EVALUATION OF PREDICTORS OF HIV/AIDS TREATMENT FAILURE IN PATIENTS WITH SUSPECTED VIROLOGICAL AND IMMUNOLOGICAL FAILURE FROM COMPREHENSIVE CARE CLINIC IN NAIROBI, 2013

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Evaluation of predictors of HIV/AIDS treatment failure in patients with suspected virological and immunological failure from Comprehensive Care Clinic in Nairobi, 2013

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2016

DECLARATION

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

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This thesis has been submitted for E-xamination with our approval as university supervisors:

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DEDICATION

This work is dedicated to my mother Dinah Luvai who has always supported me in all that I do, my siblings Jullie Ochieng' and Diamond Luvai for their continuous assistance.

I also dedicate to Simon Mugabi for his unfalteringly encouragement in absolutely everything that l do, and finally to the countless millions who have suffered at the hands of AIDS epidemic.

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LIST OF ABBREVIATIONS AND ACRONYMS

µg	Microgram
µl	Micro litre
3TC	Lamivudine (2’, 3’-dideoxy-3’-thiacytidine)
ABC	Abacavir
ABI	Applied Biosystem International
AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral Therapy
ARV	Antiretrovirals
AZT	Zidovudine
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CCR5	Chemokine Receptor 5
CD4+	Cluster of differentiation 4 (T-helper cells)
cDNA	Complementary Deoxyribonucleic acid
CRF	Circulating Recombinant Forms
CXCR4	Chemokinine Receptor 4
D4T	Stavudine

DDI Didanosine

DLV Delavirdine

dN nonsynonymous subsititution

DNA Deoxyribonucleic acid

dNTPs Deoxynucleotide Triphosphates

dNTPs Dioxyribonucleic acid triphosphates

DR Drug resistance

dS synonymous subsititution

DTT Dithiothreitol

env Envelope (gene)

ETV Etravirine

FTC Emtricitabine

Gag Group antigen gene

Gp group antigen gene (glycoprotein)

HAART Highly Active Antiretroviral Therapy

HIV Human Immunodeficiency Virus

HLA Human Leucocyte Antigen

IV Intravenous

KEMRI Kenya Medical Research Institute

LTR Long Terminal Repeat

M group Major group of viruses

MA Matrix

MAP Mitogen Activated Protein

$MgCI_2$ Magnesium Chloride

Nef Negative regulatory factor

NNRTI Non-Nucleoside Reverse Transcriptase Inhibitors

NRTI Nucleoside Reverse Transcriptase Inhibitors

NVP Nevirapine

PCR Polymerase Chain Reaction

RNA Ribonucleic Acid

RT Reverse Transcriptase

SIV Simian Immunodeficiency Virus

STIs Sexually Transmitted Infections

TAMs Thymidine analog mutations

Tat Trans-Activator of Transcription

TDF Tenofovir

UNAIDS Joint United Nations programme for AIDS

USD United States Dollars

Vif Viral Infection Factor

VL Viral load

Vpr Viral Protein R

Vpu Viral Protein Unique

WHO World Health Organization

ABSTRACT

Global access to antiretroviral therapy (ART) is continuously increasing hence it is estimated that in four years of therapy 5-10% of patients will have their first line of therapy failing, due to development of resistance. Predictors of treatment failure include: plasma viral load above 1000 copies/mL, fall of cluster of differentiation 4 (CD4) count below 350 cells/mm3 in spite of therapy. This study was carried out to evaluate the reliability of these predictors in the detection of treatment failure. Eighty one (81) subjects were recruited from the comprehensive care clinic (CCC), Nairobi, based on the inclusion criteria. The *reverse transcriptase* region (RT) of the HIV-1 genome was amplified using nested polymerase chain reaction (PCR) and a total of 80 samples were directly sequenced. The sequences were individually blasted into the Stanford HIV Drug Resistance data base *insitu* tools, where genotypic drug resistance was defined by one or more resistant-related mutations. Isolates showed that the majority of patients who showed extreme treatment failure (CD4 count <100cells/µl and high viral load $>100,000$ copies/ml) were directly related to mutations such as T215Y, MI84V, M41L, K65KN, K103N, V90I, G190A, Y181C, and H221Y. The positive predictive value (PPV) of viral load was 100% reliable in the prediction of treatment failure however CD4+ count was 89% reliable. The Pearson correlation coefficient of CD4 count and viral load, was observed to be significant at the 0.01 level, $r = -0.762$ hence $R^2 = 0.58$. The correlation coefficient is indicative of a weak correlation between viral load and CD4+ count. The HIV-1 subtypes in this study were as follows: subtype A1 was the highest 47(58%) subjects, subtype D 22(27%), subtype C 5(6.25%) subjects and subtype G present in one subject respectively. There were two circulating recombinant factor (CRF01_AE and CRF02_AG). The data obtained suggests that viral load is reliable in the detection of treatment failure, however HIV-1 genotypic drug resistance test should be highly considered for patients suspected to be failing therapy before they are switched.

CHAPTER ONE

INTRODUCTION

1.1 Introduction to the background

Global HIV/AIDS statistics show that as of 2014, there were 36.9 million new HIV-1 infections (UNAIDS, 2015). Sub-Saharan Africa accounts for more than 70% of the global prevalence of HIV, with women and the youth carrying the biggest burden of the disease (Kenya AIDS, 2014). Since the discovery of HIV-1 in Kenya in 1984, it continues to be a big healthcare burden for the country. The roll out of antiretroviral therapy (ART) has led to a significant reduction in HIV/AIDS related mortality. It is estimated that the cost of first line ART in some low and middle-income countries has been greatly reduced to around US $140 per person per year (UNAIDS, 2015). In 2013 the World Health Organization (WHO) revised the guide- lines of ART initiation meaning that the number of eligible people for treatment initiation increased to approximately 28.6 million (Kiertiburanakul *et al.*,2014).

As of December 2013, there were 656,000 patients on ART in Kenya (UNGASS, 2014). The increase in ART coverage in the country is expected to lead to an increase in drug-resistant strains (Nyamache *et al.,*2011). Stigma and cultural practices that affect ART uptake in Kenya may affect ART compliance, resulting in an increased emergence of drug-resistant mutants, which are a potential source of drug resistance. To mitigate against the development of HIV/AIDs drug resistance, the government of Kenya developed a guideline for clients who are due for initiation of ART. These include providing the clients with information on importance of ART and adherence to medication. Currently the first-line treatment of HIV-1 and AIDS is based on the combination of two nucleoside reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI), which is also known as highly active antiretroviral therapy or HAART (Hill *et al.*, 2013). Most resource-limited countries use the standard first line regimens that were recommended

by WHO (Gilks *et al.,* 2006). In Kenya, initiation of ART in drug naïve clients is based on Tenefovir + Lamivudine + Efavirenz/Nevirapine. In pregnant women where clients are unable to tolerate tenofovir then Zidovudine + Lamivudine + Efavirenz/Nevirapine have been used (Rapid advice, 2014). The most important enzyme in HIV multiplication in the body is reverse transcriptase (RT). The two main types of drugs used to inhibit RT are nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). NRTIs resemble nucleosides and are activated by the host to convert them to their triphosphate form. When bound in the active site, they terminate polymerization because they lack a 3' hy-droxyl group (Otto *et al.,* 2004). Resistance to NNRTIs occurs when there are mutations in the binding pocket, and affinity for the drug is reduced. According to the Kenya HIV-Drug resistant report of 2011, in order to establish treatment failure the following should be seen: New or recurrent WHO stage 4 condition or certain WHO clinical stage 3 conditions e.g. tuberculosis, plasma viral load above 1000 copies/ml and fall of CD4 count to baseline or persistent CD4 levels below 100 cells/mm^3. Virological failure occurs earlier, which is later followed by immunological and clinical failure. Diagnostically, the reverse is true in resource-limited settings, because of availability diagnostic resources. Viral load is the most sensitive prognostic tool to identify treatment failure and is used in resource- rich settings (Barry *et al.,* 2006). Although resistance testing is not widely employed in developing countries due to the costs involved, the need will increase due to emergence of drug resistance as antiretroviral therapy is scaled up (WHO, 2013).

1.2 Statement of the problem

In 2003, only 6,000 people living with HIV were accessing ART, by 2013 this had increased to 656,000 (UNGASS, 2014). However, development of HIV drug resistance is inevitable in patients on ART despite adherence and plasma drug levels (Calmy *et al*., 2007; Kantor *et al*., 2009). As such, minimizing development of resistance is a priority that requires early detection of the resistance so as to intervene by insuring optimal tolerance, adherence and potency. The timing and accuracy of identifying treatment

failure is fundamental. Delayed detection of treatment failure may increase drug toxicity, may lead to the accumulation of drug resistance–associated mutations and may result in increased morbidity and mortality (Anderson *et al*., 2006, Calmy *et al*., 2007). Studies have shown acquired HIV drug resistance may be on the rise in Africa, and unless HIV-1 drug resistance testing is increased, the continent's treatment programs could suffer (Kenya HIV DR Report, 2011). There is therefore a need to systematically describe the emergence of drug resistance among patients treated with ART in resource-constrained settings and especially sub-Saharan Africa, where the burden of HIV/AIDS is enormous.

1.3 Justification

Genotypic tests for HIV-1 drug resistance are the gold standard for testing for resistance which costs 140 USD or more. This is way beyond the reach of most Kenyans. As an option, virological, immunological and clinical prognostic markers are used as indicators of treatment failure in the public sector (WHO, 2013). This is because of their availability and comparative low costs.

The World Health Organization recommends that countries put in place strategies to assess and prevent drug resistance (WHO, 2013). Therefore, it is important to determine how accurate these markers are in the prognosis. This study aims to evaluate several cases of prognostic failure by comparing them to the genotypic drug resistance testing which is the gold standard. The findings will help to strengthen the importance for the use of the prognostic markers in predicting treatment failure thus improving program performance of ART.

1.4 Objectives of the study

1.4.1 General objectives

To evaluate prognostic markers of HIV/AIDS treatment failure in a cohort study in a Comprehensive Care Clinic in Nairobi

1.4.2 Specific Objectives

1. To determine drug-specific mutations that confer resistance to ART in the study population.

2. To evaluate the use of CD4 and viral load prognostic tests as a tool for prediction of ART treatment failure.

3. To determine the HIV-1 subtypes circulating among patients with suspected treatment failure in the cohort.

CHAPTER TWO

LITERATURE REVIEW

2.1 The diversity of HIV

The error-prone reverse transcriptase (RT) enzyme is responsible for a phenomenon called hypermutations (Fitzgibbon *et al.,* 1993). Hypermutations result when an excessive number of substitutions in DNA, usually from the DNA base Guanine (G) to the base Adenine (A), occur (Fitzgibbon *et al.,* 1993). Such substitutions are often induced by host cellular defence mechanisms to produce replication-incompetent viruses (Fitzgibbon *et al.,* 1993; Rose and Korber, 2000; Mangeat *et al.,* 2003) and are not restricted to HIV (Wain-Hobson *et al.,* 1997; Ngui *et al.,* 1999).

