MOLECULAR CHARACTERIZATION OF HIV AND DRUG RESISTANCE MUTATIONS AMONG HIV POSITIVE CHILDREN ATTENDING BUSIA COUNTY REFERRAL HOSPITAL, KENYA

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Molecular Characterization of HIV and Drug Resistance Mutations Among HIV Positive Children Attending Busia County Referral Hospital, Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this thesis to my parents Mr & Mrs Jacob Lel, my husband Shadrack Yego, my children Joybell, Leroy, Kalya. Thanks for your entire support, words of encouragement and push for tenacity.

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AIDS	Acquired immunodeficiency syndrome
ANC	Antenatal Clinic
ART	Anti Retroviral Therapy
ARV	Antiretroviral Drug
CD4	Cluster of Differention 4
CXCR4	Chemokine receptor 4
CCR5	Chemokine receptor 5
cDNA	Complementary DNA
DNA	Deoxy Ribonucleic Acid
DNA pol	DNA Polymerase
dNTPs	Deoxynucleotide triphosphates
ddH_20	Double distilled water
EDTA	Ethylene diamine tetra acetic acid
EID	Early Infant Diagnosis
Env	Envelope gene
Gag	Group specific antigen gene

Gp41	Glycoprotein 41
Gp120	Glycoprotein 120
Gp160	Glycoprotein 160
HIV 1	Human Immunodeficiency Virus type 1
HIV 2	Human Immunodeficiency Virus type 2
ID	Identification
KAIS	Kenya AIDS Indicator Survey
KDHS	Kenya Demographic and Health Survey
KEMRI	Kenya Medical Research Institute
LTR	Long terminal repeats
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium chloride
mM	milli molar
MOH	Ministry of Health
nef:	Negative regulator factor
NRTI	Nucleoside reverse transcriptase inhibitor
NNTRI	Non Nucleoside reverse transcriptase inhibitor

PCR	Polymerase Chain Reaction
PI	Protease Inhibitors
PMTCT	Prevention of Mother-to-Child Transmission
Pol	polymerase gene
Rev	Regulator of virion protein expression
RNA	Ribonucleic acid
RPM	Revolutions per Minute
RT- PCR	Reverse Transcription Polymerase Chain Reaction
Taq	Thermus aquaticus
Tat	transactivator of transcription
µl	micro litre
µM	micro mol
UNAIDS	Joint United Nations Programme on HIV and AIDS
vif	viral infectivity factor
vpu	viral protein U
vpr	viral protein R
WHO	World Health Organization

ABSTRACT

Drug resistant Human Immunodeficiency Virus type 1 (HIV-1) variants are the main threat to current treatment programs. Overall, the emergence of HIV drug resistance is a complex and multifaceted problem, whose true extent has not been described in Kenya. Treatment failure is a major problem among those on antiretroviral therapy (ART) in Kenya. The resistance is associated with failure to adhere to therapy and low potency of some antiretroviral regimens. Monitoring HIV drug resistance is an important component of the World Health Organization's (WHO) global HIV program. It is important to monitor for drug resistance in HIV positive children whose mothers have been on ART. The objectives of this study were to determine HIV drug resistant mutations and subtype diversity in HIV positive children born to HIV infected mothers attending Busia County Referral Hospital. To achieve this, archived plasma collected in 2011 from 65 HIV positive children aged between 6 weeks and 5 years were used. Nucleic acid (RNA) was extracted from plasma samples using the Qiagen RNA extraction kit according to the manufacturers' instructions. One step reverse transcriptase-polymerase chain reaction (RT-PCR) and nested PCR were performed using primers targeting the protease and reverse transcriptase in the HIV-1 *pol* gene. Amplicons were further sequenced using the Big Dye chemistry, according to the manufacturer's instructions. The Stanford University HIV drug resistance database and International AIDS Society (IAS) algorithm were used to determine the presence of drug resistant mutations. HIV subtype diversity was determined using Basic local alignment search tool (BLAST) as well as clustal W alignment and neighbor joining methods. The results showed that 16% (9/53) of the children had resistance mutations against Nucleoside reverse transcriptase inhibitor drugs and 24 % (13/53) had resistance mutations against Non-nucleoside reverse transcriptase inhibitor drugs. For protease inhibitor (PI) related mutations, minor mutations were found in 41.5 % (22/53) of the children. HIV-1 subtypes found included subtype A in 64 % (34/53) of the population, while 17 % (9/53) were subtype D and 5.7 % (3/53) were subtype C. Possible

recombinants found included subtype A/C at 1.8 %(1/53), subtype A/D at 5.7 % (3/53), subtype K/A at 1.8 %(1/53) and subtype B/D 3.8 %(2/53). The observed drug resistant (DR) mutations call for continuous surveillance among children and establishment of HIV subtypes circulating among HIV infected mothers who are likely to transmit the virus to their infants.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Human immunodeficiency virus (HIV) disease continues to be a serious health issue globally. Advances have been made towards understanding HIV, its prevention and treatment. Despite this most people with HIV and those at risk continue to face challenges of access, prevention, care and treatment (Sepkowitz, 2006). Despite substantial progress in the development of strategies for the prevention of transmission of HIV-1 from mother to child, infection has continued to increase globally, particularly in resource-poor settings (Tudor *et al.,* 2013).

In 2015, 1.8 million children were living with HIV and 150,000 of the children were newly infected. Only 49% of this children accessed antiretroviral therapy (UNAIDS, 2016). The children mostly acquire HIV from their HIV positive mothers *in utero,* intrapartum (during labour) or (through breastfeeding) postpartum (Bennett *et al*., 2009; Lakshmi *et al.*, 2010).

Reduction in maternal viral load in breast milk and indirect infant prophylaxis by ingestion of antiretroviral drugs in breast milk are some of the mechanisms used to protect infants from getting infected by their mothers. Studies have shown that antiretroviral drugs taken by nursing women are present in breast milk (Moodley *et al.*, 2003, Mirochnick *et al.*, 2001).

Mothers who are not on medication can transmit HIV to their children at a range of 25%- 40%, while those on short-course antiretroviral drugs during the peripartum period transmit 8%-10% of HIV. For mothers on triple therapy throughout pregnancy, transmission is less than 5% (Dabis & Ekpini, 2002). Infants who become infected before or during breastfeeding with mothers on antiretroviral drugs in breast milk, may

induce the development of drug resistance due to viral replication in the presence of low drug concentration (Zeh *et al*., 2011).

Mutations that occur in the HIV genome and lead to resistance can be classified into primary and secondary mutations. Primary mutations lead to a several-fold decrease in sensitivity to one or more HIV drugs (Hirsch *et al.*, 2000; Hirsch & Richman., 2000). Secondary mutations may not result in a significant decrease in drug sensitivity but are associated with restoration of the original viral fitness in the presence of existing HIV drug inhibitors (Hirsch *et al* 2000; Hirsch & Richman, 2000).

Transmission of resistant HIV variants has serious implications for the progression to AIDS among infected infants since it might reduce the choices of active drugs (Moira *et al.,* 2007).This study intended to determine commonly transmitted HIV-1 drug resistance mutations in children attending Busia County Referral Hospital.

1.2 Statement of the Problem

In spite of remarkable achievements with the use of antiretroviral drugs, HIV drug resistance remains a significant barrier in effective control of HIV replication. Prevention, monitoring and response to HIV drug resistance is very critical in building and sustaining gains in HIV treatment scale-up, and achieving the global 90-90-90 targets for treatment (WHO,2017). These targets involves diagnosing 90% of all people with HIV infection, providing treatment to 90% of those diagnosed and making sure that 90% of infected people are on treatment (Maheu *et al*.,2017). Increase in the rates of transmitted HIV drug resistance among drug naïve populations, suboptimal viral suppression and poor adherence among drug experienced populations are becoming a real threat to treatment programs globally and in Kenya. Sub-optimal drug levels in breast milk exposes infants to increased selection of drug resistant HIV-1 populations. This complicates regimen options in pediatric cases (Zeh *et al*., 2011). There is need therefore to determine prevalence of HIV-1 drug resistance as well as diversity in order to address challenges in pediatric HIV management and recommend individualized care.

1.3 Justification of the study

In Kenya, there has been a rapid scale up of ART which has also been seen in prevention of mother to child transmission (PMTCT) programs (UNAIDS, 2014).This has led to challenges of emergence of drug resistance even in children. Resistance to HIV drugs is a major threat to treatment since it is associated with therapeutic failure. The other challenge with drug resistance is that it can be transmitted to newly infected individuals including from mother to child. This minimizes the availability of effective drugs for treatment (Dineke *et al.,* 2012). There is need for monitoring drug resistance patterns in HIV positive individuals including children, as HIV-1 genetic diversity is a hurdle in vaccine development and clinical management. HIV-1 subtypes may exhibit differences in drug resistance development, replication capacity, disease progression and rates of transmission (Ireen *et al.,* 2012). Many questions regarding the molecular evolution and genetic diversity of HIV in children remain unanswered. Therefore there is need for research to identify circulating subtypes and presence of drug resistance mutations in children population in Kenya. This study determined HIV-1 drug resistance mutations in children born to HIV-1 positive mothers in Busia County Referral Hospital and the HIV-1 subtypes circulating in this population. Such information provides insight on the HIV treatment options for the affected patients.

1.4 Research questions

1. What is the prevalence of HIV-1 drug resistance mutations in children of mothers who are on ART attending Busia County Referral Hospital?
2. What are the common HIV-1 drug resistance mutations in children of mothers who are on ART attending Busia County Referral Hospital?
3. What are the common HIV-1 subtypes circulating among children of mothers who are on ART attending Busia County Referral Hospital?

1.5 Objectives

1.5.1 General Objective

To determine HIV drug resistant mutations and subtype diversity in HIV positive children born to HIV positive mothers on ART attending Busia County Referral Hospital.

1.5.2 Specific Objectives

i. To determine the prevalence of HIV-1 drug resistant mutations in HIV positive children born to HIV-positive mothers on ART attending Busia County Referral Hospital.
ii. To characterize HIV-1 drug resistant mutations in HIV positive children born to HIV-1 positive mothers on ART attending Busia County Referral Hospital.
iii. To determine HIV-1 subtypes circulating among HIV positive children born to HIV-1 positive mothers on ART attending Busia County Referral Hospital.

CHAPTER TWO

LITERATURE REVIEW

2.1 Etiology of HIV/AIDS and transmission

Acquired immunodeficiency syndrome (AIDS) is a disease of the human immune system caused by infection with human immunodeficiency virus (HIV) (Sepkowitz, 2001).HIV is transmitted mainly in three ways: by sexual contact, by blood (through transfusion, blood products, or contaminated needles), or by passage from mother to child (WHO, 2014). Pregnant women infected with HIV can pass the infection to their babies *in utero* or during birth. Breast milk from infected mothers has also been shown to contain high levels of the virus (WHO, 2014).

