing children and adolescents complicates ARV dosage in this population. One of the outcomes of sub-optimal dosage is that it can potentially increase the risk of developing drug resistance due to sub-therapeutic drug concentration. The consequences of these events on HIV treatment among children and adolescents who are maintained on life-long treatment are of great concern (Sigaloff *et al.*, 2011).

HIV drug resistance in children is accelerated partly by prenatal exposure to nevirapine (NVP) included in the Prevention of Mother to Child Transmission (PMTCT) regimen (Muri *et al.*, 2017). The risk of developing drug resistance and subsequent failure of standard first-line regimens is significantly high in those children who get infected despite the administration of PMTCT (Muri s*et al.*, 2017). Additionally, drug resistance among children and adolescents is associated with the scarcity of the pediatric-formulated ARV, sub-optimal adherence to treatment, psychosocial factors and inadequate treatment monitoring (Naidoo, 2017). Children and adolescents are, therefore, more vulnerable to virologic failure (VF) and the emergence of drug resistance (Castro *et al.*, 2011).

HIV is a major public health threat that demands the adoption of the public health approach to solving some of the challenges associated with it (Ford *et al*., 2018). HIV diagnosis, treatment and treatment monitoring can be optimized through the adoption of expanded approaches. Improving treatment and laboratory monitoring may also to track patients and maintain them on treatment thereby ensuring positive treatment outcomes (Ford *et al*., 2018). Lost to follow-up is another major problem in RLS that can easily be managed through the use of expanded approaches in HIV management programmes (Ford *et al*., 2018). While HIV-1 drug resistance genotyping has not been widely applied in RLS such as Kenya, it bears great potential to enhance the efficacy of antiretroviral treatment through the

determination of the appropriate switching pattern of medication from a failing regimen to second and/or third-line regimen. The lack of such an important tool in RLS has resulted in empirical switching of children from first to second and/or third-line drugs without the knowledge of the true cause of their treatment failure.

Currently, there is limited data on HIV-1 subtype diversity in Kenya yet the diversity of HIV subtypes affect treatment outcomes (Lihana *et al*., 2009). Furthermore, HIVDR testing assays currently used were developed for HIV subtype B, which may lead to underestimation of HIV drug resistance when these assays are used in regions predominated by nob-B HIV subtypes (Jagdzinski *et al*., 2003; Saravanna *et al*., 2009). The predominant HIV subtype in Kenya is subtype A underscoring the need to assays optimized for subtype A (Inzaule *et al*., 2013). Additionally, routine HIV drug resistance drug resistance testing has not been implemented in RLS, Kenya included, due to the prohibitive cost associated with the assays (Inzaule *et al*., 2016). The lack of HIVDR genotypic assays that are cost-effective and optimized for predominant subtypes in Sub-Saharan Africa presents a shortcoming in the monitoring of HIV drug resistance. This study characterized drug resistance mutations in the reverse transcriptase and protease gene among children and adolescentsexhibiting virologic failure (VF).

1.2 Statement of the problem

The emerging challenge of HIV drug resistance has significant implications for the clinical management of the disease. Drug resistance is a major cause of increased morbidity and mortality in people living with HIV. Furthermore, drug resistance aggravates poor treatment outcomes, such as virologic failure (VF), which increases the risk of opportunistic infections, HIV associated deaths, and high rate of HIV transmission. Children and adolescents must be sustained on life term treatment increasing the need to monitor HIV drug resistance in this subpopulation because prolonged exposure to ART increases the chances of developing drug resistance. In addition, it is not possible to distinguish cases of virologic failure due to drug resistance mutations from non-adherence without HIV drug resistance testing. In either case, the absence of drug resistance testing, compel clinicians to switch patients to costly and less tolerable (toxic) second or third-line treatment regimen without establishing the actual cause of virologic failure. Furthermore, the high cost associated with the existing HIV drug resistance assays derails the implementation of routine drug resistance testing services in RLS.

1.3 Objectives

1.3.1 General objective

To characterize HIV drug resistance mutations and describe HIV subtype diversity of isolates obtained from children and adolescents exhibiting virologic failure in Kenyatta National Hospital

1.3.2 Specific objectives

i. To characterize drug resistance mutations in the reverse transcriptase and protease gene of HIV isolates obtained from children and adolescents exhibiting virologic failure
ii. To investigate the relatedness of successfully genotyped sequences using phylogenetic approaches
iii. To modify and assess the performance characteristics of the Thermofisher HIV drug resistance testing assay

1.4 Hypotheses

i. There are no HIV-1 drug resistance-associated mutations in the reverse transcriptase and protease gene
ii. There is no relationship in the sequences from successfully genotyped viral isolates
iii. The performance characteristics of the modified assay are not the same as those of the original assay

1.5 Justification

The emergence of drug resistance is a barrier to HIV management initiatives since it leads to poor treatment outcomes such as virologic failure high mortality and morbidity, and increased risk of opportunistic infections (OI). Suboptimal drug pressure due to non-adherence to treatment may increase the risk of developing drug resistance. When patients fail to take medication as prescribed, the virus continues to replicate in the presence of sub-therapeutic drug concentration. Exposing the virus to sub-optimal drug pressure increases the risk of acquiring resistance associated mutations. Patients with drug resistance exhibit higher viral load and decreased CD4+ T cell count; suggestive of a compromised immune system. Opportunistic infections such as tuberculosis and pneumonia sets in when the patient's immunity is compromised. Monitoring HIV patients by routine HIV drug resistance testing can provide important information to inform clinical decisions such as determining the need to switch patients to a more effective regimen. Furthermore, DRT results enable clinicians to determine if virologic failure is caused by the development of drug-resistant viral isolates or

due to non-adherence to treatment. Such information helps to avoid premature switching of treatment therapy from one regimen to another when truly resistant variants do not exist. Avoiding unnecessary switching of treatment from one line of treatment to another would significantly reduce the cost of HIV management in resource-limited settings. Despite the huge burden of HIV in sub-Saharan Africa, there is a dearth of data on the prevalence of HIVDR in children and adolescents. The increasing evidence of multiclass resistance (MCR) in children due to limited treatment options for this sub-population necessitates the need to develop more effective ARVs. The high cost of HIVDR testing in resource-limited settings can be addressed by adopting a modified, cost-effective assay to enhance HIVDR testing service delivery.

CHAPTER TWO
LITERATURE REVIEW

2.1 Human Immunodeficiency Virus

Human Immunodeficiency Virus (HIV) is classified is a lentivirus under the retroviruses type in the family of retroviridae and is the cause of Acquired Immunodeficiency Syndrome (AIDS) (Dahlberg, 1988). Viruses in the retroviridae family possess a single-stranded RNA as the genetic material and are named after the enzyme reverse transcriptase, which is essential for their life cycle (Dahlberg, 1988). This virus can survive in the human body without being cleared by the immune system due to its ability to adapt and evade host immunity and its remarkable genetic diversity (Stevenson, 2003). HIV-1 and HIV-2 are thought to have originated from chimpanzees and Sooty Mangabey respectively. This is because of the similarity between HIV-1 to Simian Immunodeficiency Virus (SIV) that infect Chimpanzee, and HIV-2 resemblance to SIV infecting the Sooty Mangabey (Sharp & Hahn, 2011). The researchers who discovered the close resemblance postulated that chimpanzees and Sooty Mangabey were the original sources of HIV-1 and HIV-2 and that at some point the virus crossed species to humans.

The history of HIV dates back to 1984 when the National Cancer Institute announced its breakthrough toward finding the cause of AIDS, the Human T-Lymphocyte retrovirus (HTLV-III). Since then, the prevalence of HIV has rapidly increased; 7,699 cases were reported in 1984 (Bartlett & Smith, 1991). There was 3,665 AIDS-associated mortality in the USA while 762 cases were reported in Europe (Bartlett & Smith, 1991). The US Food and Drug Administration (FDA) approved the use of a rapid test kit (ELISA) for the detection of HIV antibodies in blood. Towards the end of 1985, HIV cases had been reported in every part of the world (Bartlett & Smith, 1991). Generally, there were approximately 20,303 cases across the world (Bartlett & Smith, 1991). The name of the virus causing AIDS was changed from HTLV-III to HIV in May 1986 (Sharp & Hahn, 2011). As the numbers of HIV prevalence continued to inflate, the virus attracted attention from different local and international organizations (Bartlett & Smith, 1991). This saw the World Health Organization (WHO) launch The Global Program on AIDS to increase awareness, formulate bold policies, provide support (both technical and financial) to countries, undertake research, and safeguard people living with HIV (PLHIV) (Bartlett & Smith, 1991). The number of PLHIV has continued to increase over the years. The current statistics by the United Nations Programme on HIV/AIDS (UNAIDS) show that in 2019 up to 37.9 million people are living with HIV of whom 1.8 million are children

(United Nations Programme on AIDS, 2019). The majority of people living with HIV reside in Sub Saharan Africa, where an estimated 70% of HIV cases occur. The concerted efforts by various AIDS control programs have led to a significant decline in the rate of HIV new infections with 1.7 million cases reported globally in 2018 compared to 2.9 million in 1997 (United Nations Programme on HIV/AIDS, 2019). Approximately 150,000 cases of new infections were reported in 2018 in children below 15 years (United Nations Programme on HIV/AIDS, 2019).

2.2 Pathogenesis of HIV

The interplay of multiple viral and host factors determine the outcome of HIV infection and progression of the disease in different patients (Naif, 2013). HIV research in the past 2 and half decades has revealed the role played by cellular tropism and receptor-coreceptor in defining the pathogenesis of HIV and disease outcome. Cellular tropism determines viral phenotype while receptor-co-receptor facilitates cell invasion by HIV (Moir *et al.,* 2011).

HIV infection begins with ill-feeling symptoms caused by small changes in the immune system. This phase can persist for over three months after infection until HIV antibodies can be detected in blood. Disease progression also varies from individual to individual (Moir *et al.,* 2011). During the initial stages of infection, the individual may remain asymptomatic while the virus is actively replicating in lymph node and bloodstream; gradually damaging the immune cells and the viral load significantly increases (Moir *et al.,* 2011). The late phase of disease progression is characterized by extensive damage to the immune system (Di-Mascio *et al.,* 2009). During this stage, patients become immunocompromised and susceptible to opportunistic infection including tuberculosis, pneumonia, candidiasis, and toxoplasmosis (El-Atrouni *et al.,* 2006). The infection progresses into AIDS when the patients' CD4+ T cells levels drop below 200 cells/mm^3 and viral load levels raise high. HIV infects and bursts host CD4+ T cells reducing the number of CD4+ T cells; as a consequent, the immune system is greatly weakened (immunodeficiency) (Chun & Fauci, 2012). The depletion of CD4+ T cells results in the activation of immune system elements causing functional immunosuppression. A state of chronic inflammation and coagulation accompanies these events increasing the risk of opportunistic diseases to set in (Sauce *et al.,* 2013). The prevailing state of compromised immunity also results to an inadequate immune response to the HIV infection allowing ample time for the virus to continue replicating (Sauce *et al.,* 2013).

2.2.1 Stages of infection

The infection progresses through various stages to AIDS including the acute HIV infection, clinical latency, and finally AIDS. Stage 1 is the acute HIV infection, which commences 2-4 weeks after infection. During this stage, the patient may experience a flu-like illness lasting for a few weeks. The patients exhibit a high VL level and can easily transmit the virus (Picker & Watkins, 2005). During this phase, it is impossible to diagnose the infection using serological tests such as ELISA since the antibody titer is still very low. However, more sensitive fourth-generation or nucleic acid (DNA-PCR) tests can be used to diagnose the infection in early stages (Naif, 2013). The next stage is referred to as the clinical latency where the virus is inactive. The rate at which HIV produces its copies is very low and the patient may not show any sign of sickness. For some patients, this stage may last for over a decade while others progress faster through this phase (Naif, 2013).

Towards the end of the dormancy phase, the viral load, VL starts to increase again as the CD4+ cell count drops ushering in third stage HIV infection known as AIDS. This is a severe phase of the infection, which leads to complete damage to the immune system paving way for numerous opportunistic infections (Naif, 2013). If not treated, the patient may not survive beyond 3 years. Symptoms characteristic to this stage of infection include fever, chills, sweats, swollen lymph glands, weakness and weight loss (Naif, 2013). Patients who have progressed to AIDS often have a CD4+ count than 200 cells/mm^3 and develop certain opportunistic illnesses such as tuberculosis (Naif, 2013).

2.3 HIV life cycle

The life cycle of HIV begins with the virus attaching its envelope proteins (GP 120) to CD4 receptors then to co-receptors; chemokine receptor (either CCR5 or CXCR4). The sequential binding triggers the fusion of the viral and host cell membranes. (Walker & Colledge, 2013). Upon fusion of the membranes, HIV empties its single-stranded RNA genome and some enzyme (reverse transcriptase and protease) into the host cell cytoplasm. While in the host cell cytoplasm, the reverse transcriptase enzyme facilitated the copying of the viral RNA into a cDNA (Walker & Colledge, 2013). The process cDNA synthesis has been extensively targeted to develop some of the most potent anti-HIV drugs (Nucleoside and Non-nucleoside reverse transcriptase inhibitors). The enzyme reverse transcriptase provides a favorable target for HIV drug development since the enzyme is unique to the virus. The resultant viral cDNA is translocated via the nuclear pore into the host cell nuclease where the enzyme integrase facilitates ints integration into the host cell genome (Walker & Colledge,

2013). The integrated HIV DNA is referred to as proviral DNA. Proviral DNA is transcribed along with the cellular DNA leveraging cellular mechanisms to create many copies of viral RNA (Walker & Colledge, 2013). The viral genetic material is translocated back to the cytoplasm where translation of mRNA into viral proteins occurs (Walker & Colledge, 2013). Translation leads to a large viral protein referred to as poly-protein. For the HIV poly-protein to be packaged into a viable virion, the viral enzyme protease mediates proteolytic cleavage of the poly-protein into individual proteins (Walker & Colledge, 2013). The processed proteins are assembled together with copies of the viral RNA during the assembly process to generate an active virion. The protease inhibitors ARVs inhibit this process of viral maturation. The final step in the HIV replication cycle entails budding out from the host cell. During budding, the new virus takes with it a piece of the cellular membrane, which acts as the outer membrane of the mature virus. Viral proteins (GP120 and GP41) are inserted in the outer membrane to act as receptors for another cycle of host cell infection (Figure 1).

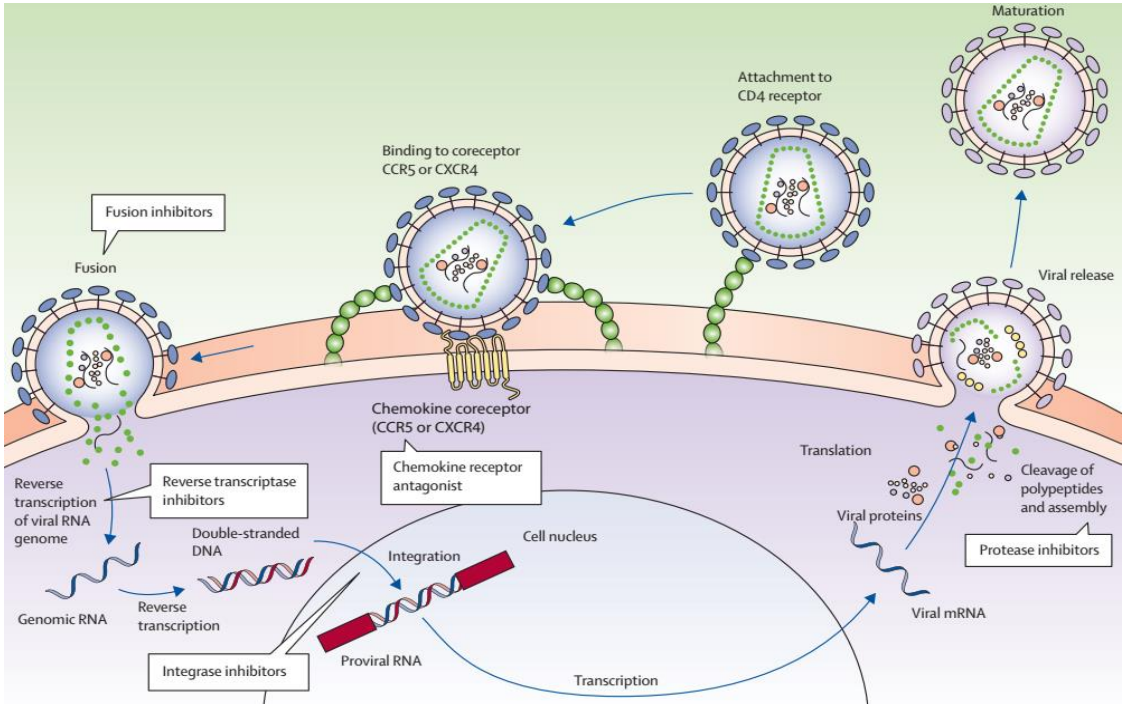

Figure 1: HIV life cycle. The stages of the HIV lifecycle targeted by inhibitors used as anti-HIV drugs have been shown. Fusion inhibitors target the events that occur during the attachment of the virus to the host cell receptors. The reverse transcriptase inhibitors inhibit the activity of the enzyme reverse transcriptase while the integrase inhibitors prevent the incorporation of HIV cDNA into the host cell genome. Lastly, the protease inhibitors prevent the protease enzyme from proteolytic cleavage of the viral polyprotein. The image was adopted from Walker & Colledge, (2013).