The phylogenetic analyses of numerous strains of HIV-1, sequenced from diverse geographical origins, have revealed that they can be subdivided into groups, subtypes and sub-subtypes (Robertson *et al.,* 1999). HIV-1, the variant responsible for the majority of HIV/AIDS infections (Moore *et al.,* 2001), has been further divided into four groups: M (major), N (New, or non-M, non-O), O (outlier) and recent detected group P in Cameroon (Peeters, 2001; Plantier *et al.,* 2009). Within group M, at least nine subtypes have been identified (A-D, F-H, J and K) (Peeters, 2000), approximately equidistantly related with intrasubtype divergence up to 20% and intersubtype divergence between 25% to 35%, for the e*nv* amino acid sequences (Robertson *et al.,* 2000; Triques *et al.,* 2000; Achkar *et al.,* 2004).

Separate sub-clusters are distinguished within subtypes A and F (A1, A2, A3 and A4, F1 and F2) each pair of sub-subtypes being more related to each other than with other subtypes (Rambaut *et al.,* 2004). Subtypes B and D should be the same subtype, but their original designation as different subtypes has been retained for consistency with earlier published work (Thomson *et al.,* 2002).

2.2 HIV-1 in Sub-Saharan Africa

In southern Africa, subtype C accounts for 92% of urban HIV cases (Williamson *et al.,* 2003) while subtype B plays a minor role, about 7% of cases. Subtype C is primarily transmitted heterosexually, and subtype B is associated with homosexual transmission (Bredell *et al.,* 2000) (**Figure 2.1**). Possibly because of limited heterosexual and homosexual risk group interaction, little recombination has occurred between these two subtypes (Bredell *et al.,* 2000). The majority of other subtypes in South Africa occur in immigrant populations from elsewhere in the continent (Bredell *et al.,* 2002). Despite the relatively minimal viral diversity, HIV prevalence is quite high in southern Africa, up to 27% (Williamson *et al.,* 2003). Ironically, in Central Africa where HIV diversity is highest, the viral prevalence is lower (Butler *et al.,* 2007).

2.3 Distribution of HIV subtypes in East Africa

In East Africa, subtypes A and D have dominated Uganda from mid 1980s (Hu *et al.,* 2000). However, HIV-1 subtype D on a localized scale accounts in Uganda, Tanzania and Kenya 34% to 53% of infections. With HIV subtype D being generally limited to East and Central Africa, some sporadic cases have been observed in Southern and Western Africa too (Piot *et al.,* 2002; Janssens *et al.,* 1997; Delaporte *et al.,* 1996). In southern Tanzania town of Mbeya, the predominant subtype is C (Hoelscher *et al.,* 2001). This probably reflects introduction from southern African countries where subtype C is the predominant type (De Baar *et al.,* 2000). HIV-1 subtypes A and D have been isolated from other parts of Tanzania (Blackard *et al.,* 1999). HIV-1 subtype G and its A/G recombinant viruses have been observed in Eastern Africa as well as in West Africa and central Europe (Piot *et al.,* 2002) though at low levels.

2.4 Distribution of HIV subtypes and circulating recombinants forms in Kenya

The great majority of strains circulating in Kenya belong to group M and have been characterized according to the HIV nomenclature (Songok *et al.,* 2003). Currently there

are 48 CRFs (Li, *et al.,* 2010) of which about 4 have been reported to be occurring in Kenya, hence the presence of diverse HIV-1 subtypes and recombinants (Lihana *et al.,* 2006). Subtype A predominates in both non-recombinations (55%) and recombinants based on near full-length sequences that have been generated (Dowling *et al.,* 2002).

Ethiopia is dominated mainly by HIV-1 subtype C, which incidentally is the dominant subtype in the town of Moyale, which borders Ethiopia, indicative of cross-border movements influencing circulation of subtypes in Northern Kenya (Khamadi *et al.,* 2005). According to Lihana *et al.,* (2006), on circulating HIV subtypes in STI (Sexually Transmitted Infection) in Nairobi based on *pol* Gene sequence, subtype A1 was the major subtype (64%) followed by D (17%), C (9%), G (1%), and recombinants AD (4%), AC (3%), CRF02–AG (1%), and CRF16–A2D (1%). These results suggest that there is increasing number of infections due to recombinant forms of the virus (Wainberg *et al,* 2004). Any use of ARVs may therefore require initial testing for *de novo* resistance before commencement of treatment and/or management (Lihana *et al.,* 2006). It is therefore important to monitor the emerging genetic diversity of HIV-1, not only because it has implications for vaccine development, diagnosis, screening of blood products, and the selection of optimal treatment regimens but also because it will facilitate epidemiological investigation of transmission patterns and help define strategies for preventing the spread of infection (de Oliveira *et al.,* 2006; Gifford *et al.,* 2007).

2.5 Antiretroviral therapy

Since the roll out of antiretroviral therapy (ART), it has led to decrease in mortality rate and decrease in the progression of HIV to AIDS, and there are now more than 20 drugs licensed for clinical use (Cane, 2009). Until the level of quantification due to usage of life-long continuous combination antiretroviral therapy (cART) has radically changed HIV infection and its complication from an invariably fatal to a chronic illness (Pallela *et al.,* 1998). There were 656,000 patients on ART in Kenya as of 2013 (UNGASS,

2014). Some of the success of combined ART has been the development of new classes and new drugs within old classes of antiretroviral therapy (Pett *et al.,* 2010). Most resource-limited countries use the standard first line regimens that were recommended by World Health Organization (Beck *et al.,* 2006). Currently the first-line treatment of HIV-1 and AIDS is based on the combination of two nucleoside reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI), which is also known as highly active antiretroviral therapy or HAART (Hammer *et al.,*2006) (Figure 2.1).

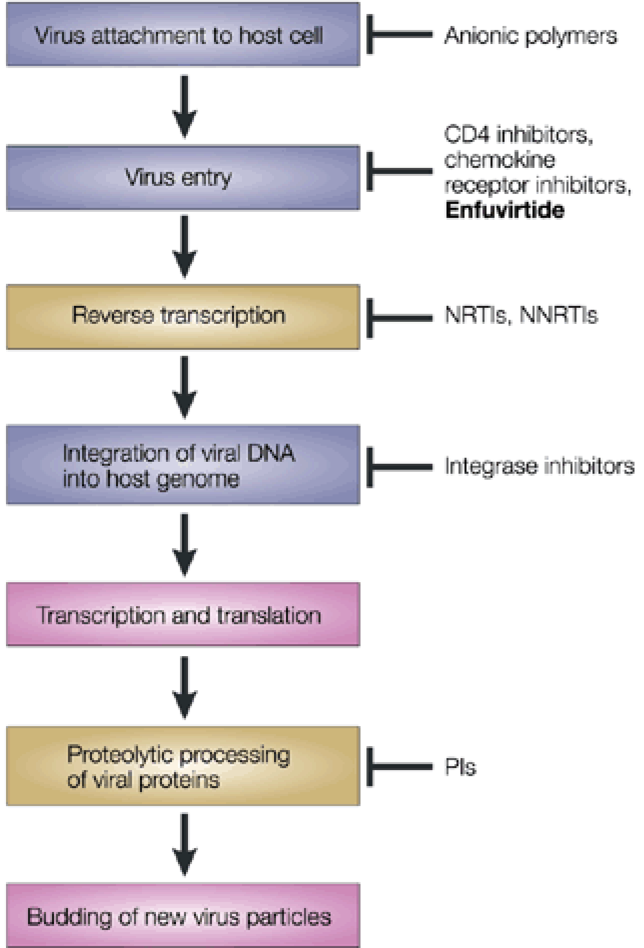


Figure 2.1: HIV-1 life cycle showing stages targeted by antiretroviral therapy

2.6 Mechanism of action of antiretroviral drug classes

2.6.1 Reverse transcriptase inhibitors

The most important enzyme in HIV-1 is reverse transcriptase (RT). Reverse transcriptase converts the RNA to DNA in the cytoplasm and can be integrated into the host DNA for storage (Zdanowicz, 2006). RT was the first target in drug therapy and is still important in the treatment of HIV infected patients (de Jonge *et al.,* 2005). The two main types of drugs used to inhibit RT are nucleos(t)ide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs)(Clavel, 2004).

2.6.1.1 Nucleos(t)ide Reverse Transcriptase Inhibitors (NRTI)

The NRTIs resemble nucleosides and are activated by the host to convert them to their triphosphate form (Pauwels, 2004). When bound in the active site, they terminate polymerization because they lack a 3' hydroxyl group (Figure 2) (Otto *et al.,* 2004). Some disadvantages of NRTIs include interference with mitochondrial DNA synthesis and other anti-retroviral drugs (Clavel and Hance, 2004). The nucleoside analogues such as zidovudine (Azidothymidine (AZT), Lamivudine (3TC), Didanosine(ddI), Zalcitabine (ddC), Stavudine (d4T), Abacavir (ABC)) are comprised of a base (thymidine in the case of AZT) that attaches to a ribose sugar in which the normal 3' hydroxyl get replaced by an azido group (Zdanowicz, 2006).

2.6.1.2 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

The NNRTIs include any drugs that are not nucleos(t)ide analogues. The NNRTIs bind near the catalytic site of reverse transcriptase and alter the enzymes ability to change conformation (Zdanowicz, 2006). This increased enzyme rigidity prevents its normal polymerization function. The side effects of the NNRTIs are generally less than those of the nucleoside analogues. Most NNRTIs bind non-competitively to a hydrophobic binding pocket (Pauwels, 2004, Shen *et al.,* 2003). Tyr-181/Tyr-188 are amino acids in RT that hydrophobically interact with the aromatic rings present on the inhibitor (Shen

et al., 2003). These amino acids act as clamps, holding the inhibitor in place (Shen *et al.,* 2003). When bound to the inhibitor, the p66 thumb of RT loses flexibility and mobility, hindering polymerization activity (Shen *et al.,* 2003, Clercq, 2004). Interactions with amino acids on the p66 subunit also occur (Harpstead *et al.,* 2006). When bound, these drugs destabilize the interactions between the two subunits decreasing the activity of RT (Harpstead *et al.,* 2005).

2.6.2 Protease Inhibitors

Newly assembled HIV particles are not fully functional or infectious until they have undergone a final "maturation." This maturation involves cleavage of viral protein precursors by HIV protease enzymes into small pieces that go to infect new cells. These enzymes are encoded by HIV and offer a unique and attractive target for preventing HIV maturation (Zdanowicz, 2006). HIV protease enzymes are symmetrical dimers with a central core that binds the peptides that are to be modified by the enzyme (Zdanowicz, 2006). Protease inhibitors (PIs) target and inhibit the enzymatic activity of the HIV-1 PR. They inhibit protease activity to the extent that is sufficient to prevent cleavage events in Gag and Gag-Pol that result in the production of non-infectious virus particles (Wensing *et al.,* 2010). The development of a second mechanistic class of antiretroviral drug, which made HAART combination therapy possible. Protease inhibitors have remained a key component of HIV-1 patient treatment regimens right up to the current day (Wensing *et al.,* 2010).

2.6.3 Intergrase inhibitors

Intergrase inhibitors are active against viruses resistant to other ARV classes, such as nucleoside and nonnucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs, respectively), protease inhibitors (PIs) and fusion inhibitors (Engelman *et al.,* 1991). They act by targeting the integrase protein. Raltegravir an intergrase drug, is a 1-N-alkyl-5-hydroxypyrimidinone. During infection and replication of the virus, Raltegravir binds the Pre-Intergration Complex (PIC), blocking the strand transfer of HIV cDNA to

cellular DNA by removing a dinucleotide from the 3' ends before intergration (Shafer, 2008).