2.2 Epidemiology of HIV

2.2.1 HIV globally

Since the beginning of the pandemic, around 76.1 million people worldwide have been infected and about 39 million have died of AIDS related causes. Approximately 35 million people were living with HIV in 2016 globally (UNAIDS, 2017). In 2016, 1.8 million people became newly infected with HIV worldwide with 160,000 of this being children. In the same year 1 million people died in the world from AIDS related causes. Eastern and Southern Africa remains most severely affected, with 19.4 million people living with HIV in 2016. Women account for 59% of this number and the incidence is especially high in adolescents and younger women (UNAIDS, 2017)**.** Estimates of 790,000 people in Eastern and Southern Africa region were newly infected in 2016 accounting for almost 43% of the global total of new HIV infections. A total of 77,000 of these new HIV infections were among children. People who died of AIDS related causes in the region were about 1 million in the same year (UNAIDS, 2017).

Western and Central Africa were also heavily affected in 2016 with 6.1 million people living with HIV infection (UNAIDS, 2017). Other areas significantly affected by HIV include Asia and the Pacific with 5.1 million people living with HIV and Latin America which had 1.8 million infected. Western and Central Europe and North America had 2.1 million people with HIV in 2016 while Eastern Europe and Central Asia had 1.6 million people infected. The Carribean had 310,000 with the virus while Middle East and North Africa had 230,000 people with HIV (UNAIDS, 2017).

2.2.2 HIV in Africa

In many parts of Africa, HIV/AIDS is a major public health concern and cause of death. Africa is home to about 15.2% of the world's population yet sub-Saharan Africa alone, the hardest hit region, accounts for more than two-thirds of people living with HIV (UNAIDS, 2014).

North Africa and countries in the Horn of Africa have lower HIV prevalence rates (Ali-Akbar *et al.,* 2007; Bozicevic, 2013). Most children (94%) with HIV live in Sub-Saharan Africa and almost all of the region's nations have their national HIV prevalence greater than 1%. Countries in Southern Africa region are worst affected on the continent (Gouws *et al.,* 2008). South Africa has the highest number of people living with HIV in the world (5.6 million) while Swaziland has the highest prevalence in the world which is 26.0% (Abu-Raddad *et al*., 2010; Iliffe**,** 2006).

2.2.3 HIV in Kenya

Human immunodeficiency virus (HIV) remains a major health burden in Kenya despite numerous efforts to contain the scourge. HIV prevalence in 2015 was 5.9% in Kenya with 1,517,707 people living with the virus. HIV prevalence among females was higher in this year at 6.4% (775,939) compared to males at 5.5% (643,598) .Children aged between 0 to 14 years had 98,170 of them living with HIV in 2015 (NACC, 2016).

HIV prevalence in Kenya also varied by region, with the highest prevalence in Nyanza region at 26%, and lowest prevalence in the North Eastern region, Wajir county at 0.4%. Kenya has an estimated 71,034 new HIV infection among adults and about 6613 among children annually (NACC, 2016).

2.2.4 HIV in Women and Children

Women represent about half of all people living with HIV worldwide and more than half in sub-Saharan Africa. HIV is the leading cause of death among women of reproductive age (Shetty, 2013). Gender inequalities, differential access to services, and sexual violence increase women's vulnerability to HIV (Mondal & Shitan, 2013). In 2016, about 76% of pregnant women living with HIV in low- and middle-income countries received ART to prevent HIV transmission to their infants (WHO,2017).

According to WHO, 2.1 million children were living with HIV in 2016 and the number who got newly infected in the same year was 160,000 with most of these children living in Sub-Saharan Africa. (UNAIDS, 2017).

In Kenya, 96% of women aged 15-54 years who reported a live birth within five years attended an antenatal clinic (ANC) during their pregnancy, and 92% of those who attended ANC were tested for HIV as part of their antenatal care (KAIS, 2012).

Transmission of HIV during breastfeeding still represents a major public health challenge in middle and low income countries like Kenya. A number of viral, maternal, immunological, genetic and maternal-infant host factors as well as type of infant feeding may influence the risk of transmission of HIV (Shetty, 2013). For HIV positive mothers in resource limited settings, breastfeeding is crucial for infant survival yet there is a considerable risk of HIV transmission (Kilewo *et al*., 2009). Through the prevention of mother to child transmission program, positive women are taught how to avoid transmitting the virus to their infants and the infants are also tested for HIV using PCR (KAIS, 2012; Schouten *et al.,* 2011). WHO recommends that infants should be breastfed

exclusively for the first six months postpartum while their mothers should be on ARV and complementary feeding can then follow (Onono *et al.,* 2014).

A study done in Kenya showed the safety and feasibility of maternal triple-antiretroviral regimen from late pregnancy through six months of breastfeeding for PMTCT when compared to single dose NVP regimen (Thomas *et al,* 2011).

2.3 HIV Classification

Human Immunodeficiency Virus (HIV) belongs to lentivirus family and one of a group of viruses known as retroviruses with single stranded RNA genome. HIV is classified into HIV-1 and HIV-2 which have similar genetic structures but differ up to 40% at the DNA sequence level (Robertson *et al.,* 2000). Globally HIV-1 has been responsible for most of the AIDS pandemic. However HIV-2 is also an important cause of disease in a number of regions in the world. At first it was dominantly found in West Africa but it has now spread to other parts of Africa, Europe, India and the United States (Omobolaji *et al.,* 2011).

During HIV replication there is a relatively high frequency of replication errors due to lack of proofreading function by the retroviral reverse transcriptase enzyme resulting in heterogeneity. Genetic recombination of HIV during replication also contributes to global genetic diversity (Jacobs *et al*, 2014). The distribution of HIV-1 subtypes is significant for epidemiological purposes as well as in clinical settings (Taylor *et al.,* 2008). Different subtypes have been shown to have some difference genotypically and phenotypically. The genetic variation of HIV-1 may influence diagnostic assays, viral replication capacity and antiretroviral therapy outcomes (Abecasis *et al.,* 2005; Camacho *et al.,* 2007).

The strains of HIV-1 are classified into three major groups due their variability: The "major or main" group M, the "outlier" group O and the "new" group N which are non-M and non-O. More than 90% of the HIV-1 infections worldwide belong to HIV-1

group M while group O and N are mainly restricted to central Africa (Roquebert *et al.,* 2009; Wainberg, 2004). HIV-1 group M is known to have at least nine genetically distinct subtypes (or clades) which include subtype A,B,C,D,G,H,J and K (Santos & Soares, 2010). In addition to subtypes there are subsubtypes which are made of groups of HIV-1 viral isolates with genetically and phylogenetically related sister clades. Examples of these include clade A classified to subsubtypes (A1, A2 ,A3,A4) and clade F classified into, subsubtypes F1 to F2 (Taylor *et al.,* 2008).

Patients co-infected with strains from two different subtypes can yield inter-subtype recombinants (Abecasis *et al*, 2013). Unique recombinant form (URFs) are inter-subtype recombinant genomes found only in one dually or multiply infected individual patient in which they arose. Circulating recombinant form (CRFs) are inter-subtype recombinant HIV-1 genomes transmitted to three or more people who are not epidemiologically related (Hemelaar *et al.,* 2011). To date 79 CRFs and several URFs have been identified according to the Los Alamos HIV database. The widespread of recombinant forms may affect the global pandemic of HIV-1 (Lau & Wong, 2013). The outcomes of various epidemiological studies have illustrated the presence of almost all subtypes, CRFs and several URFs in sub-Saharan Africa. For the other parts of the world it has been observed that HIV-1 subtypes have specific geographical distribution (Buonaguro *et al*., 2007; Jacobs *et al*, 2014).

Human Immunodeficiency Virus (HIV-1) subtypes A, B and C have been observed in studies to be the most prevalent forms globally and 50% of these infections have been found in subtype C. HIV-1 Subtype C is commonly found in Southern Africa and India (Jacobs *et al.,* 2009). In eastern and central Africa which includes countries like Kenya, Uganda, Tanzania and Rwanda, HIV-1 subtype A has been dominating. Subtype A has also been observed in eastern European countries. Subtype B is predominant in western and central Europe, America and Australia and has also been found in Southeast Asia, northern Africa and in the Middle East (Buonaguro *et al*., 2007).

2.4 The structure of HIV

Human immunodeficiency virus (HIV) is an enveloped RNA virus (**Figure 2.1**). Protruding from the envelope are peg-like structures that the viral RNA encodes (Pancera *et al*., 2010). Each peg is composed of three gp120 envelope glycoproteins attached noncovalenty to three gp41 transmembrane molecules. Nucleocapsid is found inside the envelope and is composed of protein and two enzymes which all play important roles in the HIV life cycle (Pancera *et al.,* 2010).

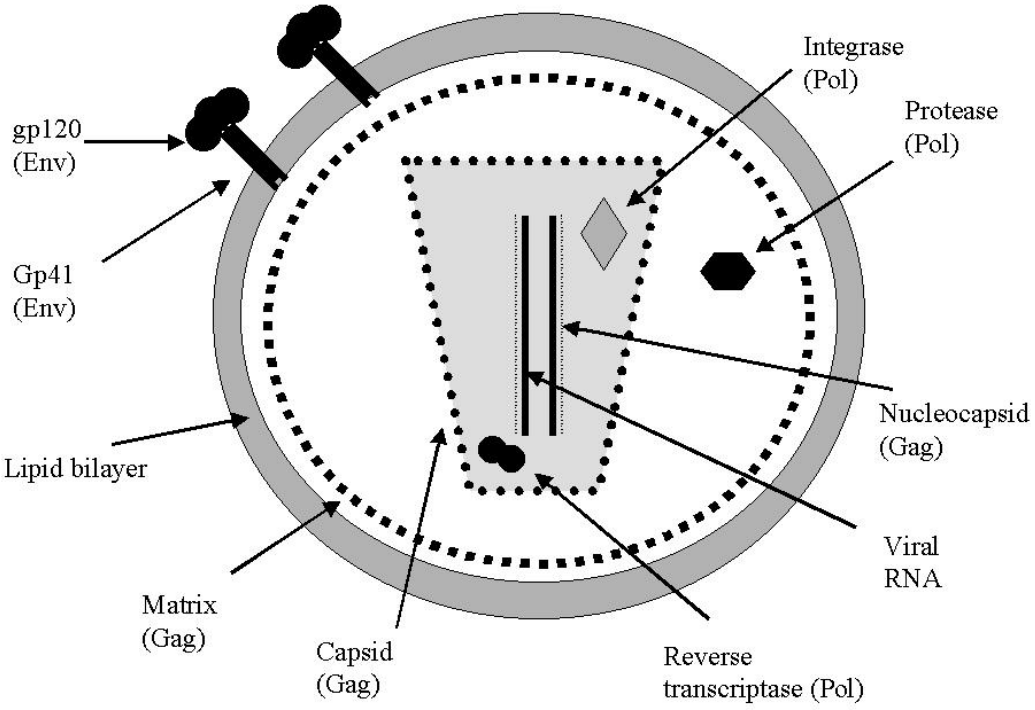

Figure 2.1: Human Immunodeficiency Virus structure (Derek & Mike, 2016)