2.4 HIV infection in children

Most infants are infected with HIV through vertical transmission, which is defined as the transmission of the infection from infected mother to child during birth or breastfeeding (Siegfried *et al*., 2011). The transmission can occur as intrapartum or post-partum (Goulder *et al.,* 2016). The intrapartum transmission occurs mostly in the third trimester and can be diagnosed through point-of-care testing (Jani *et al.,* 2014). In both cases of child infection, HIV can be diagnosed using DNA PCR also referred to as Early Infant Diagnosis (EID). Children who are diagnosed and treatment initiated instantly may achieve viral remission (Hocqueloux *et al.,* 2013).

Major progress has been achieved towards understanding the difference that occurs in HIV infection in children and adults (Saloojee & Violari, 2001). For instance, HIV infection in children has been shown to progress much more rapidly due to the high viral loads especially when diagnosis is delayed. Furthermore, children, immunity is easily damaged by the virus compared to adults' immunity. Children have also been shown to exhibit persistent bacterial infections and other opportunistic infections (Saloojee & Violari, 2001). In resource-limited setting, an estimated 40% of the women living with HIV and about 25-48% of their children are infected *in-utero* or at birth (Saloojee & Violari, 2001). In order to accelerate the global target to eradicate HIV, there is a need to strengthen systems and policies for the prevention of mother to child transmission. Various approaches including administration of PMTCT drugs during pregnancy, achieving viral suppression in pregnant women, and HIV vaccination can be used to avert vertical transmission (Shah, 2005).

2.5 HIV patient monitoring

Optimal HIV treatment programme incorporates the element of treatment monitoring. Patients on ARV treatment are required to periodically report to their clinics for assessment of various clinical indicators (Moore *et al.,* 2008). The standard approach for monitoring HIV patients is the CD4+ T cell and viral load (VL) quantification (Volberding & Deeks, 2010). The CD4+ cell count is a clinical indicator of state of immune system in HIV patients and it was initially used to guide the commencement of ART treatment in newly diagnosed patients (Chow *et al.,* 2015). The WHO 2015 guidelines for HIV treatment recommended HIV treatment to be initiated in patients whose CD4+ cell count had dropped to 350 cells/mm^3 (World Health Organization, 2016a). However, these guidelines were revised in 2016 to adopt the test and treat approach regardless of CD4+ T cell count (World Health Organization, 2016b).

HIV patient monitoring is also performed through the quantification of plasma viral load (pVL), which is a clinical predictor of drug efficacy. Patients responding well to treatment usually exhibit undetectable plasma viral load levels whereas those responding poorly to treatment exhibit elevated VL levels. The WHO consolidated care and treatment guidelines for HIV recommends VL measurement as the preferred approach for monitoring of virologic outcome for patients on ART (World Health Organization, 2014a).

2.5.1 WHO guidelines for treatment monitoring

The WHO guidelines for HIV treatment monitoring have evolved over time. Previous guidelines recommended initiation of treatment only after the patients CD4+ cell count had dropped to 350cell/mm^3 and below (World Health Organization, 2016). The guidelines also set 200cell/mm^3 as a cutoff criterion for defining the progression of the infection to AIDS. As part of the strategy to meet the post-2015 UNAIDS global targets, the WHO revised HIV treatment guidelines to include the 'test and treat approach' regardless of the patient CD4+ cell count (World Health Organization, 2016b). The new WHO guidelines have led to tremendous increase in the number of people recruited to treatment with a consequent decline in new infections (United Nations Programme on HIV/AIDS, 2018).

The primary role of plasma VL quantification is to aid in monitoring of treatment response (Mermin *et al.*, 2011). ART treatment is targeted to achieve suppression. The WHO recommends periodic quantification of VL in patients under treatment (World Health Organization, 2016b). According to the WHO treatment guidelines, patients whose viral load levels are reduced below 1000copie/ml are considered virally suppressed whereas patients with VL more than 1000 copies/ml experience virologic failure (World Health Organization, 2016b). Patient's VL levels not only indicate treatment progress but it also provides insights into the emergence of drug resistance (Derdelinckx & Boucher, 2008). Patients with consistent high viral after 6 months of treatment with optimal adherence are considered to be exhibiting virologic failure (VF) and are required to be tested for HIV drug resistance testing (Palmer *et al.*, 2008).

2.6 HIV drug resistance surveillance

HIV drug resistance can emerge even in settings where HIV treatment programmes are optimized. Furthermore, drug-resistant virus can be transmitted to previously uninfected/infected individuals. Thus, the WHO has provided HIV treatment guidelines that seek to facilitate the implementation of robust systems for surveillance and monitoring of HIV drug resistance among patients on cART (World Health Organization, 2014b). Five key pillars

make up the WHO drug resistance monitoring strategy. Firstly, the early warning indicators of HIV drug resistance must be monitored, this includes monitoring patient viral load levels. Viral load levels above 100copies/ml after a treatment period of 6 months with adherence to treatment is a signal for possible drug resistance (Boucher *et al.,* 2018). In developed settings, patients with suspected drug resistance are referred for HIVDR testing. On the contrary, due to unavailability or scarcity of drug resistance testing services in RLS, these patients are empirically switched to another line of treatment without the knowledge of the presence of HIVDRM (Boucher *et al.,* 2018). Secondly, WHO recommends the surveillance of HIV drug resistance in recently infected individuals to find out if the patients harbor transmitted drug-resistant mutations. Information about transmitted drug resistance is important in planning treatment programs for patients who harbor such mutations. Thirdly, surveillance of HIV drug resistance mutations among treatment naïve patients just before the initiation of cART is important in determining pre-treatment drug resistance (Chimukangara *et al.,* 2019). Both adults and children receiving cART should be monitored for acquired drug resistance to help guide their treatment programs by ensuring timely switching of a failing regimen due to acquired drug resistance. Finally, HIV treatment in children is of particular interest as they must be maintained on life-long treatment and are at a high risk of developing drug resistance.

2.7 HIV treatment

The Ministry of Health (Kenya) provides recommendations for HIV treatment in pediatrics and adolescents. The guidelines emphasize the 'test and treat' approach recommended by WHO (Ministry of Health, 2016). In addition, the current HIV treatment recommendations specify the regimen to be used in both children and adolescents (Table 1).

Table 1: Guidelines for ART treatment in children and adolescents

AGE	BODY WEIGHT	REGIMEN
Birth to 4 weeks		AZT + 3TC + NVP
4 weeks - < 3 years		ABC + 3TC + LPV/r
3 - 14 years	<35kg	ABC + 3TC + EFV
≥ 15 years	≥35kg	TDF + 3TC + DTG (or TDF + 3TC + EFV)

The regimens are provided based on the patient's age and weight. AZT Zidovudine, 3TC - Lamivudine, NVP- Nevirapine, ABC- Abacavir, LPV/r - Lopinavir, EFV – Effeverenz, TDF – Tenofovir, DTG – Dolutegravir. The table was extracted from the Ministry of Health Government of Kenya, [MOH] treatment guidelines, 2016 edition.

2.7.1 Prevention of mother to child transmission

The PMTCT should be offered as routine antenatal care where lifelong ART is initiated in all pregnant and breastfeeding women living with HIV. The preferred first-line cART combination for pregnant and breastfeeding women is Tenofovir (TDF) + Lamivudine (3TC) + Efavirenz (EFV). These ARVs have proved to be efficacious in the prevention of vertical HIV transmission (Colvin *et al.,* 2007). However, the use of NVP as part of the PMTCT regimen bears serious negative implications for infants who contract the infection through vertical transmission (Antunes *et al.,* 2015). These children are more likely to develop high-level drug resistance to NVP which is included in their first-line regimen. In RLS, single-dose NVP is used as the main drug for PMTCT, which increases the risk of selection of HIV-1 resistant mutations in both mothers and infants (Antunes *et al.,* 2015). To avert this problem, the WHO has recommended the use of Dolutegravir (DTG) as an alternative to NVP in the first-line regimen for infants. However, due to the uncertainty with regards to the efficacy and safety of DTG in pregnancy, this drug is not widely used in RLS (Pham *et al.,* 2018).

Studies have also reported a positive association between *in utero* ARV exposure and infant neurological dysfunction (Barret *et al*., 2003; Ekouevi *et al.,* 2006). Mitochondrial toxicity has been proposed as a mechanism through which neurological toxicity occurs in exposed infants (Brogly *et al.,* 2007; Williams *et al.,* 2016). The adverse outcomes associated with *in utero* exposure to ARV manifest as abnormalities in hematologic and liver function, myopathy, and disorders of the central nervous system (Coelho *et al*., 2017). Evaluation of the effects of Efavirenz on cultured neuron cells revealed direct toxicity of this drug (Decloedt & Maartens, 2013). Further, emerging evidence suggests that *in utero* exposure to ARV may

result in neurocognitive impairment (Blanche *et al*., 1999; Williams *et al*., 2015) and delayed language in 1-year-old children (Rice *et al*., 2013). Depletion of mitochondrial DNA, which is a marker for mitochondrial dysfunction has been detected in leukocytes of children exposed to AZT *in utero* (Coelho *et al*., 2017). The effects persisted for up to 2 years after birth (Poirier *et al*., 2003). The effects on mental development in children were characterized by white matter hypersignalling, brain atrophy, and neurologic symptoms in newborns (Blanche *et al*., 1999). These adverse effects associated with *in utero* exposure to ARVs presents a challenge to both PMTCT programmes and the 'test ad treat' approach in infants.

2.7.2 The challenge of HIV treatment in children and adolescent

The treatment of HIV in children is often faced with challenges apart from the low rates of access to the ART. The scarcity of efficient HIV rapid test kits for children and limited monitoring of treatment hinder HIV control programs in children. Immunosuppression at the initiation of ART further compounds the problem of HIV treatment in children (Bratholm *et al*., 2010; Bortich, 2016). The problem of sub-optimal adherence to medication is also significant in this sub-population due to stigma. Together, these challenges facilitate the development and spread of HIVDRM (Sigaloff *et al.,* 2011). Studies have revealed that the use of routine immunological and clinical criteria are not effective enough to detect early virologic failure among infants and adolescents (Kantor *et al.,* 2009; Mutwa *et al.,* 2014). The situation is further aggravated by the limited HIV viral load monitoring in RLS (World Health Organization, 2010). Coupled with the few available HIV treatment drugs in RLS, these problems increase the risk of HIVDRM emergence (Abela *et al.,* 2019). HIV drug resistance in SSA is likely to result in poor clinical outcomes and reduced survival (Muri *et al.,* 2017). A study in Tanzania investigating virologic response among children on treatment reported a treatment failure rate of 38.8% among children (Ciaranello *et al.,* 2009). Furthermore, the majority of the children (84.3%) were reported to harbor HIVDRM (Ciaranello *et al.,* 2009).

2.8 Mechanism of action of HIV treatment drugs

HIV treatment drugs act by blocking various stages of HIV replication cycle (Maartens *et al.,* 2014). The principle of combination therapy is based on the approach employing more than one drug each targeting different life cycle stages of the virus (Walker & Colledge, 2013). This implies that if the virus survives through the initial stages of replication, the drug targeting the subsequent step of the life cycle can block its replication (Walker & Colledge, 2013).

2.8.1 Nucleoside reverse transcriptase inhibitors (NRTIs)

The NRTIs must first enter the cell and become phosphorylated to synthetic substrate for reverse transcriptase (RT) enzyme (Sarafianos *et al.,* 2009). This class of ARVs can reduce the infection of susceptible cells but have no effect on the already infected cells. The inhibitors resemble the natural RT substrate (dNTPs) 3′-OH. The close resemblance in structure enables the inhibitors to compete with natural substrates for the binding site (Sarafianos *et al.,* 2009). If incorporated in the growing (nascent) DNA chain, they terminate DNA chain elongation. Therefore, this class of ARVs act by mimicking the natural substrate of the enzyme RT. Once incorporated into the growing chain, a stable dead-end complex (DEC) is formed permanently terminating elongation DNA synthesis (Sarafianos *et al.,* 2009) (Figure 2).

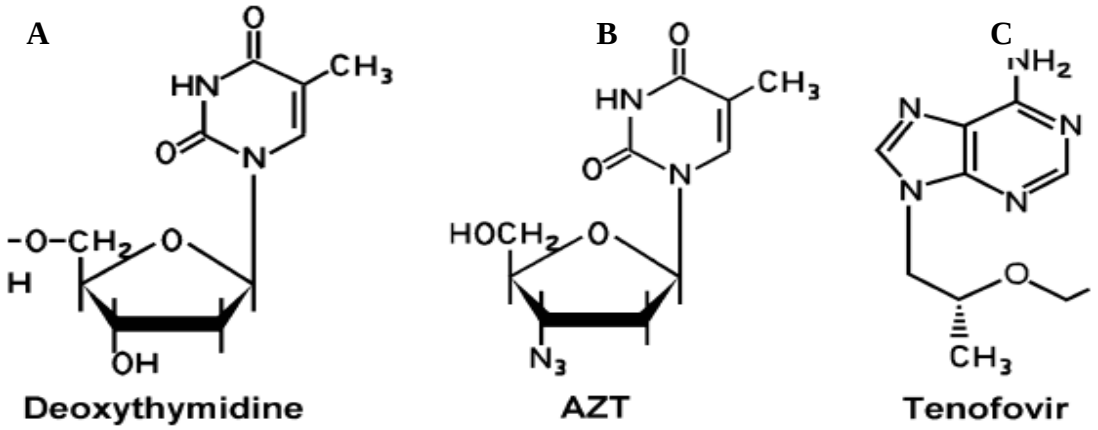

Figure 2: The structure of nucleoside, and the substrate of HIV RT enzyme. A - a normal nucleoside with the 3′-OH required for chain extension during viral cDNA synthesis. B - a nucleoside analogue (Zidovudine) lacking 3′-OH thus, when incorporated during DNA synthesis leads to chain termination. C - Tenofovir a modified nucleoside analogue with the same mechanism of action as zidovudine. The image was adopted from Sarafianos, (2009).

2.8.2 Non-nucleotide reverse transcriptase inhibitors (NNRTIs)

The second category of ARVs consists of drugs that differ in structure from the NRTIs since they do not mimic the RT substrate and therefore do not bind at the actives site of the enzyme RT (Usach *et al.,* 2013). These drugs bind at a site adjacent to the reaction site of RT and reduce the ability of the RT enzyme to change in conformation. These drugs are referred to as non-competitive inhibitors since their binding does not affect the binding of the RT substrates at the catalytic site. By increasing the rigidity of the enzyme, the NNRTIs prevents its polymerization functions (Usach *et al.,* 2013). The side effects of NNRTIs have been shown to be lower than those of nucleoside analogs but the virus is able to rapidly develop resistance to this class of drugs. As a result, it is not advisable to use NNRTs as a single therapy (Usach

et al., 2013). Some of the commonly used drugs in this category include nevirapine, efavirenz, dapivirine, etravirine, rilpivirine and delavirdine (Usach *et al.,* 2013).

2.8.3 Protease inhibitors (PIs)

The newly assembled HIV particles need to undergo protease maturation for them to be infectious (Farady & Craik, 2010). This process requires the enzyme protease. This enzyme functions to cleave the viral proteins into an active virion. The enzyme protease is uniquely present in HIV offering a specific target for altering the maturation of the new virions (Farady & Craik, 2010). The enzyme protease is symmetrical dimmers and has a central core that facilitates binding to the peptides to be cleaved. Protease inhibitors bind to the catalytic site of protease strongly blocking enzyme activity (Farady & Craik, 2010).

2.8.4 Fusion inhibitors

Fusion inhibitors encompass a category of HIV drugs that block the initial fusion process of T cell invasion; blocking HIV entry into the host cell (Briz *et al.,* 2006). The initial fusion of HIV membrane with the receptors on the host cell is a determining step in cell invasion. The virus first attaches to the CD4 receptors on the cell via its glycoprotein (GP120) (Briz *et al.,* 2006). Once the virus is attached to the host cell membrane, GP41 embeds into the membrane of the target cell. The GP41 is made up of two subunits; HR1 and HR2 that facilitate the host cell invasion. The HR1 subunit sliding over HR2 subunit drawing the virus and the host cell in close proximity during invasion. The GP41 further undergoes conformational changes bringing the virus and the cell in contact (Briz *et al.,* 2006). Fusion pores are then formed to enable the viral particle to empty its content into the target cell. Enfuvirtide is a synthetic peptide that binds to the HIV gp41 blocking it from undergoing conformational changes that would facilitate host cell infection (Briz *et al.,* 2006).

