EVALUATION OF HIV-1 EVOLUTION AND ITS ROLE IN DEVELOPMENT OF ANTIRETROVIRAL DRUG RESISTANCE IN NAIROBI COHORT

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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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This work is dedicated to my beloved wife Sepha Nyatichi, who has always supported me unfalteringly in absolutely everything that l do, and to the countless millions who have suffered at the hands of AIDS epidemic.

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Micro litre
3TC	Lamivudine (2', 3'-dideoxy-3'-thiacytidine)
ABC	Abacavir
ABI	Applied Biosystem International
AIDS	Acquired Immune Deficiency Syndrome
AZT	Zidovudine
ART	Antiretroviral Therapy
ARV	Antiretrovirals
BAF	Barrier to Autogration
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
C1-C5	Constant region 1 to 5
CCR5	Chemokine Receptor 5
CRF	Circulating Recombinant Forms
CXCR4	Chemokinine Receptor 4
D4T	Stavudine
DDI	Didanosine
DLV	Delavirdine
DNA	Deoxyribonucleic acid
dNTPs	Dioxyribonucleic acid triphosphates
env	Envelope (gene)

ETV	Etravirine
FL	Full Length
FTC	Emtricitabine
Gag	Group antigen gene
Gp	group antigen gene (glycoprotein)
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HMG	High Mobility Group
KEMRI	Kenya Medical Research Institute
LTR	Long Terminal Repeat
M group	Major group of viruses
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
PIC	Pre-Intergration Complex
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
STIs	Sexually Transmitted Infections

TAMs	Thymidine analog mutations
TDF	Tenofovir
TSR	Template Suppression Reagent
V1-V5	ariable region 1 to 5
Vif	Viral Infection Factor
vif	Viral Infection Factor gene
WHO	World Health Organization
LAV	**l**mphadenopathy virus
HTLV-III	human T cell lymphotropic virus III
LAS	lymphadenopathy syndrome
dN	nonsynonymous subsititution
dS	synonymous subsititution
IV	Intravenous
UNAIDS	Joint United Nations programme for AIDS

ABSTRACT

The treatment of HIV-1 infection with antiretroviral drugs has greatly improved the survival of those who are infected. However, HIV-1 diversity and drug resistance are major challenges in patient management, disease control and surveillance especially in resource-poor countries.

In this study, 188 blood samples were collected from Nairobi cohort and peripheral mononuclear cells (PBMCs) separated. Total proviral DNA was used in nested polymerase chain reaction to amplify 450*bp* HIV *env* C2V3, 288*bp* Integrase and env gp41 regions and directly sequenced . Generated sequences were aligned and phylogenetically analysed using known reference subtypes sequences and drug resistance mutations and substitutions determined.

Phylogenetic analysis based on env C2V3 region revealed A1 (59.6%), C (18.1%), D (10.6%), B (2.1%), G (2.1%), CRF02_AG (3.2%) and the rest of 6.9% were CRFs. In HIV-1 co-receptor switch showed R5 tropism (69.6%) while X4 (30.4%). In addition, 2.4% T97A that is associated with reduced susceptibility to Raltegravir and 26.2% had secondary mutations associated with resistance to intergrase inhibitors. In fusion inhibitors, the following mutations were detected; A316T/I323V (2.6%) combination, A316T (63%), I323V (1.1%) for Maraviroc, (10%) K305R, (3.2%) G321E, (35.1%) R315Q, (4.5%) K305R/R315Q, (62.8%) T320R for Vicriviroc and (1.6%) A316T+ K305R+ R315Q, (12.7%) A316T+R315Q, (3.2%) R3I5Q+A316T+I323V, (0.5%) R315+A316T+G321E for Maraviroc and Vicriviroc combinations. In addition, 4.2% intermediate resistance associated to Enfuvirtide was detected. The point mutations at; N42S was detected in 16.7% of all the samples, while N42D was detected in 4.2%, S138L /T 3.1%, L44M 2.1% and 1%

each for in the following mutations; N43I and L45V drug resistance mutations. In evolutionary rate; 12.5% had *d*N*d*S ration 1>, 88.5% *d*N*d*S ration < 1 and in those with *d*N*d*S ration ≠1.

The results indicate that HIV-1 subtypes in Nairobi cohort like the rest of the country, is predominated by HIV-1 subtype A1, though there could be possibity of an increase proportion of HIV-1 subtype C prevalences. Existence of diverse HIV-1 recombinants indicated viral mixing among the population, possibly as a result of dual infections. Evolutionary rate of the virus showed natural selection with high proportions of R5 strains suggestive of feasibility of use of maraviroc (CCR5 antagonists) in Kenya. However, multiple drug resistance mutations observed in the newly classes of drug-prior to their introduction, there is a need for constant monitoring of HIV-1 genetic diversity and drug resistance. Drug resistance seen to common drugs was minimal (2 %), indicating that we need to continue to prescribe the current used drugs. In addition, the new classes of entry, fusion and integrase inhibitors are feasible as firstline and thirdline drugs respectively. In addition, it was realised that it is not necessary to carry out resistance testing at baseline unless there is strong evidence of virological failure.

CHAPTER ONE

1.0 INTRODUCTION

1.1. The Beginning of the AIDS epidemic

Acquired Immune Deficiency Syndrome (AIDS) was first recognized as a new and distinct clinical entity in 1981 (Gottlieb *et al.,* 1981), when a clustering of an unusual opportunistic infection, *Pneumocystis carinii* pneumonia, and a rare neoplasm, Kaposi's sarcoma, was observed in young homosexual men in the United States of America (USA) (Gottlieb *et al.,* 1981). Since this new clinical manifestation involved homosexual men, it was thought that the cause of this syndrome might be related to a life-style habit unique to this cohort of people (Friedland *et al.,* 1987). Acquired Immune Deficiency Syndrome was subsequently defined as the appearance of certain dramatic and often life-threatening infections and cancers accompanied by a measurable depletion of immune competence (Ammamm *et al.,* 1983). AIDS cases were soon reported in other groups as well, including intravenous (IV) drug users (CDC, 1982), haemophiliacs (Bloom, 1984), blood transfusion recipients (Curran *et al.,* 1984), female sexual partners of men (Masur *et al.,* 1982) and infants (Oleske *et al.,* 1983) as well as heterosexual population of Zaire (now known as The Democratic Republic of Congo (DRC) (Piot *et al.,* 1984; Sepkowitz, 2001).

In Africa, the same clinical manifestations were observed, mainly among heterosexual population (Piot *et al.,* 1984**)**. These observations made it clear that an infectious aetiology for AIDS should be considered. In 1983, the first indication that AIDS could be caused by a retrovirus came, when Barre-Sinoussi *et al* (1983) recovered a reverse transcriptase containing virus from the lymph node of a man with persistent lymphadenopathy syndrome (LAS) which they later called Lymphadenopathy virus (LAV). A year later, Robert Gallo

and his colleagues independently postulated that a variant T lymphotropic retrovirus might be the causative agent of AIDS (Gallo *et al.,* 1984). Levy and co-workers (1984) also reported the identification of retroviruses, which they called AIDS-associated retroviruses (Levy *et al.,* 1984).

By 1986 the same retrovirus had three designations: LAV, ARV and HTLV-III (Popovic *et al.,* 1984). This was confusing and the International Committee on the Taxonomy of viruses decided to rename the AIDS virus HIV (Coffin *et al.,* 1986a; Coffin *et al.,* 1986b). Today, heterosexual transmission is responsible for the majority of new HIV-1 infections (Esparza and Bhamarapravati, 2000; Osmanov *et al.,* 2002) and even though Zidovudine (AZT), the first Food and Drug Administration (FDA) (USA) approved drug against HIV/AIDS, was introduced in 1987 (Fischl *et al.,* 1987), no known cure has been found to date.

1.1.1 The Origin of HIV

The evolutionary history of HIV-1 and HIV-2 has been reconstructed by inferring phylogenetic trees from primate lentiviruses (Gao *et al.,* 1992; Korber *et al.,* 2000; Yusim *et al.,* 2001). Results from these studies showed that HIV-1 is most closely related to Simian immunodeficiency virus (SIV) from chimpanzee **(Figure 1.1)** which is found in the chimpanzee sub-species *Pan troglodytes troglodytes* and *Pan troglodytes schweinfurthii* (Gao *et al.,* 1999). The habitats of *P.t. troglodytes* include the region in Africa, Cameroon, Gabon and Congo, with the greatest HIV-1 diversity and it has therefore been suggested that this was where HIV-1 first emerged (Gao *et al.,* 1992; Rambaut *et al.,* 2004).

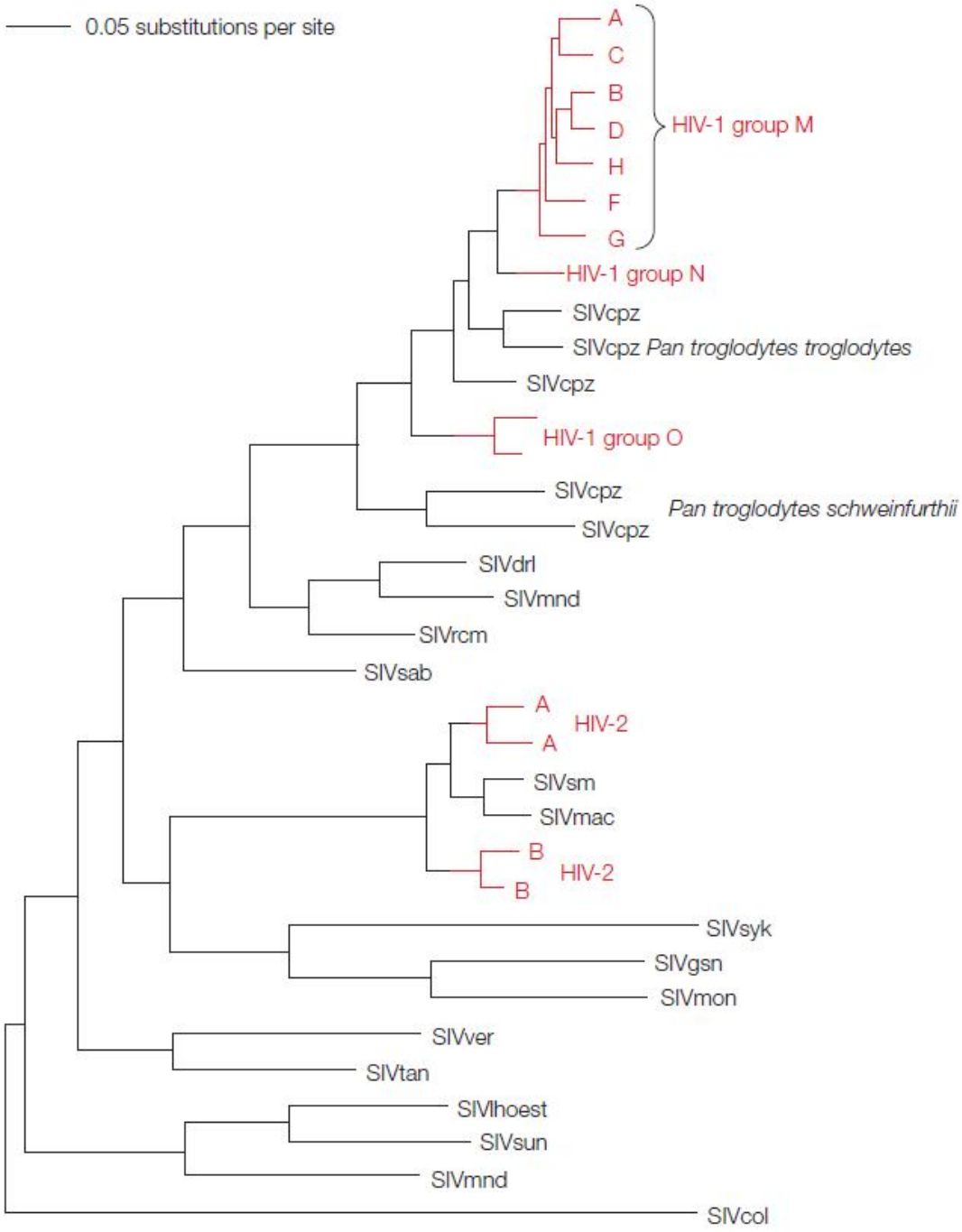

Figure 1.1 Evolutionary relationship of the primate lentiviruses (Rambaut *et al.*, 2004).

HIV-2 shares the closest genetical relationship with SIV from sooty mangabey monkeys, *Cercocebus atys*, that is most frequently found in West African countries such as Guinea Bissau, Ivory Coast, and Senegal, but has spread very little to other areas (De Cock *et al.,* 1993) **(Figure 1.1).** Therefore, it is postulated that the first cross-species transmission of HIV-2 to humans occurred in West Africa (Gao *et al.,* 1992).

It has been difficult to determine the exact number of cross-species transmission of HIV from monkeys to humans but at least four transmissions are likely for HIV-2 (Hahn *et al.,*. 2000), whereas three transmissions from chimpanzees to humans have been suggested for HIV-1 (Gao *et al.,* 1999). It is postulated that these transmissions have resulted in the three groups of HIV-1: The major (M), novel (N) and outlier (O) group (Gao *et al.,* 1999; Sharp *et al.,* 2001) **(Figure 1.1).** It has been estimated that HIV-1 group M originated in the 1930s with a range of ± 10 years (Korbe *et al.,* 2000; Yusim *et al.,* 2001). Although most estimates for the time of origin for HIV-1 are consistent, recombination events have probably biased these estimates since recombination increases apparent variation in rates among nucleotide sites and reduces the genetic distances between sequences (Schierup *et al.,* 2000; Worobey, 2001). Therefore, in order to retrieve a reliable estimate of the time of origin for HIV-1 M group, archive sequences have been analyzed. The oldest sequence of HIV-1 M is thought to have originated from 1959 (Zhu *et al.,* 1993) and it fits well with the extrapolation of the timescale obtained when the 1930 +/- 20 were calculated as the origin of HIV-1 group M as the timing of the last common ancestor of the HIV-1 group viruses (Hahn *et al.,* 2000; Korber *et al.,* 2000). The introduction of HIV-2 into the human population has been estimated to have taken place between the years 1940-1950 (Lemey *et al.,* 2006) with HIV-2 subtype A strains estimated to be 1940 +/- 16 years and that of the B strains was estimated to be 1945 +/- 14

years (Lemey *et al.*, 2003). More accurate estimation of the origin of HIV-1 and HIV-2 would require analysis of a big number of archived sequences.