2.4.1 HIV Genome organization

The human immunodeficiency virus (HIV) is composed of two copies of positive single-stranded RNA that codes for its nine genes. After viral entry into the host cell the viral RNA genome is reverse transcribed into a double-stranded DNA molecule (proviral

DNA). This proviral DNA is then integrated into the host DNA genome where its information is encoded into host DNA (Craigie & Bushman , 2012). The HIV genome at each end has long terminal repeats (LTR) which are repetitive sequence of bases that serve some structural and regulatory purposes (Guangdi *et al*., 2015). Three of the HIV genes, *gag, pol* and *env* are structural genes involved in development of structural proteins for new virus particles. The *gag* gene is a group specific antigen gene that encodes capsid proteins P24 and nucleocapsid P7 and matrix protein P17 and other internal proteins. The *pol* gene is a polymerase gene encoding the viral enzymes protease, reverse transcriptase and integrase. Reverse transcriptase copies the virus RNA genome into DNA. The protease processes proteins made from HIV genome and integrase gene integrates the DNA copy of HIV's genome into the host DNA. The *env* gene encodes *gp160* glycoprotein which is usually processed to give *gp120* an external glycoprotein and *gp41* a transmembrane glycoprotein (Rajarapu, 2014). The other six genes *tat, rev, nef, vif, vpr* and *vpu* are regulatory genes for proteins. They are involved in control of the ability of HIV to infect cells and replication. The *tat* encodes transactivator protein and it positively stimulates transcription. The *rev* encodes a regulator of expression of viral protein and stimulates the production of HIV proteins but suppresses the expression of HIV's regulatory genes. The *nef* gene encodes the negative regulator protein which retards HIV replication. The *vif* is the virion infectivity factor and suppresses resistance to HIV infection by the host. The *vpr* encodes viral protein R and accelerates HIV protein production. The *vpu* encodes viral protein u and enhances the assembly of new virus particles and budding from the host cell. It's also involved in degradation of CD4 proteins (**Figure 2.2**) (Watts *et al*., 2009).

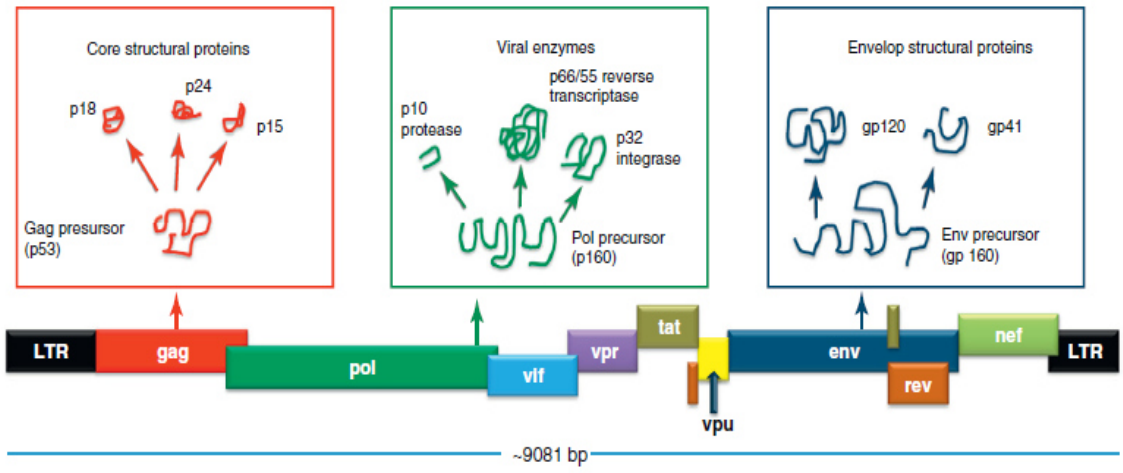

Figure 2.2: HIV 1 genome organization (Rajarapu , 2014)

2.4.2 Replication of HIV

Viral entry into the host cell is initiated by binding of *gp120* to the CD4 receptor on the cell surface. Binding to a second receptor is also required (Kondru *et al*, 2008). The CCR5 (chemokine receptor 5) is used by majority of primary HIV-1 strains while some viruses use CXCR4 (chemokine receptor 4) as a coreceptor and others use both CCR5 and CXCR4 as coreceptors (Ghalib, 2009).

Viral envelope fusion with the cell membrane occurs after the binding of HIV to the host cell allowing HIV RNA and enzymes to enter the cytoplasm. Reverse transcriptase enzyme enables the single stranded RNA of the virus to be copied leading to generation of double stranded DNA. This is followed by integration of the viral DNA into cellular chromosome facilitated by integrase enzyme (Francois & Allan, 2004). When the cell divides, HIV DNA (provirus) is replicated along with the chromosome. The integration of provirus into the host DNA provides the latency that enables the virus to evade host responses so effectively. Transcription of the provirus leads to production of viral RNA and viral proteins. This is followed by the assembly of the viral proteins using the host cell's protein making machinery (Sierra *et al.,* 2005). The protease enzyme then facilitates the processing of the newly translated polypeptides into proteins. Viral

proteins are then assembled using the host cell's protein-making machinery. The virus's protease enzyme allows for the processing of newly translated polypeptides into the proteins, which are then ultimately assembled into viral particles. The virus eventually buds out of the cell (Francois & Allan, 2004).

2.5 Antiretroviral Therapy for HIV Infection

Antiretroviral therapy is the use of pharmacologic agents (drugs) that have specific inhibitory effects on HIV replication. Highly active antiretroviral therapy (HAART) comprises of multi anti-HIV drugs and is therefore able to act on different HIV targets. The current HAART regimen suppresses viral replication and maintains the function of the immune system. This has led to a decrease in AIDS opportunistic infections and deaths (Lu & Chen, 2010; Arts & Hazuda, 2012).There has been a lot of improvement in the ART over the years which has resulted to the current therapy being more effective, easier to take and with fewer side effects (Broder, 2010). Currently the Food and Drug Administration has approved 31 antiretroviral drugs (ARVs) to treat HIV infection.

2.6 Mechanisms of Action of HIV Antiretroviral Drugs

Antiretroviral drugs act by inhibiting the viral enzymes critical to HIV replication cycle while others act on HIV host cell target (CCR5 or CCR4) which blocks the entry of the HIV virus into the cell. Currently, four classes of over 30 licensed antiretrovirals and combination regimens of these ARVs are in use clinically including: reverse transcriptase inhibitors (RTIs) (e.g. nucleoside reverse transcriptase inhibitors, NRTIs; and non-nucleoside reverse transcriptase inhibitors, NNRTIs), protease inhibitors (PIs), integrase inhibitors and entry inhibitors such as fusion inhibitors and CCR5 antagonists (Lu & Chen, 2010).

2.6.1 Reverse Transcriptase (RT) Inhibitors

Nucleoside reverse transcriptase inhibitors (NRTI) and nucleotide reverse transcriptase inhibitors (NtRTI) are nucleoside and nucleotide analogues which inhibit reverse transcription (Maxwell *et al.,* 2012). NRTIs are chain terminators such that once incorporated, work by preventing other nucleosides from also being incorporated because of the absence of a 3' OH group and both act as competitive substrate inhibitors. Examples of NRTIs include zidovudine (AZT or ZDV) stavudine (d4T), didanosine (ddI), abacavir (ABC), lamivudine (3TC) emtricitabine (FTC) and tenofovir (Kalyan, 2013). Non-Nucleoside reverse transcriptase inhibitors (NNRTI) inhibit reverse transcriptase by binding to an allosteric site of the enzyme The NNRTIs interact with a specific 'pocket' site of HIV-1 RT that is closely associated with, but distinct from, the NRTI binding site. NNRTIs act as non-competitive inhibitors of reverse transcriptase (Balzarini ,2004; Jonckheere *et al*,2000). NNRTIs examples include delavirdine (DLV), efavirenz (EFV) rilpivirine (RPV) Etravirine (ETR) and Nevirapine NVP (Kalyan, 2013; Mbuagbaw *et al.,* 2010). Mutations in HIV-1 RT structure can lead to the emergence of drug-resistant virus strains (Jonckheere *et al*., 2000; Castro *et al.,* 2006).

2.6.2 Protease Inhibitors

Protease inhibitors interfere with the protease enzyme by blocking it yet it is important in production of mature virions upon budding from the host membrane. Mostly these drugs prevent the cleavage of *gag* and *pol* precursor proteins and thus virus particles produced in their presence are defective and mostly non-infectious (Wensing, 2010). Examples of HIV protease inhibitors are lopinavir (LPV), indinavir (IDV) , nelfinavir (NFV), amprenavir (APV) fosamprenavir (FOS-APV), atazanavir (ATV), darunavir (DRV), ritonavir (RTV), saquinavir (SQV), tipranavir (TPV) and ritonavir. Maturation inhibitors have a similar effect by binding to *gag*. Resistance to some protease inhibitors

is high. Second generation drugs have been developed that are effective against otherwise resistant HIV variants (Gulick , 2003).

2.6.3 Fusion/Entry Inhibitors

This interferes with binding, fusion and entry of the virus to the host cellular membrane by blocking one of several targets. HIV requires binding to both the CD4 molecule and a co receptor to enable entry into the cell. The chemokine receptors CXCR4 and CCR5 are used as the main co-receptors (Schols , 2004). An example of this drug is maraviroc which works by targeting CCR5. This co-receptor is located on human helper T-cells though there can be a shift in tropism which allows HIV to target an alternative co-receptor such as CXCR4. Some individuals may have a mutation in the CCR5 delta gene which results in a nonfunctional CCR5 co-receptor and resulting in resistance or slow progression of the disease (Sharon & Lieberman-Blum, 2008)**.** Another example of this drug is Fuzeon (T20) which is a peptide drug and works by preventing fusion of the virus with the host membrane (Bai, 2013).

2.6.4 Integrase Inhibitors

Integrase enzyme is inhibited by integrase inhibitors. Examples of these drugs are raltegravir (RAL), dolutegravir (DTG) and elvitegravir (EVG) (Quashie, 2013). These three integrase inhibitors bind to the catalytic core (amino acids 50-212) of the integrase enzyme and target the integration process at the strand transfer step (Pinar &Anderson, 2014).

2.7 The HIV Combination therapy

Besides long-term side effects and suboptimal drug potency, the emergence of resistant virus and the necessity of perfect therapy adherence are major concerns for obtaining a sustained control of viral replication. HIV lacks proofreading enzymes to correct errors made when it converts its RNA into DNA via reverse transcription. Its short life-cycle

and high error rate cause the virus to mutate rapidly, resulting in a high genetic variability of HIV which then leads to resistance to ARVs. To combat virus strains from becoming resistant to specific antiretroviral drugs, HIV therapy involves taking a combination of antiretroviral drugs known as highly active antiretroviral therapy (HAART) (Waters *et al.,* 2013). Combinations of antiretrovirals create multiple obstacles to HIV replication to keep the number of offspring low and reduce the possibility of a superior mutation. If a mutation that conveys resistance to one of the drugs being taken arises, the other drugs continue to suppress reproduction of that mutation (Van ,2001). Examples of combined drugs include epzicom (ABC + 3TC), trizivir (ABC + AZT+3TC), combivir (AZT + 3TC) and truvada (TDF + FTC).