2.9 HIV drug resistance

The introduction of potent ARV drugs has led to patient life quality improvement and a significant reduction of mother-to-child transmission (MTCT) (Koigi *et al.,* 2014). However, the gains of these efforts have been adversely compromised by the development of HIVDR. The problem of HIVDR is particularly high in developing countries where information about drug resistance is limited. The existence of multiple HIV-1 subtypes in resource-poor settings increases the risk of cross-resistance to the available drugs. Scarce data about HIV-1 subtypes circulating in RLS and their effect on disease outcome is available (Wallis *et al.*, 2010). In addition, data on the patterns of HIV drug resistance in Sub Saharan Africa is limited. Such

information is very crucial for the design of novel drugs for HIV treatment (De Luca *et al.*, 2013).

2.9.1 Mechanism of HIV drug resistance

Research on HIV for the last two decades has led to the generation of a list of HIV drug resistance mutations that are documented and updated by the International AIDS Association (IAS) (Richman, 2017). A public HIV drug resistance mutation database has been developed and maintained by Stanford University; the database provides previously characterized HIV drug resistance-associated mutations. The mutations have been classified into different classes based on the drug classes to which the mutations confer resistance. The mutation classes include NRTI, NNRTI and PI drug resistance-associated mutations (Richman, 2017) (Table 2).

Table 2: List of commonly encounter drug resistance mutations

NRTIs drug resistance mutations	**NNRTIs drug resistance mutations**	**PIs drug resistance mutations**
D67N*, V75IM, V106I, V108I, V118I, K65R, K70R*, K101Q, K101H, K210R, K219E* , K219E/Q*, K219Q*, T69D, T69N, T215Y, T215F*, T215FS, T215F/Y, M41L*, M184I/V, M184V, A62V, A98G, L74V, L210W*, F77L, F116, Q151M, E44D, H208Y	A98G, M230L, K101H, K101E, K101PQ, K101EQ, K101HQ, K103N, G190S, G190A, Y181I/C/V, Y181C, Y181V, V106I, V108I, V179T, Y179D, V179E, P225H	M46I, I54V, L76V, V82A, L10F, Q58E, L33F, M36I, K20R, L10I, A71V

* TAMs -Thymidine Associated Mutations. The table was extracted from Richman, 2017.

Among the mutations previously reported, M184V (substitution of the amino acid methionine with valine at position 184) is the most common mutation (Gagliardini *et al.*, 2018). This mutation confers the virus with resistance to NRTIs; Lamivudine and Emtricitabine (Gagliardini *et al.*, 2018). In general, mutations conferring resistance to NRTIs are classified into two major classes based on their phenotypic mechanism of resistance. The first class increases the selectivity of the RT enzyme for the incorporation of natural dNTP substrate versus the modified dNTP (drug) (Zelina *et al.*, 2008). This class includes mutations such as M184V, K65R, K70E, L74V, and Q151M (Zelina *et al.*, 2008). The second class is termed as Thymidine Associated Mutations. These mutations increase the ability of HIV RT enzyme to

excise from the growing chain the terminating dNTP (drug) when incorporated. Mutations in this class include M42L, D67N, K70R, L210W, T215F/Y, and K219Q/E (Zelina *et al.*, 2008).

On the other hand, K103N mutation (substitution of lysine with asparagine at position 103) is the most prevalent mutation associated with resistance to NNRTIs (Lai *et al.*, 2016). This mutation occurs in 40-60% of NNRTI-resistant viruses (Barth *et al.*, 2010). It causes resistance to EFV, NVP, and DLV. The mechanism of resistance to NNRTIs can be explained by the mutation K103N, which induces mutations by increasing the stability of the closed pocket form of reverse transcriptase enzyme. The amino acid lysine at position 103 is located near the putative entrance to the NNRTI binding pocket (NNRTIBP). The comparison of wild type and mutant enzyme has revealed the presence of minor positional adjustments in the NRTIBP (Lai *et al*., 2016). These adjustments have been associated with an extensive network of hydrogen bonding present only in the mutant enzyme. The extensive network of hydrogen bonding is responsible for stabilization of the closed pocket form of the enzyme, which excludes NNRTIs from the NNRTIBP (Lai *et al*., 2016). Another mechanism proposed for the mode of action of mutations against NNRTIs (K103N) is the alteration of hydrophobic and electrostatic interactions induced by the substitution of lysine with asparagine at position 103. Lysine possesses along aliphatic side chain (4Cs) while asparagine has a short aliphatic side chain (1C). The replacement of Lysine with Asparagine at position 103 may result in reduced affinity to the NNRTIBP (Lai *et al*., 2016) (Figure 3).

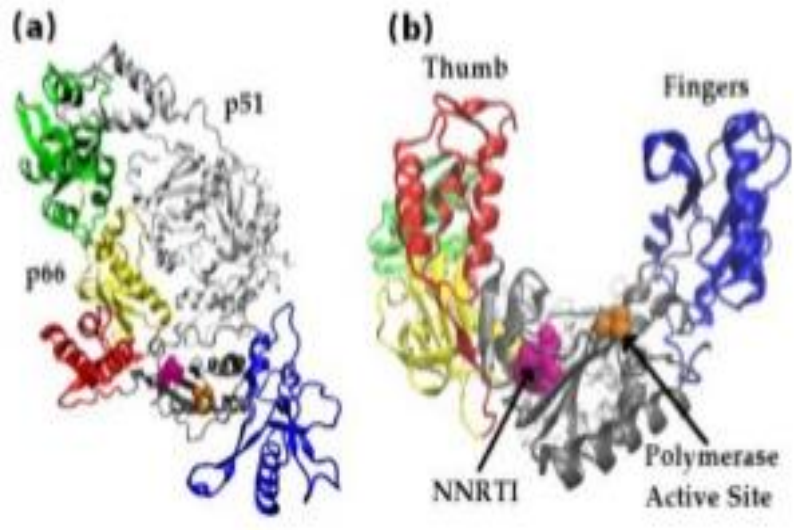

Figure 3: Closed and open pocket forms of RT. A: The closed pocket form of the RT enzyme. B: the open pocket form of the enzyme with NNRTI. The Inhibition of the NNRTIs is by non-competitive inhibition. The image was adopted from Lai *et al.* 2016

Mechanism of action of resistance to protease inhibitors acts by a concerted effort of multiple mutations. Several mutations that induce resistance to protease inhibitors (PI) have been characterized (Richman, 2017). The mutations occur in the protease gene of the virus reducing the efficacy of protease inhibitors. A complementary strategy has been proposed to explain the mechanism of resistance. Mutations in the active site reduce affinity for both drug and the substrate. The reduced affinity is due to the disruption of favorable drug-protease hydrophobic and electrostatic interactions (Piana *et al*., 2002). To compensate for the reduced affinity of the substrate to the active site, a mutation in a site located far from the active site increases the affinity o of protease to its natural substrate (Gulnik *et al*., 2000) (Figure 4).

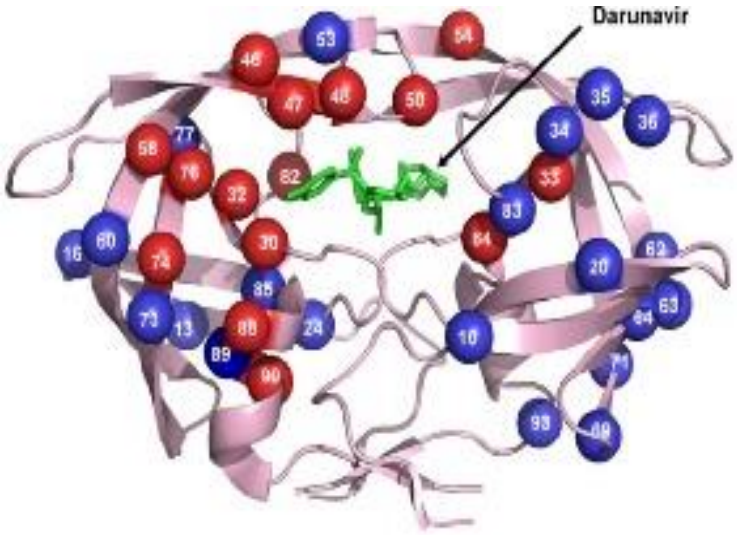

Figure 4: Protease enzyme showing sites of resistance mutations. Adopted from Weber & Agniswamy, 2009.

2.10 HIV strain and subtype distribution

A major feature of HIV is its remarkable genetic variability that is reflected in its biological characteristics. The high evolution rate of the virus calls for continuous monitoring of circulating strains (Lihana *et al*., 2009). Based on its variability, the virus has been grouped into two major strains, HIV-1 and HIV-2. The predominant HIV strain is HIV-1 accounting for over 95% of all infections worldwide (Campbell-Yesufu & Gandhi, 2011). The relatively uncommon HIV-2 is mostly found in West Africa is less infectious and progresses more slowly than HIV-1. HIV-2 results in low mortality compared to HIV-1. However, if not treated, people with HV-2 progress to AIDS and die from the disease (Campbell-Yesufu & Gandhi, 2011). Most of HIV replication inhibitors currently in the market are active against both HIV-1 and HIV-2. However, NNRTIs such as NVP and EFV are not effective against HIV-2. HIV-1 has further been classified into groups M, N, O, and P. Group M strains are responsible for the

global epidemic with different subtypes within the group circulating in different geographical areas (Campbell-Yesufu & Gandhi, 2011). Groups N, O, and P are uncommon; group O is found in countries of West Africa representing 5% of the infections in West Africa (Campbell-Yesufu & Gandhi, 2011). Groups N and P have been in few incidences been identified in Cameroon. All groups can be detected by the HIV-1 ELISA kit (Campbell-Yesufu & Gandhi, 2011).

A study to assess the diversity of circulating HIV-1 subtypes in Kenya (Lihana *et al*., 2009) reported that subtype A was the majority of subtypes in circulation. Another study in the Western region of Kenya that performed molecular characterization of HIV-1 subtypes in HIV infected participants reported 74/130 (56.9%) as subtype A (Oyaro *et al*., 2011). Additionally, the study identified a considerable prevalence of circulating recombinants forms (33.7%) (Oyaro *et al.,* 2011).

2.10.1 HIV subtypes and their impact on disease outcome

There are nine genetically distinct subtypes in group M including A, B, C, D, F, G, H, J and K (Hemelaar, 2012). Different subtypes can also combine their genetic material to form hybrid viruses referred to as circulating recombinant forms (CRFs). Approximately 89 CRFs exist (Hemelaar, 2012). Global distribution of HIV subtypes shows that the Americas, West Europe, and Australia are predominated by subtype B. The majority of research on HIV have been conducted on this subtype despite it representing only 12% of global HIV epidemics. Only a few research is available on subtype C and A yet it accounts for almost half of the global HIV epidemic. Subtype C is very common in high prevalence countries of South Africa, the Horn of Africa and India (Fox *et al.,* 2010).

The genetic variation of HIV-1 and its constant evolution bears a significant impact on HIV pathogenesis and disease outcomes (Oyaro *et al.,* 2011). A previous study has shown that some subtypes possess a greater risk of transmission and faster disease progression (Bhargava *et al.,* 2014). For instance, a study performed among Ugandan and Zimbabwean women living with HIV suggested that those infected with subtype C had a slower rate of CD4+ T cells decline and tended to progress slowly compared to those infected with subtype A or D (Venner *et al*., 2016). In another study assessing the risk factors for HIV progression, it was reported that the circulating recombinant forms (CRF01_AE) was associated with a faster progression of the disease (Venner *et al*., 2016; Chu *et al*., 2017).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

Samples for this study were collected from Kenyatta National Hospital (KNH) which is the largest public referral hospital in Kenya. Kenyatta National Hospital is a teaching hospital for the University of Nairobi, College of Health Sciences. The hospital is located in the area to the immediate west of Upper Hill in Nairobi, the capital city of Kenya. With its strategic location, the hospital receives patients from the capital city with a population of over 3 million people and its location is about 3.5 kilometers west of the city's central business district. The hospital hosts a Comprehensive Care Center (CCC) for HIV patients and offers all HIV services ranging from diagnosis, counseling, treatment, follow-ups, treatment monitoring (viral load quantification and CD4+ T cells enumeration) and HIV drug resistance genotyping.

3.2 Ethical approval

Prior to the commencement of the study, permission and ethical clearance was obtained from Kenyatta National Hospital/Kenya Medical Research Institute/Scientific and Ethics Review Unit (KNH-KEMRI-SERU). The ethical clearance reference number is KEMRI/RES/7/3/1.

3.3 Sample inclusion criteria

Archived plasma samples were used for this study. Samples were selected to be included in the study based on the viral levels. Samples from patients who were failing treatment according to WHO guidelines (VL> 1000copies/ml) (World Health Organization, 2017). Further, only samples from participants who were less than 18-year-old and were included. Samples which passed the two selection criteria but has plasma volume less than 500µl were excluded. An additional set of 26 samples with VL level ranging from 207 – 86,040 copies/ml were randomly selected for use in assay validation analysis. The samples used in this study were collected between March to December 2018.

3.4 Plasma collection and storage

Plasma was separated within 6 hours of whole blood collection by centrifugation at 2000g for 10 minutes (Thermofisher Scientific, Waltham, Massachusetts, USA) in EDTA-anticoagulant vacutainer tubes (Becton, Dickinson, San Jose, CA) and stored at -80°C until the time of batch RNA extraction at the University of Nairobi Molecular and Infectious Diseases Research Laboratory.

3.5 HIV RNA extraction from plasma

RNA was extracted from plasma samples using the PureLink extraction kit (Invitrogen, Carlsbad, CA, USA). Briefly, 25µl Proteinase K was transferred into sterilized microcentrifuge tubes and 500µl aliquot of plasma was added to the tube at room temperature. The lysis buffer provided with the kit (500µl) was added to the mixture and vortexed for 15 seconds and incubated at 56^0C for 15 minutes. Five hundred microliters of 100% ethanol were added to the reaction tube, vortexed for 15 seconds and incubated at room temperature for 5 minutes. The lysate was transferred to sterile viral spin columns and centrifuged for 1 minute at 6800xg. The spin columns were then transferred into sterile wash tubes and washed twice with 500µl of wash buffer. The viral spin columns were transferred into sterile 1.5ml recovery tubes and purified viral RNA eluted in 40µl of elution buffer. The extraction procedure was similar for both the original and the modified assay. The extracted RNA was immediately reverse transcribed to obtain complementary DNA (cDNA).

3.6 HIV drug resistance genotyping

HIV-1 drug resistance genotyping was performed at the University of Nairobi Molecular and Infectious Diseases Research Laboratory which is an approved HIV drug resistance testing site for HIV patient samples for Kenyatta National Hospital. Thermofisher genotyping assay which amplifies 1.1kb fragment covering codons 6 – 99 of the protease region and codon 1-251 of the reverse transcriptase (RT) region was used in this study. The assay has been validated for all group M subtypes and circulating recombinant forms (CRF) (Parkin *et al.*, 2012). For the RT-PCR, 10µl of the RNA extract was used with primers shown in Table 3. Superscript III one-step RT-PCR system with Platinum *Taq* high-fidelity polymerase enzyme was used to generate cDNA. The RT-PC reaction mix was prepared by adding 1µl of the Platinum *Taq* high-fidelity polymerase enzyme into 39 µl of the master-mix. Therefore, 40µl of the reaction mix was added to 10 µl of the extracted RNA to make 50 µl reaction volume. The reaction mix was vortexed for 5 seconds and centrifuged for 3 seconds to collect the component at the bottom of the tube. Thereafter, the PCR tubes containing the reaction mix were transferred to PCR room and RT-PCR performed in Veriti thermocycler (Applied Biosystems, Carlsbad, CA, USA). The reaction conditions for RT-PCR were 50^0C for 45 minutes where the first-strand cDNA was performed. Enzyme inactivation and denaturation of cDNA-RNA hybrid was accomplished by incubating the reaction at 94^0C for 2 minutes. Second strand synthesis and PCR amplification was carried out in 40 cycles of 94^0C for 15 seconds, 50^0C for 20 seconds, 72^0C for 2 minutes and a final extension for 10 minutes at 72^0C. For

nested PCR, 2µl of the RT-PCR products were used with inner primers (Table 3) (Yang *et al.*, 2010; Zhou *et al.*, 2011).