AIDS, a syndrome caused by HIV is arguably one of the most serious infectious diseases to have affected humankind (Rambaut *et al.*, 2004). Since the discovery of HIV/AIDS in 1981 there are an estimated 60 million people living with the virus (UNAIDS, 2009). Not only are an estimated 33.4 million people carrying the virus at present (UNAIDS, 2009), but its case fatality rate is close to 100%, making it an infection of devastating ferocity (Rambaut *et al.*, 2004). The HIV pandemic remains the most serious of infectious disease challenges to public health (UNAIDS/WHO, 2007). For instance, subSaharan Africa remains the region most heavily affected by HIV (UNAIDS, 2009). In 2008, sub-Saharan Africa accounted for 67% of HIV infections worldwide, 68% of new HIV infections among adults and 91% of new HIV infections among children. The region also accounted for 72% of the world's AIDS-related deaths in 2008 (UNAIDS, 2009). In the year 2008, the prevalence of HIV in Kenya was 7.8% by 2008, which was an increase from 7.4% reported in 2007. However the figure reported for 2009 was slightly lower at 7.1 % (UNAIDS, 2008; KAIS, 2009).

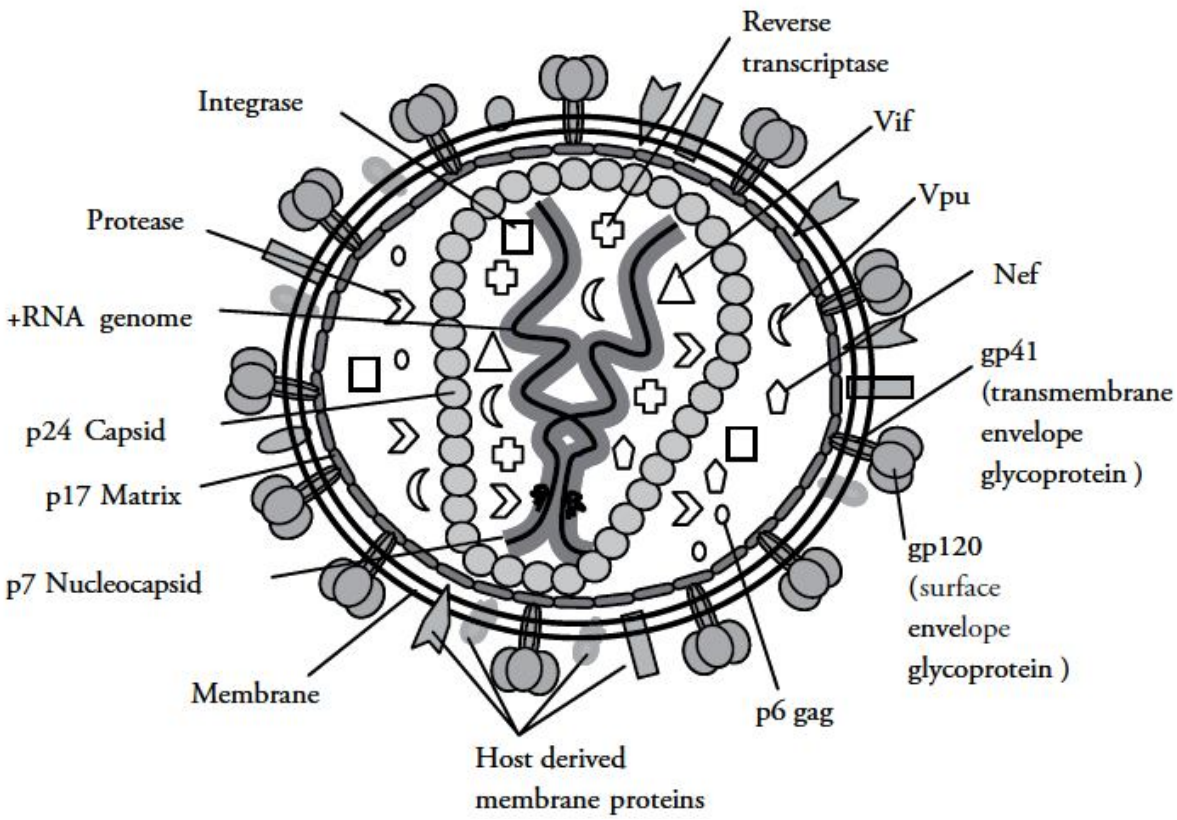

Figure 1.2 The structure of HIV-1 virion
(http://www.chemsoc.org/exemplarchem/entries/2002/levasseur/images/hiv. GIF).

1.2. HIV-1 genome organization

The genomic size of the HIV-1 virion (**Figure 1.3**) is about 9.2 *kb*, with open reading frames coding for several proteins (**Table 1.1**). HIV contains long terminal repeats (LTR) that do not encode proteins but are essential for the regulation of viral gene expression (Briggs *et al.*, 2003). The LTRs are on both sides of the HIV genes: structural genes (*gag*, *pol* and *env*), the regulatory genes (*tat*, *rev* and *nef*) and the accessory genes (*vif*, *vpr* and *vpu*) (Briggs *et al.*, 2003).

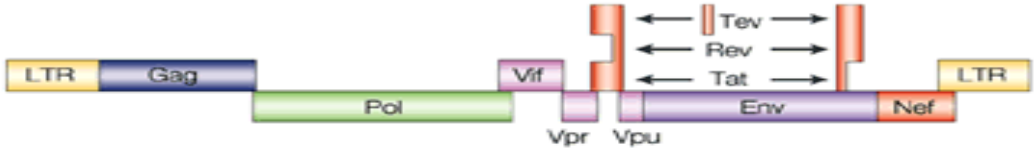

Figure 1.3 HIV-1 genome organisation (Briggs *et al.*, 2003).

KEY: The genomic organization of HIV-1. The genome is flanked by long terminal repeats (LTR). The main genes *gag*, *pol* and *env* encodes the structural proteins (p24, p17 and p7), the viral enzymes (RT, PR and IN) and envelope protein (gp120 and gp41), respectively. Regulatory proteins are encodes by *tat* and *rev*. Finally, *nef*, *vif*, *vpr* and *vpu* encode the accessory proteins.

The *gag* and *env* genes encode the capsid protein (Gag) and external glycoprotein (Env), respectively, where as the *pol* gene encode the viral enzymes necessary for replication; Reverse transcriptase (RT), Intergrase (IN), and Proteas (PR). In addition, HIV also contains at least seven accessory genes (vif, vpu, vpr, tat, rev, nef and tev) (Sakai *et al.*, 2006).

The HIV genome encodes a total of three structural proteins, two envelope proteins, three enzymes, and six accessory proteins (Brian and Michael, 1999). Efforts to control the AIDS epidemic have focused heavily on studies of the biology, biochemistry, and structural biology of HIV and on interactions between viral components and new drug candidates. (**Table 1.1**) shows some of the targeted structural proteins that codes enzymes targeted by antiretroviral drugs including the accessory proteins involved in HIV replications (Brian and Michael, 1999). Through understanding structural biology and functions of some of the HIV structures, it will enable scientists and researchers to design antagonists at various stages of HIV replication cycle.

HIV exhibits a characteristic cone-shaped core that is surrounded by a bilayer lipid envelope derived from the host cell membrane (**Figure 1.2)**. HIV-1 viral particles have a diameter of

100 nm and are surrounded by a lipoprotein membrane (Oh, 1992). The inner core is comprised of the major capsid (CA) protein *p*24 (Gag protein), which surrounds two copies of the viral RNA (Briggs *et al.,* 2003). Closely associated with the RNA strands is the viral RNA-dependant DNA polymerase (*pol*) including the protease, reverse transcriptase (RT) and integrase and the nucleocapsid (NC) proteins (Levy, 1994; Hahn, 1994; Briggs *et al.,* 2003). The inner portion of the viral membrane is surrounded by a myristolated *p*17 core (*gag*) protein that provides the matrix (MA) for the viral structure and is vital for the integrity of the virion. The Matrix (MA) is required for the incorporation of the *env* proteins into the mature virions (Levy, 1994; Goettlinger, 2001).

The surface of the virus is characteristically made up of 72 knobs containing trimers or tetramers of the envelope glycoproteins. They are derived from a *gp*160 precursor, which is cleaved inside the cell into a *gp*120 external surface (SU) envelope protein and a *gp*41 transmembrane (TM) protein (Levy, 1994; Goettlinger, 2001). These proteins are transported to the cell surface, where part of the central and N-terminal portion of *gp*41 is also expressed on the outside of the virion. The central region of the TM protein binds to the external viral *gp*120 in a noncovalent manner (Hahn, 1994). It is estimated that a single HIV-1 virion contains about 1200 molecules of *p*24, roughly 80 molecules of the reverse transcriptase and up to 280 molecules of *gp*120 (Hahn, 1994).

Table 1.1 The HIV-1 genes and their products (HIV Sequence Compendium, 2002)

Gene	Protein	Function
gag MA	p17	Membrane anchoring; *env* interaction; nuclear transport of viral core, (myristylated protein)-
CA	p24	Core capsid
NC	p7	Nucleocapsid, binds RNA
	p6	Binds Vpr
protease (PR)	p15	*gag/pol* cleavage and maturation
reverse transcriptase (RT)	p66 p51	Reverse transcription, RNase H activity
RNase H *integrase* (IN)		DNA provirus integration
env	*gp*120 *gp*41	External viral glycoproteins bind to CD4 and secondary receptors
tat	p16/p14	Viral transcriptional transactivator
Rev	p19	RNA transport, stability and utilisation factor (phosphoprotein)
Vif	p23	Promotes virion maturation and infectivity
Vpr	p10-15	Promotes nuclear localization of preintegration complex, inhibits cell division, arrests infected cells at G2/M
Vpu	p16	Promotes extra cellular release of viral particles; degrades CD4 in the ER; (phosphoprotein only in HIV-1 and SIV cpz
Nef	p27-p25	CD4 and class I down regulation (myristylated protein)
Vpx	p12-16	Vpr homologue (not in HIV-1, only in HIV-2 and SIV)

The HIV-1 genes and their products as well as their function in the life cycle of the virus (**Table 1.1**) briefly, *nef* is a regulatory protein present only in the primate lentiviruses HIV-1, HIV-2 and SIV. The *nef* gene is situated at the 3'-end of the viral genome. Initially it was believed that the *nef* gene is of little importance and that it inhibits transcription. From this characteristic the gene got its name, which is short for negative factor. However, Nef was

soon shown to be crucial in the maintenance of optimal viral replication and in the progression of infection to acquired immune deficiency syndrome (AIDS) (Geyer and Peterlin, 2001; Arold and Baur, 2001). For the env, the Infection of the host cell by HIV is initiated by interactions between SU and cell-surface CD4 molecules. SU binds to CD4 and anchors the virus to the cell surface, and additional interactions with chemokine receptors trigger a conformational change that leads to fusion of the viral and cellular membranes (Brian and Michael, 1999).

1.2.1 Genomic region of HIV

1.2.1.1 envelope glycoprotein 120 V3 loop

The *env* gene codes for the envelope glycoprotein *gp*160, which is a precursor to two glycoproteins: *gp*41 and *gp*120. This protein (*gp*120) is embedded in and extends exterior to the viral lipid membrane and is primarily responsible for host cell receptor binding and host cell tropism. Additionally, due partly to its physical location in the virion, *gp*120 contains a number of recognition sites for various adaptive immune responses, including neutralizing antibodies, (Goudsmit *et al.*,1988), helper T lymphocytes (Fenoglio *et al.*,2000), and cytotoxic T lymphocytes (Walker *et al.*,1986; Tsubota *et al.*,1989). Therefore, two cells are potentially important positive selective forces acting on the *env* gene leading to changes in optimal host cell receptor affinity and evasion of host immune responses (Modrow *et al.* 1987).

The action of natural selection on the *env* gene is evident from patterns of synonymous and nonsynonymous substitutions in *env* sequences. When the rate of nonsynonymous substitution is greater than the rate of synonymous substitution in some regions of *env*

(Yamaguchi-Kabata and Gojobori 2000), it is a clear indication of positive selection. Estimates have been obtained for the relative frequency of adaptive mutation, the strength of positive selection, and the exact location of positively selected sites (Yamaguchi-Kabata and Gojobori 2000; Ross and Rodrigo 2002).

The *gp*120 portion of *env* has been broadly categorized into five hypervariable regions (V1 to V5) with conserved regions interspersed (Starcich *et al.,* 1986; Modrow *et al.* 1987). The variable regions are mostly found within regions encoding disulfide-constrained loops and four of which (V1-V4) are exposed to the surface and to the host immune system (Leonard *et al.*, 1990) (**Figure 1.4**). Even though other *gp*120 regions play a role in predicting viral phenotype (Koito *et al.,* 1994; Carrillo and Ratner, 1996; Choe *et al.,* 1996), the V3 loop has been the focus of most researchers (Korber *et al.,* 1993; Bickel *et al.,* 1996; Hartley *et al.,* 2005). The V1/V2 region and the V3 loop protein are targets for neutralizing antibodies and also play an important functional role, with the V3 loop largely determining whether a virus uses CCR5 (R5), CXCR4 (X4), or either coreceptor (R5X4) to infect cells (Laakso *et al.,* 2007).

The C2-V5 region of *env* is usually chosen for the analysis of viral changes research because it encodes an important target for immune responses, determines co-receptor specificity, and exhibits a high degree of phylogenetically informative variability (Levy, 1993; Letner *et al.,* 1997). **Figure 1.4** Illustrates the inner domain of *env gp*120 core and *gp*41 *env* glycoprotein. The outer domain, quite variable (V1 to V5) and heavily glycosylated, is exposed on the assembled envelope glycoprotein trimer (Wyatt *et al.,* 1998).