2.8 Global Prevalence of HIV Drug resistance

By the end of 2016, 19.5 million HIV infected individuals were on ART globally. The numbers of those initiating treatment and those maintained on treatment are expected to increase in order to achieve global targets of epidemic control. However, the increase in numbers of people on treatment comes with the challenge of drug resistance (Zhou *et al*.,2016).

Pre treatment drug resistance to NNRTI was predicted to have reached different levels in 2016. In East Africa it had reached 15.5%,Latin America had 15%,11% in Southern Africa and 7.2% in West and Central Africa (WHO, 2017). Prevalence of NNRTI resistance exceeding 10% in a country requires a public health response according to WHO (WHO, 2017). Surveys were conducted on pre treatment drug resistance by WHO between 2014 and 2016. This indicated that all low and middle income countries had HIV drug resistance prevalence approaching or above the 10% mark. NNRTI resistance ranged from 8.1% in Cameroon to 15.4% in Uganda. HIV drug resistance prevalence of any drug class was 9.8% in Brazil and 23.4% in Nicaragua (WHO, 2017).

2.9 Development of Drug Resistance

Due to the introduction of highly active antiretroviral therapy in the mid- 1990's HIV related morbidities have reduced and the lifespan of people living with HIV has also been prolonged. However, the broad use of multiple antiretroviral drugs has led to drug resistance which is a common cause of treatment failure and also to transmission of drug resistant viruses (Obiako, 2010). HIV-1 is known to possess a high mutation rate and recombination of high frequency and the outcome of this could be rapid emergence of drug-resistant variants due to the viral replication not being inhibited sufficiently (Pinar & Anderson, 2014). There are also other factors that have been associated with emergence of drug-resistant HIV viruses including patient adherence to drugs, drug pharmacological factors and host immune response pressure (Obiako, 2010; Weber *et al.,* 2011).

To monitor disease progression in HIV infected individuals and measure ARV treatment success, viral replication in blood (plasma viral load) is measured and also the levels of $CD4^+$ T cells. A rise in viral load above 200 copies/ml might be a sign of emergence of drug resistance mutations (Paredes & Clotet, 2010). Prior to designing new antiretroviral regimens after treatment failure, detection and quantification of drug resistance have to be done (Weber *et al.,*2011). Drug resistance testing which involves genotyping and phenotyping techniques are crucial in the management of antiretroviral therapy since they guide the clinician in finding better drug choices and combinations with minimum risk of drug resistance (Obiako, 2010; Paul & Jorden, 2003).

2.10 Types of HIV drug resistance

Human Immunodeficiency Virus (HIV) drug resistance occurs when there is a change (mutation) in the genetic structure of HIV. This interferes with the blocking of replication of the virus by antiretroviral drugs. The emergence of drug resistant virus posses a risk of ARV drugs becoming partially or fully inactive.HIV drug resistance is divided into three main categories (WHO,2017). First is the acquired HIV drug

resistance (ADR) which emerges due to viral replication in patients receiving ARV drugs.This occurs mainly because of medication nonadherence and suboptimal treatment (De Luca & Zazzi, 2015). Secondly there is transmitted HIV drug resistance (TDR) which happens when uinfected ARV naive individuals are infected with HIV that already has drug resistance mutations (De Luca & Zazzi,2015). Thirdly is the pretreatment HIV drug resistance(PDR) which is detected at initiation of first line ART or at re initiation of first line ART. Pretreatment drug resistance can either be transmitted or acquired drug resistance and it can also be both (Kityo C., 2017).

2.11 Genotypic and Phenotypic Measurement of Drug Resistance

Genotyping tests mainly comprise polymerase chain reaction (PCR) amplification and nucleotide sequencing of protease, reverse transcriptase and integrase genes allowing the detection of resistance associated mutations in the genome (Pinar & Anderson, 2014). Phenotyping is a more direct method that involves testing the ability of a patient derived virus to replicate in the presence of antiretroviral drugs in a cell-based assay (Weber *et al.,* 2011). HIV is grown in peripheral blood mononuclear cells maintained in RPMI 1640 medium in the presence of different concentrations of antiretroviral drugs and thereafter viral growth is observed and 50% tissue culture infective dose ($TCID_{50}$) is determined (Weber *et al.,* 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area and Population

The study was conducted in Busia County Referral Hospital. Study participants were drawn from the population of children coming to the PMTCT clinic with their mothers. Children attending PMTCT are usually between 6 weeks to 5 years. The hospital is situated at the Kenya-Uganda border as shown in the **figure 3.1** below.

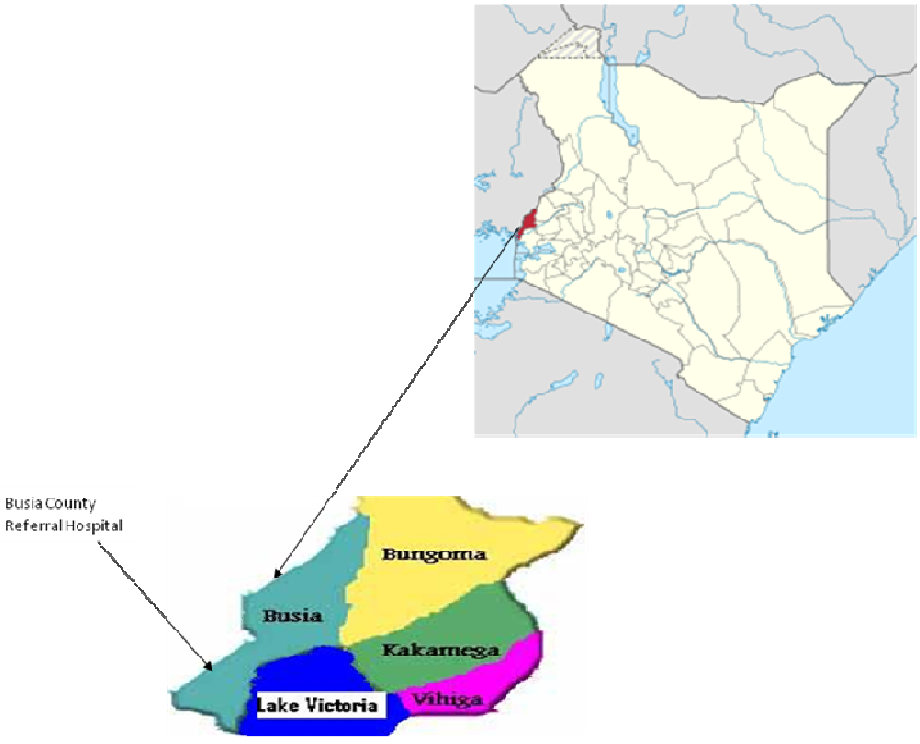

Figure 3.1: Busia County Referral Hospital Location (NCPD,2017)

3.2 Study Design

This was a retrospective study. Samples had been collected from HIV positive children in 2011.

3.3 Sample Size Determination

Using Fischer's method (Fischer *et al.,* 1998) the minimal sample size was calculated

$$N = \frac{Z^2 \times p \times q}{d^2}$$

N = Minimum sample size required

Z = Z-score for normal standard deviation for a 95% confidence interval (1.96)

p = HIV drug resistance mutation prevalence in children - 4%

(Van Zyl *et al*, 2010)

q = (1-P)

d = Significance level at 95% confidence interval (0.05)

$$N = \frac{(1.96)^2 \times 0.04 \times 0.96}{(0.05)^2} = 58.9 = 59$$

The minimum sample size was 59.

3.4 Inclusion Criteria

1. HIV positive children (confirmed by PCR)
2. Children whose mothers had been on ART
3. Children who had not been on ART.
4. Children who had detectable viral load ≥ 1000 copies/ml

3.5 Exclusion Criteria

1. Children who were HIV negative
2. Those whose parents had not consented

3.6 Assumption

It was assumed that the children in the study were between 6 weeks and 5 years which are the ages for testing HIV exposed children in PMTCT.

3.7 Laboratory Procedures

3.7.1 Sample collection

The samples used in this study had been collected as whole blood from the study participants at the health facility. They were then transported to Kenya Medical Research Institute Nairobi in a cool box. In the laboratory, they were centrifuged and plasma stored at -80°C till use.

3.7.2 Nucleic acid (RNA) Extraction

Ribonucleic acid (RNA) was extracted from plasma samples using the Qiagen RNA extraction kit (QIAamp® Viral RNA mini kit Qiagen RNA extraction protocol) according to the manufacturers' instructions. Briefly, 560 µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube. One hundred and forty microliters of plasma was added to the Buffer AVL-carrier RNA in the

microcentrifuge tube. This was mixed by pulse-vortexing for 15 seconds and incubated at room temperature (15-25°C) for 10 minutes. The sample had 560 µl of ethanol (96-100%) added to it and mixed by pulse vortexing for l5 seconds. Six hundred and thirty (630) µl of the extract was added to a QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 6000 x g (8000 rpm) for 1 minute and the supernatant discarded. The QIAamp spin column was placed into a clean 2 ml collection tube. Five hundred (500) µl of wash buffer (Buffer AW1) was added and centrifuged at 6000 x g (8000 rpm) for 1 minute. The supernatant was discarded and 500 µl of wash buffer (Buffer AW2) added and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini spin column was placed in a new 2 ml collection tube. Sixty (60) µl of elution buffer (Buffer AVE) equilibrated to room temperature was added and incubated at room temperature for 1 min. This was then centrifuged at 6000 x g (8000 rpm) for 1 min to elute the viral RNA. This RNA was kept at -80^{o}C till use (Burchard *et al*., 2014).

3.7.3 Reverse Transcription Polymerase Chain Reaction (RT PCR)

Ribonucleic acid (RNA) template was thawed together with master mix reagents. Depending on the number of samples, a master mix was prepared (**Table 3.1**).

Table 3.1: Reverse Transcription Polymerase Chain Reaction master mix

Component	**Volume**
RNase free water	5µl
5x Qiagen one step RT PCR buffer	5µl
dNTP mix (10Mm of each dNTP)	2 µl
Forward primer (0.6 µM)	1 µl
Reverse primer (0.6 µM)	1 µl
RT PCR enzyme mix	1 µl
RNase inhibitor	5µl
Total volume	20 µl

The master mix was then mixed thoroughly and 20µl dispensed to PCR tubes. The RNA template (5µl) was added to the individual PCR tubes containing the master mix. Negative and positive controls were included and the master mix was then loaded onto

the thermocycler under the following programmed conditions; reverse transcription at 50°C for 30 minutes; Initial PCR activation step 95°C for 15 minutes; 30 cycles of: denaturation for1 minute at 94°C, annealing 1 minute at 55°C; extension for 1 minute at 72°C. Final extension for 10 minutes 72°C. (Nyombi *et al*., 2008).