2.7 Antiretroviral drug resistance development

With global access to ART increasing, it is estimated that in four years of therapy 5-10% of patients will have their first line therapy fail. This is high despite plasma drug levels, plasma efficacy and adequate adherence (Calmy *et al.,* 2007). The phenomenon of HIV-DR can be attributed to poor laboratory infrastructure, inadequate staff at health centers', and poor drug procurement and delivery systems (Jordan *et al.,* 2008).

Resistance of HIV-1 to antiretroviral drugs is one of the most common causes for treatment failure in individuals infected with HIV-1; unfortunately the emergence of drug-resistant HIV-1 variants is a common occurrence (Francois *et al.,* 2004). In spite of advances in antiretroviral therapy that have transformed HIV-1 management and the control of the spread of regional epidemics (Johnston *et al.,* 2010) resistance to antiretroviral drugs has emerged in all locales in which such drugs are used (Wainberg *et al.,* 2011).

2.7.1 NRTI resistance mutations

The NRTI resistance mutations include M184V, thymidine analog mutations (TAM), mutations selected by regimens lacking thymidine analogs, multi-nucleoside resistance mutations, and many recently described non-polymorphic accessory mutations (NAM) (Shafer *et al.,* 2008). There are two biochemical mechanisms of NRTI resistance: enhanced discrimination against and decreased incorporation of NRTI in favor of authentic nucleosides, and enhanced removal of incorporated NRTI by promoting a phosphorolytic reaction that leads to primer unblocking (Shafer *et al.,* 2008). Altogether, M184V, non thymidine analog-associated mutations such as K65R and L74V, and the multinucleoside resistance mutation Q151M act by decreasing NRTI incorporation (ARV guidelines 2008, Deval *et al.,* 2002). Thymidine analog mutations, the T69

insertions associated with multi-nucleoside resistance, and many of the accessory mutations facilitate primer unblocking (Arion *et al.,* 1998, Boyer *et al.,* 2001).

2.7.2 NNRTI resistance mutations

Nearly all of the NNRTI resistance mutations are within the NNRTI binding pocket or adjacent to residues in the pocket (Sarafianos *et al.,* 2004, Ren *et al.,* 2008). There is a low genetic barrier to NNRTI resistance, with only one or two mutations required for high-level resistance (Shulman *et al.,* 2000, Lecossier *et al.,* 2005).

According to Shafer *et al.,* (2008) NNRTI resistance mutations can be classified into the following categories: (i) primary NNRTI resistance mutations that cause high-level resistance to one or more NNRTI (ii) secondary NNRTI resistance mutations that usually occur in combination with primary NNRTI resistance mutations (iii) minor non-polymorphic mutations that may occur alone or in combination with other NNRTI resistance mutations and that cause consistent but low-level reductions in NNRTI susceptibility; and (iv) polymorphic accessory mutations that modulate the effects of other NNRTI resistance mutations.

2.7.3 HIV-1 Drug Resistance in Kenya

The main issues with the management of HIV disease in Kenya is the high rate of sub-optimal adherence which may be brought about by a combination of various factors; like disruption in supplies of antiretroviral drugs, wrong dosage of drugs, forgetting to take drugs, frequently change of medication; interruptions in treatment due to financial constraints and stigma (Nyamache *et al.,* 2011) thus increasing the chances of drug resistance. Treatment options are limited in Africa and therefore it is likely that if transmission of resistance occurs, future ARV therapy in a newly infected individual may be affected. Treatment of a transmitted drug-resistant variant is associated with an increased risk of virological failure (Nichols *et al.,* 2011; Wittkop *et al.,* 2011).

Although resistance testing is not yet widely employed in developing countries due to the costs involved, the need will increase due to emergence of drug resistance as antiretroviral therapy is more widely introduced (Hirsch *et al.,* 2008). Identifying treatment failure in resource limited settings is really important but it is challenging since if the detection is delayed, it can lead to drug toxicity (Calmy *et al.,*2007), cross resistance between ARV drugs within each class(Clavel *et al*., 2004) while if there is misdiagnosis it may cause misclassification of treatment failure leading to early switching to second line of therapy which is costly (Mee *et al.,* 2008).

2.7.4 HIV-1 Drug Resistance Assays

There are two general types of resistance assays used in clinical practice: genotypic assays where by HIV-1 gene sequencing is done to detect mutations that confer HIV-1 drug resistance and phenotypic assays which is cell culture–based viral replication assays in the absence or presence of drugs (Hirsch *et al.,* 2008). Genotypic resistance assays identify specific codon mutations in the reverse transcriptase and protease genes of plasma virus by amplification and sequencing or by use of standardized probes. Results obtained are compared with databases that match particular mutations with drug resistance. Phenotypic resistance assays measure the susceptibility of chimeric viruses that contain the reverse transcriptase and protease genes of the patient's virus spliced into a retroviral (Calmy *et al.,* 2007).

Kenya has made significant progress in the area of genotyping for HIV-1 drug resistance. Both the public and private institutions are involved in training personnel and acquiring the relevant platforms required to carry out HIV resistance testing (Kenya HIV-DR, 2011). It is suggested that HIV drug resistance genotyping data be transferred electronically from the resistance genotyping laboratory to the national data centre in the form of a nucleotide sequence text file or files (UNAIDS, 2011). The database is important to keep record on the sequences conducted however, due to the cost

limitations, resistance testing is currently limited to Research institutions, the private sector and a selected number of Universities (Kenya HIV-DR, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

This study was carried out at the Centre for Virus Research using samples that were collected from a Comprehensive Care Clinic (CCC) at the Centre for Respiratory Diseases Research (CRDR), based in the Kenya Medical Research Institute (KEMRI) on 15th March 2013.

3.2 Study design

This study was a descriptive cross-sectional study. The design was selected mainly because it's usually conducted to estimate the prevalence of an outcome of interest for a given population (Levin, 2006). This design was used to determine the development of HIV drug resistance among the study participants that were on first line ART.

3.3 Ethical approval and informed consent

The study was carried out after obtaining informed consent from the patients (**Appendix 1**) and clearance under protocol No: 2451 that was approved by the KEMRI/National Scientific and Ethical Review Committees (**Appendix 2**).

3.4 Study population

The study population consisted of eighty one (81) HIV-1 patients on antiretroviral therapy who were enrolled at the CCC from KEMRI-Nairobi, Kenya. The subjects were selected based on inclusion criteria.

3.4.1 Inclusion Criteria

Samples used in the study were from patients who had met the following criteria:

1. Patients on ART regimen classified as standard first-line for more than 12 months.
2. Patients with CD4 count below 350 cells/ml and/or patients with persistently high viral load (>1000 copies/µl) in spite of taking ART, indicative of suspected treatment failure.
3. Patients above 18 years of age.
4. Patients who have given informed consent to participate in the study
5. Patients who have attended the clinic at least once within the previous 6 months

3.4.2 Exclusion criteria

1. Patients naïve or receiving other non-standard or second-line regimens.
2. Patients with CD4 above 350 cells/ml and/or patients with low viral load who are showing a significant reduction for the last 2 months (reducing to a lesser log).
3. Patients' below18 years of age.
4. Patients lost to follow-up (not attended the clinic within the previous 6 months)
5. Patients refusing to participate in the study

3.5 Sample size estimation

The samples were selected based on a criterion of treatment failure in the cohort from the patients who are failing 1st line treatment or have had one of their drugs in 1st line substituted with another drug in the same class. Using a HIV-1 prevalence of 5.6% in Kenya by 2013 (Kimanga *et al.,* 2013), the sample size was derived using (Chadha *et al*., 2006) formula as shown below.

P= HIV-1 prevalence rate by 2013, d=absolute precision required on either side of the proportion.

P and d are expressed in fractions, Z is a constant, and its value is 1.96 for 95% confidence.

Thus P=0.056% (0.056), Z=1.96, d=0.05

Therefore,

$$n = \frac{1.96^2 X\, 0.056(1-0.056)}{0.05^2}$$

$$= 81.23 \approx 81$$

Therefore by use of simple random sampling, 81 samples were selected for the study.

3.6 Sample collection and handling

The selected cryo-preserved samples from Comprehensive Care Center were put in ice-packed cool boxes. They were then transported to Kenya Medical Research Institute and immediately stored at –80ºC at the Center of Virus Research - HIV laboratory until use.

3.7 RNA Extraction

Viral RNA was extracted from plasma samples using the Qiagen® RNA extraction kit according to the manufacturer's specifications. Briefly, 140 µl of plasma was dispensed into 560 µl of RNA viral lysis buffer (AVL). This was then vortexed for 15 seconds to mix. The resulting mixture was incubated at room temperature for 30 minutes after which the entire solution was added to a spin column. The column was span down at 6,000xg for 10 minutes. The resulting supernatant was discarded and the RNA bound on the spin column washed with 70% ethanol. This was done by adding 200 µl of 70% ethanol to the column and spinning at 6,000xg for 10 minutes. The column was then transferred to a sterile eppendorf tube and extraction of the RNA facilitated by adding 200 µl of sterile distilled RNAse-free water to the column. The column was span at 6,000xg to elute the RNA into the eppendorf tube.

3.8 RNA.Reverse Transcription

Reverse transcription of the RNA was performed by priming with UNINEF7 primer (5'-GCACTCAAGGCAAGCTTTATTGAGGCTT-3') close to the 3′ end of the viral RNA. The extracted RNA (3 µl) was reverse transcribed in a total volume of 20 µl with 2 µl dNTP, 2 µl primer, 8 µl 1× RT buffer, 1 µl MgCl2, 1 µl DTT, 1.5 µl RnaseOUT and 1.5 µl SuperScript^{TM} III RNase H^{-} RT (Invitrogen, Carlsbad, CA2). The RNA primer and dNTPs were first incubated at 65°C for 5 minutes, and the remaining reagents were added for cDNA synthesis at 50°C for 2 hours, followed by 85°C for 5 minutes. Finally 1.5 µl SuperScript^{TM} III RNase H^{-} RT (Invitrogen, Carlsbad, CA) was added, and the reaction tubes were incubated at 37°C for 20 minutes followed by 70°C for 15 minutes (Khamadi *et al.,* 2005).

3.9 Nested Polymerase chain reaction

The reverse transcriptase region of the viral genome was amplified using nested PCR from the cDNA to amplify the region of interest for HIV-1 drug resistance. All reagents were thawed and placed on ice to preserve their integrity. The first-round of PCR had 25µL reaction volume with a mixture containing 3 µL of the DNA template to be amplified, 2.5 µL of 5X buffer, 0.3 µL of each RT18 and KS104 primers (**Table 3.1**), 2.0µL dNTP, 2.0 µL $Mgcl_2$, 0.2 µL of *Taq* polymerase and 14.7 µL of distilled water. The cycling conditions were 1 cycle of 95˚C for 10 min and 35 cycles of 95˚C for 30 s, annealing at 60˚C for 60 s, and 72˚C for 1 min, and final extension of 72˚C for 10 min. From the first-round PCR products, 3 µL was used as a template for the second reaction volume with the second set of primers (KS101 and KS102) (Songok *et al.,* 2004; Khamadi *et al*., 2005; Lihana *et al.,* 2009) (**Table 3.1**). The second cycling conditions was the same, except that the annealing temperature for the first 10 cycles was 65°C and the incubation at 68°C in each cycle was for 8 minutes (Songok *et al.,* 2004; Khamadi *et al*., 2005; Lihana *et al.,* 2009, Meeme *et al.,* 2013).