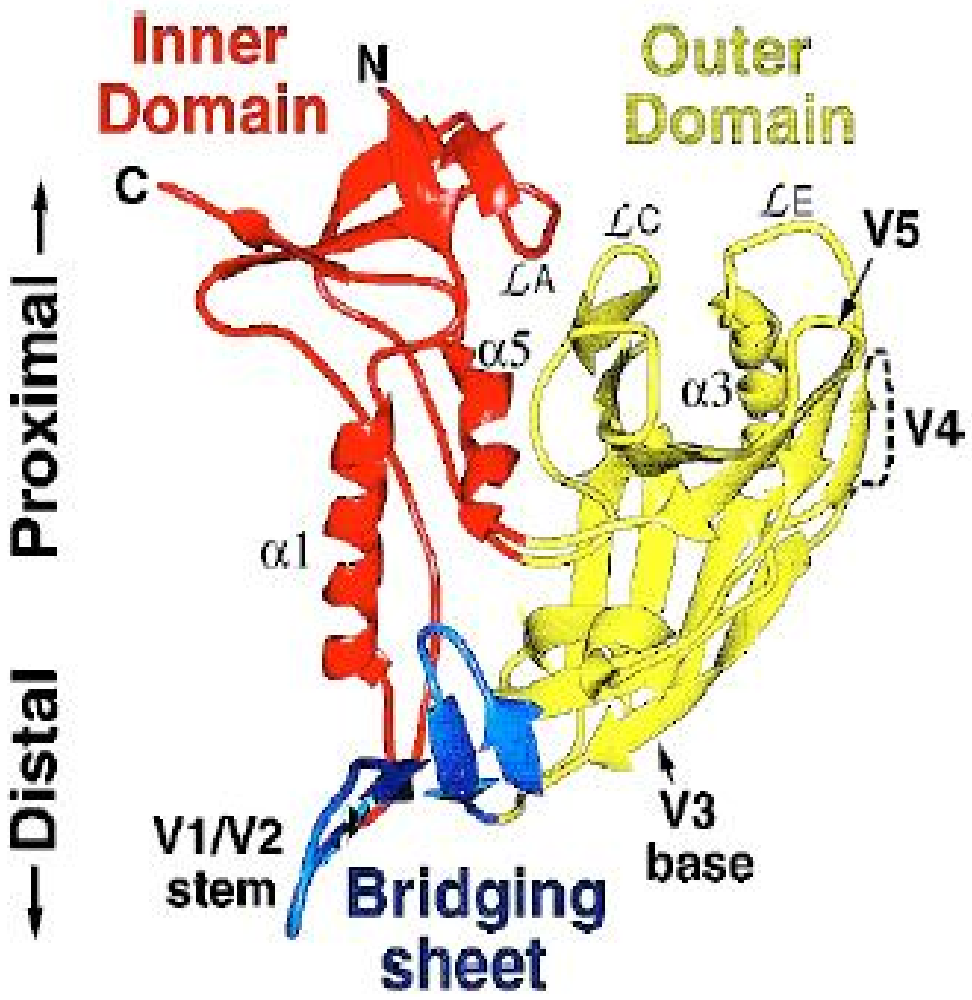

Figure 1.4 Illustration of env *gp*120 core structure
KEY: inner domain (red), outer domain (yellow), bridging sheet (blue) (Wyatt *et al.*, 1998).

1.2.1.2 The pol gene

The *pol* region of the HIV-1 genome is highly conserved amongst HIV-1 groups and subtypes (Rami *et al.*, 2003). This gene encodes for the enzymes intergrase (IN), reverse transcriptase (RT) and protease (PR) (Martin, 2006). Excessive mutations in these HIV regions reverse transcriptase, intergrase and protease genes would hamper the ability of the virus to replicate in its host cell. This is why many HIV-1 antiretroviral drugs have been aimed at inhibiting the function of these viral enzymes (Cornelissen *et al.*, 1997; Lindström and Albert, 2003). The *pol* mutations occur as a result of selection pressure caused by certain inhibiting PR and RT drugs. These drugs include nucleoside / nucleotide RT inhibitors (NRTIs), non-nucleoside RT inhibitors (NNRTIs), PR inhibitors (PIs) and intergrase inhibitors (Johnson *et al.*, 2003).

NRTIs are analogues of the body's own nucleoside or nucleotide molecules and act as alternative substrates for DNA polymerases. NNRTIs are a set of drugs which binds and physically interacts with the RT enzyme of HIV-1. However, HIV integrase, a critical enzyme in the HIV replication cycle, has recently become a viral target for antiretroviral (ARV) therapies (Lataillade *et al.*, 2006; Charpentier *et al.*, 2008). They 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. InSTI act by targeting the integrase protein. Integrase has the following two catalytic functions: (i) it removes a dinucleotide from each 3_ end of viral DNA (the 3_ processing reaction), and (ii) in the host nucleus, it mediates the transfer of the proviral DNA strand and covalently links the 3_ ends into the host DNA (the strand transfer reaction) (Engelman *et al.*, 1991). These steps create the provirus, a state that, at once, achieves

transcriptional competency for the retrovirus and provides for the stable maintenance and integrity of the viral genome throughout the life span of the infected cell and for all subsequent daughter cells (Low *et al.*, 2009). All current compounds in clinical development preferentially target the strand transfer reaction of integrase, despite representing distinct chemical classes (DeJesus *et al.*, 2006; Markowitz *et al.*, 2007). Most of the current antiretroviral treatment (ART) drugs attempt to stop viral replication by inhibiting the RT enzymatic activity, stop virus maturation by inhibiting the PR enzyme or attempt to stop the virus from entry into the host cell. ART has led to the reduction of opportunistic infections, an increased life span and an improved quality of life in many HIV-1 infected individuals. Mutations associated with drug resistance can often lead to the failure of ART in patients infected with HIV-1 (Lindström and Albert, 2003).

1.3 The HIV life cycle

1.3.1 The entry of the HIV into human cells

HIV-1 most efficiently infect cells of the T-cell and macrophage lineages (Gartner *et al.*, 1986) and enters target cells through a series of interactions between the viral glycol proteins, the cellular receptor CD4 and a coreceptor, most often CCR5 or CXCR4 **(Figure 1. 5).** The surface protein *gp*120 consists of five constant regions (C1-C5) and five variable regions (V1-V5) and both constant and variable regions are involved in the sophisticated entry process of HIV-1 (**Figure 1.4**).

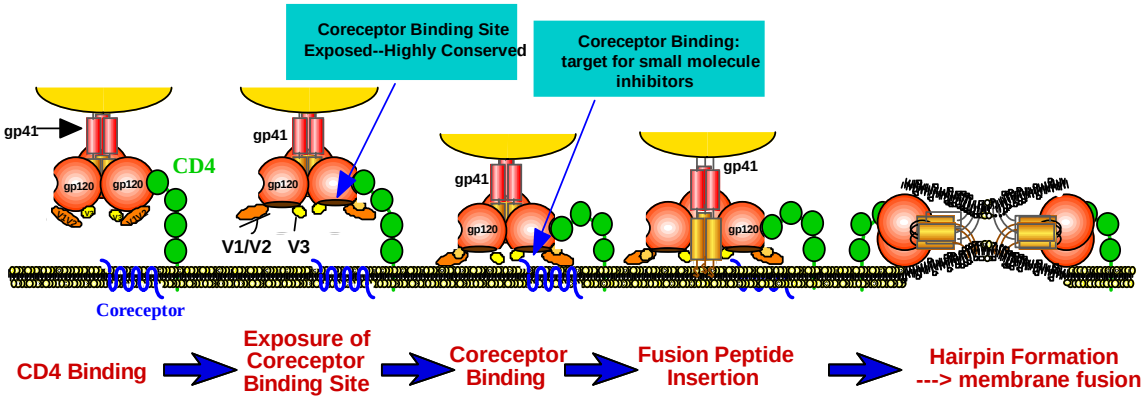

Figure 1.5 Schematic illustration of HIV-1 entry (Kindly provided by Dr. R.WDoms).

First the CD4 binding domain, key epitopes in V1/V2 stem, C2, C3, C4, V5 and C5 of *gp*120 interacts with CD4 (Kwong *et al.*, 2002) (**Figure 1.4, 1.5 and 1.6**). This interaction promotes a conformational change in *gp*120 , which results in exposure of the bridging sheet, composed of four anti-parallel ß-strands from the V1/V2 and C4 regions of *gp120* (Wyatt *et al.*,1998). The bridging sheet and the V3 region interact with the coreceptor, leading to a conformational change in *gp*41. Consequently, a fusion peptide in *gp*41 is inserted in the host cell membrane (Markosyan *et al.*, 2003). The final step is the formation of a six-helix bundle of *gp*41 that brings the viral and cellular membranes together and allows fusion to occur (Melikyan *et al.*, 2000). Dendritic cells (DC) also play an important part in HIV infection by transferring the virus to susceptible CD4-expressing cells in *trans* (Geijtenbeek *et al.*, 2000). HIV-1 *gp*120 can bind C-type lectins, for example DC-specific ICAM-3 grabbing nonintegrins (DC-SIGN), on the surface of DC. The virus is internalized and can retain infectivity up to 4 days (Geijtenbeek *et al.*, 2000). The transfer of HIV-1 from DC to T-cells occurs via the formation of virological synapses that concentrate viruses and receptors at the

site of contact between the two cells (Jolly *et al.*, 2004). It has been suggested that virological synapses protect the virus from recognition by neutralizing antibodies (Ganesh *et al.*, 2004).

1.3.2 Transcription and translation

After fusion, the capsid is delivered to the cytoplasm (**Figure 1.6**). The +ssRNA is reverse transcribed into double stranded (ds) DNA by RT, inside the semi-dissolved capsid.

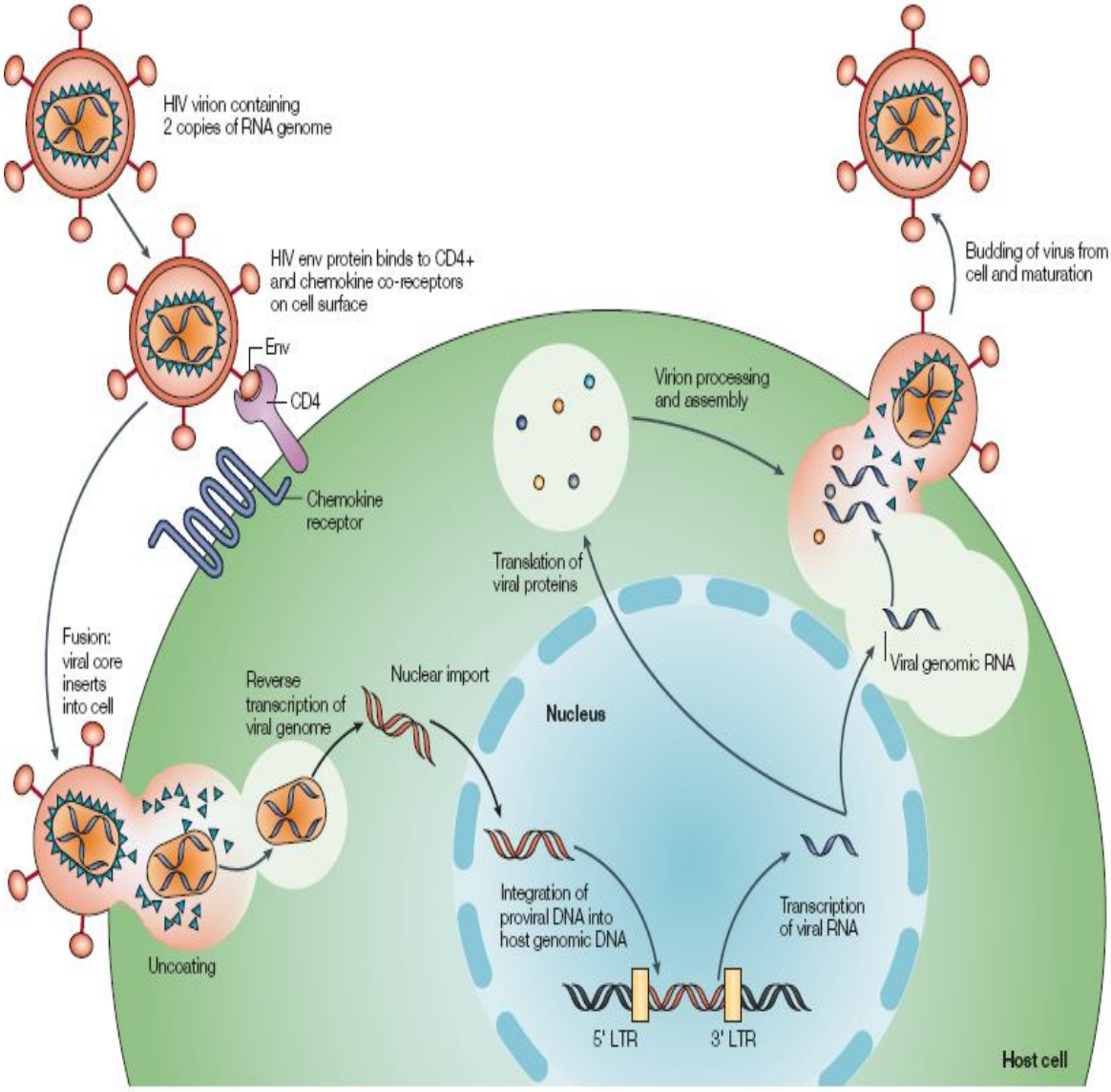

Figure 1.6 The HIV-1 life cycle (Rambaut *et al.*, 2004).

The viral proteins *Vpr*, the matrix protein *p*17 and integrase binds to the dsDNA and forms the pre-integration complex (PIC). A nuclear localization signal within p17 directs the PIC into the nucleus (Bukrinskaya *et al.,* 1996) where the provirus is integrated into the host cell genome. The viral integrase catalyzes the integration process and it has been shown that the proviral DNA preferentially integrates within gene regions (Schroder *et al.*, 2002). Once the proviral DNA is integrated, the virus fully relies on the host cell transcription machinery, since the virus lacks RNA polymerase II, required for transcription of dsDNA (**Figure 1.6**).