A portion of the HIV *pol* gene was amplified by nested polymerase chain reaction. This included protease sequence (*pol*-PR) corresponding to nucleotide position 2253 – 2550 in HIV-1_{HXB2} genome and reverse transcriptase sequence *pol*-RT corresponding to nucleotide position 2550 – 3247 in HIV-1_{HXB2} genome The primers specifications (Koizumi *et al*., 2006) used in this experiment were as follows.

Table 3.2: Protease region primers

First PCR primers
Nyupol 7(5'- GGGAATTTTCTTCAGAGCAG-3') Forward primer
Nyupol 8(5'-TCTTCTGTCAATGGCCATTGT-3') Reverse primer
Nested PCR primers
Nyupol 9 (5'-TCCTTAACTTCCCTCAAATCACT-3') Forward primer
Nyupol 10(5'-CTGGCACGGTTTCAATAGGACT-3') Reverse primer

Table 3.3: Reverse transcriptase (RT) region primer pairs

First PCR primers
RT 18(5'-GGAAACCAAAAATGATAGGGGGAATTGGAGG-3') Forward primer
KS 104(5'-TGATTGCCCAATTTAGTTTTCCCACTAA-3') Reverse primer
Nested PCR primers
KS101(5'-GTAGGACCTACACCTGTTCAACATAATTGGAAG-3')Forward primer
KS102(5'-CCCATCCAAAGAAATGGAGGAGGTTCTTTCTGATC-3')Reverse primer

3.7.4 Nesting PCR

The starting template for the nesting PCR was DNA from one-step RT PCR. All the reagents were thawed at room temperature and placed on ice bucket. The master mix was made using reagents shown in **table 3.4** (Nyamache *et al*., 2011; Koizumi *et al*., 2006).

Table 3.4: Nested PCR master mix

Component	Volume
25mM $MgCl_2$	2.8 µl
10mM dNTPs	2.0 µl
0.5 units Taq polymerase	0.2 µl
10 x PCR buffer	2.0 µl
RNase free water	10.2µl
10µM forward primer	0.4µl
10µM reverse primer	0.4µl
Total	18 µl

Two (2 µl) of DNA template (from one-step RT PCR) was pipetted into each correctly labeled PCR reaction tube and 18µl of the master mix added. The PCR tubes containing the samples were placed into the thermocycler under the following programmed conditions; Initial PCR activation step 95°C for 10 minutes; 30 cycles of: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C; extension for 1 minute at 72°C. Final extension for 10 minutes at 72°C (Nyamache *et al*., 2011).

3.7.5 Detection by Gel electrophoresis

After DNA amplification the PCR products were run on a 2% agarose gel and electrophoresed at a voltage of 100 for 20 minutes. Staining was done with ethidium bromide solution for 20 minutes and thereafter ultra violet transilluminator used to visualize the PCR products. (Kiptoo *et al.,* 2013).

3.7.6 Sequencing PCR

Using the second set of primers, the generated amplicons from the second nested PCR were sequenced. A reaction mixture containing the following reagents was used for sequencing PCR: Thirteen (13) µl RNase free double distilled H_2O,5x sequencing buffer,1.5 µl primers of 1 µM sense and antisense primer,1.0 µl big dye terminator and 1.0 µl PCR product making up a total reaction volume of 20 µl. The PCR tubes containing the samples were placed into the thermocycler under the following programmed conditions; Initial PCR activation step 96°C for 2 minutes; 30 cycles of : denaturation for 10 seconds at 96°C, annealing for 10 seconds at 50°C; extension for 4 minute at 60°C and thereafter the samples were held at 4°C (Kiptoo *et al.,*2013).

3.7.7 Purification of PCR products for Sequencing

The following reagents were added to a new 1.5ml microtube: 20 µl of the sample,2 µl of 125Mm EDTA, 2 µl 3M sodium acetate and 50 µl 100% ethanol .This was then vortexed and kept at room temperature for 15 minutes .Centrifugation was done at 14,000 rpm for 20 minutes. The supernatant was discarded, 70 µl of 70% ethanol added and mixed gently. Centrifugation at 14,000 rpm for 10min was done. Supernatant was discarded and sample dried at room temperature for about 10 min. This was followed by addition of 25 µl of HiDi formaminde to the sample then vortexing and spinning down. Sample was then incubated at 95 ºC for 2 min then placed on ice for 10 min. The sample was span down and transferred to sequencing tube and loaded into the ABI 310 DNA sequence for sequencing(Applied biosystems, Foster City, CA) (Kiptoo *et al.,* 2013).

3.8 HIV-1 subtyping, drug resistance testing and phylogenetic analysis

The Sequences derived in this study were subjected to BLAST and REGA search tools to determine subtype diversity. Stanford University HIV database and the International AIDS Society (IAS) algorithm were used to find out if there were any mutations in the

sequences. This was consequently used to determine the prevalence and characterization of drug resistance mutations present.

Reference sequences were selected from Los Alamos HIV database. The sequences were aligned and gaps removed using BioEdit. A phylogenetic tree was constructed using neighbour joining method in MEGA 6 software (Tamura *et al*.,2013;Foley *et al*.,2015). Clustal W profile alignment option was used to compare phylogenetic relationships between the sequences derived from the study with those from Los Alamos sequence database. Two parameter method of Kimura was used to calculate the genetic distances and reliability was estimated by 1000 bootstrap. The tree profile was visualized with tree view PPC version 1.65 (Kiptoo *et al.,* 2013; Foley *et al*., 2015).

3.9 Data management

Data was stored and archived in excel database in a safe and secure manner during and after the conclusion of the research project. Data was handled with integrity since it addresses concerns related to confidentiality, security and retention of research data. Electronic data was handled with integrity to ensure that data was not altered, erased, lost or accessed by unauthorized users. This softcopy files were protected by a password and data backups were made on external hard disks.

3.10 Ethical consideration

Approvals for the study were sought from the KEMRI Scientific Steering Committee (SSC No.2553) and the KEMRI National Ethical Review Committee (ERC) (Appendix 1 and appendix 2). Confidentiality was maintained by using codes for sample labelling instead of names.

CHAPTER FOUR

RESULTS

4.1 Study Population

Sixty five samples were available for this study. Nucleic acid (RNA) from all samples was extracted and amplified.

4.2 Analysis of PCR products

After amplification of the 65 samples, they were run in a gel for identification .Presence of bands with the right size of DNA (297 bp for protease and 697 for Reverse Transcriptase) was an indication of successful amplification (**Fig 4.1 and 4.2**).

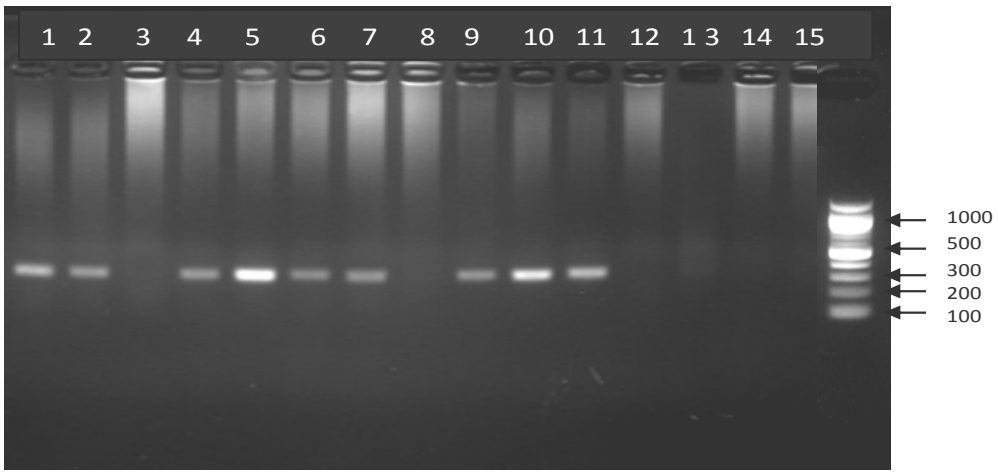

Figure 4.1: Agarose gel electrophoresis profile of *pol*-protease gene, Lane 1-Positive control, Lane 2-14 – PCR products, Lane 15 - Negative control, Last lane - Molecular marker (100 base pairs).

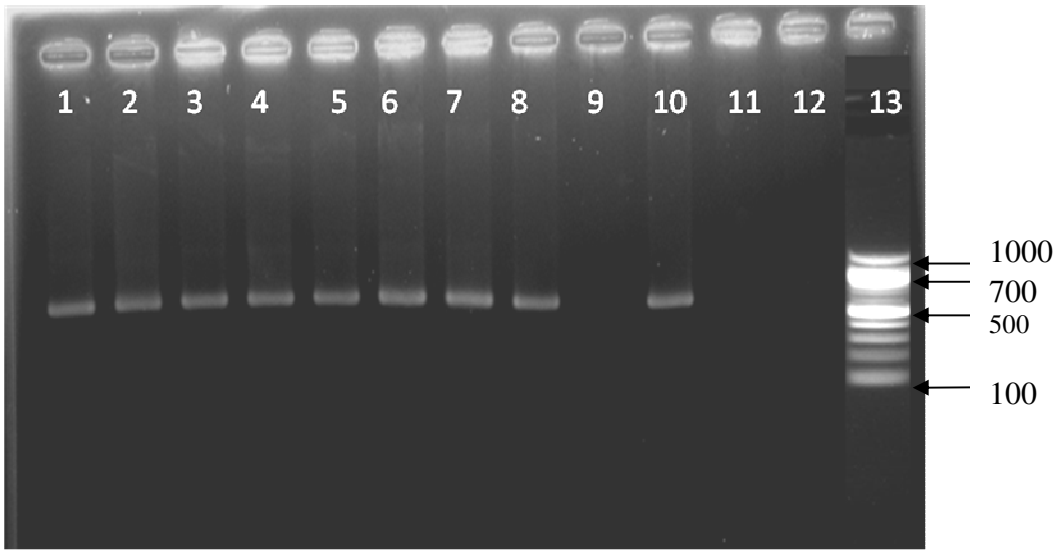

Figure 4.2: Agarose gel electrophoresis profile of *pol*-reverse transcriptase gene, Lane 1-Positive control, Lane 2-11 – PCR products, Lane 12 - Negative control, Lane 13 - Molecular marker (100 base pairs).

After electrophoresis 53 samples were found to have amplified successfully.