Table 3: Primers used in reverse transcriptase and nested PCR

	Primer Sequence (5′ – 3′)	**Step**	**Size**
1	F1a, 5′-TGAARGAITGYACTGARAGRCAGGCTAA	RT-PCR	23 base pairs
2	F1b, 5′-ACTGARAGRCAGGCTAATTTTTTAG	RT-PCR	24 base pairs
3	R, 5′-ATCCCTGCATAAATCTGACTTGC	RT-PCR	22 base pairs
4	PRT-F2, 5′-CTTTARCTTCCCTCARATCACTCT	NESTED-PCR	23 base pairs
5	RT-R2, 5′-CTTCTGTATGTCATTGACAGTCC	NESTED-PCR	22 base pairs

The amplification reaction mix was prepared by mixing 47.5µl of the nested PCR master mix with 0.5µl of the AmpliTaq Gold™ LD DNA Polymerase enzyme (Thermo Fisher, Waltham, USA). The amplification reaction mix contained 0.12 mM of each of the inner primers PRT-F2 and RTR2, 1x GeneAmp Gold Buffer II, 2 mM $MgCl_2$, 400 mM each dNTP and 0.5µl of AmpliTaq Gold LD DNA polymerase (Thermo Fisher, Waltham, USA). The reaction mix was vortexed gently for 5 seconds and spun down to collect the content at the bottom of the tube. Following the gentle mixing, 48µl of the nested reaction mix was added to each well of the labeled PCR strips and 2µl of RT-PCR products and controls added. Amplification was performed with Veriti thermocycler (Applied Biosystems, Carlsbad CA). The reaction conditions for the nested PCR were 94^0C for 4 minutes, 40 cycles of 94^0C for 15 seconds, 55^0C for 20 seconds, 72^0C for 2 minutes and a final extension at 72^0C for 10 minutes.

Nested PCR product quality was assessed by performing gel electrophoresis. In brief, 5µl aliquot of the nested PCR products was subjected to 1% agarose gel electrophoresis. DNA ladder (GeneRuler 1kb DNA ladder) was loaded in the first well followed by samples in the subsequent wells. Electrophoresis was performed for 35 minutes at 100 volts and the gel read using Gel doc. The presence of successful amplification was determined by the presence of a bright band.

To clean the nested PCR products, for the subsequent sequencing reactions, 10µl of the successfully amplified nested PCR products was aliquoted to new labeled PCR strips and 4µl of ExoSAP enzyme (Thermo Fisher, Waltham, USA) added. The strips were vortexed for 2 to

3 seconds and centrifuged at 1,000×*g* for 10 seconds and incubated in Veriti thermocycler (Applied Biosystems, Carlsbad, CA, USA) at 37^0C for 15 minutes, 80^0C and 15 minutes.

Six overlapping primers were used to sequence the nested PCR amplicons. All the sequencing mixes (F1, F2, F3, R1, R2, R3) were first thawed, vortexed for 5 seconds and 18µl of each added in the appropriate well of the 96 well plate. Two microliters of the purified nested PCR product and control were added to the sequencing mix to make 20µl reaction volume in each well and 20µl of pGEM added to the last well of the sequencing plate. The plate was then sealed and incubated in Veriti thermocycler (Applied Biosystems, Carlsbad, CA, USA). The cycle sequencing reaction conditions were 25 cycles of 96^0C for 10 seconds, 50^0C for 5 seconds and 60^0C for 4 minutes.

After cycle sequencing, the Big Dye XTerminator purification kit was used to purify the sequencing reaction by adding 90µl of the SAM solution and 20µl of Big Dye XTerminator to each well of the sequencing plate and vortexed for 30 minutes at 1,800 rpm in a plate shaker. The plate was centrifuged at 1000g for 2 minutes at room temperature. Thirty microliters of the cycle sequencing products were transferred to a new reaction plate and analyzed using ABI 3730 genetic analyzers (Applied Biosystem, CA, USA).

3.7 HIV- drug resistance genotyping assay modification and validation

HIV-1 RNA extraction was performed on 26 plasma samples following the procedures in section 3.5.

The original genotyping system: HIV drug resistance genotyping was performed according to the protocol described in section 3.6.

The modified genotyping system: For this validation, amplification and sequencing steps of Thermofisher HIV genotyping assay were modified by reducing the reagent volumes by 50% of the original reaction volume. The modified assay was then assessed for accuracy, precision, reproducibility and amplification sensitivity. The RNA extraction and cDNA generation steps remained unmodified and were performed as described in section 3.5. The modification to reduce the reagent volume by half was performed from the nested PCR downstream.

In the nested PCR, 2µl of the RT-PCR product was used with inner primers previously used by (Yang *et al.*, 2010; Zhou *et al.*, 2011). The amplification reaction mix was prepared by mixing 23.75µl of the nested PCR master mix with 0.25µl of the AmpliTaq Gold™ LD DNA Polymerase enzyme (Thermo Fisher, Waltham, USA). The amplification reaction mix contained 0.12 mM of each of the inner primers PRT-F2 and RTR2, 1x GeneAmp Gold Buffer

II, 2 mM $MgCl_2$, 400 mM each dNTP and 0.5µl of AmpliTaq Gold LD DNA polymerase (Thermo Fisher, Waltham, USA). The reaction mix was vortexed gently and spun down to collect the content at the bottom of the tube. Following the gentle mixing, 24.25 µl of the nested reaction mix was added to each well of the labeled PCR strips and 2µl of RT-PCR products and controls added. Amplification was performed using Veriti Thermocycler (Applied Biosystems, CA, USA). The reaction conditions for the nested PCR were initial denaturation at 94^0C for 4 minutes, 40 cycles of denaturation at 94^0C for 15 seconds, annealing at 55^0C for 20 seconds, extension at 72^0C for 2 minutes and a final extension at 72^0C for 10 minutes.

The quality of the nested PCR product was assessed following the protocol described in 3.6. To clean the nested PCR products, for the subsequent sequencing reactions, 5µl instead of 10µl of the successfully amplified nested PCR product was transferred to new labeled PCR strips and 2µl of ExoSAP enzyme (Applied Biosystems, CA, USA) added. The strips were vortexed for 2 to 3 seconds, centrifuged at 1,000×*g* for 10 seconds and loaded to Veriti Thermocycler (Applied Biosystems, CA, USA). Clean-up reaction conditions were digestion at 37^0C for 15 minutes and heat deactivation at 80^0C for 15 minutes.

Six sequencing mixes (F1, F2, F3, R1, R2, R3) were used in cycle sequencing reactions. The sequencing mixes were first thawed and vortexed and 9µl of each sequencing mixes added in the appropriate well of the 96 well plate. Afterward, 1µl of the purified nested PCR product or control was added to the sequencing mix followed by 10µl of pGEM, which was added to the last well of the sequencing plate. The plate was sealed and loaded to the Veriti Thermocycler (Applied Biosystems, CA, USA).

The Big Dye XTerminator purification kit was used to purify the sequencing reaction by adding 55µl of the Big Dye XTerminator/SAM working solution into all wells of the sequencing plate and shaking for 30 minutes at 1,800 rpm. After shaking, the plate was then centrifuged at 1000×*g* for 2 minutes at room temperature. Thirty microliters of each content of the sequencing plate were aliquoted to a new sequencing plate and loaded into 3730 genetic analyzers (Applied Biosystem, CA, USA).

3.8 Sequence generation and drug resistance mutation detection

All sequences were generated using RECall v 3.05 (Woods *et al*., 2012) (https://pssm.cfenet.ubc.ca) and drug resistance mutations interpreted using the Stanford genotyping resistance interpretation algorithm (Shafer, 2006):
(http://sierra2.stanford.edu/sierra/servlet/JSierra).

3.9 Subtype analysis

Subtype analysis of all sequences obtained was performed by the REGA tool for subtype analysis (Pineda-Peña *et al.*, 2013): (http://dbpartners.stanford.edu:8080/RegaSubtyping/stanfordhiv/typingtool/job/864501860/).

3.10 Phylogenetic analysis

Multiple sequence alignment was performed using Clustal W build-in Molecular Evolutionary Genetic Analysis (MEGA) software version 10.0 (Kumar *et al*., 2018). The aligned sequences were presented using ESPript online tool for sequence alignment presentation (Robert & Gouet, 2014). Phylogenetic analysis was performed using the neighbor-joining method in MEGA version 10.0 software (Kumar *et al*., 2018). The evolutionary distances were computed using the maximum composite likelihood (MCL) method in units of the number of base substitutions per site. The tree was then generated by the neighbor-joining method from a nucleotide alignment of 1050 positions by gaps removal, and tree topology was confirmed by bootstrapping analysis using 100 replicates.

3.11 Assessment of performance of the modified assay

Performance characteristics of the modified assay were assessed using the WHO/HIV ResNet guidelines including accuracy, precision, reproducibility and amplification sensitivity. (World Health Organization, 2012b).

Accuracy: Ten samples were analyzed using both methods and the degree of concordance in mutations detected was compared. Nucleotide sequence identity between the paired sequences was also assessed (World Health Organization, 2012b).

Precision: Three samples were analyzed using the modified assay method in 4 replicates and the degree of sequence similarity within replicates determined using EMBOSS (World Health Organization, 2012b).

Reproducibility: Ten samples were analyzed in duplicates using the modified assay on different days and nucleotide sequence identity determined using EMBOSS (World Health Organization, 2012b).

Amplification sensitivity: Sixteen samples with viral loads ranging between 207 and 86,040 copies/ml were analyzed using the modified assay to determine the viral load ranges at which ≥95% of the samples were successfully genotyped (World Health Organization, 2012b).

3.11.1 Cost analysis

The difference in the cost of reagents used in HIV drug resistance testing was determined by comparing the reagent cost of the original and modified assays. The reagent cost

incurred when processing one sample was considered at every step of HIV-1 drug resistance genotyping using both the original and modified assay.

3.12 Statistical analyses

Quantitative variables were expressed as mean ± standard deviations (SD). Descriptive statistics were used to summarize participants' characteristics while the frequency drug resistance mutations were analyzed and presented as proportions. The McNemar test was used to assess significance in the discordant mutations between the modified and the original assay. Precision and reproducibility were assessed using the Cohen kappa statistic. The Wilcoxon signed-rank test was used to compare the original and modified assay in base calling for mixed bases. The Analyses were performed in Graphpad Prism version 6.01 (www.graphpad.com).

CHAPTER FOUR

RESULTS

4.1 Participant demographic and clinical characteristics

A total of 50 archived samples were used for the first and second objective. For the third objective, an additional set of 26 randomly selected plasma samples with viral load ranging between 207 to 86,000 copies/ml were used to assess the performance of the modified assay. The samples were collected between March and December 2018. The characteristics of the participants for samples used in the first and second objectives are presented in Table 5. The median age for children was 9 years (interquartile range [IQR] 7- 12 years), while for adolescents was 16 years ([IQR] 14-17 years). Children had a median VL level of 24537 copies/ml ([IQR]2193-215884 copies/ml), whereas adolescents had a median VL of 1419 copies/ml ([IQR] 3258-88263).

Table 4: Participants' age, gender, and viral load distribution

Age group	Median Age (IQR)	Gender (N)		Median Viral load (IQR)
		Male	Female	
2-12	9 (7- 12)	3	9	24537 (2193-215884)
13-18	16 (14-17)	21	17	1419 (3258-88263)

4.2 Regimen distribution

The combination of drugs used by the participants has been shown in Figure 7. Regimens were constituted by combining Antiretroviral (ARV) from NRTI, NNRTI and PI classes.

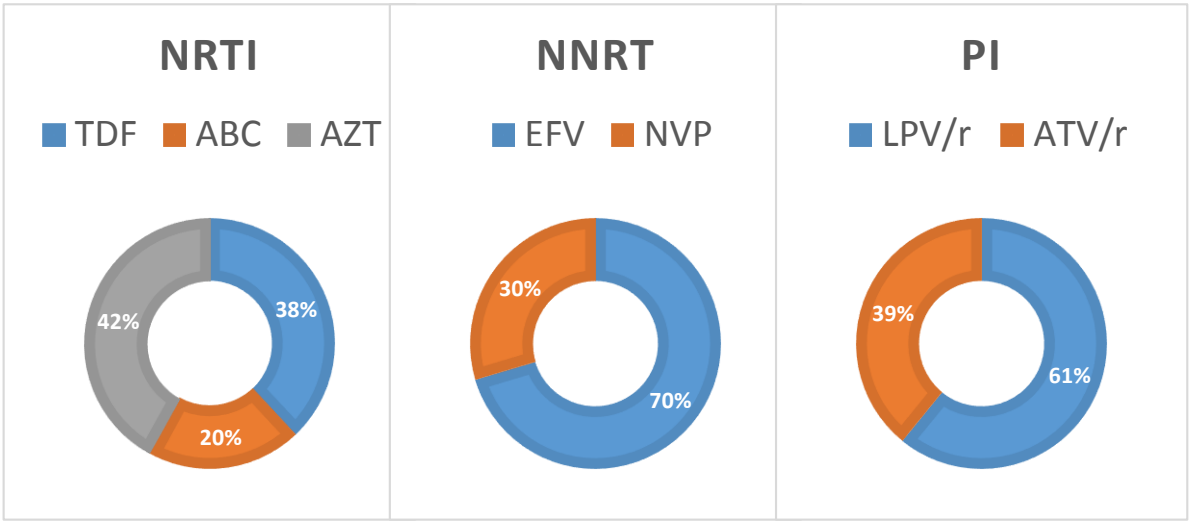

Figure 5: The frequency of different ART taken by the participants

4.3 The rate of HIV-1 drug resistance mutations

The current study showed a substantial burden of HIV-1 drug resistance mutations among children and adolescents experiencing virologic failure. Out of the 50 participants tested for HIV-1 drug resistance, 47 (94%) were successfully genotyped and 42/47 (89.4%) harbored at least one major drug resistance-associated mutation. Five, (10%) participants did not harbor major drug resistance-associated mutations. Successfully genotyped samples were defined as the samples that were amplified at PCR step and a sequence generated. Successful amplification was determined by performing gel electrophoresis and gel image observed for the presence of clear bands (Figure 6).

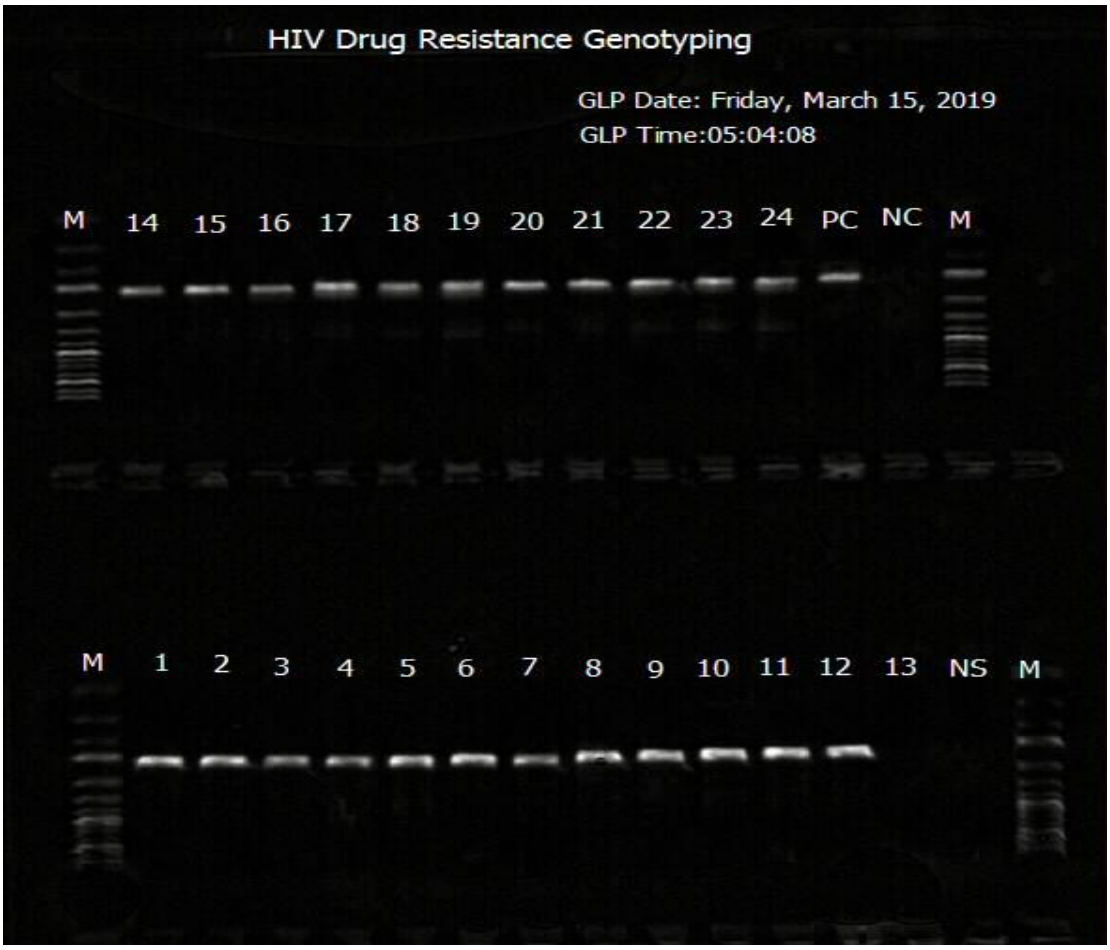

Figure 6: Gel electrophoresis image. The presence of a bright band indicated a successfully amplified sample while samples that failed the amplification step did not produce bands. A total of 24 samples were loaded of which 23 samples, the PC and M produced bands. One sample (number 13) and the NC did not produce bands. M – marker (GeneRuler DNA ladder), PC – positive control and NC – negative control. NS – No sample in the well.