Table 3.1: PCR Primers for the specific target gene region.

Pol-Reverse transcriptase region	
RT18: forward 1	5'-GGA AAC CAA AAA TGA TAG GGG GAA TTG GAG G -3'.
KS104: reverse 1	5'-TGA CTT GCC CAA TTT AGT TTT CCC ACT AA-3'
KS101: forward 2	5'-GTA GGACCTACACCT GTT CAA CATAATTGGAAG- 3'
KS102: reverse 2	5'CCC ATC CAA AGAAATGGAGGAGGTTCTTTCTGATG -3'

3.10 Analysis of PCR amplicons by gel electrophoresis

On completion of the second nested PCR, all products were viewed by conventional agarose gel electrophoresis on a 2.0% agarose (SEakem LE® agarose; FMC BioProducts, Rockland, Marine, USA) gel in 1X TAE buffer (0.04M Tris acetate, 0.001 M EDTA). The gel was prepared by dissolving in 2 g of agarose gel into 100 mL of 1X TAE buffer by heating. This was cooled to about 50°C and 70 µl of ethidium bromide added (Sigma-Aldrich®) to the gel to stain the DNA (Sharp *et al.,* 1973). A 1000 kb DNA molecular weight marker (exACTGene™ Low Range DNA ladder from Fisher scientific) was used to estimate the DNA band size. The samples were mixed with gel loading dye before loading into the wells on the gel. Electrophoresis was done at a constant voltage of 100 volts/cm using a Mupid®2 plus submarine electrophoresis system power supply source. After electrophoresis, the location of PCR DNA fragments on the gels was visualized under an ultra violet light at a wavelength of 301 nm and photographed with the syngene™ GeneGenuis computer system (Synoptics LTD, Cambridge, United Kingdom) (Khamadi *et al.,* 2005).

3.11 Sequence PCR

The generated amplicons from the second nested PCR products were then directly sequenced using the second set of primers (KS101 and KS102) (**Table 3.1**). This sequence PCR was carried in a reaction mixture of 20 µl with a dilution of 1:10. These contained 3 µl of DNA, 1.5 µl 5X sequence buffer, 2.0 µl BigDye®, 12 µl of distilled water, and 1.5 µl of forward and reverse primers (Songok *et al.,* 2004; Khamadi *et al*., 2005; Lihana *et al.,* 2009).

The amplification was carried out using a thermal cycler at following PCR conditions (denature for 5 minutes at 96 °C, and again for 10secs at 96°C, anneal at 50˚C for 5 seconds, final extension 60˚C 4 minutes for 25 cycles.

3.12 Sequencing of HIV-1 positive samples

The amplified products were labeled using the BigDye® sequence terminator kit from Applied Biosystems® and the products sequenced directly using an automated ABI 310 sequencer (Applied Biosystems, Foster City, CA). Using BigDye® sequence terminator kit, fluorescently labeled dyes were attached to ACGT extension products in DNA sequencing reactions. The dyes come in four colours red (labels Thymidine base), blue (Cytosine), black (Guanine) and green (adenine). The dyes were incorporated using 3'-dye label dideoxynucleotide terminators. AmpliTaq® polymerase was used for primer extension.

3.13 Sequencing reactions and purifications for sequencing

Subsequently on purification, 2µl of 3 mM sodium acetate was added to an eppendorf tube, 50 µl of absolute ethanol and 20 µl of the dye-labelled PCR product. This was incubated at room temperature for 15 minutes in the dark. The tube was centrifuged at 1760 xg for 30 minutes at room temperature. 500 ml of 70% ethanol was added and centrifuged at 1760 xg for 5 minutes and the supernatant discarded. The two steps above were repeated and DNA air-dried for a minimum of 45 minutes. Twenty microliters (20

µl) of template suppression reagent (TSR) was added and heated at 95˚C for three minutes and finally contents transferred into sequencing tube and loaded on the automated ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA) for direct sequencing.

3.14 Sequence analysis

The sequences generated from section 3.12 above were deposited in gene bank and allocated accession numbers **(Appendix 5).** The nucleic acid sequences were manually edited and submitted in individually into the Stanford HIV drug resistance database at (http://hivdb.stanford.edu/, accessed on January 13th 2014). Genotypic drug resistance in the *RT region* was defined as the presence of one or more resistance-related mutations, as specified by the consensus mutation figures of the International AIDS Society-USA (Johnson *et al.,* 2008) and the online tool on the Stanford HIValg-Software at http://hiv.grade.de.

3.15 HIV-1 Subtyping and Phylogenetic analysis

The sequences generated above were used in the determination of HIV-1 subtypes circulating in the population. The sequences were aligned using Muscle 3.8 (Edgar, 2010), then they were ripped and gaps removed using BioEdit (Hall, 1999). HIV-BLAST and REGA HIV-1 subtyping tool were used in subtyping and in the prediction of the unique circulating recombinant forms using the *pol*–RT sequences. Since the *RT* gene was short the subtypes and circulating recombinant forms were confirmed by the following silico tools; Recombinant Identification Program (RIP3.0) (Siepel *et al.,* 1995), Jumping HMMER (http://jphmm.gobics.de/submission), and recombinant drawing tools version 2.1.0 (http:// www.hiv.lanl.gov/content/sequence/DRAW_CRF/). Reference sequences were selected from the Los Alamos HIV database in the construction of the phylogenetic trees **(Appendix 5).** Phylogenetic analysis was done using the Maximum likelihood method by use of MrBayes version 3.1 (Ronquist *et al*., 2005) based on Bayesian phylogenetic analysis. Their reliability was estimated by 1000

bootstrap replications. The profile of the trees were visualized with Tree Graph version 1.4.0 (Stover *et al.*, 2010). This procedure was used for analysis of all the sequences generated from the *pol-RT* region of the HIV-1 genome.

CHAPTER FOUR

RESULTS

4.1 Baseline characteristics of the study subjects

All subjects were from Comprehensive Care Clinic, with age ranging from 18 to 72 years (Median age 36 years) and mean CD4 count of 177.6 cells/mm^3 (range: 10 to 349 cells/mm^3). All the patients were on first-line ART and had received a combination of 2NRTI+NNRTI treatment for 1–2 years and showed clinical signs of treatment failure with baseline CD4 count below 350 cells/mm^3 and viral loads ranging from 1.23E3 to 1.03E7copies/ml (Table 4.1)

Table 4.1 Summary of the baseline characteristics of the study population

	All (N=81)	Female (n=45)	Male (n=36)
Ages			
Median (Range)	36 (18-72)	35 (18-56)	39 (18-72)
CD4+ T-cell count	177.6	185.8	162.9
Mean (Cells/mm^3) Range	10-350	12-349	10-324
350-200 cells/mm^3	35	25	18
> 199 cells/mm^3	46	20	19
Viral load count	514,929	356,124	799,105
(Copies/mm^3) (Range)	(1,234 - 10,344,012)	(1,234 - 2,465,798)	(3,000- 10, 344,012)
<100,000 N (%) Copies/mm^3	50 (61.7%)	30 (67%)	20 (55.5%)
>100,001 Copies/mm^3	31 (38.3%)	15 (33%)	16 (44.5%)
EFV Containing regimen	**37**	**22**	**15**
TDF/3TC	5	3	2
AZT/3TC	25	15	10
D4T/3TC	7	4	3
NVP Containing regimen	**44**	**23**	**21**
TDF/3TC	10	6	4
AZT/3TC	9	5	4
D4T/3TC	25	14	13

4.2 Analysis of the generated amplicons

Eighty samples were successfully amplified at the *pol-RT* gene region. The PCR products were 697 base pairs in size.

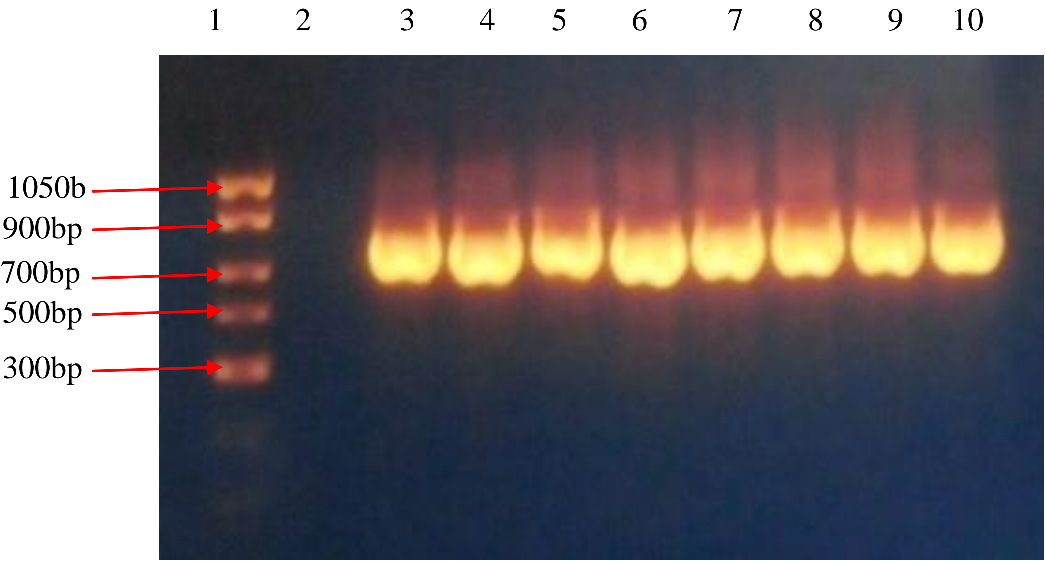


Figure 4.1: A 2.0% agarose gel electrophoresis profile of the *pol-RT* gene. Number 1 is the molecular weight marker ladder while numbers 3-9 are some of the PCR products that were generated in the study, negative control (2) and positive controls (10).

4.3 Drug-specific mutation that confer resistance to ART

There were eighty successfully sequenced samples, all of which were from patients who were on first-line of ARV treatment. Fifty four percent (n=44) of the patients were on Nevirapine (NVP) containing regimens, while 46% (=36) were on Efavirenz (EFV) drug combination. The drug combinations were: 6 patients (7.4%) were on TDF+3TC+EFV, AZT +3TC +EFV for 25 (30.9%), D4T+3TC+EFV for 6 (7.4%), TDF+3TC+NVP for 9 (11.1%), AZT+3TC+NVP for 10 (12.3%) and D4T+3TC+NVP for 25 (30.9%).

It was noted that 14.8% (n=12) of the patients did not have any drug related mutations that could confer treatment failure. This patients who showed no drug resistance related mutations were on the following drug treatment: 3TC+AZT+EFV 25% (n=3), 3TC+D4T+NVP 25% (n=3), 3TC+D4T+NVP 33% (n=4), and 3TC+AZT+NVP 16% (n=2). They had viral load range of between 1.04E3 copies to 5.8E4, while a CD4 count below 350 cells/mm^3.