4.3 HIV-1 drug resistant mutations found in the study

The prevalence of reverse transcriptase (RT) drug resistant mutations in this study was 24 % (13/53). No major protease inhibitor (PI) related mutations were found. Minor PI mutations were found in 41.5 % (22/53) of the samples at position L101/V. With in-depth analysis of the reverse transcriptase region, major and minor mutations for the Nucleoside reverse transcriptase inhibitors (NRTI) and Non-Nucleoside reverse transcriptase inhibitors (NNRTI) were found. Mutations associated with resistance to non-nucleoside reverse transcriptase inhibitors (NNRTI) were the most frequent with 13 of the children being found with resistant mutations (24%, 13/53).

The results showed that 16 % (9/53) samples had HIV viruses with mutations against the common NRTI drugs such as lamivudine (M184VI), abacavir (M184V), tenofovir (K70E) and Zidovudine (M41L; K70R; L210W; K219Q). Mutation M184V was the

most common among NRTI being found in seven cases. Thirteen (24%) of the samples were found with mutations resistant to NNRTI drugs like efavirenz (K103N) and nevirapine (K103N; V108I). Mutation K103N was the most common among NNRTI and was found in seven cases. Mutations against both NRTI and NNRTI drugs were found in 16% (9/53) of the samples. Minor NNRTI mutations was found in 3.8 % (2/53) of the population and this was E138A mutation which causes low level resistance to rilpivirine a NNRTI drug. (**Table 4.1**).

Table 4.1: HIV-1 drug resistance associated mutations found in the study

	Major Mutations	
Sequence ID	**NRTI Mutations**	**NNRTI Mutations**
PaedBusia 001	None	K103KN
PaedBusia 004	M41L, T69N, K70R, V75M, M184V, L210W, T215Y	L100I, K103N, V179T
PaedBusia 005	M41L, T69N, V75M, M184V, T215N, K219N	K103N, V106I, V108I, P225H, M230
PaedBusia 007	M184V	K103N
PaedBusia 008	M184V	K103N, V108I, K238T
PaedBusia 010	D67G, K70R, M184V, K219Q	K101E, G190S
PaedBusia 011	M184V	K103N, P225H
PaedBusia 013	A62AV, T69NT, V75I, F77FL, F116Y, Q151KLMQ, M184IM	K103KN, Y181
PaedBusia 014	None	K103KN
PaedBusia 015	V75M, M184I, T215N	K103N, V106I, M230L
PaedBusia 019	None	K103N
PaedBusia 046	M41LM, M184V	V106A, F227L
PaedBusia 051	None	K103KN
	Minor mutations	
PaedBusia 37	None	E138A
PaedBusia 041	None	E138A

NRTI : Nucleoside reverse transcriptase inhibitor; **NNTRI:**Non Nucleoside reverse transcriptase inhibitor

4.4 HIV-1 subtype distribution among the children

All the sequences derived in this population were found to be of HIV-1 group M. They were further classified into subtypes. Subtype A was observed in 64 % (34/53) of the population of the children making it the most prevalent in this population. Subtype D was found in 17 % (9/53) while 5.7 % (3/53) of the population had subtype C. Possible recombinants found included subtype A/C at 1.8 %(1/53), subtype A/D at 5.7 % (3/53), subtype K/A at 1.8 %(1/53) and subtype B/D 3.8 %(2/53) (**Table 4.2**).

Table 4.2 Distribution of HIV-1 subtypes among HIV-infected children in Busia County Referral Hospital

Subtype	No. of samples
Subtype A	34
Subtype D	9
Subtype C	3
Subtype A/C	1
Subtype A/D	3
Subtype K/A	1
Subtype B/D	2

4.5 Phylogenetic analysis

Phylogenetic tree is illustrated in **Figure 4.3** with reference sequences labelled using their gene bank accession number and country of origin sequences .Study sequences with HIV-1 subtype A clustered with those of Cyprus (CP), Congo (CNG), Uganda (UG) and Kenya (KE).The samples with HIV-1 subtype C clustered with those of Tanzania (TZ) ,South Africa (SA), Ethiopia (ETP) and Malawi (ML) .Samples with HIV-1 Subtype D clustered with those of Tanzania, Congo and Uganda.

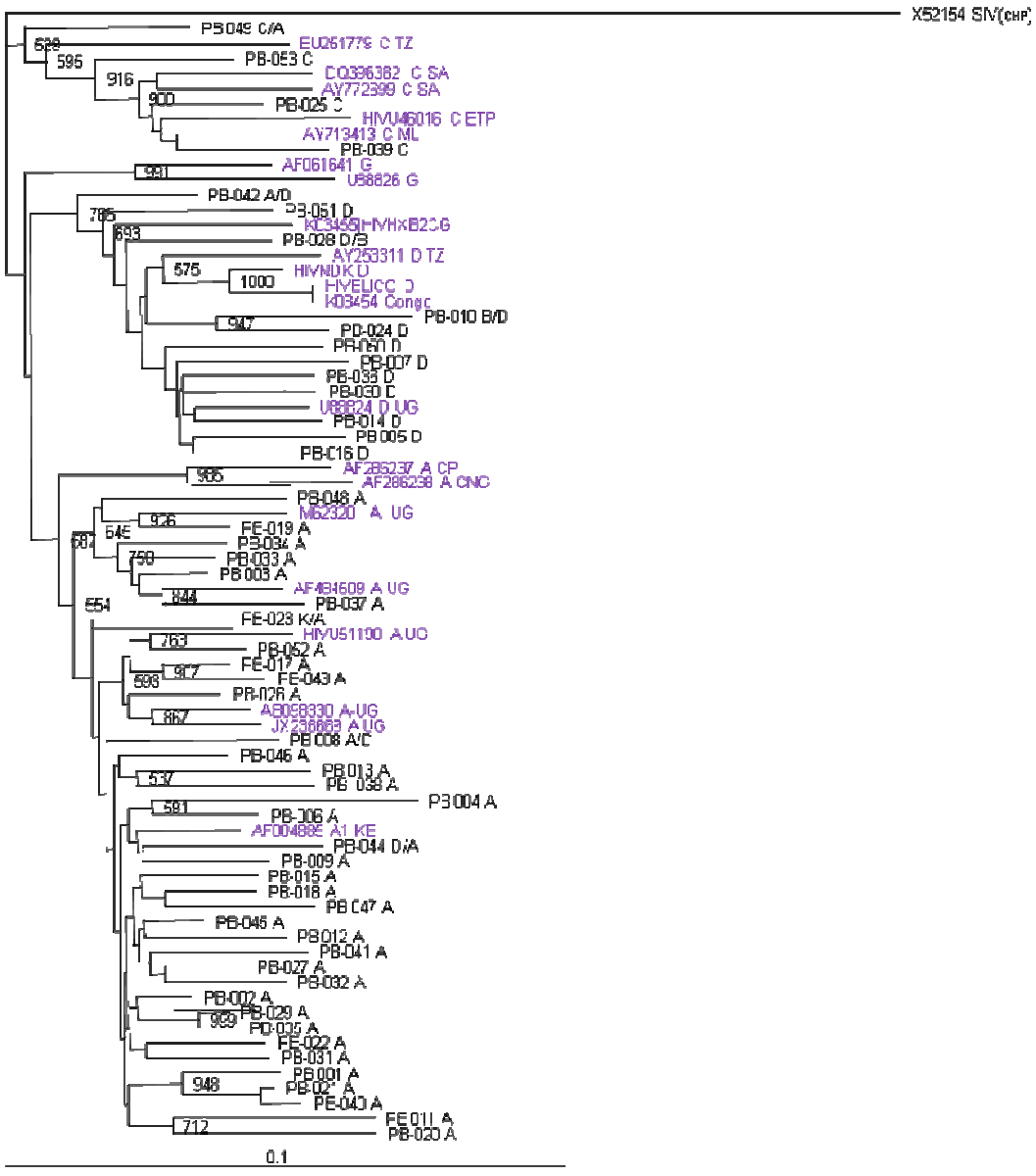

Figure 4.3: A rooted Phylogenetic tree illustrating the evolutionary relationships of HIV-1 subtypes found in the study in relation to reference sequence from Los Alamos database. The internal branches defining a subtype were supported by 1000 bootstraps. Reference sequences are in purple.

CHAPTER FIVE

DISCUSSION ,CONCLUSION AND RECOMMENDATION

5.1 Discussion

5.1.1 HIV-1 Drug Resistant Mutations and Its Impact

The children in this study most likely were infected with HIV vertically from their mothers. They were found to have developed drug resistance mutations despite not having initiated antiretroviral therapy at the time their samples were collected. In infants who become infected before or during breastfeeding, the presence of antiretroviral drugs in breast milk have been suggested to induce the development of drug resistance due to viral replication in the presence of low drug concentration (Zeh *et al*.,2011) Antiretroviral drug resistance has been shown to reduce available treatment options and increases the chances of virological and clinical failure (Michael *et al*., 2008).

This study showed minor protease inhibitor (PI) mutations to be present in the population. This was L101/V mutation that was found in majority of the study participants. This is a accessory (polymorphic) mutation and when present with other PI resistance mutations it may increase virus replication or reduce PI susceptibility (Johnson *et al* , 2013).

Some of the NRTI mutations found included M41L which was detected in two participants. This mutation is known to increase zidovudine (AZT) resistance when present with T215Y or T215F. This mutation has been observed in HIV subtype D individuals in another study (Shafer *et al*., 2007). Mutation D67E was observed in one child in the study. Mutation D67E/G is known to occur in heavily treated patients (Johnson *et al*, 2013). Mutation T69N observed in two cases causes zalcitibine (ddC) and didanosine (ddI) resistance and may cause low level stavudine (d4T) resistance particularly when present in isolates with zidovudine (AZT) mutation (Johnson *et al* , 2013).This mutation in some studies has been observed in persons with subtype B and F sequences (Shafer *et al*.,2007). Mutation V75M also observed in two cases is known to

cause d4T resistance and may cause low level resistance to ddC and ddI (Johnson *et al* , 2013). Fifteen percent (8/53) of the study participants harbored M184V/I making it the highest NRTI mutation present in this population. Mutation M184V is among a group of mutations that promote resistance by selectively impairing the ability of reverse transcriptase to incorporate an analogue into DNA (François & Allan, 2004). In M184V mutation, methionine is replaced by valine at position 184 of the reverse transcriptase. This mutation causes high level resistance to (3TC) lamivudine and with use of lamivudine as single agent, resistant strains overtake wild-type virus in a few weeks. Mutation M184V almost always emerges as the first mutation when lamivudine is used as part of a failing regimen of HAART (François & Allan, 2004). Mutation M184V also causes low level resistance to ddC, ddI and (ABC) Abacavir. It also partially suppresses T215Y mediated AZT resistance (Johnson *et al* , 2013). Mutation L210W seen in one case in this study is known to increase AZT resistance when present with mutations at position 215 (Johnson *et al* , 2013). Mutation K219Q which is known to increase AZT resistance when present with K70R or T215Y/F was found in one child in this study. Mutation K219N which occurs commonly in heavily treated NRTI patients was found in one participant (Johnson *et al* , 2013).