4.3.1 Mutations detection in sequences

Mutations in sequences were identified by performing pairwise sequence alignment of individual sequences against HIV-1 reference sequence obtained from GenBank, accession number: MH355048.1. The aligned sequences were presented using ESPript online tool for sequence alignment presentation (Robert & Gouet, 2014) (Figure 7). Positions of the paired sequence alignment with different nucleotide bases represented areas where mutations may have occurred in the sequence.

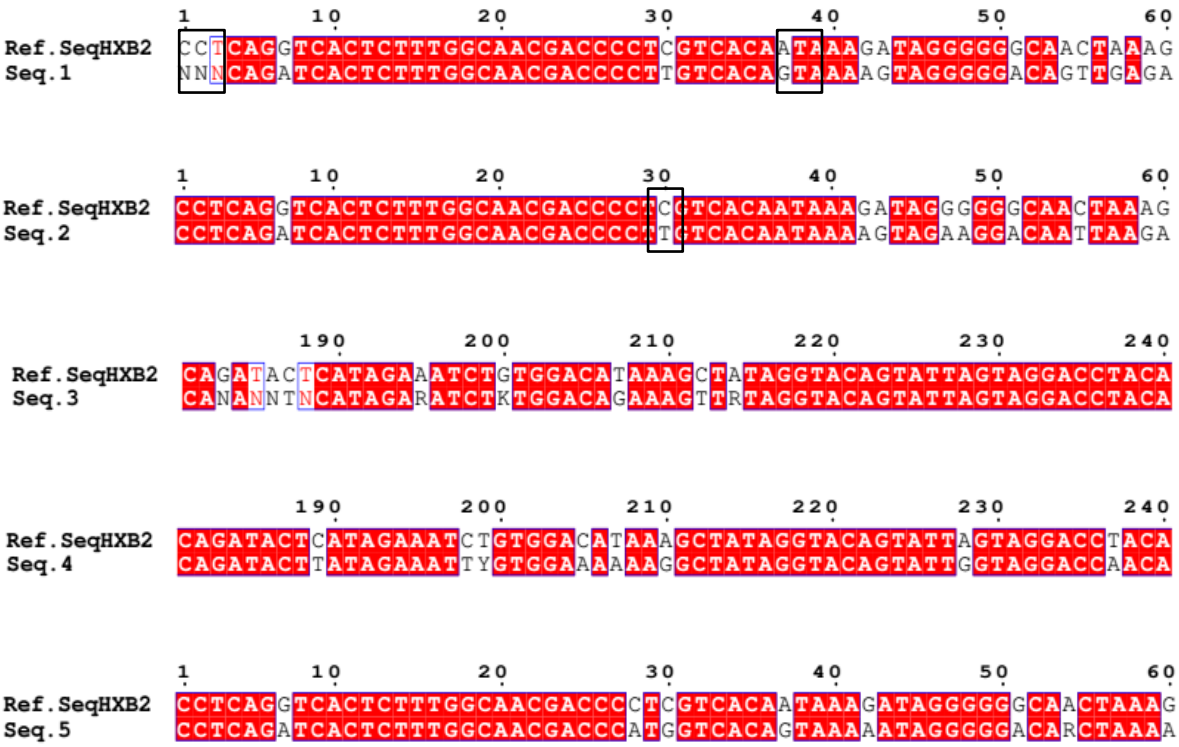

Figure 7: Pairwise sequence alignments. The unshaded regions of the alignments depict the positions of the sequence where mutations may have occurred. The areas highlighted in red are the stretches of the sequence without mutations (conserved regions).

Out of the 42 participants with drug resistance mutations, 34/42 (81%) and 38/42 (90%) harbored mutations to NRTIs and NNRTIs respectively. Majority of the participants 31/42 (74%) harbored dual-class drug resistance-associated mutations (NRTIs and NNRTIs). Two participants harbored drug resistance mutations to protease inhibitors (PIs) (Figure 8).

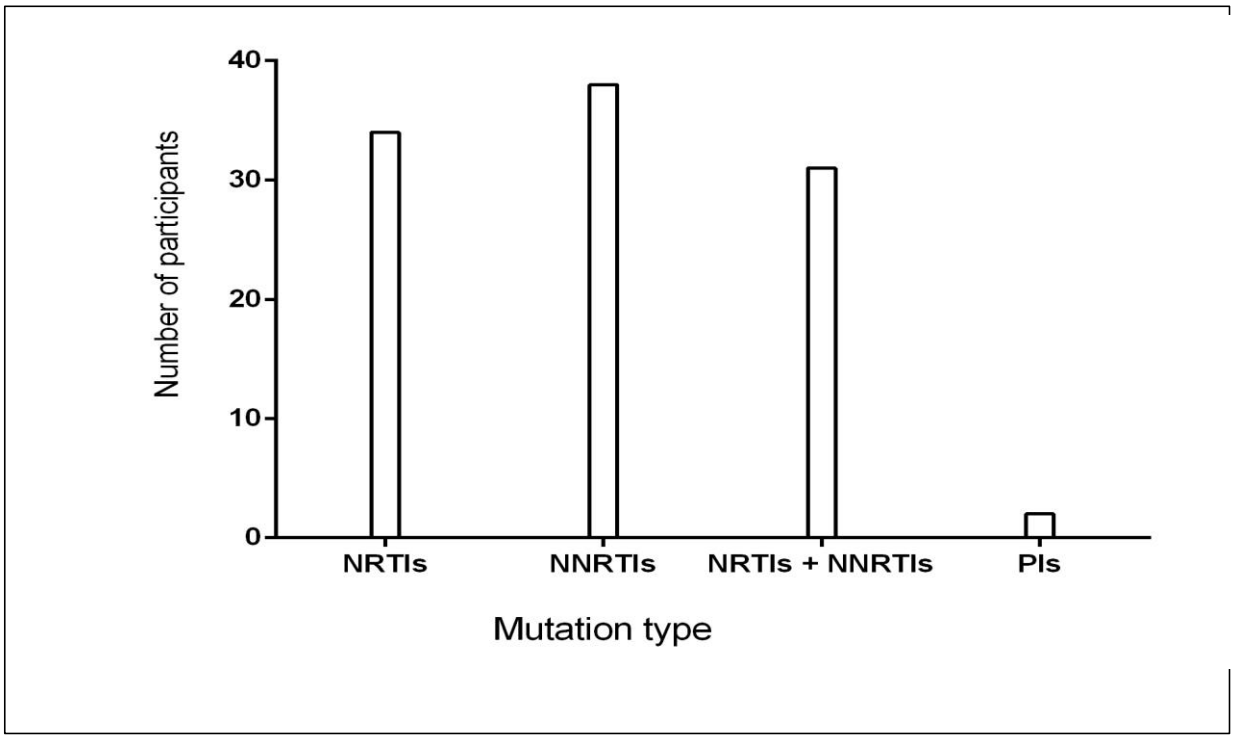

Figure 8: Prevalence of drug resistance mutations to the three drug classes

A broad range of drug resistance-associated mutations were identified among the participants who were successfully genotyped. Currently, there are 45 drug resistance mutations to NRTI occurring at 19 RT codons, which have been documented by (Richman, 2017). Out of the 45 mutations to NRTI, 33 (73%) were detected among the participants. The mutation M184V (substitution of methionine with valine at position 184) was the most common resistance mutation to NRTIs with 21/42 (50%) participants harboring this mutation. The second most common mutation to NRTIs was the D67N which was observed in 7/42 (17%) of participants harboring drug resistance-associated mutations.

A total of 36 NNRTIs resistance-associated mutations are listed on the in the IAS mutation (Richman, 2017); of the 36 mutations to NNRTI, 33 (92%) mutations were detected. The mutation K103N which is a non-polymorphic mutation selected both NVP and EFV was the most common NNRTI mutation observed in 15/42 (34%) of the study participants (Reuman *et al.*, 2010). The second most common mutation in this class was the G190A that was observed in 8/42 (19%) of the participants with drug-resistant mutations to NNRTIs. G190A mutation confers high-level resistance to NVP and EFV (Reuman *et al.*, 2010). The one patient that harbored major PI mutation had D30DN mixed base mutation and 3 accessory mutations (K20T, I50IM, and N88D) while the second patient harboring only PI accessory mutation (L33LF) (Table 6).

Table 5: HIV drug resistance mutations detected

		Reverse transcriptase gene		Protease gene
Patient ID	**Sub type**	**Mutations to NRTIs**	**Mutations to NNRTIs**	**Mutations to PIs**
1	D_G	None	G190A	None
2	A_D	M184MV	None	None
3	C_D	None	K103KN	None
4	A	L210LW	None	None
5	D	M41L, D67DN, L74LI, M184MV, L210W, T215Y	K101KE, V108VI, G190GA	D30DN K20T[1], I50IM[1], N88D[1]
1	A	None	None	L33LF[1]
7	A	None	None	None
8	A	F116FY, M184V	G190A	None
9	D	M41L, E44ED, D67N, L74I, M184V, L210W, T215Y	K103N, V108VI, E138Q, P236PL, K238T	None
10	A	M184V	K103N, V108I, E138G, P225H, K238T	None
11	A	None	K103N	None
12	C	None	K103N, P225PH	None
13	D	K65R, M184IV	L100I, K103N	None
14	A	K65R, K70KE, Y115F, M184V	V106I, Y181YC, Y188L	None
*15				
16	A	None	Y181YD	None

17	A	None	None	None
18	A	D67N, K70R, L74I, M184V, K219Q	A98G, K103N, V108I, E138Q, V179L, K238T	None
19	A	L74V, Y115F, M184V, K219KQ	Y181C, Y188L, H221Y	None
20	A	None	None	None
*21				
22	A	L74V, Y115F, M184V, K219N	G190E	
23	A	M184MV	None	None
24	A	None	None	None
25	A	L74V, Y115F, M184V	K101HPQ, V106M, Y181YC, G190A	None
26	A	D67N, T69DN, K70R, L74LI, Y115YF, M184V, L210LW, T215F, K219Q	A98G, K101H, V108VI, G190A	None
27	D	D67N, K70R, M184V, T215F, K219E	A98G, K103N, Y181C, G190A	None
28	A	L74I, M184V	K103N, V108I	None
29	C	M41L, D67N, K70R, M184V, T215Y, K219E	Y181IS	None
*30				
31	A	None	K103N	None
32	A	T69D	Y181C	None
33	A	M41L, M184V, T215Y	A98G, G190A	None
34	A	M184MV	K103N	None

		M41L, E44EA, D67N, T69TADN, L74LI, V75VIM,		
35	A	M184V, L210W, T215Y	A98G, K101E, E138A, G190A	None
36	A	D67N, K70R	E138EK	None
37	A	None	V106VI, Y188YFHL	None
38	D	M184MV	K101E, E138A, G190A	None
39	D	M184V	K103N, E138A	None
40	A	M184V	K103KN, Y188L	None
41	C	M184V	K103N	
42	A	M184MIV, L210LW, T215TNSY	K103N, V106VI, Y188YF, M230ML	None
43	A	None	None	None
44	A	M184V, K219KR	K101E, K103N, E138A, P225H	None
45	A	M41L, E44D, M184V, L210W, T215Y	V108I, Y181I, H221Y	None
46	A	D67N, K70R, M184V, T215FY, K219E	A98G, G190A	None
47	C	K65R, L74I, Y115F, M184V	V106M, Y181C, G190A	None
48	D	M184V	K103N, G190A	None
49	A	K65R, M184V	L100I, K103N, E138A	None
50	A	M41L, E44ED, D67N, K70R, L74I, M184V, L210W, T215Y	K103S, G190A	M46I, I54V, L76V, V82A, L10F, Q58E

*Samples that failed at the amplification step, [1]PI accessory mutation. Some of the samples failed the amplification step.

4.4 Resistance mutations associated with resistance to multiple drugs

Mutations that confer drug resistance to multiple drugs were determined using the Stanford University drug resistance database guideline. Among the mutations, obtained in this study, 4 mutations (M184V, L210W, D67N, and K65R) caused resistance to multiple drugs including 3TC, FTC, ABC, TDF, DDI, AZT, d4T, and ABC. The mutation M184V causes cross-resistance between 3TC and FTC. Both children below the age of 12 years and adolescents 13-17 years had these mutations. On the other hand, thirteen mutations conferring resistance to multiple NNRTIs were identified (Table 7).

Table 6: Mutations associated with resistance to multiple drugs

Age group (years)	**< 12 years**	**13 – 17 years**
N	4	22
Gender	1 (Male) 3 (Female)	14 (Male) 8 (Female)
Mutations to multiple NRTIs	M184V, L210W	D67N, M184V, K65R, L210W,
Mutations to multiple NNRTIs	L100I, K103N, E138A, V108I, Y181I, G190A	K103N, K238T, V108I, A98G, Y181C, G190E, G190A, V106M, V108I, K101E, E138A, Y188L, L100I
NRTI drugs	3TC, FTC, ABC, TDF, DDI	3TC, FTC, AZT, d4T, TDF, ABC, DDI
NNRTI drugs	NVP, EFV, DOR, RPV	NVP, EFV, DOR, ETR, RPV

3TC – Lamivudine, FTC - Emtricitabine ABC - Abacavir, TDF - Tenofovir, DDI - Didanosine

4.5 Drug resistance profile

The analysis of drug resistance to the commonly used drugs was performed using the Stanford HIV drug resistance database (https://hivdb.stanford.edu/hivdb/by-sequences/). The drug resistance profile revealed that the participants experiencing virologic failure were resistant to the majority of the drugs included in both first and second-line (Figure 9).

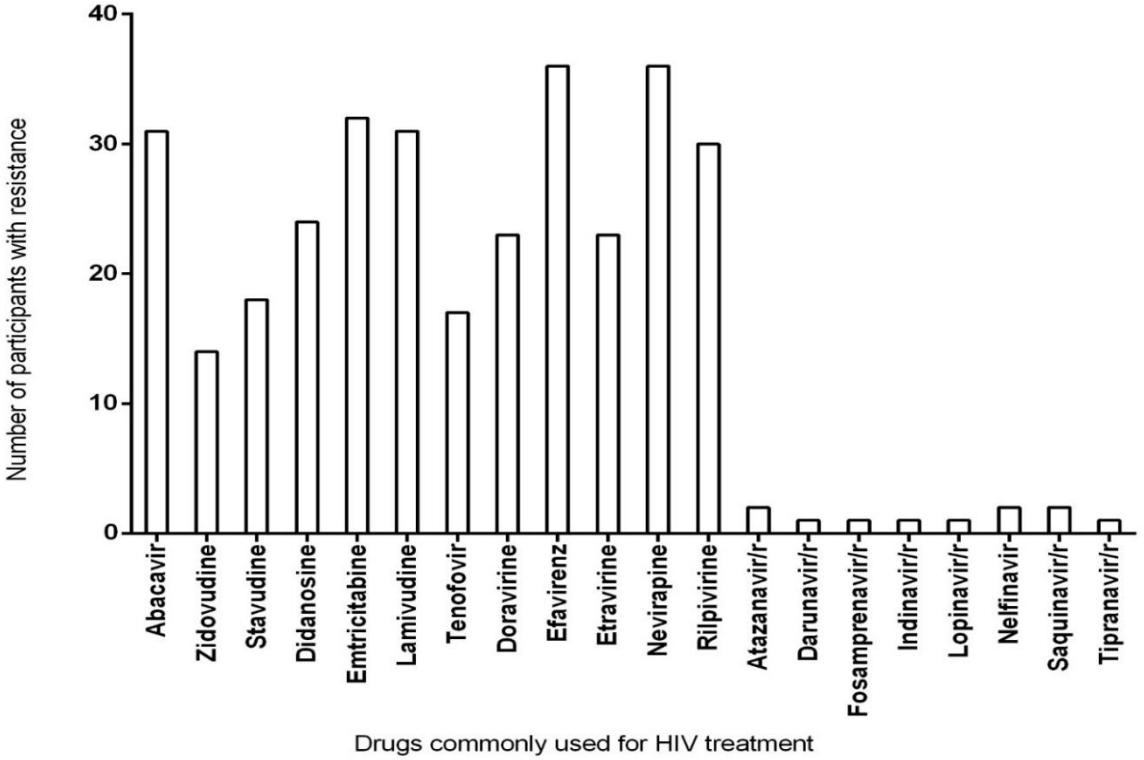

Figure 9: Number of study participants with resistance to commonly used drugs

4.6 Genetic diversity of HIV-1 isolates

Subtype analysis of all sequences obtained was performed by the REGA software for subtype analysis (Table 8). The majority of the patients harbored subtype A 73% while only three circulating recombinant forms were detected in all sequences analyzed.