4.3.1 Nucleoside Reverse Transcriptase Inhibitors (NRTI) drug resistance related mutations

Among the study subjects 85.1% (n=69) of the patients exhibited major mutations that could confer resistance to treatment. The most common NAM was M184V 42 (51.8%) then K65R was present in 19 (23.4%) while L74V was seen in 8 patients (9.8%). Thymidine analog mutations accumulated in two distinct but overlapping patterns type I pattern: M41L, L210W, and T215Y and type II pattern: D67N, K70R and K219Q/E. The most common type I pattern was T215F/Y 25 (30.8%), while K219Q/E 18 (22.2%), was the most observed type II pattern (Table 4.2). It was observed that Nevirapine (NVP) containing regimen had a higher prevalence rate in mutations as compared to Efivarenz (EFV) containing regimen.

Table 4.2: A summary of Nucleoside RT Inhibitors (NRTI) drug resistance mutations in patients treated with duo nucleoside RT inhibitor plus a NNRTI drugs regimens

	Pol RT Gene Mutation	***No. of patients N/80 (%) EFV containing regimen***	***No. of patients N/80 (%) NVP containing regimen***
NAMs	M184V	16 (20%)	26 (32.5%)
	K65R	10 (12.5%)	9 (11.25%)
	L74V	3 (3.75%)	5 (6.25%)
Type I TAMS	M41L	4 (5%)	11 (8.75%)
	L210W	1 (1.25%)	4 (5%)
	T215F/Y	11 (13.75%)	14 (17.5%)
Type II TAMS	D67N/G	7 (8.75%)	17 (12.5%)
	K70R	6 (7.5%)	6 (7.5%)
	K219Q/E	5 (6.25%)	13 (16.25%)

4.3.2 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI) drug resistance related mutations

The NNRTI resistance mutations observed were classified into the following categories: Primary NNRTI resistance mutations, secondary NNRTI resistance mutations and minor non-polymorphic mutations. The primary NNRTI resistance mutations seen in this study include: K103N, V106A/M, Y181C/F/L/V, Y188C, and G190A/ G. The secondary mutations that occurred in the study were the following P22H/P, F22Y/L, K101P, P225H, and K238T. The minor NNRTI mutation were as following A98G, H22I/Y, V90I, V108I, and V179D/E. The highest primary mutation observed in this study was K103N seen in 41 patients (51.25%), while K101EK 9 (11.1%) was the highest secondary mutation. Lastly H22I/Y was observed to be highest minor mutations detected in 17 patients (20.9%) (**Table 4.3**).

Table 4.3: A summary of Non-nucleoside RT Inhibitors (NNRTI) drug resistance mutations in patients treated with duo nucleoside RT inhibitor plus NNRTI drugs regimens

	Pol RT Gene Mutation	*No. of patients N/80 (%) EFV containing regimen*	*No. of patients N/80 (%) NVP containing regimen*
Primary Mutations	K103N	19 (23.75%)	22 (27.5%)
	V106A/V	2 (2.5%)	4 (5%)
	Y181C/F/L	4 (5%)	11 (13.75%)
	Y188C/I/L	2 (2.5%)	4 (5%)
	G190A/G	4 (5%)	10 (12.5%)
Secondary Mutations	P22H/P,F22Y/L, K101E/K,P225C/S, K238T	12 (15%)	26 (32.25%)
Minor Mutations	H22I/Y, V90I, A98G, V108N/C, V179D/T	9 (11.25	15 (18.75%)

4.4 Evaluation of prognostic assays by use of HIV-1 genotypic test

Genotypic testing is a gold standard test used to detect failure of HIV-1 drug therapy and it is used to guide the best regimen for the patients who have failed treatment. This tool was used in this study to evaluate the prognostic assays used to predict treatment

failure. Prognostic assays used in the prediction of treatment failure are viral load (VL) count and CD4 count. Thirty samples were selected that had high level drug resistance. The samples selected, sensitivity and specificity of the prognostic assays were calculated based on the drug resistance test as the gold standard. The sensitivity of viral load was 95% while CD4 count was 94%, the specificity was 100% for VL while CD4+ count was lower at 83%. The positive predictive value (PPV) of VL was 100% while CD4+ count was 89%, while the negative predictive value (NPV) were the same for both at 91%.

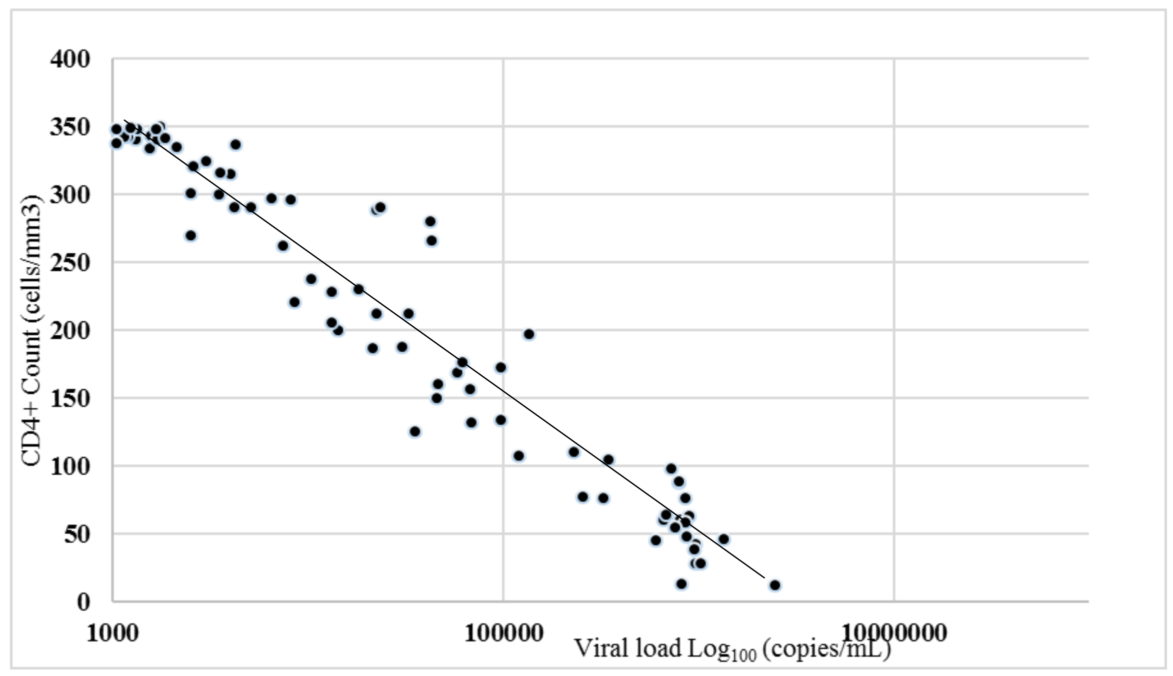
Table 4.4: Predictive values of the prognostic assays

	Positive	Negative	SENSITIVITY	SPECIFICITY	PPV	NPV
VL (30)	19 TP 0 FP	1 FN 10 TN	19/20*100=**95%**	10/10*100=**100%**	19/19*100=**100%**	10/11*100=**91%**
TOTAL	**19**	**11**				
CD4+(30) count	17 TP 2 FP	1 FN 10 TN	17/18*100=**94%**	10/12*100=**83%**	17/19*100=**89%**	10/11*100=**91%**
TOTAL	**19**	**11**				

4.5 Correlation between Viral load and CD4 count

It was observed that extremely low CD4/CD8 count (<100 cells/mm^3) and high viral load (>100,000 copies/ml) indicated very high level of resistance to both NNRTI and NRTI. However a fairly average amount of CD4/CD8 count (300 -349 cells/mm^3) and a minimal amount of viral load (1000-10,000 copies/ml) displayed a slight susceptibility to NNRTI and/or NRTI. Pearson correlation coefficient was used to establish the correlation between CD4 count and viral load, correlation was observed to be significant at the 0.01 level, $r = -0.762$ hence $R^2 = 0.581$. An inverse relationship was observed between viral load and CD4 count (Figure 4.2).

Figure 4.2: A Scatter plot illustrating the correlation between viral load and CD4 count



4.6 Sub-type variation within the study population

From the 80 samples successfully amplified and sequenced. Overall, HIV-1 subtype A1 was the most prevalent (47 samples) followed by HIV-1 subtype D (22), HIV-1 subtype C (5), HIV-1 and subtype G (1). There were two circulating recombinants CRF01-AE and CRF02-AG. (**Table 4.5**).

Table 4.5: Circulating subtypes within the study population

Subtypes	Subjects N (%)
A1	47(58.25%)
A2	3(3.75%)
D	22 (27.5%)
C	5 (6.25%)
G	1 (1.25%)
CRF01-AE, CRF02- AG	3 (2.75%)

4.7 Relationship between the HIV-1 subtypes and major drug related mutations

The M184V nucleoside reverse transcriptase inhibitors related mutation was observed in all subtypes, while K103N was highly prevalent in subtype D. Due to the low numbers of some subtypes within the study group (subtypes A2, C, G and the CRFs), the prevalence rate of the mutations were high. **(Table 4.6)**

Table 4.6: Prevalence rate of the major drug related mutations within specific subtypes

	Prevalence rate of major NRTI drug related mutations				Prevalence rate of major NNRTI drug related mutations		
SUB-TYPES	**M184V**	**K65R**	**M41L**	**T215F/Y**	**K103N**	**Y181C/F/L**	**G190A/G**
A1	61.7%	29.8%	17.0%	46%	42.5%	12.7%	10.6%
A2	66.6%	-	33.3%	-	-	-	-
D	45.4%	27.2%	18.1%	27.2	77.2%	18.1%	13.6%
C	40%	20%	-	40%	60%	-	20%
G	100%	100%	100%	-	100%	-	100%
CRF	1 50%	-	-	-	-	-	-

4.8 Phylogenetic analysis of the study samples

Phylogenetic analysis revealed five main clusters which were HIV-1 subtype A1, A2, C, D, G and two CRFs, A1 being the predominant HIV-1 subtype with a prevalence of 58.5%. Human Immunodeficiency Virus subtype A1 being the most common subtype in the population clustered with reference sequences from various countries (Tanzania, Kenya, Rwanda, Uganda and Democratic Republic of Congo) at 0.72 bootstrap values. However numerous sub-clusters were observed in the HIV-1 subtype A1 cluster (**Figures 4.3 and Figure 4.4**). Subtype C clustered with sequences from Tanzania, Djibouti, and Zambia. The reference sequences HIV-1 subtype D Democratic Republic of Congo and Kenya.