HIV-1 gene expression is regulated by both cellular and viral proteins. The transcription is initiated by binding of cellular transcription factors, including NF-B, to the enhancer region in the LTR (Nabel and Baltimore, 1987). The early transcripts are mainly short transcripts (Kao *et al.*, 1987). These are multiply spliced mRNAs and encode the Tat, Rev and Nef proteins. Tat binds to the transactivation-responsive RNA (TAR) element positioned downstream from the enhancer element in the LTR, and facilitate efficient elongation. Therefore, the transcription terminates prematurely in the absence of Tat (Karn, 1999). Consequently, at early time points a high level of *Tat, Rev a*nd *Nef* is observed. Accumulation of Rev results in a shift from early to late transcripts. In addition, Rev binds to the rev responsive element (RRE) in the *env* region and activates the transport of unspliced mRNA to the cytoplasm. There are two types of late transcripts. The first encodes the structural Gag proteins and the viral enzymes. The second type of transcripts consist of 5 singly spliced mRNA that encodes the *gp*160 Env precursor protein and the accessory proteins Vif, Vpr and Vpu (Frankell and John, 1998).

All mRNAs are translated in the cytoplasm or in close proximity to the endoplasmatic reticulum. The Env proteins are heavily glycosylated and cleaved by cellular proteinases into *gp41* and *gp120* in the Golgi apparatus, before being inserted into the membrane. The viral particle is assembled at the plasma membrane and buds off from the cell, thereby acquiring the cell derived envelope (**Figure 1.6**). The final maturation step occurs after budding and is performed by the viral protease that cleaves the *Gag-Pol* polyprotein into smaller proteins. The generation time of HIV has been estimated to 1.2-2.6 days (Fu, 2001).

1.4 Statement of Problem

AIDS is arguably the most serious acquired immune disease to have affected human kind (Rambaut *et al.,* 2004). Not only are an estimated 33.4 million people carrying the virus as by 2008, but its case fatality rate is close to 100%, making it an infection of devastating ferocity (Rambaut *et al.,* 2004). In 2002 alone, five million people became infected and, of these, 70% were living in sub-Saharan Africa and currently averagely 1.2 million. Although a success of antiviral agents has made HIV/AIDS a more manageable disease in some industrialized nations, and several vaccines about to enter Phase III of clinical trials, HIV will doubtless continue to impose a terrible burden of morbidity and mortality (Rambaut *et al.,* 2004).

Development of anti-HIV drugs, microbicides and vaccines still remains a frustrating exercise, no cure yet. Moreover, many aspects of HIV pathogenesis remain unclear, even though research into the origins and evolution of the virus has proven more fruitful (Rambaut *et al.,* 2004). The overall rate of evolution of HIV-1 is the highest documented for viruses to date (Karolina *et al.,* 2007). Several mechanisms contribute to this phenomenon, amongst

them the high error rate of the viral reverse transcriptase (RT), which lacks a 3'→5'exonuclease proofreading capacity, the short generation time, and the high rate of recombination between viral genomes (Jetzt *et al.,* 2000). Formation of complex recombinants and through its general evolution dynamics has led to development of unique mutations that confer drug resistance to current Antiretroviral drugs (ARV). Moreover, the rapid evolution of the virus has now led to adaptation mechanisms of the virus resulting into virus escape immune cell response and with it diverse genetic variation now makes it complex in vaccine development. New classes of drugs targeting different steps in HIV replications continue to be developed but drug resistance development even to these new drugs is still not an exceptional.

Understanding the evolution of the HIV is therefore crucial for reconstructing its origin, deciphering its interaction with the immune system and developing effective control strategies. The ability to predict the spread of drug-resistance and immune-escape mutations depends on understanding how HIV evolution differs within and among hosts and the role played by positive selection. This forms the basis of this study.

1.5 Justification

The development of HIV genetic diversity and increasing levels of migration may result in the global HIV-1 epidemic becoming increasingly heterogeneous (Gifford *et al.,* 2007). As the AIDS pandemic progresses, an increasingly broad range of genetic diversity is being reported within the main (M) group of HIV-1 viruses (Gifford *et al.,* 2007). The intermixing of diverse HIV subtypes around the world is making it more complex in terms of management due to development of mutants that confer drug resistance that are not captured

by reference HIV subtype B. This diversity is further increased by viral recombination making more complex in understanding the Circulating recombinant form (CRF) or unique circulation forms (UCF) being developed in response to therapy.

Consideration of this genetic diversity and potential consequences on drug resistance are of paramount significance in treating both B and non B HIV-1 strains. Antiviral drug regiments presently in use have been designed against HIV-1 clade B infections that are less susceptible to HAART and statistically associated with a more rapid post HAART progression of mutational patterns than subtype B isolates (Cocchi *et al.,* 1995). The emergence of resistance has fuelled the search for new drug classes with novel mechanisms of action. Different classes of entry inhibitors and intergrase have therefore been developed as alternatives for those failing therapy (de Mendoza *et al.,* 2007). Effort to target other steps in the virus lifecycle and to develop viable treatments for HAART-resistant HIV patients, entry inhibitors have emerged as a new target for anti-retroviral (ARV) therapy (Ray, 2008). However, viral adaption that leads to mutations that are associated with drug resistance has remained a challenge even to the newly introduced drugs. Therefore, this study was aimed at determining circulating HIV-1 subtypes in a Nairobi cohort. In addition, this study also sought to determine the evolutionary rates of the infecting viruses and its effects on development of drug resistance. This was to help shed light on the role of viral evolution on emergence of drug resistance.

1.6.0 Hypothesis

1.6.1 Null hypothesis

There is no relationship between HIV-1 genetic diversity and development of drug resistance.

1.7.0 General objective

To determine the association of HIV-1 evolution on development of antiretroviral drug resistance in a cohort study

1.7.1 Specific objectives

1. To determine the circulating subtypes of HIV-1 in cohort of patients attending comprehensive care clinics in Nairobi, during the year 2008-2009.
2. To determine the levels of viral recombination in the cohort.
3. To determine HIV-1 tropism in the cohort.
4. To identify mutations associated with development of antiretroviral drug resistance.
5. To determine the rates of viral evolution in this cohort

CHAPTER TWO

LITERATURE REVIEW

2.0. The diversity of HIV

The error-prone reverse transcriptase (RT) enzyme is also responsible for a phenomenon called hypermutations. Hypermutations result when an excessive number of substitutions in DNA, usually from the DNA base Guanine (G) to the base Adenine (A), occur. 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.,* 1995; Ngui *et al.,* 1999).

Therefore, no two HIV strains are alike and even within a single individual, HIV is present as a 'quasispecies' - a swarm of micro variants that are highly related, yet genetically distinct from each other (Goodenow *et al.,* 1989; Vartanian *et al.,* 1992). Diversity is further increased by the short replication time of HIV, which results in the fast turnover of new viruses and some selective forces within environmental host further contributes to high genetic diversity of the virus (Coffin, 1995; Spira *et al.,* 2003; Gao *et al.,* 2004). Recombination events also contribute to the diversity of these viruses (Robertson *et al.,* 1995). Dual infections by genetically diverse viruses have been linked to higher levels of viral replication and faster depletion of CD4+ T-cells. Infections with multiple HIV-1 variants have been associated with faster disease progression (Sagar *et al.,* 2004).

HIV is a member of the retroviridae family and has a single stranded RNA genome. It exists as two major types HIV-1 and HIV-2 (Robertson *et al.,* 2000). The high genetic variability of

HIV-1 together with the forces driving its evolution has resulted in the emergence of several different viral lineages spreading throughout the world (Lemey *et al.,* 2007). 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 (99% worldwide) (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 and Essex, 2000; Jacqueline *et al.,* 2004).

In addition, the *env* proteins of groups M and O may differ by as much as 30–50%. The N subtype, in turn, appears to be phylogenetically equidistant from M and O (Gurtler, *et al.,* 1994; Gao, *et al.,* 1994; Renjifo *et al.,* 2002). Generally, the *pol* region of HIV-1 is two to three times less divergent than *env* because this region encodes two critically important enzymes, Reverse transcriptase (RT) and Protease, which, if excessively mutated, render the virus inoperative. *Gag* sequences are even further intolerant of mutations, seeing as they encode for relatively inflexible core protein sequences. Subsequent to the designation of group M subtypes, it was realized that certain sequences do not display a single subtype cluster pattern when different regions of their genomes where phylogenetically analysed (Robertson *et al.,* 1999; Moore *et al.,* 2001). These mosaic HIV-1 genomes have been identified in several, apparently unlinked, individuals and some (A/E; B/C etc) play a major

role in the global AIDS epidemic and are now designated circulating recombinant forms (CRFs) (Robertson *et al.,* 1999; Moore *et al.,* 2001).

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 (Thomson *et al.,* 2002), but their original designation as different subtypes has been retained for consistency with earlier published work (Thomson *et al.,* 2002). The identification of new clades of HIV-1 and the realisation of the existence of CRFs, characterised by full-length genome sequence analysis, has led to several re-adjustments in the taxonomy of HIV-1 (Thomson *et al.,* 2002). The intermixture of HIV-1 variants that circulate together within a geographical region provides an opportunity for recombination of virus strains within dually infected individuals. Currently, forty eight (48) circulating recombinants forms (CRFs) and numerous unique mosaic strains of HIV-1 have been identified (Yue *et al.,* 2010). Most of the M group HIV-1 viruses are found in one area or another of Africa, while other subtypes predominate in other regions of the world (Lihana *et al.,* 2006).

The distribution of HIV-1 genetic diversity with respect to epidemiological factors such as risk group and geographic location is highly dynamic; novel genetic diversity is continually being generated through mutation and recombination, and travel and migration promote the transfer of diverse viral strains between populations, often across large distances (Lasky *et al.,* 1997; Thomson *et al.,* 2005). The speed at which genetic diversity is generated by HIV-1 presents a challenge to standard phylogenetic classification systems, as reflected in the growing number of unclassifiable and complex recombinant sequences being reported (Yang

et al., 2002; Takebe *et al.,* 2003; Gomez-Carrillo *et al.,* 2004; Tee *et al.,* 2006; Tovanabutra *et al.,* 2007), the proliferation of "sub-subtype" nomenclature (Gao *et al.,* 2001; Meloni *et al.,* 2004), and accumulating evidence that at least some of the established groupings are artifacts of sampling (Anderson *et al.,* 2000; Abecasis *et al.,* 2007).

It is 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 (Peeter *et al.,* 2003; Hue *et al.,* 2004; Bennet *et al.,* 2005; Hue *et al.,* 2005; de Oliveira *et al.,* 2006)

2.1 Distribution of HIV-1 subtypes

HIV-1 subtype diversity is the highest in the world (Diminitrios *et al.,* 1999). With ongoing generation of viral genetic diversity and increasing levels of human migration, the global HIV-1 epidemic is becoming increasingly heterogeneous (Gifford *et al., 2007*). As the AIDS pandemic progresses, an increasingly broad range of genetic diversity is being reported within the main (M) group of HIV-1 viruses (Gifford *et al.,* 2007).

2.1.1 Distribution of HIV-1 subtypes in Europe

There are clear differences in geographical distribution of HIV subtypes. In Europe, HIV-1 subtype B is predominant in homosexuals, while a variety of subtypes are found in a relatively small numbers of people infected through heterosexual contact in Europe and the countries of the former Soviet Union (Ortiz *et al.,* 2000) (**Figure 2.1**). However in recent

years, all group M subtypes as well as O strains have been isolated in these regions, with exception of the N group, mainly in immigrants or in travelers from HIV-1 endemically infected areas (Ortiz *et al.,* 2000; Fleury *et al.,* 2003). A phylogenetic exploration of transmission dynamics indicated that the majority of non-B infections in the United Kingdom reflect separate introductions through travel and migration (Gifford *et al.,* 2007). For instance, HIV subtype A may have been spread into UK in the late 1980s and early 1990s spreading within the United Kingdom probably via the route of men having sex with men from East Africa that are usually associated with heterosexual infections (Gifford *et al.,* 2007). HIV-1 subtype C that ranks second to HIV-1 subtype B in the United Kingdom has had its prevalence increasing steadily since the early 1990s (Tatt *et al.,* 2004). The overwhelming majority of HIV-1 subtype C infections in the UK occur in individuals whose reported exposure risk is heterosexual contact, and who were likely infected in Southern or Eastern Africa (Dougan *et al.,* 2005).