Table 7: HIV-1 subtypes circulating among the successfully genotyped samples

Subtypes	A	C	D	CRF_A_D	CRF_C_D	CRF_D_G
Frequency	33	4	7	1	1	1
Percentage	73.%	9%	12%	2%	2%	2%

Sequences generated from this study together with 10 sequences other sequences obtained from multiple regions (downloaded from GenBank) were used to perform multiple sequence analysis in Clustal W. The alignments have been shown in figure 12.

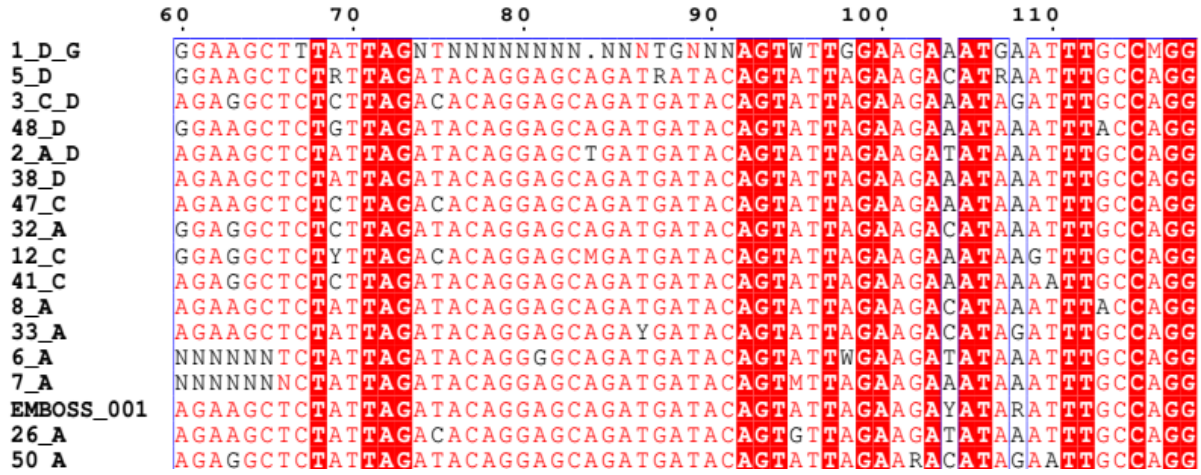

Figure 10: Multiple sequence alignment. The red shade represents regions in the conserved regions in all the sequences aligned.

4.6.1 Phylogenetic analysis

Phylogenetic inference for the circulating viruses was conducted using the Maximum Likelihood method and the Tamura-Nei model (Tamura & Nei, 1993). The tree was generated to scale with the branch lengths estimated with the number of substitutions per site with bootstrap support of 100. The branch lengths were measured in the number of substitutions per site. The evolutionary analyses were conducted with MEGA 10 (Kumar *et al.*, 2018) (Figure 11).

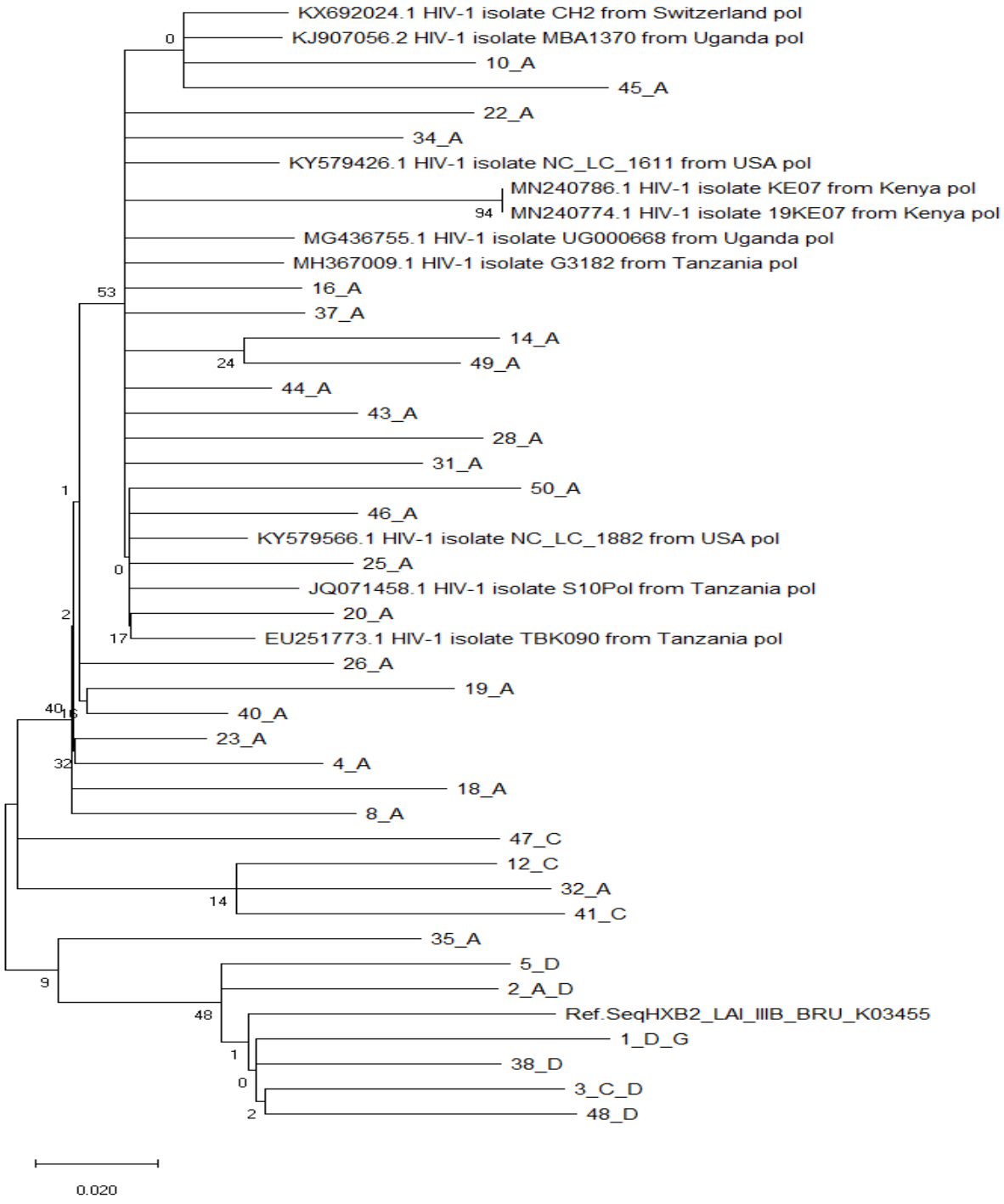

Figure 11: Phylogenetic tree. The tree shows the relationship between the circulating HIV-1 subtypes detected in the study participants. The numbers represent the unique identifier assigned to samples while the letters represent the HIV-1 subtypes detected. The sequences obtained from GenBank have been labeled with the sequence name and country of origin.

4.7 HIV drug resistance assay validation

4.7.1 Accuracy

The accuracy of the modified assay was assessed by comparing 10 paired nucleotide sequences generated by the original and the optimized assay using the EMBOSS pairwise alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). The pairwise sequence alignments were presented using ESPript online tool for sequence alignment presentation (Robert & Gouet, 2014) (Figure 12). The mean nucleotide identity was 98.5% (CI, 97.92 – 99.08%).

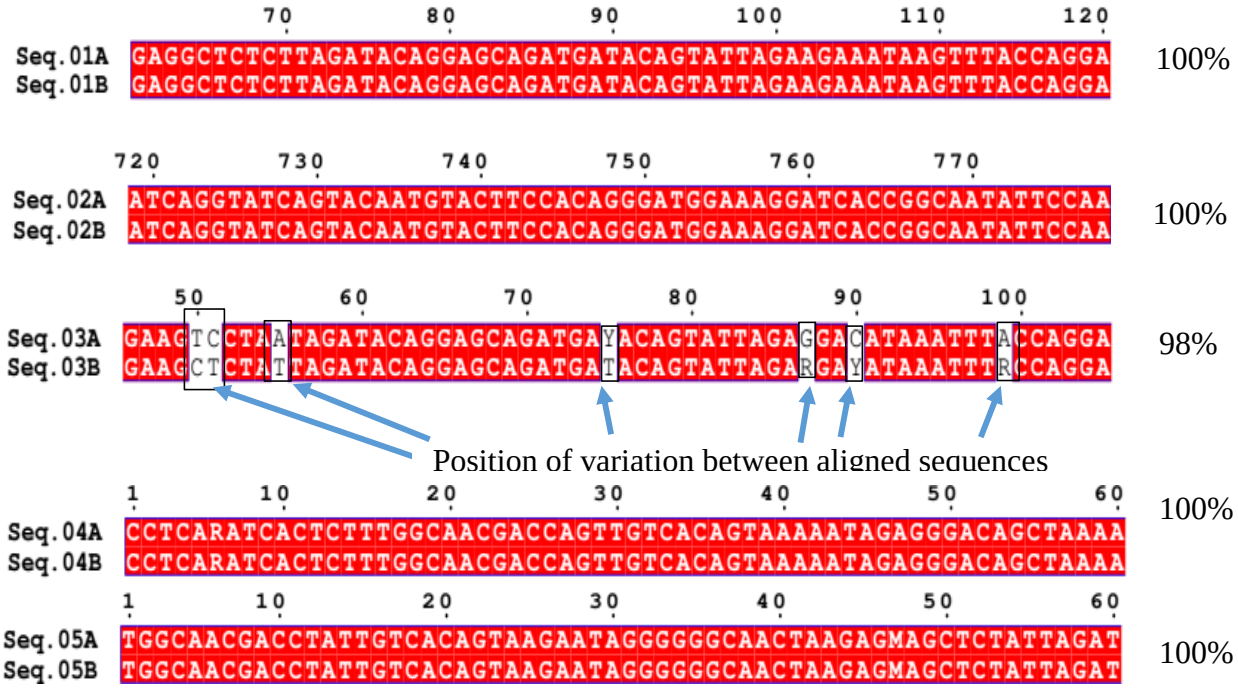

Figure 12: Pairwise sequence alignment . The sequences were viewed in ESPript. Sequences generated by both original and modified assay were compared and positions where variations occurred identified.

The concordance of the two assays in detection of drug resistance-associated mutations revealed that 68 drug resistance mutations detected including 9 (13%) mutations in the protease gene and 59 (87%) in the RT gene (Table 8). The original assay detected 67 DR mutations including 2 mutations (V106I and K225H) not detected by the optimized assay. On the other hand, the modified assay detected 68 DR mutations including four mutations (D67DG, K101KPQT, K70KN, and I50IL) not detected by the original assay. Six mutations were discordant (V106I, P225H, D67DN, I50IL, K101KPQT, and K70KN). Of the six discordant

DR mutations, two were complete discordant (V106I and P225H) while four (D67DN, K101KPQT, K70KN, and I50IL) were partial discordant due to mixed base mutations. Mixed mutations represent mutations occurring in positions in DNA sequence where two or more bases are identified. Three of the discordant mutations (D67DN, K101KPQT, and K70KN) were detected as mixtures in the modified assay but were non-mixtures (D67N, K101P, and K70N) in the original assay (Table 9).

Table 8: Pairwise comparison of drug resistance mutations detected by the two assays

	Reverse transcriptase gene		Protease gene	
ID	**Original**	**Modified**	**Original**	**Modified**
1	M184V, H221Y	M41L, M184V, H221Y		
2	V106VM, V179E, Y181C, Y188YC,	A62V, V75I, D67DN[b], K219KE, V106VM, V179E,		
3	A62V, V75I, K219KE	Y181C, Y188YC		I50IL[b]
4	M41L, M184V, L210W, T215FY, A98G, V106I[a], Y188L	M184V, L210W, T215FY, Y188L, K238KT	M46I, I84V	M46MI, I84V
5	M184V, K103N, V108I, P225H[a]	M184V, K103N, V108I		
6	K65R, Y115F, M184V, V106M, G190A	K65R, Y115F, M184V, V106M, G190A		
7	D67N, K70R, L74I, M184V, K219Q, A98G, K103N, V108I, E138Q, V179L, K238T, L74V, Y115F, M184V	D67N, K70R, L74I, M184V, K219Q, A98G, K103N, V108I, E138Q, V179L, K238T, L74V		
8	K219KQ, Y181C, Y188L, H221Y, M41L	L74V, Y115F, M184V, K219Q, Y181C, Y188L, H221Y		
9	D67N, K70R, M184V, T215F, K219E, A98G, V108VI, Y181C, Y181YC	D67N, T215F, K219E, A98G, K101KPQT[b], E138Q, Y181YC, K238N	M46I, I54V, V82A	M46I, I54IV, V82A

10	D67DG, K70N, M184V, K101P, E138Q	D67DG, K70KN[b], M184V, T215F, K219E, A98G, K101P, Y181YC	M46MI, I54IV, V82VA	M46MI, I54IV, V82VA

[a]Two discordant mutations were detected by the original assay but missed in the modified assay. [b]Discordant mutations detected by the modified assay

Analysis using McNemar test on paired results for drug resistance mutations supported the good performance of the optimized assay (χ^2 = 2.358, df=1, p > 0.05). The minor differences observed in sequence identity were mainly caused by mixed bases. Among the 10 paired sequences, we identified 29 mixed bases of which 18 (62%) were detected by the optimized assay while 11 (38%) by the original assay (Table 10).

The difference in drug resistance mutations detected by the optimized and original assay resulted from the detection of mixtures affecting the concordance of HIV drug resistance-associated mutations detected by both assays. We, therefore, determined the significance of the mixture base calling between the two assays by performing a Wilcoxon signed-rank test. The results showed no significant difference in mixtures detection by the two assays at $p < 0.05$.

Table 9: Summary of the paired sequence comparisons

Pairwise sequence identity analysis	
Basis of comparison	Original vs Optimized
Number of samples	10 vs 10
Nucleotide identity	98.5% (CI, 97.92 – 99.08%)
Number of mutations detected	67 vs 68
Number of mixtures	11 vs 18
Number of discordant mutations	6 (2[a] vs 4[b])

[a]Discordant mutations detected by the original assay; [b]discordant mutations detected by the optimized assay.

4.7.2 Precision and reproducibility

Further assessment to examine the precision and reproducibility of the optimized assay was performed by analyzing 3 samples each with four replicates all in one test run for precision. The sequence identity among the replicated ranged from 96-100% with the mean sequence identity being 98.67% (CI, 98.06 – 99.28). Reproducibility was assessed by testing 10 samples in duplicate on different days. The sequences obtained by testing the same samples at different time points were tested for nucleotide sequence identity using the EMBOSS program for pairwise alignment (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). The sequence identity was 98.6% (CI, 98.17 – 99.03). The overall agreement drug resistance mutations detected in both precision and reproducibility substantial with a kappa value of 0.792 ($p < 0.05$) and 0.778 ($p < 0.05$) respectively (Table 11).

Table 10: Precision and reproducibility of genotyping using the two assays

Quality	Number of samples	Replicates	kappa value	Nucleotide identity
Precision	3	12	0.792	98.67%
Reproducibility	10	20	0.778	98.60%

4.7.3 Amplification sensitivity

Of the 16 samples used in the assessment of amplification sensitivity, 8 samples had VL values ranging from 207 to 999 copies/ml while the remaining 8 samples had 1000 to 86, 040 copies/ml. The modified assay showed an amplification sensitivity of 62.5% for viremia between 200 and 999 copies/mL and 100% for viremia above 1000 copies/ml (Table 12).

Table 11: Amplification sensitivity of the modified assay

Sample ID	**VL (Copies/ml)**	**Subtype**
1	86, 040	A1
2	12,155	A1
3	2,802	G
4	2,700	C
5	1,717	A1
6	1.547	A1
7	1,100	C
8	1,040	G
9	625	G
10[b]	612	
11	514	C
12[b]	459	
13	385	A1
14	312	A1
15	214	A1
16[b]	207	

[b]Sample failed to amplify

4.8 Reagent cost comparison between the original and modified assay

Following a 50% reduction in reagent volumes in the amplification and sequencing reactions steps, the cost of reagent was compared between the original and the modified assay. The cost of reagents at the amplification step (nested PCR) was reduced from $37.32 to $19.23 per test, while the sequencing reactions reagent cost was reduced $54.4 to $34.42 per test. Taken together, the cost of analyzing one sample from extraction to sequencing was reduced from $98.7 to $60.72. This represented a 38.5% cost reduction (Table 13).