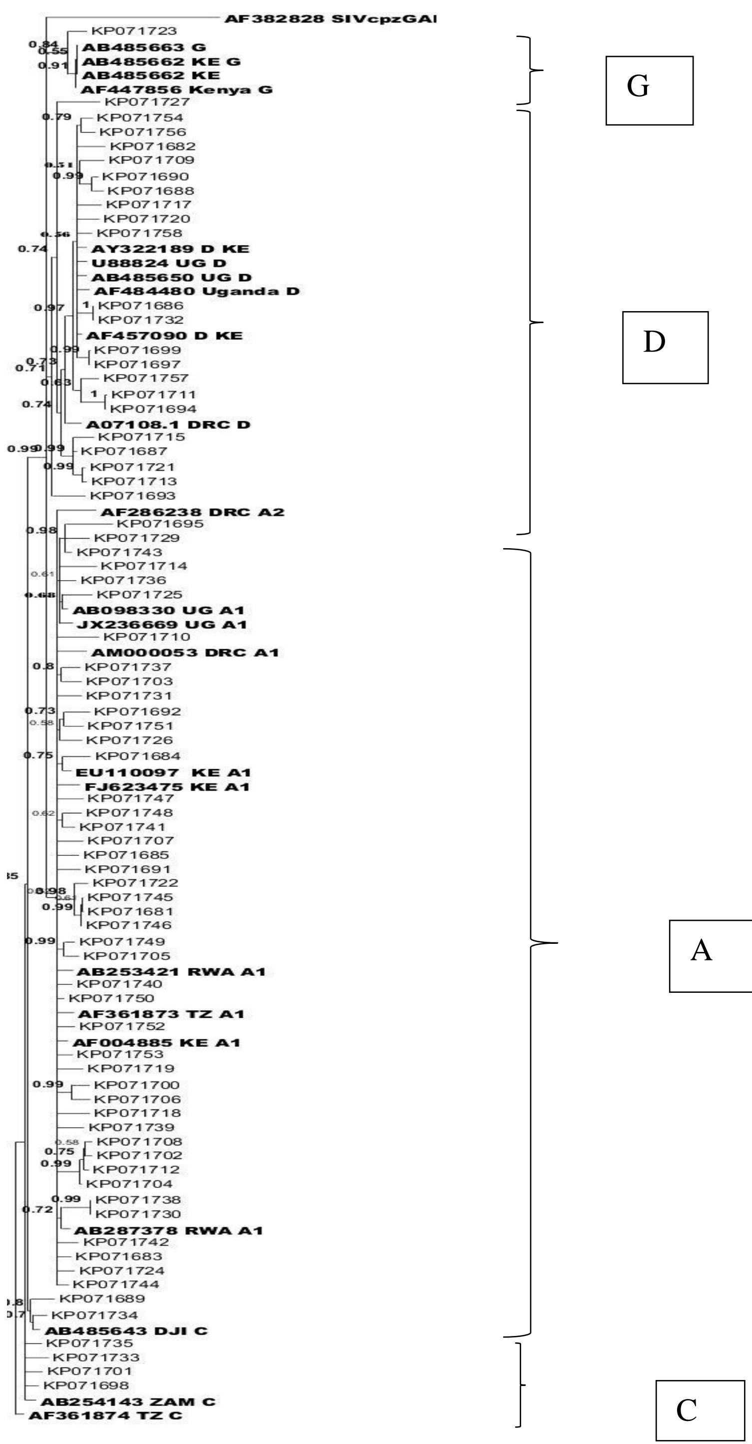


Figure 4.3: A phylogenetic tree of the HIV pol-RT gene showing HIV-1 subtypes in relation to reference sequences from Los Alamos database (Boldface).

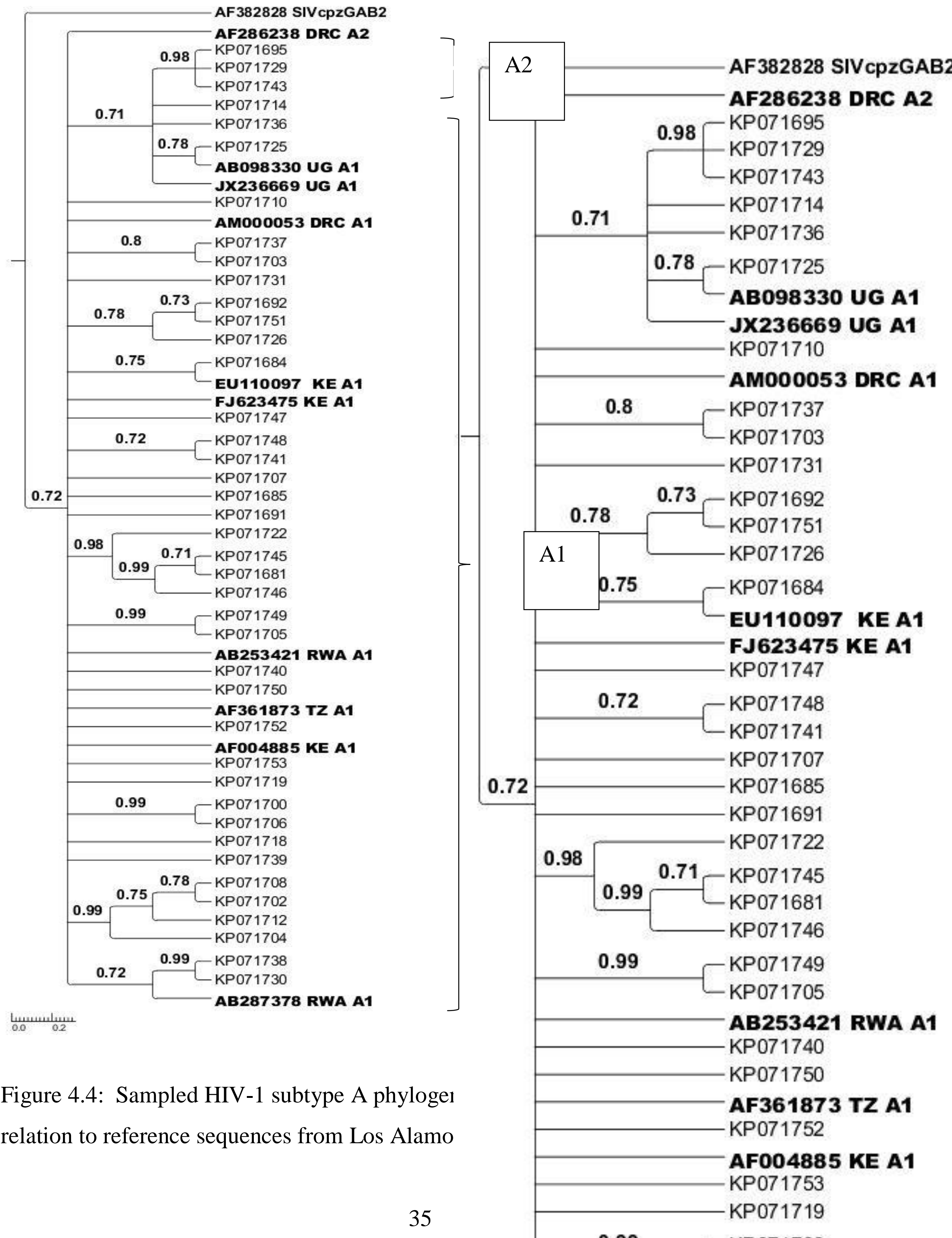


Figure 4.4: Sampled HIV-1 subtype A phyloge

relation to reference sequences from Los Alamo

CHAPTER FIVE

DISCUSSION

5.1 Analysis of Nucleoside Reverse Transcriptase Inhibitors (NRTIs) Drug Resistance associated mutations

In this study major non-thymidine analog containing NRTI mutations (NAMs) were observed to be M184V (51.8%), followed by K65R (23.75%) then L74V (10%). Approximately 17.5% (14) of patients that had the M184V mutation, were taking AZT+3TC+EFV drug combination. Nine patients on the same drug regimen were indicated to have K65R mutations.

A similar study conducted by Lanier *et al.,* 2004, it was observed that the most common mutations in patients developing virologic failure while receiving a non-thymidine analog containing NRTI backbone include M184V alone or M184V + K65R or L74V. However M184V mutation have favorable effects on the susceptibility of some other NRTIs hence they can delay clinical and immunologic progression (Marcelin *et al.,* 2006). Eight study subjects were observed to have L74V mutation, this mutation has been known occasionally to be selected by TDF (Lanier *et al.,* 2004). Thymidine analog mutations (TAMs) was observed to occur in 2 patterns. In this population study, type 1 pattern mutation T215Y was observed in 31.25%, followed by 13.75% of patients who had M41L mutation. Mutations such as M41L and T215Y have been known to confer high-level resistance to AZT and d4T (Shafer *et al.,* 2008) hence high mutations were observed to occur in patients who were taking D4T+3TC+NVP and AZT+3TC+EFV combination. However d4T based regimens are being phased out because of their association with higher toxicities leading to poor adherence, consequently treatment failure (Kenya HIV DR report, 2010-2011). The most observed mutation within the type II pattern was K219Q/E (22.5%), followed by D67N/G (21.25%) then K70R (15%). According to (Rhee *et al.,* 2005 and Shafer *et al.,* 2008) the clinical significance of the type II TAM is not as well characterized. Similar studies conducted Type I TAM has

been known to cause higher levels of phenotypic and clinical resistance to the thymidine analogs and cross-resistance to ABC, ddI, and TDF than do the type II TAM (Lanier *et al.,* 2004, Miller *et al.,* 2004, Cozzi-Lepri *et al.,* 2005, and Marcelin *et al.,* 2006). Therefore patients with type I TAM mutation require regimen change to treatment that will be more effective in suppression of the virus.

5.2 Analysis of Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) Drug Resistance associated mutations

The NNRTI mutation profiles differed between patients failing EFV compared to those on NVP containing regimens. Approximately 51.25% (n=41) of the study subjects had K103N mutation with the highest frequency occurring on NVP containing regimens. Generally a higher frequency of mutations was observed in NVP drug combination as compared to EFV. This findings disagree with (Koigi *et al.,* 2014) which was observed that EFV seemed to select for a wider range of mutations compared to NVP.

Other primary NNRTI mutations that were observed in a number of the study subjects include Y181C/F/L at 18.75% (n=15), G190A 17.5% (n=14), Y188C/I/L 7.5% (n=6) and V106A/V 7.5% (n=6). All primary NNRTI mutations are known to cause high-level resistance to NVP and variable resistance to EFV, ranging from about 2-fold for Y181C, 6-fold for G190A and 20-fold for K103N (Rhee *et al.,* 2006). K103N mutation observed to be the highest among patients on NVP combination, explains the high level of resistance observed in the group.

In this study the secondary NNRTI mutation were less frequent as compared to the primary NNRTI mutations. The most frequently observed secondary NNRTI mutations was K101P at 11.25% (n=9), followed by K238T 7.5% (n=6) and P225H 3.75% (n=3), the other mutations F22Y/L and P225C/S had a frequency of less than 2%. Secondary NNRTI mutation usually occur in combination with one of the primary NNRTI resistance mutations (Shafer *et al*., 2008). For instance according to Rhee *et al.,* (2006) a mutation such as K101P if it occurs in combination with K103N it further decreases

NVP and EFV susceptibility from 20-fold with K103N alone to more than 100-fold. Others such P225H and K238T/N usually occur in combination with K103N and synergistically reduce nevirapine and efavirenz susceptibility (Pelemans *et al.,* 1997, Parkin *et al.,* 2006). In addition F227L occurs in combination with V106A, leading to a synergistic reductions in nevirapine susceptibility (Balzarini *et al.,* 1998). These findings from other studies conducted explains the high level of resistance observed in both NVP and EFV in the study population.

5.3 Evaluation of the prognostic assays

All the subjects selected for this study were predicted to have treatment failure according to the prognostic assays, which showed virological failure (viral load above 1000 copies/ml) and fall of CD4/CD8 count (<350 cells/mm^3) in spite of treatment. The positive predictive value of viral load was 100% which indicates that the prognostic assay is 100% reliable in the prediction of treatment failure while 91% reliable in the prediction susceptibility to treatment. On the other hand CD4+ count had a positive predictive value of 89% while a negative predictive value of 91%. According to the predictive values obtained, VL is shown to be more reliable in the prediction of treatment failure as compared to the CD4+ count however both VL and CD4+ count are 91% reliable in the prediction of susceptibility of treatment.