Out of all NRTIs detected in the study, six were found to belong to thymidine analogue mutations (TAMs) family. Major TAMs observed in this study included M41L and T215Y which are associated with higher levels of resistance (Jiong *et al.,* 2013). Other TAMs present were K70R, L210W and K219Q. The TAMs are known to reduce the antiviral activity of both d4T and AZT and in NRTI exposed patients, TAMs M41L, L210W and T215Y can lead to increased susceptibility to NNRTIs (Robert *et al*, 2001). Mutation M41L+T215Y have been shown to reduce the stimulatory potential of NVP and EFV (Jiong *et al.,* 2013). Some study observed K70R in persons with subtype G while V75M has been found in subtype CRF01_AE individuals and K219Q was found in subtype D individuals (Shafer *et al*., 2007).

It has been observed that people with NNRTI resistance have challenges in achieving viral suppression, more prone to virological failure or death, more likely to discontinue treatment and more likelly to acquire new HIV drug resistant mutations (WHO,2017).The prevalence of NNRTI mutations was high in this study which is similar to a study that has shown NNRTI mutations to be highest in drug naïve patients when compared to the other classes of antiretroviral drugs (Omobosola, 2004). Among the NNRTI mutations observed in this study K103N mutation was the highest. This is similar to a study where K103N and Y181C mutations has been found be the most common NNRTI mutations (Arts & Hazuda, 2012). Mutation K103N is a major resistance mutation among NNRTIs and is known to confer resistance to almost all drugs in this class. This mutation causes resistance to nevirapine (NVP) which has been in use in many parts of Africa for the prevention of mother to child transmission of HIV-1.This explains the existence of such a mutation in this population (Omobosola, 2004). Mutation K103N also causes resistance to DLV and EFV (Johnson *et al* , 2013). One child in the study harbored K101E which is a nonpolymorphic mutation associated with intermediate resistance to each of the NNRTIs. (Wang *et al*., 2010). Mutation K101E in combination with M184I is known to reduce susceptibility to RPV (Tambuyzer *et al*., 2010, Reuman *et al*., 2010).

Mutation V106A which was observed in one child is a nonpolymorphic mutation that causes high level resistance to NVP, intermediate resistance to DLV and low level resistance to EFV (Parkin *et al*., 2004). A combination of V106A with F227L which was observed in one case in the study causes high level resistance to both NVP and EFV (Melikian *et al.,* 2014). Mutation V106I which was also found in one case is a polymorphic accessory mutation that occurs occasionally in patients receiving NNRTI and in combination with V179D it causes low-level resistance to NVP, EFV, ETR (Johnson *et al.*, 2013; Gatanaga *et al.,* 2010). Mutation V108I observed in two cases is a relatively nonpolymorphic mutation which causes low level resistance NVP and EFV (Wu *et al.,*2012) while G190S a nonpolymorphic mutation was found in one child and has been known to cause high level resistance to NVP and EFV (Wang *et al*.,2010).

Mutation P225H which is a secondary mutation was observed in two cases in the study and has been associated with EFV resistance when present with K103N (Bacheler *et al.,* 2000). It confers hyper susceptibility to DLV (Johnson *et al*, 2013; Bacheler *et al.,* 2000). Mutation F227L is a nonpolymorphic mutation that is associated with resistance to DLV,NVP and EFV when present with other NNRTI mutations and in this study it was observed in one child (Reuman *et al.,* 2010).When F227L is present with V106A it confers high level resistance to NVP and EFV(Reuman *et al.,* 2010). Mutation M230L which is a uncommon nonpolymorphic mutation that causes intermediate to high level resistance to each of the available NNRTI was also observed in one child as well (Melikian *et al*., 2014). Mutation E138A which is a polymorphic mutation has low level resistance to rilpivirine and was found in two children in this particular study (Johnson *et al*, 2013; Xu *et al*., 2013).

Mutation V179T is a rare nonpolymorphic mutation conferred to one child in this study and is associated with minimal reductions in ETR and RPV susceptibility (Melikian *et al*., 2014).

Development of drug resistance has been shown to vary with exposure to different drugs. An example of this is when nevirapine or lamivudine is initiated as monotherapy, high level resistance has been shown to emerge within days or weeks. This is because of the proliferation of variants containing K103N and M184V mutations for nevirapine and lamivudine resistance respectively. These two mutations were seen to be the highest in prevalence in this study. The fact that these drugs are given in PMTCT programs could be the reason behind the presence of drug resistance mutations in the study participants.

5.1.2 Impact of HIV-1 Subtype Diversity on the population

In this study population participants with HIV-1 subtype A were the majority. This is similar to what has been found in other studies that have been done in Kenya (Lihana *et al.,* 2009 ; Nyamache *et al,* 2013). Many of such studies have shown a broad range of HIV-1 genetic diversity in Kenya with subype A being dominant (Lihana *et al.,* 2009; Nyamache *et al.,* 2013).The second most prevalent subtype in the study was subtype D, but a study by Khamadi *et al* (2008) found it to be less prevalent than subtype C. Compared to other studies, this study had a significant percentage of viruses that were subtype D , which is much higher than what has been reported in other parts of Kenya (Kageha *et al.,* 2012). Busia County Referral Hospital borders Uganda which is known to be having a higher prevalence of HIV-1 subtype D as compared to Kenya (Gonzalo *et al*., 2015). This population in Busia may have a higher prevalence of subtype D due to their interaction with neighboring Uganda population. This study also observed presence HIV-1 subtype C in the population. This subtype which is predominant in Southern African countries (Lihana *et al.*, 2012) has been reported to be transmitted more frequently from mother to child in the absence of antiretroviral prophylaxis when compared to subtype D. Sexual transmission of subtype C is also suggested to be more than that of subtype A and D (Abecasis *et al.,* 2013).

Some recombinants were observed in this study. This included subtype A/C of HIV-1 a recombinant of subtype A and C, subtype A/D which is a recombinant of subtype A and D , Subtype K/A a recombinant of subtype A and K and subtype B/D a recombinant of subtype B and D . Occurrence of such mixed subtypes points out that HIV subtypes in Kenya are slowly evolving and genetically diverging maybe due to dual infections (Khoja *et al*., 2008).

5.1.3 Study limitations

Data on the actual ART given to the mothers was not available. Therefore study was not able to link drug resistance mutations to ART regimen administered to the mothers. There was also a lack of information about the age and sex of the children which would have been an important contributor to the social demographic analysis in this study.

5.2 Conclusions

1. The prevalence of HIV-1 drug resistant mutations found in this study was 24% (13/53). Reverse transcriptase mutations found were at a prevalence of 16 %(9/53) for NRTI mutation while for NNRTI the prevalence was 24 %(13/53).
2. The most common major NRTI mutation found was M184V/I at 15% (8/53). For the NNRTIs seven (13%) children harbored K103N mutation which was also the most common major mutation. The only mutation found in PI was L101 at 41.5% (22/53) which is a minor mutation.
3. The most prevalent HIV-1 subtype was A at 64 % (34/53) while HIV-1 subtype D was found in 17 % (9/53) of the participants. Subtype C was found in 5.7 % 3/53) of the population. Subtype A/C was found in 1.8 %(1/53) of the children, subtype A/D in 5.7 % (3/53), subtype K/A in 1.8 %(1/53) and subtype B/D in 3.8 %(2/53).This shows the genetic diversity of the HIV-1 subtypes in this population.

5.3 Recommendations

1. This study recommends the close monitoring of drug resistance before initiation of treatment and even during treatment.
2. There is need of regular studies on HIV diversity and HIV molecular epidemiology.

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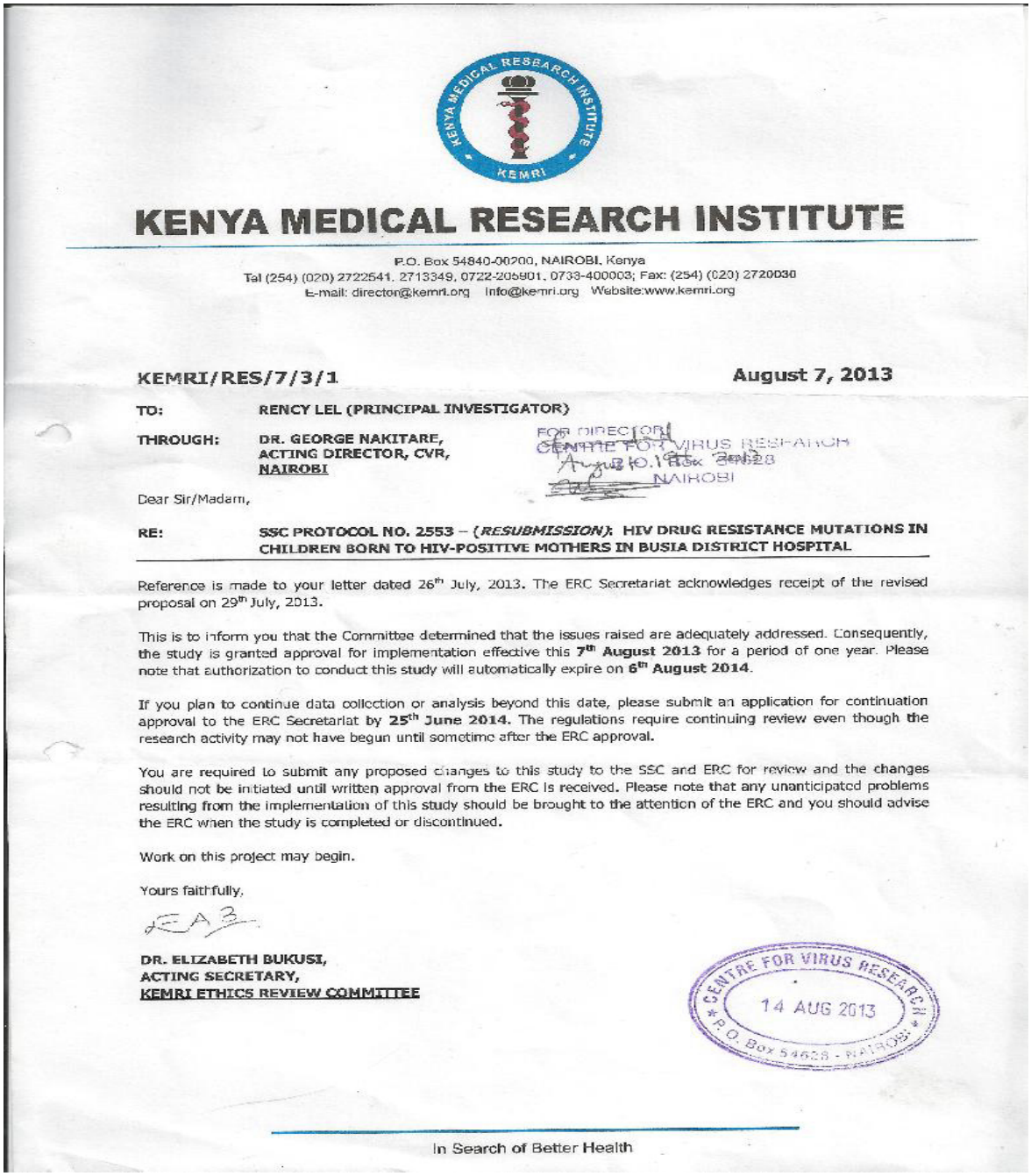
APPENDICES

Appendix 1: KEMRI Ethical Review Committee approval



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KEMRI/RES/7/3/1 August 7, 2013

TO: RENCY LEL (PRINCIPAL INVESTIGATOR)

THROUGH: DR. GEORGE NAKITARE,
ACTING DIRECTOR, CVR,
NAIROBI

FOR DIRECTOR
CENTRE FOR VIRUS RESEARCH
P.O. Box 54628
NAIROBI

Dear Sir/Madam,

RE: SSC PROTOCOL NO. 2553 – (*RESUBMISSION*): HIV DRUG RESISTANCE MUTATIONS IN CHILDREN BORN TO HIV-POSITIVE MOTHERS IN BUSIA DISTRICT HOSPITAL

Reference is made to your letter dated 26th July, 2013. The ERC Secretariat acknowledges receipt of the revised proposal on 29th July, 2013.