Table 12: Cost reduction at various steps of HIV drug resistance testing

Reaction Steps	**Original Assay**	**Modified assay**
RNA Extraction	4.17	4.17
DNA/RNA amplification	37.23	19.23
Gel electrophoresis	1.15	1.15
Sequencing	54.4	34.42
Sample collection	1.74	1.74
Total cost	**98.7**	**60.72**

CHAPTER FIVE
DISCUSSION

In the current study, a high rate of drug resistance mutations among children (below 12 years) and adolescents (13 to 17 years) was reported. Out of the 47 of participants who were successfully genotyped, 42 of them harbored at least one major drug resistance-associated mutations. These findings confirmed the results reported in Mali (73%; N=97) (Germanaud *et al.*, 2010) and Ethiopia (81%; N=94) (Tadesse *et al.*, 2018). Considering the complexities associated with the management of HIV in children and adolescents, the prevalence of drug resistance is likely to remain high in children and adolescents who are not virally suppressed (Workneh, 2009; Schlatter, 2016).

Other than drug resistance-associated mutations, numerous factors have been associated with high drug resistance in children and adolescents. Firstly, children have been shown to have high VL levels at the time of treatment initiation (Tadesse *et al.*, 2018). This can be explained by their premature (developing) immune system that allows the virus to rapidly replicate and in the process of acquiring mutations. Young children are more likely to be immunosuppressed and exhibit coinfections with bacterial infections giving the virus ample time to replicate and accumulate mutations (Bachou *et al*., 2006). The problem of high viral loads in children and adolescents is further compounded by the inadequate treatment monitoring in this population (Tadesse *et al*., 2018). Secondly, poor HIV management programs may also present systematic challenges such as lack of access to HIV Comprehensive Care Center (CCC) or long distance to the CCC. These challenges result in poor adherence to treatment and lost to treatment due to missed appointments. Such patients are bound to develop drug resistance mutations. Thirdly, psychosocial factors such as stigma and lack of support from family members may act as prerequisites for poor adherence to treatment hence drug resistance. HIV associated stigma has been found to be profound among adolescents, hindering them from adhering to treatment (McHenry *et al*., 2017). In particular, school-going children are at a high risk of skipping their drugs due to fear that other children may know their HIV status. All these factors act in synergy to increase the risk of drug resistance development in HIV patients. These findings further underpin the need to implement timely drug resistance testing to inform clinical decision making.

In the context of HIV drug resistance mutations reported in this study, more mutations were detected in the reverse transcriptase (RT) region than in the protease region (206 versus 11). This can be explained by the larger size of the RT gene, approximately 251 codons versus

the smaller protease gene, 99 codons (Shafer *et al.,* 2000). This difference in size implies that there are more positions for the selection of random as well as drug-induced mutations to occur in the RT gene compared to the shorter protease gene. Additionally, this study revealed that more participants harbored resistance to NNRTIs (38/42) versus NRTI (34/42). Studies examining the structure of mutant RT enzyme have shown that the mutations conferring resistance to NNRTIs are located in the hydrophobic pock*et al*so known as NNRTI binding pocket (NNRTIBP) (Shafer *et al.,* 2000). A single mutation in the NNRTIBP results in high-level resistance to multiple NNRTIs, accounting for the high prevalence of resistance to NNRTIs (low genetic barrier to resistance). Drug resistance to NNRTI also emerges rapidly when NNRTI monotherapy is administered in patients exhibiting virologic failure (VF) (Shafer *et al.,* 2000). On the contrary, few mutations to PIs were detected in this study which can be explained by the high genetic barrier to resistance to the development of resistance. The virus has to undergo multiple mutations to develop resistance to a single PI agent. Drug resistance to PIs occurs through the compensatory mechanism, where mutation at the active site of protease reduces favorable interaction between the drug and the protease substrate (Gulnik *et al*., 2000). To compensate for the lost affinity to the natural substrate, the virus has to undergo other mutations at a different site that preferentially restore affinity to the natural substrate (Gulnik *et al*., 2000).

Mutations conferring resistance to multiple NRTIs and NNRTIs were identified in this study. There were slightly more mutations that caused cross-resistance between NVP and EFV. A total of 13 mutations (L100I, K103N, E138A, V108I, Y181I, G190A, K238T, A98G, Y181C, G190E, V106M, K101E, Y188L) caused resistance to multiple NNRTIs. On the other hand, 4 mutations (M184V, L210W, D67N, K65R) were associated with multiple resistance to NRTI. The mutation M184V has been shown to confer drug resistance to 2 NRTIs (3TC, FTC) (Markowitz *et al*., 2005). These drugs are vital components of both first and second-line regimens. In addition, high-level cross-resistance between EFV and NVP has previously been described (Markowitz *et al*., 2005). One immplication of these findings is that if timely interventions are not implemented to manage drug resistance, it will lead to exhaustion of the available ARVs. Apart from, the development of mutations causing cross-resistance between multiple drugs, this study also reported a significant proportion (31/50) of participants who exhibited dual-class drug resistance NRTI and NNRTI. These findings are important to the national HIV management program as they underscore the need to urgently implement routine

HIV drug resistance testing to help control the emergence of HIV-1 drug resistance (Tadesse *et al*., 2018).

All the major HIV-1 drug resistance mutations reported in this study resulted from amino acid substitution at a specific position in the RT and protease genes. The amino acid substitution occurred as a result of nucleotide changes causing a codon change and hence amino acid. For instance, at position 40 of sequence 1; aligned against the reference sequence (Figure 7), the change of nucleotide base from A to G led to codon change from ATA to GTA. The codon ATA codes for amino acid isoleucine whereas GTA codes for valine (Shafer *et al*., 2000). This implies that the mutant viruses had a valine at this position as opposed to isoleucine in the wild type virus. A mutation of this nature resulted in the alteration of the viral protein's primary structure by changing the amino acid sequence. On the other hand, a minor mutation at position 30 on the same sequence resulted in a codon change from CTC to CCT. This change did not alter the amino acid sequence as both codons code for the amino acid leucine. These kinds of mutations are referred to as silent mutations and they did not change the primary structure (Telwatte *et al.,* 2015). In this study, all major HIV drug resistance detected led to primary structure change while the minor mutations were silent mutations.

The viral RT enzyme lacks proofreading and error correction mechanism (Hu & Hughes, 2012). During the rapid replication of the virus, opportunities exist for the virus to accumulate mutations that confer resistance to ARVs. The mutations K65R has previously been reported to occur in treatment naïve patients (Muri *et al.,* 2017). Our findings showed that 2 of the four participants with K65R were on TDF containing regimen, while the other two had been exposed to AZT. Therefore, the emergence of K65R mutation in participants who did not have prior exposure to TDF may be due to the random accumulation of mutations as a result of the error-prone HIV reverse transcriptase enzyme (Hanping *et al.*, 2016).

The mutation M184V selected by lamivudine (3TC) was the most common NRTIs resistance mutation occurring in 74% of the participants harboring NRTI drug resistance mutations. The drug 3TC is an important ART as it is included in all first and second-line cART regimens (Petrella *et al.*, 2004). The high prevalence rate of resistance to 3TC emphasizes the need for the national HIV management program to implement strategies that will ensure timely detection of emerging HIV drug resistance in this population. Such information will help to make important clinical decisions such as switching patients to a more effective treatment regimen with evidence of drug resistance mutations.

Consistent with the WHO recommendations for the first-line regimen, the Ministry of Health (Kenya) recommends the inclusion of two NRTIs and one non-nucleoside reverse transcriptase inhibitors (either NVP or EFV) in the first-line treatment regimen and one protease inhibitor (PI) (Ministry of Health, 2016). This study revealed that the majority of participants 38/42 had drug resistance mutations to NNRTIs and 34/42 had resistance mutation to NRTIs. This high rate of drug resistance mutations to the two drug classes are likely to hinder the attainment of the UNIDS 90-90-90 global targets. The Ministry of Health guidelines for HIV treatment recommends the use of 3TC in both first and second-line regimen (Ministry of Health, 2016) based on the replicative cost of M184V mutation. Studies have shown that the mutation M184V selected by 3TC potentially increases the susceptibility of the mutant virus to ABC and TDF and AZT (Wainberg, 2004). This drug is included in the HIV treatment regimen despite the high burden of drug resistance to this drug. Protease inhibitors on the other are administered to patients who fail NVP/EFV-based second regimen due to their high genetic barrier.

The analysis of the circulating HIV-1 subtypes revealed that subtype A was the most prevalent circulating subtype. These findings were in agreement with the results obtained by other studies (Hassan *et al.,* 2014) where subtype A was the most prevalent. Correlation of HIV-1 subtypes with disease progression (drug resistance burden and viral load levels) did not show an association between the subtypes and disease progression. Previous studies have correlated HIV-1 subtypes with disease progression (CD4+ cell decline) (Venner *et al*., 2016). However, there has not been consensus on whether HIV-1 subtypes affect disease progression as conflicting findings have been reported (Bhargava *et al.*, 2014).

Phylogenetic analysis of the circulating HIV subtypes revealed a significant evolutionary relationship among the viruses detected in the successfully genotyped viral isolates. Subtype A sequences clustered together confirming that they shared their immediate ancestor. Additionally, the subtype A sequences clustered in the same clade with sequences from different geographical regions obtained from GenBank, which suggested that the sequence obtained from GenBank were also subtyped A. On the other hand, subtype A sequences are distantly related to the reference sequence suggesting that significant evolution had occurred since the time they shared a common ancestor. The reference sequence clustered on the same clade with subtype D and CRF_DG.

The performance characteristics of the modified assay (accuracy, precision, reproducibility, and amplification sensitivity) were comparable to those of the original assay.

The study reported 98.5%, 98.7% and 98.6% sequence similarity for accuracy, precision, and reproducibility respectively. Similar findings were also supported by previous studies (Chaturbhuj *et al.*, 2014; Yang *et al.*, 2010; Zhou *et al.*, 2011). The WHO guidelines for assay validation require that for an assay to pass validation test, sequence similarity of more than 90% must be achieved (World Health Organization, 2012b). The high sequence similarity reported was further supported by the concordance of mutations generated by the two assays. However, despite the high sequence similarity, more mixed-base mutations were detected by the modified assay. Previous studies that reported similar findings have proposed a number of factors including quasispecies primer binding preference and location, Taq polymerase misincorporation and sequence quality to contribute to the detection of mixed base mutations (Galli *et al.*, 2003). Of significance among these factors is quasispecies, which is defined as a viral population with a significantly variant genome (Hedskog *et al.*, 2010). Sanger sequencing approach used on this study applies population sequencing approach, that sequences every viral variant present in the population, thus there is a possibility of sequencing viruses with different mutations at the same position, hence the mixed bases (Saravanan *et al.*, 2009).

Amplification sensitivity, defined as the percentage of samples successfully genotyped at a given VL range was assessed using 16 samples with viral load levels ranging from 207 to 86,040 copies/ml. The modified assay reported an amplification sensitivity of 62.5% for viremia between 200 and 999 copies/mL and 100% for viremia above 1000 copies/ml. The WHO criteria for recommending HIV patients for drug resistance testing is when a patient presents with viral load levels of 1000 copies/ml (World Health Organization, 2017); the modified assay successfully genotyped all the samples with viral loads >1000 copies/ml with 100% efficiency. These findings demonstrated the feasibility of successfully utilizing the modified assay in RLS to scale up routine surveillance of HIVDR. Additionally, the modified assay can be used to genotype HIVDR low-level viremia (LLV) samples.

Cost comparison between the two assays revealed a cost reduction of 38.5% when the modified assay is used. Owing to the fact that the high cost of HIV molecular genotyping is a major barrier to the implementation of routine HIVDR testing services in RLS, the adoption of a cost-effective method can significantly enhance the delivery of this service.

Despite the new insights achieved by the current study, a limitation encountered was the relatively small sample size warranting further investigations using large scale studies. Furthermore, the use of the population Sanger sequencing approach might have led to an underestimation of drug resistance mutations.

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The high burden of HIV-1 drug resistance among HIV positive children and adolescents derail progress achieved through ART scale-up in this population. The majority of drug resistance-associated mutations were detected in the reverse transcription gene. Notably, there was a high rate of M184V mutation, which conferring resistance to lamivudine, a backbone drug in both first and second-line regimens. Among the circulating HIV subtypes, HIV subtype A was noted to be the most prevalent. The performance characteristics of the modified assay were satisfactory and attained the WHO set criteria for assay validation. These findings underscored the potential of utilizing the modified cost-effective assay as an approach to enhance the scale-up of routine HIVDR testing in resource-limited settings.

6.2 Recommendations

Although this study has successfully characterized most drug resistance mutations among HIV positive children and adolescents exhibiting virologic failure, the following recommendations will help to improve HIV treatment and monitoring.

i. Children and adolescents on ART should be tested for drug resistance; especially those with high viral loads.
ii. There is need for continuous monitoring of circulating HIV subtypes since the virus evolves at a high rate.
iii. Studies focusing on HIV drug resistance genotyping assay modification and validation are needed to come up with cost-effective methods as a step towards implementing routine HIV drug

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APPENDICES

Appendix I: Abstract

F1000Research F1000Research 2019, 8:1518 Last updated: 18 SEP 2019

Check for updates

RESEARCH ARTICLE

Performance characteristics of a modified HIV-1 drug resistance genotyping method for use in resource-limited settings [version 1; peer review: 1 approved]

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Abstract

Background: HIV-1 drug resistance (HIVDR) assays are critical components of HIV clinical management programs in the face of emerging drug resistance. However, the high costs associated with existing commercial HIVDR assays prohibit their routine usage in resource-limited settings. We present the performance characteristics of a modified commercial HIVDR testing assay.
Methods: A total of 26 plasma samples were used to validate and assess the accuracy, precision, reproducibility and amplification sensitivity of a modified HIVDR assay by HIV genotyping. In addition, a cost comparison between the original and the modified assay was performed using the ingredient costing approach.
Results: The performance characteristics of the modified assay were in agreement with the original assay. Accuracy, precision and reproducibility showed nucleotide sequence identity of 98.5% (confidence interval (CI), 97.9–99.1%), 98.67% (CI, 98.1–99.23) and 98.7% (CI, 98.1–99.3), respectively. There was no difference in the type of mutations detected by the two assays ($\chi^2 = 2.36$, $p = 0.26$). Precision and reproducibility showed significant mutation agreement between replicates (kappa = 0.79 and 0.78), respectively ($p < 0.05$). The amplification sensitivity of the modified assay was 100% and 62.5% for viremia ≥1000 copies/ml and <1000 copies/ml respectively. Our assay modification translates to a 39.2% reduction in the cost of reagents.
Conclusions: Our findings underscore the potential of modifying commercially available HIVDR testing assays into cost-effective, yet accurate assays for use in resource-limited settings.