The prognostic assays in this study showed that samples that had high viral load (>100,000 copies/ml) and low CD4/CD8 count (<100 cells/mm^3), had high levels of resistance to the treatment. The Pearson correlation coefficient of .581 is indicative of a weak inverse relationship between viral load and CD4 count. The weak inverse relationship basically is indicative of CD4 count being a poor predictor of treatment failure as compared VL. The CD4 count is used to assess a patients' immunologic response to ART while VL testing is an established surrogate marker for treatment response (Thiebaut *et al.,* 2000).

However according to a consensus statement developed by the International AIDS Society-USA panel (Hirsch *et al*., 2008) it was recommended resistance testing as a guide for the treatment of patients that therapy has failed. In addition resistance testing at low-level viraemia was shown to be of clinical benefit, with results accurately predicting the risk of an increase in viral load to above 1000 copies/ml and the virological failure of antiretroviral therapy (Sanjeev, 2013). In a slightly similar study conducted in India it re-empasizes the importance of genotyping prior to the start of second line treatment for rational selection of regimen to have better efficacy of the therapy (Toor *et al*., 2012).

Eleven respondents (13.75%) did not show treatment failure, whereas 26.25% (n=21) showed high resistance to both NNRTI and NRTI, thus genotypic tests can help the clinician on the proper combination of regimen of the second line of therapy which comprises of 2 or 3 NRTI drugs and one protease inhibitor.

In this study EFV 10% (n=9) containing regimens was observed to have less resistance as compared to NVP 15% (n=12) drug combinations. According to (Toor, 2012) EFV containing regimen is associated with better survival, fewer AIDS-related opportunistic infections and a decreased likelihood of experiencing virologic failure. Conferring to a previous study done (Sanjeev *et al.,* 2013) there was a trend in increase in mortality within the nevirapine group as compared to that of the efavirenz group (19 · 4% vs. 14 · 7%); this difference, however, was not significant (P = 0 · 46).

5.4 Circulating HIV-1 subtypes

This study agreed with molecular epidemiological studies conducted whereby HIV-1 subtypes A1, C and D are the main subtypes of HIV-1 circulating in Kenya, like the rest of the other East Africa countries of Uganda and Tanzania, occurring in different proportions in different populations (Hoelscher *et al.,* 2001).

The sequences analysed were found to harbour diverse HIV-1 subtypes with majority belonging to HIV-1 subtype A1. This finding was in concurrence with previous studies

conducted in Nairobi and Kenya in general (Songok *et al.,* 2003; Yang *et al.,* 2004; Songok *et al.,* 2004; Allison *et al.,* 2008; Lihana *et al.,* 2006; Khoja *et al.,* 2009; Steegen *et al.,* 2009; Lihana *et al.,* 2009).

The findings on circulating HIV-1 subtypes from this study concurred with previous studies conducted in Kenya including the rest of East Africa in general (Neilson *et al.,* 1999; Hoelscher *et al.,* 2001; Lihana *et al.,* 2009) where 92.8% pure subtypes and 2.4% CRFs; 71% pure subtypes and 29% CRFs respectively.

Surveillance studies have been carried out in different parts of Kenya to establish HIV-1 subtypes. It has been carried out mainly in Nairobi, Central Kenya, Western and Nothern parts of the country (Poss *et al.,* 2001; Dowling *et al.,* 2002; Songok *et al.,* 2003; Yang *et al.,* 2004). HIV-1 subtype C is the most prevalent worldwide (Hemelaar *et al.,* 2004) and a major HIV-1 subtype in some countries of southern and eastern Africa, Asia and the Middle East, where most infections occur (Fontella *et al.,* 2008).

5.5 Relationship between the HIV-1 subtypes and major drug related mutations

From this study it was observed that certain subtypes had a high prevalence rate of drug associated mutations. Subtype A1 was observed to have a high number of drug associated mutations, M184V was the highest, this mutation is known to confer very high resistance against NRTI. A similar study conducted in Botswana, it was observed that certain mutations seem to occur more frequently in non-B subtypes in particular in response to a didanosine or stavudine based regimen, virus containing K65R mutations developed within 8 months, more rapidly than is seen in patients infected with subtype B (Frater *et al.,* 2001). In this study most of the subtypes had K65R mutations however it was higher in subtype A1. It was noted that subtype C had a 60% prevalence rate of developing K103N mutation. A study conducted by (Grossman *et al.,* 2004) showed that Subtype C viruses also develop resistance against nonnucleoside reverse-transcriptase inhibitors through either the K103N or V106M mutations, whereas subtype B viruses rarely develop V106M mutations.

Subtype D was observed to have a prevalence rate of 77.2% in developing K103N mutation which is known to cause rapid decline in the susceptibility of NNRTI treatment. A study conducted by Easterbrook *et al.,* (2010), observed that subtype D had a four-fold faster rate of CD4 decline and a higher rate of virological rebound on ART compared to other subtypes. Due to the variation of drug related mutations, observed within the subtypes it is paramount to detect the different subtypes so as to determine effective treatment.

5.6 Phylogenetic analysis

As the prevalence of HIV-1 increases, it has led to diversification of the geographic distribution of subtypes hence the greatest genetic variation in HIV-1 has been found in regions where the HIV epidemic is oldest such as the regions of sub-Saharan Africa where most of the HIV-1 subtypes and many of the CRFs have been identified (McCutchan, 2000).

Phylogenetic analysis revealed that 97.51% were pure HIV-1 subtypes and 2.5% CRFs. It was observed that analysis of samples showed that HIV-1 subtype A1 was the dominant virus with a prevalence of 58.25%. The low prevalence HIV-1 subtype G detected in this work also concurred with previous published reports (Yang *et al.,* 2004). These results suggested that HIV-1 continues to evolve among the Kenya population with new HIV-1 subtypes being introduced and the existing ones increasing in their transmission fitness (Bello *et al.,* 2010).

In this study, HIV-1 subtype D had increasing prevalence (27.5%) that surpassed HIV-1 subtype C (2.5%). These findings concurs with the previous studies conducted particularly in Nairobi (Neilson *et al.* 1999; Songok *et al.,* 2003; Yang *et al.,* 2004; Lihana *et al.,* 2006; 2009; Allison *et al.,* 2008; Khoja et al., 2008).

In the phylogenetic analysis of HIV-1, subtype A1 strains among the study subjects revealed that they were highly divergent, though the clustering with reference HIV-1 subtypes was not ideally significant from the low bootstrap values.

Only one HIV-1 subtype G was observed to cluster with reference sequences from Kenya. However, it is difficult to tell whether these viruses originate from here or are from other parts of continent or world. Nevertheless HIV-1 subtypes G have been isolated from West Africa (Laurent *et al.,* 2002).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

- The mutations observed in this study are related to the HIV-1 treatment failure, however some mutations showed favorable effects on the susceptibility of the drugs administered to the patients.
- The results showed that the viral load is reliable in the prediction of treatment failure, though it should not be entirely relied upon as a means of detecting treatment failure.
- It was observed in the study that patients on Nevirapine containing regimen had a higher level of resistance to drug therapy, while Efavirenz drug combination showed lower levels of resistance.
- This study has shown that HIV-1 subtypes in Nairobi are diverse with HIV-1 subtype A1 as the predominant subtype.
- The subtypes were observed to cluster with reference sequences from different countries from other continents it being an indicator of an increase to diversification of the geographical distribution.
- The limitation of this study is that the prevalence rate given of the mutations within each subtype was dependent upon the numbers within the study group. Due to the fact that numbers of each subtype was variable, the prevalence rate cannot be generalized within the entire population.

6.2 Recommendations and Further Studies

- Viral load testing is important in the evaluation of treatment failure hence they should be all incorporated in the evaluation of therapy on a regular basis. However resistance testing should be used as a guideline of selecting the best regimen

combination for the patient. Not only is resistance testing important for treatment guidelines but also gives specific mutations which will affirm whether the mutations are drug related or not.

- With continued migrations and increasing populations in Nairobi, there is need for a constant monitoring of HIV-1 subtypes.
- Information on the circulating HIV-1 strains is very important, it is therefore critical to have a continuous monitoring of HIV-1 subtypes and circulating recombinants in Kenya including surrounding countries of East Africa.
- This study recommends further studies on the constant monitoring of drug resistance levels among the population. Since the sample population of this study was based on individuals presumed to have undergone treatment failure, it may not reflect HIV dynamics in the general population at the time of sampling. This calls for continued monitoring of circulating HIV-1 subtypes and drug resistance in the general population as treatment options become increasingly available in resource-poor settings. This will eventually help in understanding the dynamics of HIV strains locally and in the region for better management of the infected.

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APPENDICES

APPENDIX 1

ETHICAL CONSIDERATIONS

Evaluation of Predictors of HIV/AIDS treatment failure in a cohort study from Comprehensive Care Clinic in Nairobi, 2013.

Purpose of the study

This is a research study designed to establish the efficacy of prognostic tests done to evaluate drug resistance in HIV-1 patients failing treatment

Sample collection

a) Plasma to be used will be primarily acquired under study protocol SCC No. 2451 from consenting participants.

b) Full informed consent will be sought from participants

Benefits

Any important results that can be used in the management of the patient will be relayed to the patients' doctor. Moreover, information gained from the study will be used to guide effective drug management of the HIV patients. Data will be provided to ministry of health as part of evaluation of rate of development of HIV drug resistances, circulating HIV-1 subtypes and the existing HIV-1 recombinants.

Risk of subject

There are no known major physical, psychological or social associated with participating in the study. However due to the fact that the samples being used are samples that were earlier cryopreserved the samples were de-identified and given unique codes so as to protect the information of the patients. The data was handled with uttermost confidentiality and all information obtained was stored in computer data base which was password protected.

APPENDIX 2

INFORM CONSENT FORM.

1. **<u>Name of the study</u>:** Evaluation Of Predictors Of HIV/AIDS treatment failure in a cohort study in Nairobi

Cohort Identification Number: ______________________.

Today's Date (dd / mm / yyyy): _______/________/_______.

Name/ Surname (participant): ___________________ ___________________.

Name/ Surname (interviewer): __________________ ___________________.

Hello, my name is _________________________. I am working with KEMRI NAIROBI). I am going to give you information and invite you to be in this study. Before you decide, you can ask me any questions you may have or talk to anyone else that you feel comfortable with. If I use any word that you do not understand or any part of my questions is not clear for you, please let me know and I will explain to you in more detail. If you have questions later, you can ask any KEMRI NAIROBI staff or health facility.

This study aims to evaluate whether the normal tests that are used to detect whether you have undergone HIV drug resistance are reliable. This will help your physician to know whether it is necessary to change your drugs to another group of drugs or retain you on the same group of HIV drug therapy but different combination.

Your participation and your decision to participate in this study are entirely voluntary. If you choose not to participate, all the services you receive at the health facility will continue to be offered to you. You may also choose to change your mind later and stop participating, even if you had earlier agreed to take part and changed your mind, and the services you receive at the clinic will continue to be offered to you.

The sample that will be used is the sample taken from you at the Comprehensive Care Clinic in which was collected for CD4 cell count and/viral load testing. The samples will be taken to Kenya Medical training Institute in Nairobi to test for HIV drug resistance test.