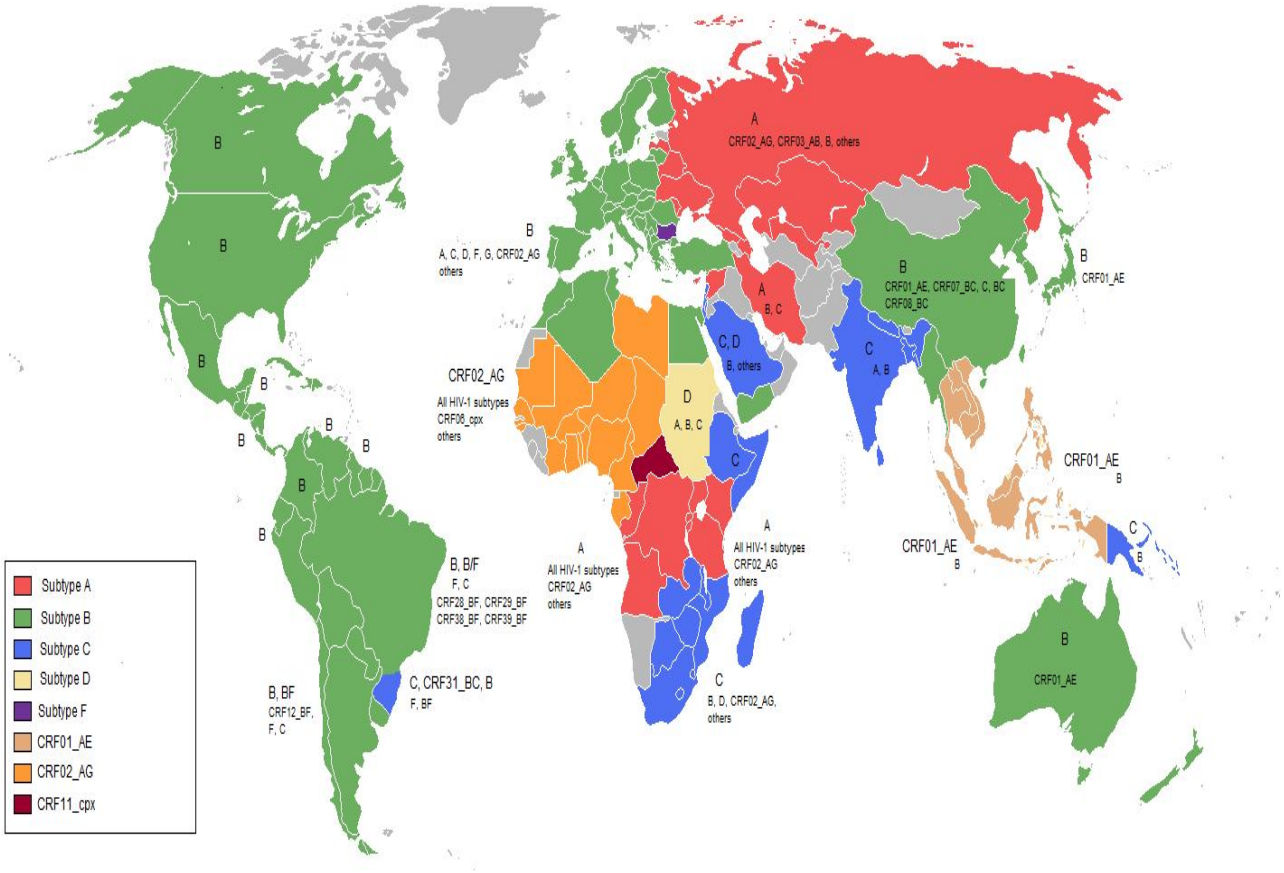

Figure 2.1 World regional spread of HIV-1 subtypes and circulating recombinant forms (Butler et *al.*, 2007)

2.1.2 Distribution of HIV subtypes in Asia

HIV-1 Subtype B has also been noted in Indonesia, the Philippines, and Taiwan (Ortiz *et al.*, 2000) (**Figure 2.1**). In Thailand, HIV-1 subtype E predominates, while minority of B infections occurs in drug users. This HIV-1 subtype B strain has also been found in drug users in Myanmar (Burma), Malaysia, and southeast China. In parts of South East Asia HIV-1 subtypes B and CRF01_AE have been detected , while in India and the predominant subtype is C while in small proportions in Russia (Perrin *et al.*, 2003) with small numbers of A and B infections (McCutchan *et al.*, 2000). However, Fontella *et al.*, (2008), indicates that HIV-1 subtype C was spread from Ethiopia to Israel (Gehring *et al.*, 1997) while to India

from South Africa (Dietrich *et al.,* 1995) and from India to China (Qiu *et al.,* 2005). HIV-1 subtype B is found in India that ranks second in HIV-1 subtype predominance (Butler *et al.,* 2007). Recombinant viruses including genes derived from subtype C have been increasingly recognized in China, Thailand, and Taiwan, where phylogenetic studies indicate that complex BC subtype recombinants, such as circulating recombinant forms CRF_07 and CRF_08, comprise much of the current epidemics (Dalai *et al.,* 2008). However, other subtypes and CRFs have been found to circulate, although at low levels (Bou-Habib *et al.,* 2000; Montano *et al.,* 2002). Although early in its epidemic many strains circulated in the former Soviet Union, today HIV-1 subtype A, which first gained a foot hold among IDUs, is now the predominant strain in Russia and most of its western neighbours (Bobkov *et al.,* 1996).

2.1.3 Distribution of HIV-1 subtypes in North and South America

The predominantly circulating strains in South America have been HIV-1 subtypes B and F (Russell *et al.,* 2000; Hierhozer *et al.,* 2002; Montano *et al.,* 2002) (**Figure 2.1**). In the Americas (North, South and Central), as well as in Australia, New Zealand, and Japan, subtypes B is most common with HIV-1 subtype C occurring in a small proportion in Brazil (McCutchan *et al.,* 2000) (**Figure 2.1**). HIV subtype C prevalence has been increasing in Brazil (Pando *et al.,* 2007; Dilernia *et al.,* 2007). In addition, studies by Fontella *et al.,* (2008) indicate that the entry of the subtype C in Brazil and South America may have occurred in a single episode or in multiple episodes of genetically close viruses, possibly from a country of Eastern Africa, Burundi (Fontella *et al.,* 2008; Bello *et al.,* 2008). Nevertheless, HIV

subtype C has continued to be spread to Brazil to Argentina and Uruguay (Fontella *et al.*, 2008).

2.1.4 Distribution of HIV subtypes in Australia and Oceania

In the mid-90's, all reported Australian cases of HIV-1 were subtype B, as were those of New Zealand and Papua New Guinea (Womack *et al.*, 2001). Several years later, although B still accounted for 74% of cases, other subtypes emerged, including C (12%), CRF01_A/E (9%), and limited cases of A and G. Different risk groups tended towards different subtypes, with B accounting for 100% of cases among IDUs and 84% of homosexually transmitted cases. Non-B, however, accounted for 57% of heterosexually acquired cases and 22% of vertical transmissions, mostly among immigrants. Thus, although the majority of Australian HIV remains subtype B, continued immigration will likely facilitate the spread of new subtypes into the general population (Butler *et al.*, 2007).

2.1.5 Distribution of HIV subtypes in Africa

In Africa, where the effects of HIV-1 have been most devastating (Neilson *et al.*, 1999), all HIV subtypes have been found with HIV-1 subtype A and subtype C being the most prevalent (Diminitrios *et al.*, 1999) (**Figure 2.1**). Africa makes an ideal setting in which to examine in more detail the diversity and mixing of viruses of different subtypes on a population basis (Neilson *et al.*, 1999). Although broad diversity is concentrated in areas of West and Central Africa, where infection is longest established (Ndembi *et al.*, 2004; Vidal *et al.*, 2003; Zhong *et al.*, 2003), it is increasingly evident elsewhere as infections expand globally (Gomez-Carrillo *et al.*, 2004; Hemelaar *et al.*, 2004). The distribution of different subtypes within African populations is generally not linked to particular risk behaviours

(Neilson *et al.,* 1999) unlike in some Asian countries where the spread of HIV-1 subtype E (CRF01_AE) is linked to intravenous drug users (Weniger *et al.,* 1994).

2.1.6 Distribution of HIV subtypes in West and Central Africa

Central Africa is thought to be the origin of all subtypes of HIV-1 (Vidal *et al.,* 2000) (**Figure 2.1**). The initial diversification of group M viruses may have occurred within or near the territory of the DRC, where the highest diversity of group M has been reported (Vidal *et al.,* 2000), and the earliest case of HIV-1 infection been documented (Nahmias *et al.,* 1986). HIV Subtype G and its A/G recombinant viruses have been documented in many West and Central African countries, whereas subtype H was only found in Central Africa (Peeters *et al.,* 1999; Triques *et al.,* 2000; Bikandou *et al.,* 2000; Piot and Bartos, 2002). In certain populations and regions where multiple HIV-1 subtypes co-circulate, many combinations of intersubtype recombinant viruses have been documented (A/C, A/D, B/F, A/G/I/J) (Montavon *et al.,* 1999), and even an intergroup M/O recombinant virus has been recently isolated from a Cameroonian patient (Peter *et al.,* 1999). HIV diversity in Central Africa remains a dynamic process, as subtype C is on the rise in the DRC, up from 2% of HIV cases in 1997 to 10% in 2002 (Vidal *et al.,* 2005), and other subtypes continue to diverge in both inter- and intra-subtype distances (Yang *et al.,* 2005). West Africa shares a similar epidemiological pattern with Central Africa. CRF02_A/G is the predominant strain, reaching 59% prevalence in Ghana and neighboring countries, and several other CRFs have now emerged (Fischelli *et al.,* 2004).

2.1.7 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).

2.1.8 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.

2.1.9 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 (Yue, *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). According to Robbins *et al.,* (1999), in a study based on analysis of the *env C2-V3* region, majority of sequences analyzed were subtype A (71-87%), with significant components of subtype D (7-29%) and subtype C (7-17%). Therefore, subtype A predominates in both non-recombinations (55%) and recombinants based on near full-length sequences that have been generated (Dowling *et al.,* 2002) (**Figure 2.1**). HIV-1 Subtypes C and D occur as no recombinant (2% each) but to a much lesser extent than HIV-1 subtype A that also occur in recombinants (Dowling *et al.,* 2002).

A full-length subtype G has been sequenced from isolates collcted in Kenya (Carr *et al.,* 1998), with recombinants between A1, A2, and D; A2 and D; A1 and D; A1 and G; A1 and C; A1, C, and D (Dowling *et al.,* 2002). According to Khamadi *et al.,* (2005), the HIV-1 subtypes circulating in the northern region of Kenya were subtype A: 50%, subtype C: 39% and subtype D: 11% based on partial *env* sequences. An indication that subtypes A and C are the dominant HIV-1 subtypes in circulation. 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 (Oleivera *et al.,* 2006; Gifford *et al.,* 2007).

2.2.0 HIV Recombination

Recombination is a shared feature among retroviruses (Levy *et al.,* 2004). It occurs by template switching of the reverse transcriptase between the two RNA templates in the diploid virion, generating a daughter DNA provirus that is a mosaic between the two parental genomes (Hu and Temin., 1990). The Reverse transcriptase enzyme (RT) jumps back and forth, switches between alternative genomic templates during replication (Jetzt *et al.,* 2000) **(Figure 2.2).** It has been estimated that HIV-1 undergoes approximately two to three recombination events per replication cycle (Jetzt *et al.,* 2000). Genetic recombination is thus an integral part of the HIV lifecycle and hence making the virus the fastest evolving of all organisms (Karolina *et al.,* 2007), with an estimated three recombination events occurring per genome per replication cycle (Zhuang *et al.,* 2002), thereby exceeding the mutation rate per replication. Recombination events may lead to major genome rearrangements, and therefore exceed the impact of nucleotide substitution on HIV-1 variation. This makes

recombination the most important contributor in the generation and diversification of HIV-1 subpopulations (Rambaut *et al.,* 2004).

The discovery that most infected cells harbour two or more different proviruses (Jung *et al.,* 2002), and the evidence for dual infection (Jost *et al.,* 2002; Koelsch *et al.,* 2003), set the stage for recombination to have a central role in generating HIV diversity. Indeed, recombination has now been detected at all phylogenetic levels: among primate lentiviruses (Salemi *et al.,* 2003; Bailes *et al.,* 2003), among HIV-1 groups (Takehisa *et al.,* 1999), among subtypes (Robertson *et al.,* 1995) and within subtypes (Crandall and Templeton., 1999). Prevalent inter-subtype recombinants are denoted 'circulating recombinant forms' (CRFs). There are forty eight currently recognized CRFs that show a broad range of complexity and are widely distributed. In some geographical regions, CRFs account for at least 25% of all HIV infections (Yue *et al.,* 2010; Los Alamos , 2010).

Recombination has important implications for understanding the HIV epidemic. In particular, many evolutionary inferences about HIV are made after the reconstruction of phylogenies, which can be greatly affected by recombination. Therefore, analyses of phylogenetic relationships, the timing of events, demographic processes or natural selection, are all potentially affected by recombination (Schierup *et al.,* 2000; McVean *et al.,* 2002; Posada *et al.,* 2002). For example, although the data set used for dating the origin of HIV-1 group M to the 1930s was 'cleaned' for known recombinants before analysis (Korber *et al.,* 2000), the presence of recombination still seems evident, raising concerns about the accuracy of this estimate (Schierup *et al.,* 2003). Because recombination is so frequent, it cannot be factored out by simply identifying recombinants and excluding those from the analysis. Indeed,

today's subtypes might comprise old and successful recombinant lineages that trace back to a shared ancestral population. HIV should therefore be studied with methods that are robust to the occurrence of recombination, or that explicitly take recombination into account. The development of such methods will doubtless prove difficult, but is necessary to make reliable inferences on many aspects of HIV-1 evolution (Posada and Crandall, 2001; Wain-Hobson *et al.,* 2003).