Open Peer Review

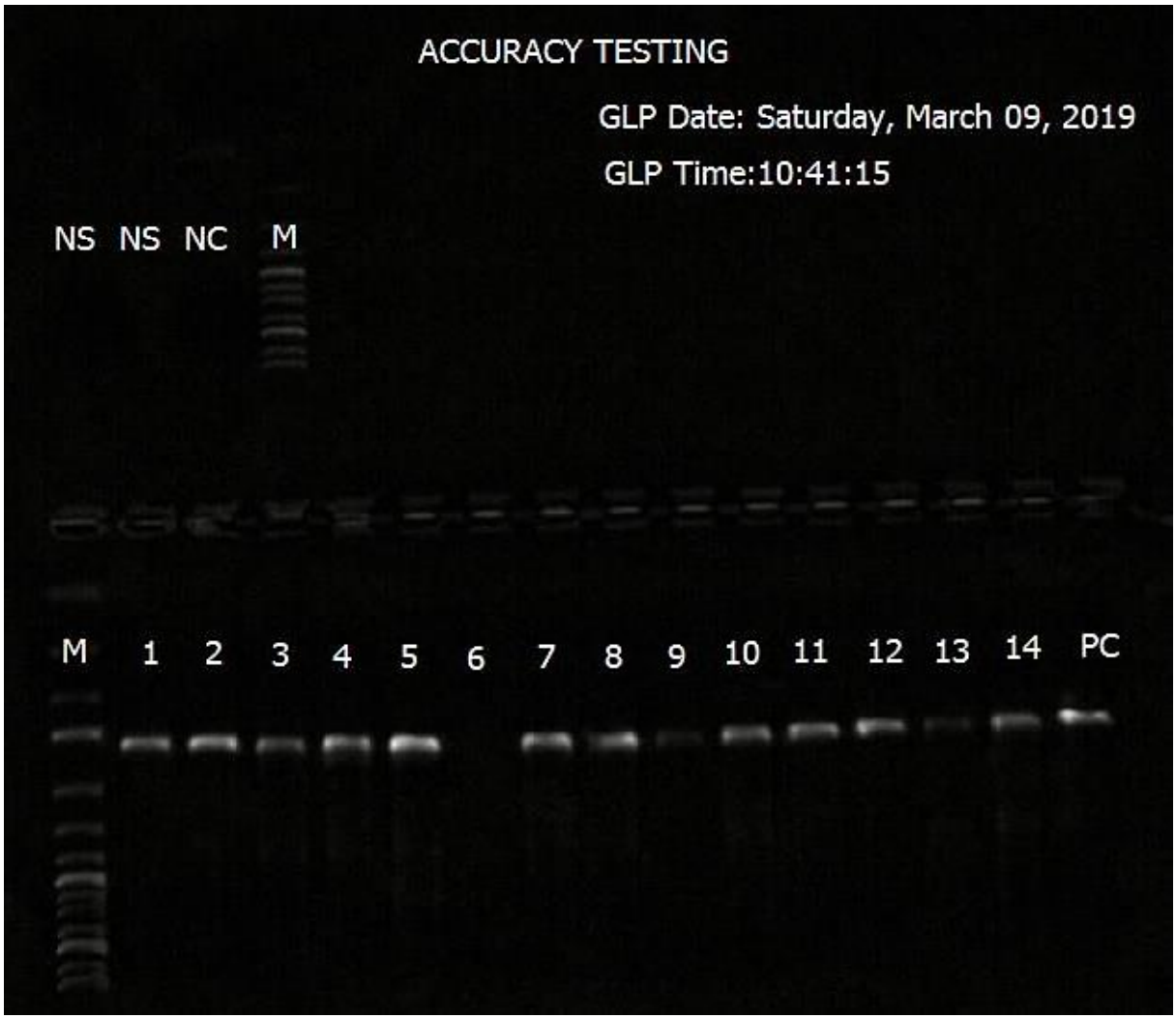
Reviewer Status ✓

	Invited Reviewers
	1
version 1 published 28 Aug 2019	✓ report

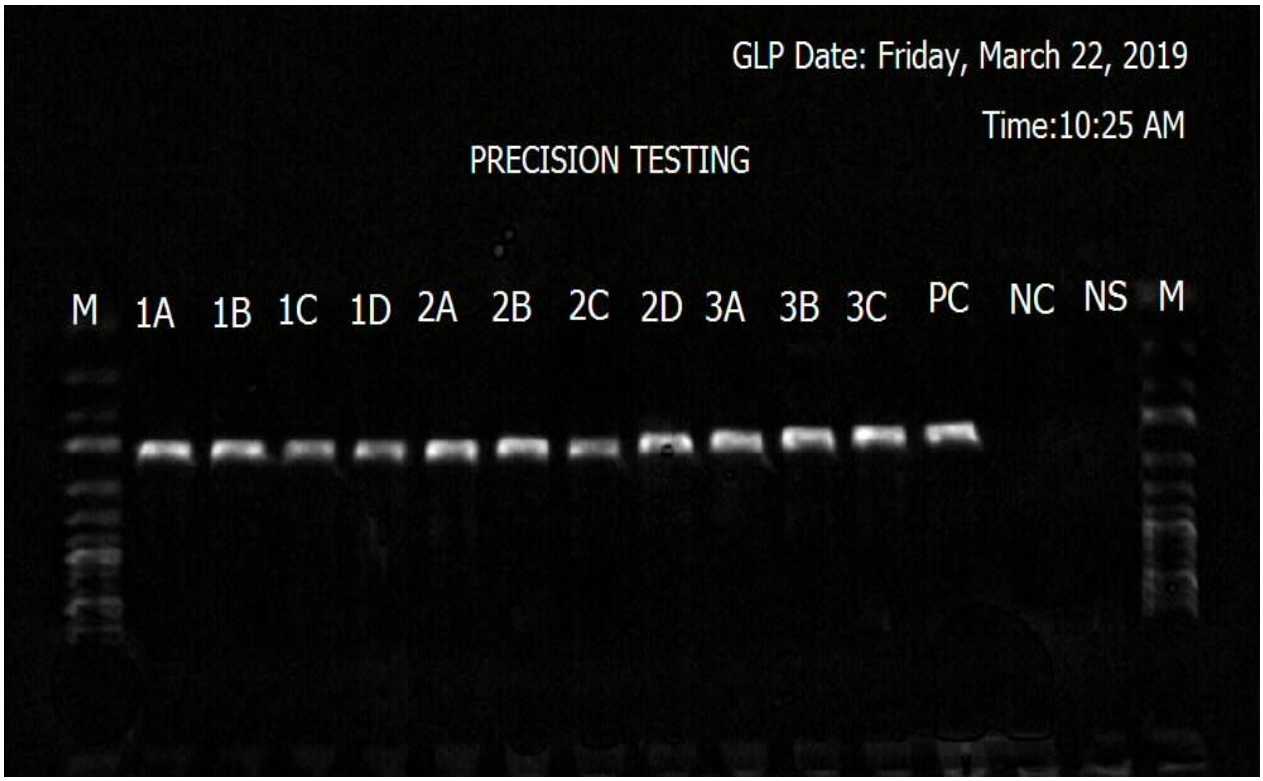
1 **Michael Walekhwa**, Kabarak University, Nakuru, Kenya

Any reports and responses or comments on the article can be found at the end of the article.

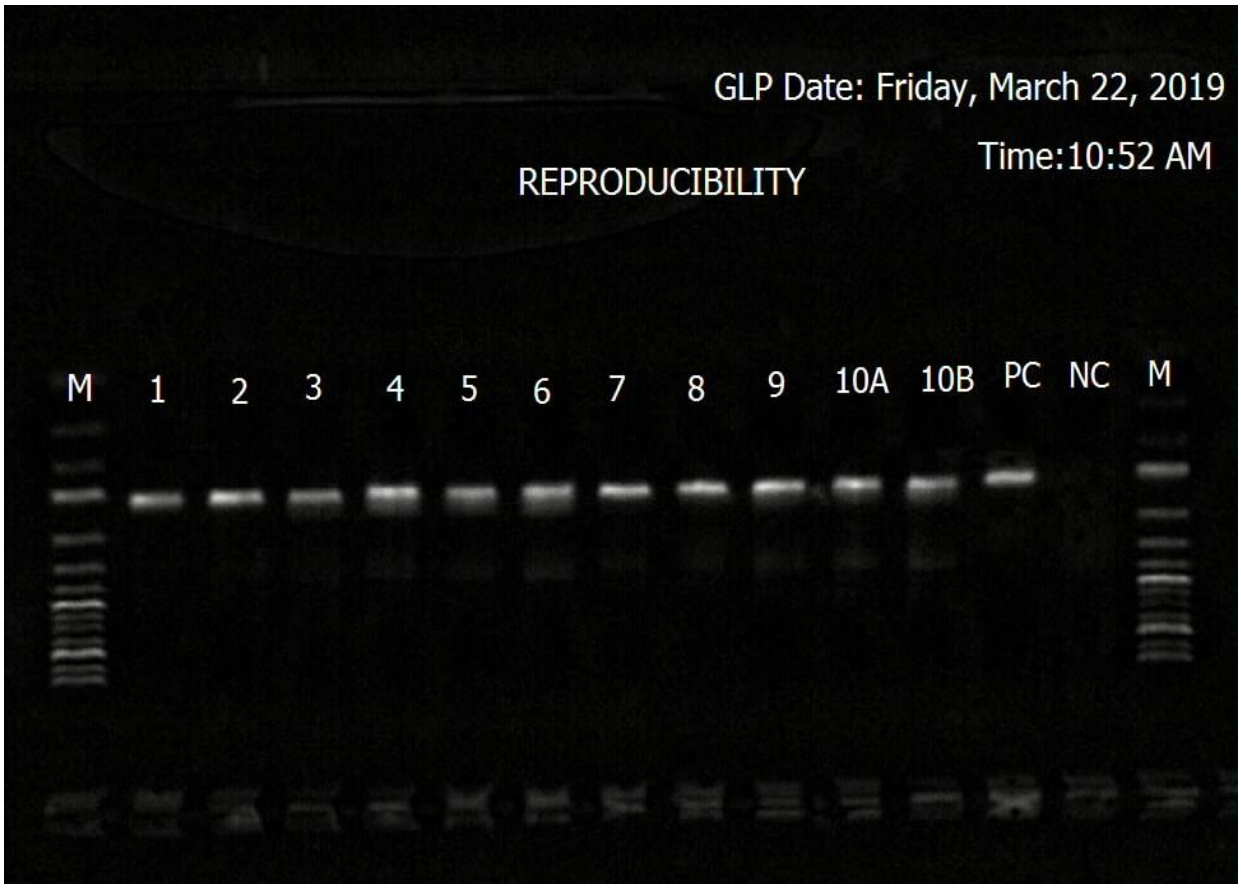
Appendix II: Gel electrophoresis image for accuracy. Successfully amplified samples produced bands while samples which failed amplification did not produce band. Out of 14 samples, 12 produced clear bands, one (sample 13) produced a faint band while sample 6 failed to produce a band. M – Molecular marker (geneRuler 1 kb DNA ladder); PC – Positive control, NC – Negative control; NS – No sample loaded.



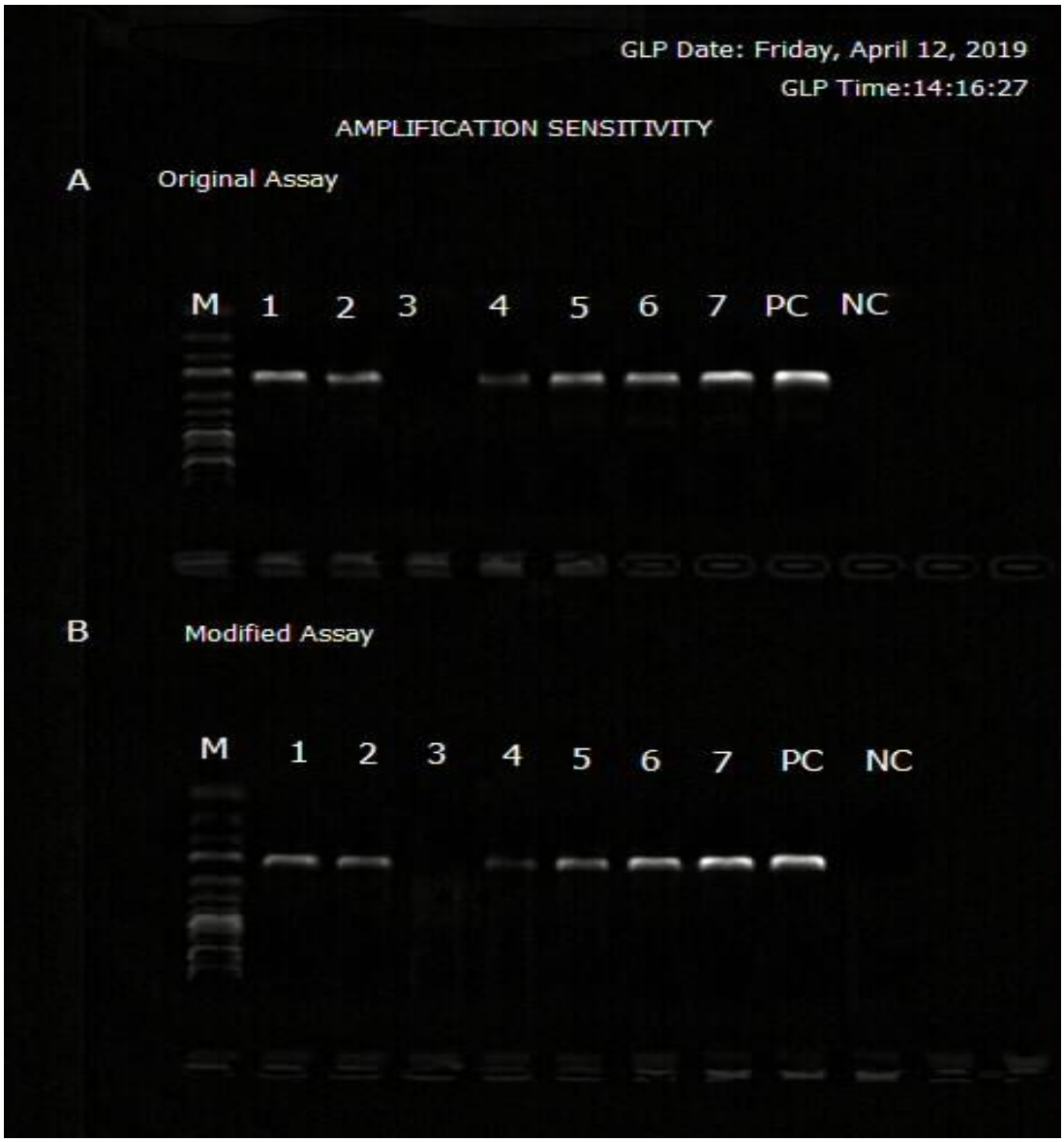
Appendix III: Gel electrophoresis images precision. Successfully amplified samples produced bands while samples which failed amplification did not produce band. Sample 1 and 2 were run in quadruplicate while sample 3 was run in triplicated. All the samples were successfully amplified including PC and M. The NC did not produce any band. M – Molecular marker (geneRuler 1 kb DNA ladder); PC – Positive control, NC – Negative control; NS – No sample loaded.



Appendix IV: Gel electrophoresis images for reproducibility. Successfully amplified samples produced clear bands while samples which failed amplification did not produce any band. A total of 10 samples were subjected to gel electrophoresis and all samples produced clear bands including PC and M suggesting that amplification was 100% successful. The NC did not produce a band. Sample 10 was loaded twice. M – Molecular marker (geneRuler 1 kb DNA ladder); PC – Positive control, NC – Negative control.



Appendix V: Gel electrophoresis images for reproducibility amplification sensitivity. Successfully amplified samples produced clear bands while samples which failed amplification did not produce any band. In total 7 samples were loaded on the gel for both original and modified assay. In both assays, all samples produced bands except for sample 3, which together with NC failed to amplify in both cases. M – Molecular marker (geneRuler 1 kb DNA ladder); PC – Positive control, NC – Negative control.



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APPENDIX VI: Ethical approval

KENYA MEDICAL RESEARCH INSTITUTE

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Tel: (254) (020) 2722541, 254 (020) 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2720030
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KEMRI/RES/7/3/1 **March 31, 2016**

TO: **OCHWOTO MISSIANI,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. ROSEMARY SANG,**
ACTING DIRECTOR, CVR,
NAIROBI

FOR DIRECTOR
CENTRE FOR VIRUS RESEARCH
P.O. BOX 54628
NAIROBI.
1st April 2016

Dear Sir,

RE: PROTOCOL NO. KEMRI/SERU/CVR/001/3211 (*RESUBMISSION2 OF INITIAL SUBMISSION*): CHARACTERIZATION OF HIV DRUG RESISTANCE MUTATIONS AND SUBTYPE DIVERSITY OF ISOLATES FROM CHILDREN AND ADOLESCENTS EXPERIENCING VIROLOGICAL FAILURE IN KENYATTA NATIONAL HOSPITAL

Reference is made to your letter dated 24th March, 2016. KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised protocol on the 29th March, 2016.

This is to inform you that the Committee notes that the issues raised by the Expedited Review Team have been adequately addressed.

Consequently, the study is granted approval for implementation effective on **31st March, 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **March 30, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **February 13, 2017**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

CENTRE FOR VIRUS RESEARCH
01 APR 2016
P.O. BOX 54628 - NAIROBI

APPENDIX VII: Biomedical research training certificate

CITI PROGRAM

Completion Date 12-Jul-2017
Expiration Date 12-Jul-2018
Record ID 23618191

This is to certify that:

EDWIN MAGOMERE

Has completed the following CITI Program course:

Biomedical Research - Basic/Refresher (Curriculum Group)
Biomedical Research - Basic/Refresher (Course Learner Group)
1 - Basic Course (Stage)

Under requirements set by:

Kenya Medical Research Institute

CITI
Collaborative Institutional Training Initiative

Verify at www.citiprogram.org/verify/?w96e20d86-64eb-4977-a068-c8f5581078c9-23618191

APPENDIX VIII

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **994240**

Date of Issue: **06/November/2019**

RESEARCH LICENSE

This is to Certify that Mr.. Edwin Magomere of Egerton University, has been licensed to conduct research in Nairobi on the topic: CHARACTERIZATION OF HIV DRUG RESISTANCE MUTATIONS AND SUBTYPE DIVERSITY OF ISOLATES FROM CHILDREN AND ADOLESCENTS FAILING VIRAL SUPPRESSION for the period ending : 06/November/2020.

License No: **NACOSTI/P/19/2538**

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Director General
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