Based on your approval and the approval of the Ethical Review Committee in KEMRI the results obtained after the testing of the HIV drug resistance will be compared with the predictive tests that were used to detect the drug resistance (CD4 and/Viral load).

The results of this testing on your blood will help us determine whether the predictive tests used to detect drug resistance are reliable or another method should be in cooperated with it. This study will also assist in changing your treatment if your doctor feels that it is necessary, to an appropriate drug combination. All data obtained will be handled with confidentiality and will only be accessible to the researcher and your doctor only.

If you have any questions you may ask me now or later, even after the study has started. If you wish to ask questions later, you may call or write to the following persons:

1. Elizabeth Ajema Luvai: Principal Investigator, Telephone: 0721606252. Email: ajemaluvai@gmail.com
2. The Kenya Medical Research Institute. National Ethical Review Committee. P.O. Box 54840-00200.Nairobi, Kenya. Telephone: 0722205901.

Consent signing:

I have read the above information, or it has been read it to me. I have had the opportunity to ask questions about the study and I am satisfied with the information I have been given. I consent voluntarily to participate as a participant in this study and understand that I have the right to withdraw the study at any time without in any way affecting my medical care. I accept for my sample to be tested genetically for resistance to medication, I hereby do give consent for my sample to be transferred to KEMRI (tick where appropriate):

Yes: ☐

No: ☐

Write name of participant here: ______________________________________.

Today's Date (dd / mm/ yyyy): _____/_____/_______.

Signature of participant: ___________________________________.

OR

Thumbprint of participant:

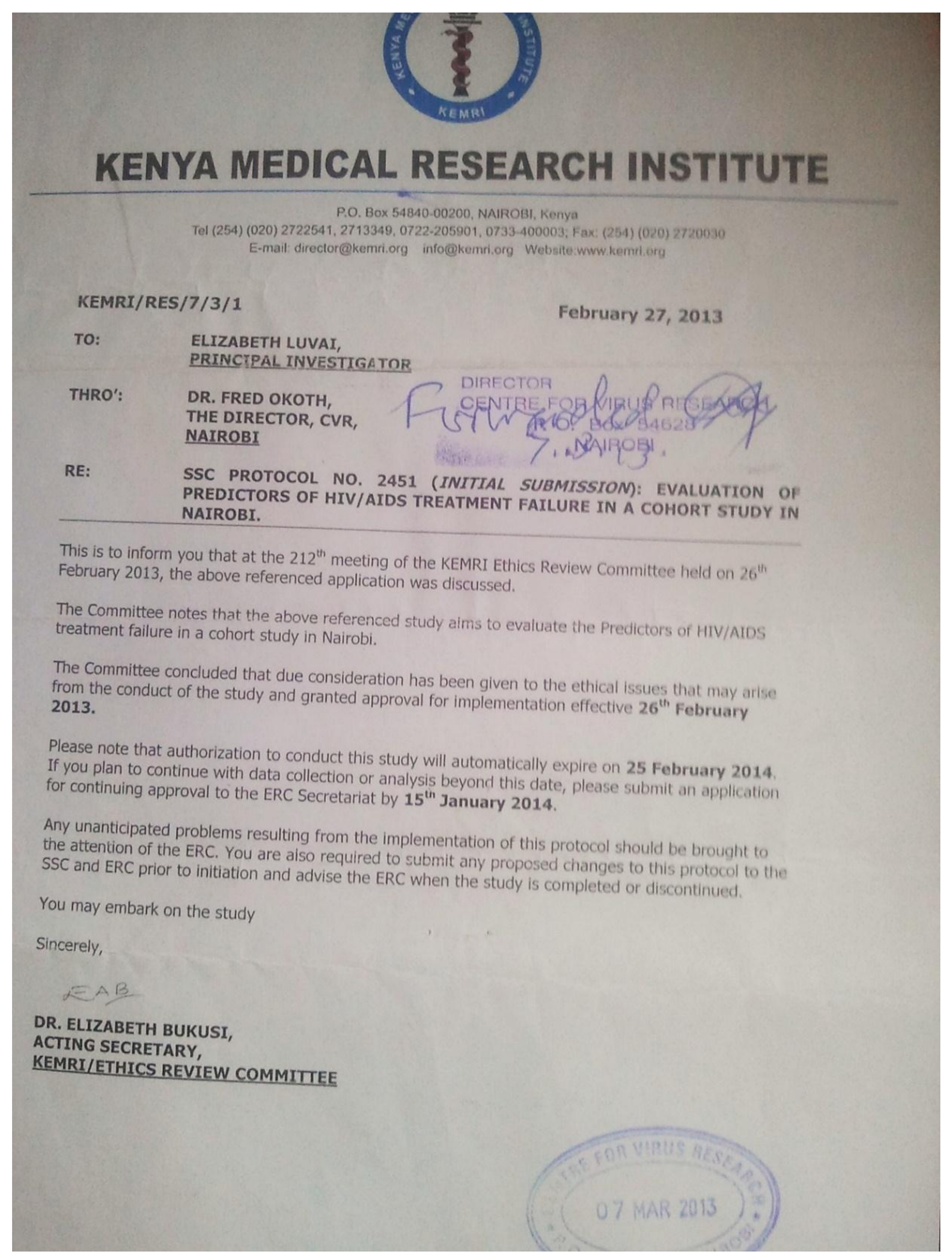
Witness: (only to be used if the patient is not able to read the consent form and has given his/her thumbprint). I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely. I also witnessed that a copy of this Informed Consent Form has been provided to the caregiver.

Print name of witness_______________________________

Signature of witness._______________________________

APPENDIX 3:

ETHICAL APPROVAL LETTER



KEMRI

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KEMRI/RES/7/3/1 **February 27, 2013**

TO: **ELIZABETH LUVAI,**
PRINCIPAL INVESTIGATOR

THRO': **DR. FRED OKOTH,**
THE DIRECTOR, CVR,
NAIROBI

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

RE: **SSC PROTOCOL NO. 2451 (*INITIAL SUBMISSION*): EVALUATION OF PREDICTORS OF HIV/AIDS TREATMENT FAILURE IN A COHORT STUDY IN NAIROBI.**

This is to inform you that at the 212th meeting of the KEMRI Ethics Review Committee held on 26th February 2013, the above referenced application was discussed.

The Committee notes that the above referenced study aims to evaluate the Predictors of HIV/AIDS treatment failure in a cohort study in Nairobi.

The Committee concluded that due consideration has been given to the ethical issues that may arise from the conduct of the study and granted approval for implementation effective **26th February 2013.**

Please note that authorization to conduct this study will automatically expire on **25 February 2014.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **15th January 2014.**

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study

Sincerely,

EAB

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

CENTRE FOR VIRUS RESEARCH
07 MAR 2013

APPENDIX 4

Reference sequences accession numbers from the Los Alamos HIV database

ACCESSION #	SEQ ID	SUBTYPE	COUNTRY	YEAR
AF382828	SIVcpzGAB2	CPZ	GABON	1988
AB253421	92RW008	A1	RWANDA	1992
AB253421	92RW008	A1	RWANDA	1992
AB098330	UG031	A1	UGANDA	1995
FJ623475	06KECst_006	A1	KENYA	1997
U88824	94UG114	D	UGANDA	1994
AF457065	KNH1135	A1	KENYA	1999
AF484493	99UGG03379	A1	UGANDA	1999
AM000054	97CD_KTB13	A1	DEM REP OF CONGO	1999
AY253305	A173	A1	TANZANIA	2001
AF004885	Q23_17	A1	KENYA	1994
A07108	ELI_patent	D	DEM REP OF CONGO	1983
AB254143	02ZM112	C	ZAMBIA	2002
AB485646	ZAM18	C	ZAMBIA	1989
U88824	94UG114	D	UGANDA	1994

AB485662	HH8793	G	KENYA	1993
AB485663	HH8793	G	KENYA	1993
AF361874	97TZ04	C	TANZANIA	1997
A07108	ELI_patent	D	DEM REP OF CONGO	1983
AB485650	UG270	D	UGANDA	1991
AB485651	UG270	D	UGANDA	1991

APPENDIX 5

GENERAL GENERATED SEQUENCES

The generated sequences were deposited in the gene bank and assigned the following accession numbers.

GenBank: KP071681-KP071732

Sequence name	GenBank Sequence ID
Luvai RT sequences.sqn 544	**KP071681**
Luvai RT sequences.sqn 1393	**KP071682**
Luvai RT sequences.sqn 1508	**KP071683**
Luvai RT sequences.sqn 1591	**KP071684**
Luvai RT sequences.sqn 1788	**KP071685**
Luvai RT sequences.sqn 2017	**KP071686**
Luvai RT sequences.sqn 2096	**KP071687**
Luvai RT sequences.sqn 2174	**KP071688**
Luvai RT sequences.sqn 047	**KP071689**
Luvai RT sequences.sqn 096	**KP071690**
Luvai RT sequences.sqn 105	**KP071691**
Luvai RT sequences.sqn 126	**KP071692**

Luvai RT sequences.sqn 171	**KP071693**
Luvai RT sequences.sqn 252	**KP071694**
Luvai RT sequences.sqn 288	**KP071695**
Luvai RT sequences.sqn 307	**KP071696**
Luvai RT sequences.sqn 384	**KP071697**
Luvai RT sequences.sqn 412	**KP071698**
Luvai RT sequences.sqn 433	**KP071699**
Luvai RT sequences.sqn 439	**KP071700**
Luvai RT sequences.sqn 466	**KP071701**
Luvai RT sequences.sqn 604	**KP071702**
Luvai RT sequences.sqn 652	**KP071703**
Luvai RT sequences.sqn 687	**KP071704**
Luvai RT sequences.sqn 867	**KP071705**
Luvai RT sequences.sqn 023	**KP071706**
Luvai RT sequences.sqn 054	**KP071707**
Luvai RT sequences.sqn 111	**KP071708**
Luvai RT sequences.sqn 142	**KP071709**
Luvai RT sequences.sqn 145	**KP071710**
Luvai RT sequences.sqn 157	**KP071711**

Luvai RT sequences.sqn 167	**KP071712**
Luvai RT sequences.sqn 309	**KP071713**
Luvai RT sequences.sqn 322	**KP071714**
Luvai RT sequences.sqn 430	**KP071715**
Luvai RT sequences.sqn 498	**KP071716**
Luvai RT sequences.sqn 516	**KP071717**
Luvai RT sequences.sqn 605	**KP071718**
Luvai RT sequences.sqn 627	**KP071719**
Luvai RT sequences.sqn 661	**KP071720**
Luvai RT sequences.sqn 758	**KP071721**
Luvai RT sequences.sqn 872	**KP071722**
Luvai RT sequences.sqn 948	**KP071723**
Luvai RT sequences.sqn 031	**KP071724**
Luvai RT sequences.sqn 792	**KP071725**
Luvai RT sequences.sqn 061	**KP071726**
Luvai RT sequences.sqn 106	**KP071727**
Luvai RT sequences.sqn 119	**KP071728**
Luvai RT sequences.sqn 9427	**KP071729**
Luvai RT sequences.sqn 10615	**KP071730**

Luvai RT sequences.sqn 11480	**KP071731**
Luvai RT sequences.sqn 12256	**KP071732**