This is to inform you that the Committee determined that the issues raised are adequately addressed. Consequently, the study is granted approval for implementation effective this **7th August 2013** for a period of one year. Please note that authorization to conduct this study will automatically expire on **6th August 2014**.

If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **25th June 2014.** The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

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

Work on this project may begin.

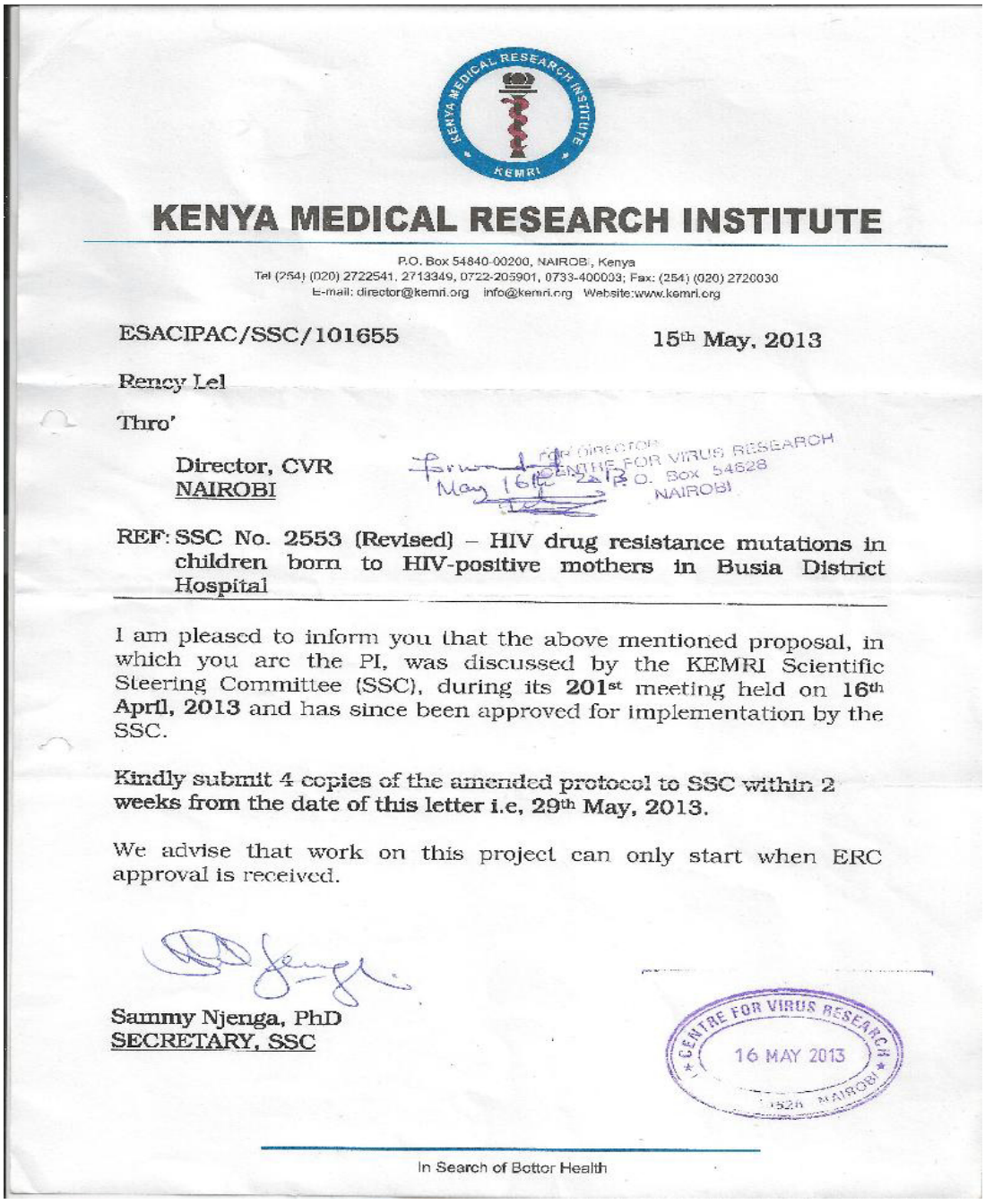
Yours faithfully,

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

CENTRE FOR VIRUS RESEARCH
14 AUG 2013
P.O. Box 54628 - NAIROBI

In Search of Better Health

Appendix 2: KEMRI Scientific Review Committee approval



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ESACIPAC/SSC/101655 15th May, 2013

Rency Lel

Thro'

Director, CVR
NAIROBI

FOR DIRECTOR
CENTRE FOR VIRUS RESEARCH
P. O. Box 54628
NAIROBI

REF: SSC No. 2553 (Revised) – HIV drug resistance mutations in children born to HIV-positive mothers in Busia District Hospital

I am pleased to inform you that the above mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its 201st meeting held on 16th April, 2013 and has since been approved for implementation by the SSC.

Kindly submit 4 copies of the amended protocol to SSC within 2 weeks from the date of this letter i.e, 29th May, 2013.

We advise that work on this project can only start when ERC approval is received.

Sammy Njenga, PhD
SECRETARY, SSC

CENTRE FOR VIRUS RESEARCH
16 MAY 2013
NAIROBI

In Search of Better Health

Appendix 3: Publication in peer reviewed journal

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 30, Number 00, 2014

DOI: 10.1089/aid.2014.0158

HIV-1 Drug Resistance Mutations Among Infants Born to HIV-Positive Mothers in Busia, Kenya

Rency Lel,[1,2] Jane Ngaira,[2] Raphael Lihana,[1,2] and Samoel Khamadi[1]

Abstract

To determine HIV-1 subtypes and transmitted HIV-1 drug-resistant mutations among HIV-1-positive children born to HIV-positive mothers in Busia County, blood samples were collected from 53 children aged between 6 weeks and 5 years in 2011. Their mothers were HIV-1 positive and on antiretroviral therapy at the time the children were born. The samples were analyzed for HIV-1 drug resistance and subtypes through sequencing of portions of the HIV-1 *pol* gene. The generated sequences were analyzed for subtype diversity using the REGA and BLAST subtyping tools. HIV-1 drug resistance was determined using the Stanford University HIV database. Of the 53 samples that were successfully amplified and sequenced, 69.8% (37/53) were determined to be HIV-1 subtype A, 22.6% (12/53) were subtype D, 5.6% (3/53) were subtype C, and 1.8% (1/53) were subtype A_{1C}. The prevalence of HIV-1 drug resistance mutations of any kind was 22.6% (12/53).

MOTHER-TO-CHILD TRANSMISSION (MTCT) of HIV is one of the important means of new transmission of HIV. In 2009 alone, it is estimated that 370,000 infants acquired HIV infection through MTCT, i.e., *in utero*, during the peripartum period and via breastfeeding.[1,2] In Kenya as in other sub-Saharan African countries, efforts have been made to prevent HIV transmission from mothers to children using prophylactic antiretroviral medication.[3,4]

Reduction in maternal viral load in breast milk and indirect infant prophylaxis by ingestion of antiretrovirals in breast milk are some of the mechanisms used to protect infants from becoming infected with HIV from their mothers. Studies have shown that antiretroviral drugs taken by nursing women are present in breast milk.[5,6] Between 25% and 40% of mothers who are not on medication can transmit HIV to their children while those on short-course antiretrovirals during the peripartum period have transmission rates of between 8% and 10%.[7] For mothers on triple therapy throughout pregnancy, transmission is less than 5%.[8] However, for infants who become infected before or during breastfeeding, the presence of antiretroviral (ARV) drugs in breast milk may induce the development of drug resistance due to viral replication in the presence of low drug concentrations.[7]

Mutations that occur in the HIV genome and lead to resistance can be classified into primary and secondary mutations. Primary mutations lead to a several-fold decrease in sensitivity to one or more drugs. Secondary mutations may not result in a significant decrease in drug sensitivity but are associated with increases in viral fitness in the presence of existing drug resistance mutations.[9] Transmission of resistant HIV variants has serious implications among infected infants since it might reduce the choices of active ARV drugs.

This study was carried out to determine the common transmitted HIV-1 drug resistance mutations in children who were born to HIV-infected mothers in Busia District. This was a retrospective study conducted in Busia County in 2011. After obtaining informed consent from the parents of the children, 5 ml of blood was collected in EDTA tubes and plasma was separated and stored for future analysis. The blood samples were collected from children aged between 1 and 5 years. Scientific and ethical approval was sought from the KEMRI National Ethical Review Committee (ERC).

RNA was extracted from 140 μl of plasma samples using the Qiagen RNA extraction kit according to the manufacturer's instructions. One-step reverse transcriptase polymerase chain reaction (RT-PCR) and nested PCR were performed using in-house primers targeting the protease and reverse transcriptase enzymes in the HIV-1 *pol* gene as previously described.[10] Both the reverse transcriptase and protease genes were amplified and sequenced.

The generated sequences were analyzed for subtype diversity using the REGA subtyping tool[11] and BLAST tool.[12] Drug resistance was determined using the International Aids Society (IAS) algorithm and the Stanford University HIV

[1]Centre for Virus Research, Kenya Medical Research Institute, Nairobi, Kenya.
[2]Institute of Tropical Medicine and Infectious Diseases (ITROMID), Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.

Appendix 4: Generated sequences

The 53 sequences generated from this study were submitted to the Genbank under accession numbers KM016171-KM016223 .Some of the sequences are shown below.

>PaedBusia_001

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>Paed Busia-002

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>Paed Busia_003

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>Paed Busia_004

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>Paed Busia_005

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>Paed Busia-006

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>Paed Busia-007

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