The **(Figure 2.2)** illustrates intersubtype recombination resulting from a simultaneous infected cell with two viruses of different subtypes, allowing the encapsidation of one RNA transcript from each provirus into a heterozygous virion**.** During subsequent infection of a new cell, the strand-jumping polymerase will generate a mosaic provirus that is recombinant between the two parental subtypes. In addition, **(Figure 2.2),** show **t**wo genetically distinct (red and black) virus particles infecting the same cell. During assembly both genomes are packed in the same particle. Strand transfer event during reverse transcription in the second cell results in a mosaic genome and consequently a recombinant progeny (Mild *et al.*, 2007)

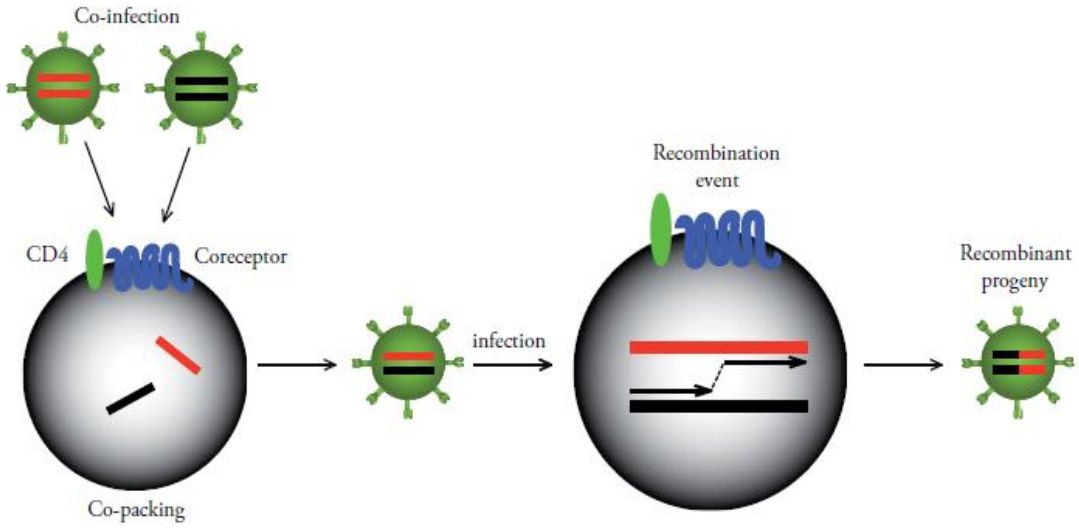

Figure 2.2 HIV-1 recombination illustration (Mild *et al.*, 2007)

2.3.0 HIV receptors

In 1984, the CD4 molecule was identified as a receptor for HIV-1 (Dalgleish *et al.*, 1984). It soon became evident that CD4 was not enough to confer HIV-1 entry. The discoveries in late 1995 that the CC-chemokines could inhibit HIV-1 replication (Cocchi *et al.*, 1995), and in early 1996 that the CXC-chemokine receptor CXCR4 was a coreceptor for some HIV-1 strains (Feng *et al.*, 1996) had an extraordinary impact on the HIV research field. Within a year several groups identified a second co-receptor for HIV-1, CCR5 (Dragic *et al.*, 1996) and it was discovered that a 32-base pair (*bp*) deletion in the coding region of the CCR5 gene (CCR5-Δ32) was associated with resistance to HIV-1 infection (Liu *et al.*, 1996). Since the finding that CCR5 and CXCR4 are the major co-receptors for HIV-1, more members of the seven-span transmembrane of minor co-receptors have been identified for HIV-1, such as CCR2b, CCR3 (Choe *et al.*, 1996), CCR8 (Rucker *et al.*, 1997) and US28, CXCR6 (Deng *et al.*, 1997), and chemokine receptor-like orphan molecules STRL33 or BONZO or TYMSTR, V28, *gp*r1 (Shimizu, *et al.*, 1999[a]), *gp*r15 or BOB (Deng *et al.*, 1997), RDC1 (Shimizu, *et al.*,

2000) and APJ (Choe *et al.,* 1996) (Dragic *et al.,* 1996; Alhatib *et al.,* 1996; Zhang *et al.,* 1997). However, when these minor receptors are used *in vitro* it is always in combination with CCR5 and/or CXCR4 and the *in vivo* role of these minor receptors is unclear. The chemokine receptors belong to the seven transmembrane spanning G-protein-coupled receptors that are involved in signal transduction (Rollins, 1997). The amino-terminus (N-terminal) part of the receptor is located outside the cell and the carboxy-terminus (C-terminal) part is coupled to a signal transducing G-protein and is located inside the cell. The N- and C-terminal are separated by three extracellular loops and three intracellular loops (Zlotnik and Yoshile, 2000). Disulfide bonds between the first and second extracellular loops bring the extracellular domains together, resulting in a stable barrel-like structure. Chemokines receptors can be divided into four families: C, CC, CXC and CX3C, chemokines, based on the arrangement of conserved cystein (C) residues (Zlotnik and Yoshile, 2000).

Chemokines are small peptides (92-125 amino acids (AA) involved in leukocyte trafficking (Baggiolini *et al.,* 1997). The chemokines that bind to CCR5 are RANTES (Regulated on activation, normal T-cell expressed, and secreted), Macrophage inflammatory protein (MIP) -1 and MIP-1 and the natural ligand for CXCR4 is stromal cell-derived factor 1 (SDF-1). They exhibit suppressive effect of HIV-1 by down regulating coreceptor expression and through competitive binding (Alkhatib *et al.,* 1996). This has been employed in the development of HIV-1 inhibitors. It has been shown that different parts of CCR5 and CXCR4 are involved in HIV-1 binding. The N-terminal and the first extracellular loop seem to be most important for HIV-1 binding to CCR5 (Lee *et al.,* 1999), while the first and second extracellular loops are the key epitopes in CXCR4 (Brelot *et al.,* 2000). The binding

of HIV-1 to CCR5 and CXCR4 results in a signal cascade that affects the gene expression of the infected cell. The major group of genes affected is involved in the cell cycle and the cell is forced into an active state (Cicala *et al.,* 2006). This promotes transcription of HIV-1 genes and results in increased viral production. In addition, viruses that bind to CCR5 and CXCR4, respectively, seem to induce distinct gene expression in peripheral blood mononuclear cells (PBMC) (Cicala *et al.,* 2006).

HIV-2 and Simian Immunodeficiency Virus (SIV) have been reported to enter cells in a CD4-independent manner (Reeves *et al.,* 1999). In addition, some laboratory adapted HIV-1 strains have been shown to exhibit the same properties as HIV-2 and SIV (Hoffman *et al.,* 1998; LaBranche, *et al.,* 1999) while primary CD4-independent HIV-1 has not been isolated until recently (Zerhouni *et al.,* 2004). The discrepancy in the ability of CD4 independent infections between HIV-1 and HIV-2 and SIV could be a consequence of differences in the conformational changes needed during the entry process. It is possible that HIV-2 and SIV envelopes have a more open conformation compared to HIV-1 and therefore the initial binding step to CD4 is not essential for exposure of the coreceptor binding site (Hoffman *et al.,* 1999). However, the more open structure could also make the virus more vulnerable to neutralizing antibodies, a correlation that has not been fully investigated (Edwards *et al.,* 2001; Thomas *et al.,* 2003).

2.3.1 Co-receptor Usage

HIV-1 viruses can be characterized into two phenotypes referred to as syncytium inducing (SI) and non – syncytium inducing (NSI) (Koot *et al.,* 1992) or fast or slow replicating in the peripheral PBMC (Asjo *et al.,* 1986; Fenyo *et al.,*1988). These phenotypes have different

cellular tropisms and appear during different stages of infection (Shankarappa *et al.*, 1999). The macrophage tropic NSI phenotype is predominant during the early stages of infection (Connor and Ho, 1994) while the T cell tropic SI phenotype is often observed later during and around the time of progression to AIDS (Koot *et al.*, 1999). Therefore, detection of SI strains in infected individuals was an indicator of poor prognosis since it was associated with accelerated loss of CD4+ T-cells and a more rapid progression to AIDS (Karlsson *et al.*, 2004).

2.3.2 HIV Co-receptor usage in phase of antiretroviral drugs

Primate immunodeficiency viruses enter target cells by fusion at the cell surface membrane (Chen *et al.*, 1997; Berger *et al.*, 1997; Pleskoff *et al.*, 1997). HIV-1 coreceptor usage plays a critical role in the virus tropism (Dragic *et al.*, 1996; Alhatib *et al.*, 1996; Zhang *et al.*, 1997). Therefore based on the coreceptor usage, HIV-1 variants are classified as CCR5-tropic (R5 variants), CXCR4-tropic (X4 variants), and dual tropic (R5=X4 variants) (Berger *et al.*, 1998). The R5 variants are responsible for the establishment of HIV-1 infection and predominate in the early stage of HIV-1 infection (Shankarappa *et al.*, 1999) while X4 variants emerge later as disease develops (Hunt *et al.*, 2006). A switch in HIV-1 coreceptor usage from CCR5 to CXCR4, which correlates with the subsequent accelerated decrease in CD4+ T cell count and disease progression, occurs in the late stage of HIV-1 infection in about half of HIV-infected individuals (Koot *et al.*, 1993).

Recently several longitudinal studies regarding the effect of highly active antiretroviral therapy (HAART) in the dynamics of evolution of HIV tropism in patients under HAART have been carried out and report a higher prevalence of X4 variants in those HIV-1-infected

individuals exposed to HAART than in drug-naive individuals (Timothy *et al.,* 2007). In addition, effective HAART has been reported to enhance CCR5 to CXCR4 coreceptor switch (Pierre *et al.,* 2005). However, the dynamics of viral tropism during the course of HIV-1 infection in persons exposed to antiretroviral therapy (ART) still remain unclear (Lwembe *et al.,* 2009). These co receptors provide a potentially useful and novel target for antiretroviral therapy (Pierson *et al.,*2004) and could help to overcome some of the problems associated with highly active antiretroviral therapy (HAART) such as patients adherence to treatment (Ickovics *et al.,* 2002) and the emergence of drug resistant variants (Kutilek *et al.,* 2003).

Antiretroviral therapy (ART) using nucleoside- and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs) as well as protease inhibitors (PIs) has sharply reduced HIV morbidity and mortality in developed countries but has created the problem of drug resistance. Drug resistance mutations associated with most regimens have previously been described (Johnson *et al.,* 2008). The emergence of resistance has fuelled the search for new drug classes with novel mechanisms of action. Different classes of entry inhibitors have therefore been developed as alternatives for those failing therapy (de Mendoza *et al.,* 2007). Effort to target other steps in the virus lifecycle and to develop viable treatments for HAART-resistant HIV patients, entry inhibitors have emerged as a new target for anti-retroviral (ARV) therapy (Ray, 2008). Entry inhibitors, a new class of regiment do target the viral entry process including the following; receptor binding, coreceptor engagement, and membrane fusion. Amongst these candidates the drugs that are farthest along in clinical development include the fusion inhibitors and the CCR5 coreceptor inhibitors (Ray, 2008). Enfuvirtide, a peptide fusion inhibitor that targets a conformational intermediate of the fusion process was the first molecule to obtain approval (de Mendoza *et al.,* 2007). While

Enfuvirtide was the only entry inhibitor on the market until late 2007, the use of this drug has been complicated by its need for twice daily injection, leading to some significant problems with regards to compliance and injection site reactions. Owing to these issues, the use of Enfuvirtide has been restricted to treatment-experienced patients who are failing HAART, and are on salvage therapy (Ray, 2008). CCR5 antagonists are another new class of entry inhibitors under development. These inhibitors block the *Env*: CCR5 interaction leading to their antiviral effects (Ray, 2008). The approval of maraviroc, a CCR5 antagonist is promising and evidence of its success has been reported (Saag *et al.,* 2009; Lihana *et al.,* 2009). Thus, the use of co-receptor antagonists among patients failing ART has been successful in industrialised countries, but has yet to reach patients in resource-poor countries. Likewise, a number of small positively charged compounds, such as T22 and AMD3100, have been observed to block CXCR4 - tropic HIV-1 cell entry (Schols *et al.,* 1997). Inhibiting CXCR4 usage in this way could theoretically prove useful during the later stages of infection to slow down or potentially stall disease progression. However CXCR4 is critical for haematopoiesis, cardiac function and cerebellar development (Zou *et al.,* 1998) and so using it as a target could prove difficult due to severe side effects (Scozzafava *et al.,* 2002).

In addition, in clinical trials the presence of the CXCR4-using phenotype pre treatment with maraviroc is predictive of failure as this phenotype will emerge once treatment begins (Westby *et al.,* 2006). In this study, we aimed at determining HIV-1 co-receptor usage among Kenyan patients before the introduction of Maraviroc or Vicriviroc (fusion inhibitors) as antiretroviral therapy among drug naive and those already under therapy. We therefore investigated co-receptor use among viral strains isolated from a clinical cohort in Nairobi and surrounding regions using online bioinformatic tools. Furthermore, characterisation of co-

receptor tropism was aimed at evaluating feasibility of introduction new class of CCR5 antagonist as alternative therapy with envisaged treatment failure since determining co-receptor use present within an individual will be vital in determining the probability of success.

2.4.0 Mechanism of action of antiretroviral drug classes

2.4.1 Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

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 (Martin, 2006). The presence of the 3' OH is required for elongation of the growing DNA chain. Replacement of the OH at the 3' position prevents disulphide bonds from being formed with this nucleoside. Incorporation of AZT into the growing DNA chain in place of the normal nucleoside leads to a "chain termination." Anti-HIV nucleoside inhibitors are also referred to as nucleoside reverse transcriptase inhibitors (NRTIs) since they are more potent inhibitors of HIV RT than human DNA polymerases (Martin, 2006).

Both nucleoside and non-nucleotide RT inhibitors must enter the cell and become phosphorylated in order to act as synthetic substrates for RT. Both classes of agents can prevent infection of susceptible cells but will have no effect on cells that already harbor HIV. Likewise, this class of agent targets the active site on RT that is involved in DNA polymerization (Martin, 2006).

Resistance to NRTI's occurs through two mechanisms: the first is mutation of the residues that results in reduced incorporation of the NRTI into the growing DNA chain. While some of these mutations arise in the actual catalytic site of RT, a number of these mutations are actually proximal to the active catalytic site of RT but are still able to cause a conformational change in the enzyme that impairs binding of the drug to the active site (Martin, 2006).

The second mechanism of NRTI resistance is associated with enhanced removal of drug from its site of attachment at the end of the DNA chain. These RT mutations allow ATP or pyrophosphate (both of which are in high concentration within the cell) to bind at the active site adjacent to the bound nucleoside analog. The high energy ATP or pyrophosphate can then attack the bond that binds the drug to DNA, thereby liberating the drug and terminating its effect (Martin, 2006).

2.4.2 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Drugs in this class are structurally different from the nucleoside RT inhibitors, this include Delavirdine and Efavirenz. The NNRTIs bind near the catalytic site of reverse transcriptase and alter the enzymes ability to change conformation (Martin, 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. However, the main drawback of these agents is the rapid development of resistance. As a result, the NNRTIs are never used for monotherapy of HIV infection. Resistance to this class of agents occurs mainly through mutation of hydrophobic RT residues within the binding pocket for the NNRTIs (Martin, 2006). Since all of the NNRTIs bind to essentially at the same region of RT, mutations in this area will affect binding of all of the agents in this class to some extent. This may in part

explain the high rates of HIV cross-resistance within this class of agents (Brennar *et al.*, 2003).

2.4.3 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 (Martin, 2006). HIV protease enzymes are symmetrical dimers with a central core that binds the peptides that are to be modified by the enzyme (Martin, 2006). Protease inhibitors such as Saquinavir, Indinavir, Ritonavir, Nelfinavir and Amprenavir are designed to fit and bind at the catalytic site of the HIV protease enzyme with high affinity and thereby block the viral enzyme from cutting the viral proteins molecules into the correct sizes. This means the virus cannot make copies that can infect new cells. This happens at end of the life cycle of the virus (Martin, 2006).

2.4.4 Fusion Inhibitors

A drug that acts by blocking the fusion and entry of HIV into the host cells (enfuvirtide) has been used clinically. Fusion of HIV with the host cell membrane is an essential step in viral entry into the cell (Martin, 2006). HIV attaches specifically to CD4 molecules on the host cell membrane through glycoprotein *gp120* on the HIV peplomer. Once attachment to the host cell occurs, *gp41*, which constitutes the stalk of the HIV peplomer, embeds itself in the host cell membrane (Martin, 2006).

The *gp41* peplomer is comprises of two adjoining subunits, HR1 and HR2. The embedding of the *gp41* involves the HR1 subunit of *gp41* "sliding" over the HR2 subunit to draw the

HIV and host cell membranes closer together. The *gp41* fusion peptide now undergoes a further conformational change that brings the HIV and host cell membranes in contact with one another. Fusion "pores" are formed that facilitate entry of the HIV nucleocapsid (protein capsid + HIV genome) into the host cell (Greenberg *et al.*, 2004).

The drug enfuvirtide is a synthetic peptide that binds directly to the HIV *gp41* and prevents it from undergoing the conformational change that leads to fusion of HIV and host cell membrane. Although just introduced to clinical practice, varying susceptibility of different HIV strains to enfuvirtide has already been documented (Greenberg *et al.*, 2004). Enfuvirtide inhibitors have high genetic barrier, they require more than one mutation on the *gp*41 gene to develop resistance to Fusion inhibitors. The development of resistance to Enfuvirtide centres round a 10 amino acid motif in the envelope glycoprotein to which the drug binds. However, by virtue of the fact that the 10 amino acid motif is critical for viral function, Enfuvirtide-resistant mutants show poor replicative capacity and revertance to a drug-susceptible state following drug withdrawal has been reported (Poveda *et al.*, 2002).

While clinical resistance to enfuvirtide has not yet been observed, amino acid mutations between residues 36 and 45, or around positions 36-38 of *gp*41 region located in the heptad repeat region HR1, which are part of the binding site for enfuvirtide, region identified to confer some acquired resistance to the drug (Rimsky *et al.*, 1998). However, since the region in which the mutations occur is required for viral function, enfuvirtide-resistant mutants still replicate poorly and revert back to full-drug susceptibility once the agent is stopped (Martin 2006).

2.4.5 CCR5 antagonists

The process by which HIV infects a host cell is complicated and requires multiple steps. First, the env protein (gp120) on the surface of the virus binds to cellular CD4 receptors. The binding of gp120 leads to a conformational change that exposes the V3 loop; the exposed V3 loop of gp120 then interacts with and binds to a coreceptor on the host cell (either CCR5 or CXCR4) (Latinovic *et al.,* 2009). After the coreceptor is bound, another conformational change in the viral envelope unmasks gp41, which can then insert into the cell's membrane (Lieberman *et al.,*2008). This step brings the virus into close proximity with the cell, leading to fusion of the virus with the cell (Lieberman *et al.,*2008). Small-molecule chemokine receptor antagonists are a new class of antiretrovirals (ARVs) that target interaction of virus and the host co-receptor usage step.

CCR5 antagonists bind to the CCR5 receptor and induce a conformational change to it such that the V3 loop of the viral gp120 is unable to recognize and bind (Kondru e*t al.,* 2008). CCR5 antagonists act as allosteric, non-competitive inhibitors of the receptor (Latinovic *et al.,* 2009). CCR5 antibodies work by binding to the extracellular domain of the CCR5 receptor and thereby inhibit interaction between gp120 and the coreceptor (Castagna *et al.,* 2005). The result of binding of either an antagonist or an antibody is blockade of the binding interaction which prevents HIV from entering the host cell (Gilliam *et al.* 2010). Although several CCR5 antagonists have been evaluated in clinical trials, only maraviroc has been approved for clinical use in the treatment of HIV-infected patients (Gilliam *et al.* 2010). However drug resistance has been detected against CCR5 antagonists. Evolution of drug resistance developes first is through selection of minority variants of CXCR4 or dual/mixed

tropic virus. The second is through development of mutations in the gp120 V3 loop, elsewhere in gp 120, or in gp41 (Anastassopoulou *et al.*, 2009).

2.4.6 Intergrase inhibitors

HIV integrase, a critical enzyme in the HIV replication cycle, has recently become a viral target for antiretroviral (ARV) therapies (Charpentier *et al.*, 2008). They 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). InSTI act by targeting the integrase protein. Integrase has two catalytic functions; firstly it removes a dinucleotide from each 3_ end of viral DNA (the 3_ processing reaction), and secondly, it mediates the transfer of the proviral DNA strand in the host nucleus and covalently links the 3_ ends into the host DNA (the strand transfer reaction) (Engelman *et al.*, 1991). These steps create the provirus, a state that, at once, achieves transcriptional competency for the retrovirus and provides for the stable maintenance and integrity of the viral genome throughout the life span of the infected cell and for all subsequent daughter cells (Low *et al.*, 2009). All current compounds in clinical development preferentially target the strand transfer reaction of integrase, despite representing distinct chemical classes (Markowitz *et al.*, 2007). Raltegravir an intergrase drug, is a 1-N-alkyl-5-hydroxypyrimidinone . During infection and replication of the vrus, Raltegravir binds the Pre-Intergration Complex (PIC), blocking the strand transfer of HIV cDNA to cellular DNA by removing a dinucleotide from the 3^1 ends before intergration (Evering and Markowitz, 2008).

2.5 Evolution and mechanisms of drug resistance

Viral resistance develops largely because of changes (mutations) in the genetic material that codes for the HIV reverse transcriptase (RT) and protease enzymes. Both these enzymes are required for viral reproduction, and current antiretroviral drugs impede their activity (Brumme *et al.,* 2003). Most mutations are lethal or neutral and do not confer drug resistance. However, under conditions in which treatment does not completely inhibit viral replication, viruses with drug resistant mutations can develop and replicate, resulting in treatment failure. In general, it is theoretically possible for every single drug-resistant mutation to be generated daily. For some drugs (e.g. NNRTIs), a single mutation is associated with a high level of drug resistance. Such a mutation is referred to as a "major" mutation. For other drugs (e.g. most protease inhibitors), a combination of mutations is often required to confer resistance. Such mutations are known as "minor" mutations (Public health Canada, 2004).

The high rate of replication that is found throughout the course of HIV infection and the variability of HIV, coupled with the relative inaccuracy of the enzyme, are the main reasons for frequent occurrence of copying errors in the transcription of viral genetic information (WHO, 2002). HIV replicates at the rate of around 10^8 to 10^{10} virus particles per day, probably giving daily rise of about $3x10^{-3}$spontaneous changes (mutations) in its genetic sequence. The ultimate size of a viral population containing a mutation is probably determined by three concurrent factors: the forward mutation frequency, the replicative capability of the mutated virus and the “age” of the viral population containing the mutation or how long ago this population was generated (WHO, 2002).

With the ongoing production of genetic variants of HIV, there is then a continuous selection for the "fittest" virus population. Sub-optional ART regiments that allow replication of HIV to continue in the presence of antiretroviral drugs encourage the growth of viral populations that are carrying a specific mutation, which protects against these drugs (WHO, 2002). It is likely that many of these drug resistance mutations already exist before any antiretroviral drug is introduced and are further encouraged to proliferate under the selective pressure exerted by drug treatment (WHO, 2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.0 Study area

This study was conducted in six locations within Nairobi (**Figure 3.1**). These were Kenyatta National hospital that serves approximately 3, 138,295 people by 2010 National census (Kenya, 2010). Kangemi health facility which serves informal settlement and is situated on the west part of Nairobi, Kasarani health facility situated on the North Eastern part of the city, Ngong facility that serves Ngong region that is in the Rift Valley bordering Nairobi and Kitengela health facility that serves Kitengela area that is on the Eastern province but bordering Nairobi. The Kamiti Maximum Prison clinic serves inmates in the prison and the Prison police and is situated in Central Province and borders Nairobi. Therefore in this study, six study sites were involved but with varying numbers of recruited study subjects.

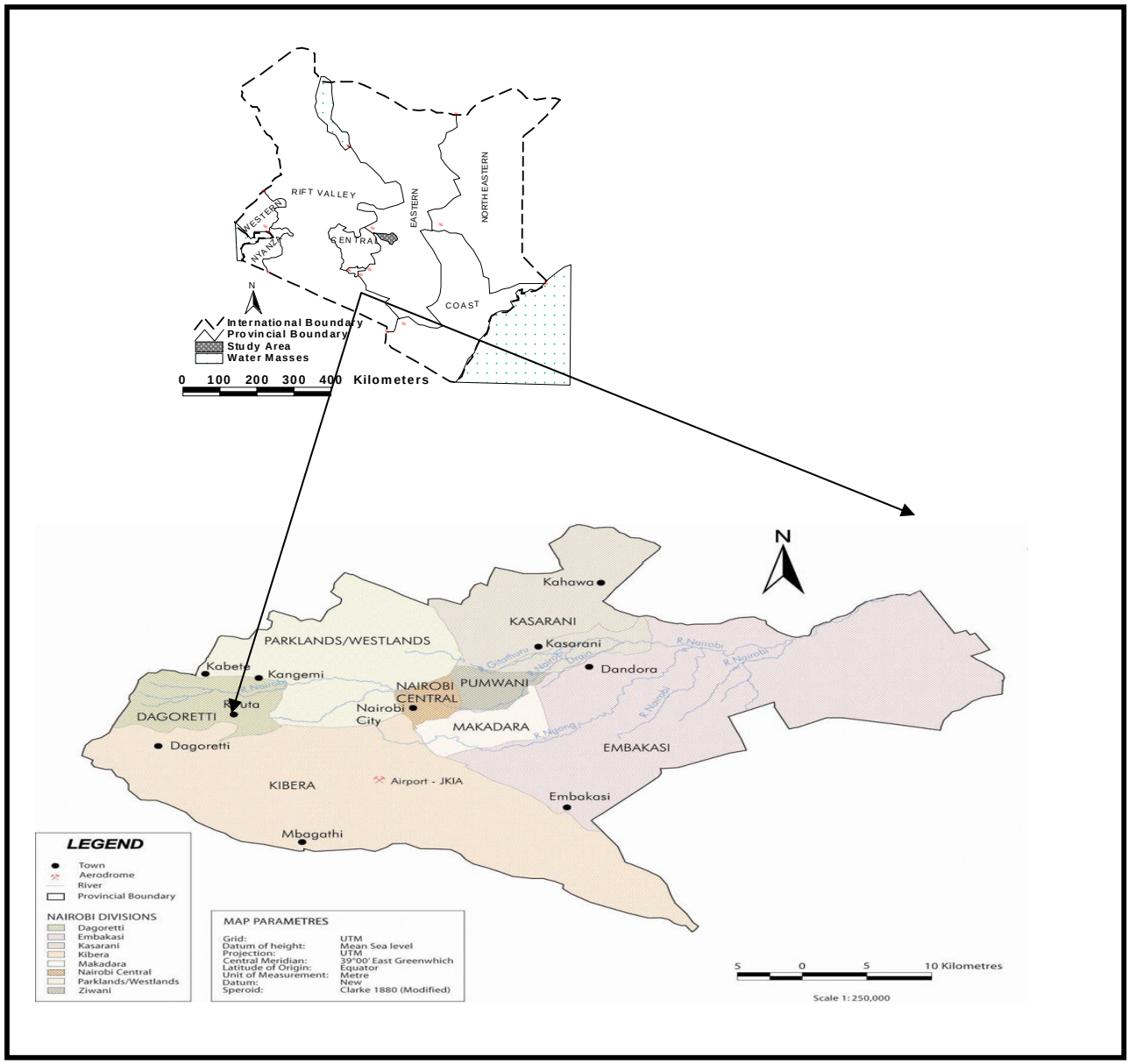

Figure 3.1: Map of Kenya showing location of sampling sites.
(http://www.mapsofworld.com/kenya/kenya-political-map.html retrieved on 3rd Feb 2008)

3.1 Biodata of HIV-infected patients in Nairobi, Kenya

A questionnaire was prepared and used to collect information on age and sex of respondents.

A specific code for name of participating individuals and study sites was used (**Appendix2**).

3.2 Study design

This was a prospective cohort study design.

3.3 Study population

The study was conducted on 188 subjects both male and female. All, one hundred and eighty eight patients both antiretroviral naive and those on antiretroviral treatment, were recruited from comprehensive HIV care clinics in Nairobi and its surrounding environs between 2008 and 2009. The recruitment sites were randomly selected and they included the medical facilities in the following locations in Nairobi and the surrounding; Kasarani, Kamiti Maximum prison clinic, Ngong clinic, Kangemi, Kitengela clinic and Kenyatta National Hospital. In total, 1500 visiting patients were approached and explained the objectives of the study. Out of these, 250 subjects met the inclusion criteria. The patient's basic characteristics and CD4 counts were determined and their age and CD4 counts mean determined (**Table 3.1**).

Table 3.1. Baseline Characteristics of study subjects prior to ARVs Therapy

GENDER			
	ALL	**FEMALE**	**MALE**
	N=188	**(n=101)**	**(n = 87)**
Age (years)			
Mean (Range)	**33.8 (3.5-73)**	**30.9 (5-69)**	**38.8 (3.5-73)**
CD4+ T cell count (Cells/mm3)			
Mean (Range)	**420(6-1155)**	**407 (69-1155)**	**394 (6-1063)**
Range			
<300	**56**	**38**	**18**
301-400	**25**	**13**	**12**
400-500	**22**	**14**	**8**
>500	**85**	**36**	**49**

3.4 Inclusion criteria

Patients attending comprehensive HIV care clinics and willing to participate in the study for a period of three years were eligible for the study. Those who gave informed consent were recruited to participate.

3.4.1 Exclusion criteria

Patients who attended comprehensive HIV clinics and were not willing to participate in the study for a period of three years. Those who did not give informed consent.

In this study only 188 (72.2%) of the respondents provided consent to participate and were recruited into the study. Counseling of the subjects was conducted within the health facility framework including the signing of the consenting forms. This study was approved by the KEMRI/National Ethical Review Committee with approval Number SSC No: 1394 (**Appendix 2**).

3.4.2 Sample size Determination

A simple random sampling technique was used in prospective cohort study design. This was applied in general population regardless of age, sex or race. Using a HIV-1 prevalence of 10% in Kenya (Khamadi *et al.,* 2005), and the sample size was derived using (Fischer *et al.,* 1922; Zodpey *et al.,* 2004) formular as shown below:

$$N = \frac{Z^2\ 1\text{-}ap\ (1\text{-}P)}{d^2}$$

Where:

N= minimum sample size required

Z =1.96 (STD)

A= level of significant at 5%

P =0.10 (10%) HIV-1 prevalence rate

D =0.05 (5% absolute precision)

Therefore:

$$N=\frac{1.96^2(1-(0.05x0.1)\ (1-0.1)}{0.05^2}=\textbf{138 samples}$$

Therefore, the working samples size was 138, however the used sample size of 188 was used to exclude occlusion errors.

3.5 Blood collection and handling

Using a sterile syringe and a needle, five-milliliter of venous blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) tubes as well as with anonymous epidemiological data, including age, sex, drug naive and those on antiretroviral therapy using an approved protocol. The samples were confirmed to be positive for HIV-1 antibodies using a rapid detection kit (Determine HIV1/2; Abbot, Japan) and Bioline HIV1/2; Republic Korea).

3.6.0 Peripheral Blood Mononuclear cells (PBMCs) separation

To a falcon tube containing 10 ml of 0.84% ammonium chloride, 5 ml of whole blood was added and vortexed to allow complete mixing and incubated at 37°C for 5 - 10 minutes. This was then centrifuged at 226 xg for 10minutes at room temperature and supernatant discarded. To the resulting pellet, 10 ml of 0.84% ammonium chloride was then added and the procedure repeated three times after which the final supernatant was discarded. The PMBC pellet was drawn into 1.5 eppendorf® tubes and stored for DNA extraction (Khamadi *et al.,* 2005).

3.7.0 Proviral DNA extraction

To the PBMC pellet extracted by the above protocol, 500 ul of DNAzol genomic DNA extract reagent (Gibco BRL®) was added. This was pipetted up and down to ensure adequate mixing. Two Vol (1ml) of chilled, cooled 4°C absolute ethanol was added to the dissolved pellet & mixed gently. This was spun at 906 xg at 4°C for 15 minutes and supernatant discarded and 1000 µl of 70% ethanol added to the pellet and vortexed thoroughly. The mixture was centrifuged again at 906 xg at 4°C for 15 minutes and supernatant discarded. The pellet was air dried at room temperature at safety carbinate and 100 µl distilled RNase free water added and stored at -20°C for subsequent analysis (Khamadi *et al.,* 2005).

3.8.0 RNA isolation from Plasma

RNA was extracted from plasma using Qiagen® RNA extraction kit according to manufacturer's instructions. Briefly, 140 µl of plasma was put into 560 µl of RNA viral lysis buffer (AVL) vortexed for 15 seconds and incubated for 10 minutes at room temperature. This was then centrifuged at 176 xg for 10 minutes and 560 µl ethanol added, pulse vortexed and centrifuged. Approximately 630 µl of the solution was applied to QIAamp® spin column in 2 ml column tube without wetting the rim and centrifuged for 1 minute. The QIAamp® spin column tube was then placed in a clean 2 ml collecting tube. The tube was closed to avoid contamination and centrifuged at 2817 xg. The tube containing filtrate was discarded. The QIAamp® column was opened, centrifuged and filtrate discarded. If sample solution obtained was more than 140 µl, the step was repeated till all lysate had loaded into column. To this column, 500 µl of wash buffer was then added and centrifuged for 1 minute and the column placed in a clean 2 ml collection tube and filtrate discarded. About 500 µl of wash

buffer was added to the collecting tube cap closed and centrifuged at 15,339 xg 3 minutes. This procedure was repeated to remove any wash buffer carryovers. The column was placed in a sterile 2 ml collection tube and the old collection tube discarded plus the filtrate and centrifuged again at full speed for 1 minute. The QIAamp® spin column was placed in a clean 1.5 ml microcentrifuge tube and 60 µl of elution buffer added. This was equilibrated at room temperature and the cap closed and incubated at room temperature for 1 minute and finally centrifuged at 704 xg for 1 minute to elute the RNA. The yielded viral RNA was stored at -20°C or -70°C for later use in Reverse transcriptase polymerase chain reaction (RT–PCR) (Ping *et al.*, 2003).

3.9.0 Genotypic Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The starting template for genotypic reverse transcriptase polymerase chain reaction (RT-PCR) was from viral RNA extracted from plasma for *pol*-intergrase and *env* regions. All reagents were thawed and put on ice. The PCR amplification conditions and list of primers used in this study are indicated in (**Appendix 4**) (Ping *et al.*, 2003; Songok *et al.*, 2004; Khamadi *et al.*, 2005; Lihana *et al.*, 2009).

The PCR amplifications were performed in a total volume of 20 µl containing 2.0 µl of 20 ng template DNA, 2.0 µl of 2.5 mM dATP, dGTP, dTTP, dCTP, 2.0 µl PCR buffer, 0.4 µl each of 400 µl/ml forward and reverse primer, 0.2 units *Taq* polymerase (Promega®) and 10.2 µl distilled water. The amplification was performed using a PTC-100™ (MJ Research, Inc), a PTC-200™ (MJ Research, Inc) or a GeneAMP®PCR System 9600 (Applied Biosystems) thermocycler. All the PCR amplifications included a 10 minute denaturation step at 95 °C, followed by 35 cycles of 30 sec 94 °C, 30 sec at 45-60 °C annealing temperature (**Appendix**

4), 1 minute at 72 °C and a final extension step at 72°C for 10 minutes (Janssens *et al.,* 1994; Ping *et al.,* 2003; Songok *et al.,* 2004; Khamadi *et al*., 2005; Lihana *et al.,* 2009).

3.10.0 Analysis of PCR amplicons products by gel electrophoresis

On completion of the second nested PCR all products were viewed by conventional agarose gel electrophoresis on (Khamadi *et al.,* 2005) a 0.8% agarose (SEakem LE® agarose; FMC BioProducts, Rockland, Marine, USA) gel in 1X TAE buffer (0.04M Tris acetate, 0.001 M EDTA). Five micro-litres ethidium bromide 0.5 µg per ml (Promega®, Madison, Wisconsin, USA) was added to the gel to stain the DNA (Sharp *et al.,* 1973). A 1 kb DNA molecular weight marker (Promega, Madison, Winsconsin, USA) 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^{TM} GeneGenuis computer system (Synoptics LTD, Cambridge, United Kingdom) (Khamadi *et al.,* 2005).

3.1.1.0 Sequencing of HIV-1 positive samples

The generated amplicons from the *env* and *pol* regions were then directly sequenced using same forward and reverse primer as in nested PCR (**Table 3.1**). This sequence PCR was carried with reaction mixture of 20 µl. These contained 3 µl of DNA, 5X sequence buffer, 2.0 µl BigDye®, 10.5 µl of distilled water, and 1.5 µl of forward and reverse primers (**Table 3.1**) (Songok *et al.,* 2004; Khamadi *et al*., 2005; Lihana *et al.,* 2009).

The amplification was carried out using PTC-100^{TM} a MJ Research, Inc thermal cycler at following PCR conditions (denature for 5 minutes at 96 $^{\circ}$C, and again for 10secs at 96°C, anneal at 50˚C for 5 seconds, final extension 60˚C 4 minutes for 25 cycles. The amplified products from the nested PCR were labeled in PCR reaction 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 are 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 are incorporated using either 5'-dye label primers or 3'-dye label dideoxynucleotide terminators. AmpliTaq® polymerase is used for primer extension. The dyes are used to perform sequencing PCR with the template being the PCR products to be sequenced.

3.12.0 Sequencing reactions and purifications for sequencing

From the second nested PCR products, directing sequencing was performed the primer M3 Forward and M8 Reverse for C2V3, Unipol 1 forward and Unipol 2 reverse for intergrase and gp46 Forward and *gp*47 reverse for *gp*41 env region as shown (**Table 3.1**). Subsequently on purification, to an eppendorf tube was added 2µl of 3 mM sodium acetate, 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. 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.

3.13.0 Sequence analysis

One hundred and eighty eight samples were successful sequenced using reverse transcriptase polymerase chain reaction targeting regions of the *pol*-intergrase genes, *env*- C2V3 and *gp*41 genes**.** The nucleic acid sequences were were manually edited and translated to amino acids sequences and submitted directly to HIV drug resistance database and examined for reverse transcriptase resistances associated substitution as compared to references in HIV drug resistances database using HIV seq (HIV search engine for queries) of the Stanford HIV drug resistance database at (http://hivdb.stanford.edu/, accessed on 13th July 2010) and bioinfo at http://integrase.bioinf.mpi-inf.mpg.de/index.php (accessed on 12th July 2010). Genotypic drug resistance in the *pol*-intergrase region and env *gp*41 regions were 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), http://g2p-454.bioinf.mpi-inf.mpg.de and by use of **G**enotypic **R**esistance-**A**lgorithm **De**utschland. (GRADE) online tool on the Stanford HIValg-Software at http://hiv.grade.de. HIV-1 co-receptor usage analysed using three different *in silico* online tools; (i) CPSSM http://indra.mullins.microbiol.washington.edu/webpssm/, (ii) (ds) Kernel http://genome.ulaval.ca/hiv-dskernel/ and (iii) http://coreceptor.bioinf.mpi-inf.mpg.de/index.php (Neogis *et al.*, 2010).

3.14.0 HIV-1 tropism, Subtyping and Phylogenetic analysis

The successfully generated sequences were used for subtyping in determination of HIV-1 subtypes and co-receptor usage. HIV BLAST and REGA subtyping were used in subtyping and in circulating forms and unique circulating g forms using both *env* and *pol* sequences. Sample sequences were converted into the PHYLIP format and gap stripped using Molecular Evolutionary Genetic Analysis software (MEGA 4.1) (Kimura, 1980), aligned using ClustalW version 1.6.6 and evolutionary distances inferred using the neighbor-joining method (Saitou and Nei, 1987). Their reliability was estimated by 1000 bootstrap replications. The profile of the trees was visualized with Tree View PPC, version 1.6.6 (Institute of Biomedical and Life Sciences, Scotland, UK). This procedure was used for analysis of all the sequences generated from the two regions (*pol* and *env*) of the HIV-1 genome.

Using the generated sequences, HIV tropism was also determined using *in silico* on-line programs; Geno2pheno, ds Kernel and C-PSSM bioinformatic tools (Sing *et al.,* 2007). Genotypic methods use simple rules, such as the 11/25 rule which predicts X4 merely on the basis of the presence of basic side chains in residues 11 or 25 of the V3-loop (35 amino acids). This method also uses the overall net charge of the 35 amino acids in the V3 loop; showing R5 viruses with a net charge of 5 or less and X4 viruses with a net charge of more than five (Wolinsky *et al.,*1992). V3 loop that is a 35 amino acid (AA) long structure held together by disulphide bonds between the cysteins in position 1 and 35, A few amino acid changes in V3 can change the coreceptor usage from CCR5 to CXCR4 (De Jong *et al.,* 1992). High V3 loop charge and presence of basic aa in position 11 and 25 (*gp*120 at sites 306 and 320) been associated with CXCR4 usage (Fouchier *et al.,* 1995), whereas negative or uncharged AA in positions 11, 25, or 28/29, resulting in a low V3 charge, or presence of a

glycine-proline-glycine motif in position 15-17 has been associated with CCR5 usage (De Jong *et al.,* 1992; Regoes *et al.,* 2005). Based on this, classification into CCR5 (R5), CXCR4 (X4) or dual/mixed tropic (R5X4) is possible. Dual classification into X4 and non-X4 is also possible and most useful as it closely reflects the clinically relevant problem (Regoes *et al.,* 2005).

3.15.0 Synonymous and nonsynonymous substitutions analysis of envelope gene

The rate of HIV-1 envelope gene evolution was determined by analyzing the ratio of synonymous (*d*s) to non-synonymous (*d*n) substitutions using online tools; synonymous and nonsynonymous substitutions per site (SNAP). The dN/dS ratio compares non-synonymous to synonymous substitutions in order to study the kinetics of evolution. A dN/dS value equal to one indicates neutrality; purifying selection occurs when a dN/dS ratio is substantially less than one and positive selection when dN/dS ratio is more than one (Hong *et al.,* 2005). The subjects were sampled at three points of six months intervals for two and half years.

CHAPTER FOUR

RESULTS

4.1 Molecular HIV Type 1 Genetic Diversity in Nairobi cohort

In this study, from samples collected, Nairobi had the highest number of recruited subjects (100) while Kitengela had the lowest (10).Most of the clients were recuited from comprehensive HIV care clinics where they access comprehensive health care.. Nairobi site had the highest number of HIV-1 subtype A1 (62) followed by Kamiti site (18). HIV-1 subtype A1 was the highest among the study sites. Overall, HIV-1 subtype A1 was the most prevalent (112 samples) followed by HIV-1 subtype C (34), HIV-1 subtype D (20) and HIV-1 subtype G (6). Circulating recombinant forms recorded low threshold levels of between 0.5% and 3.2% prevalence. A unique circulating form was also detected (**Table 4.1**).

Table 4.1 A summary of HIV subtypes and circulating recombinants recorded in the six study sites

	HIV-1 SUBTYPES												
STUDY SITE	**A1**	**B**	**C**	**D**	**A1D**	**02_AG**	**01A1**	**G**	**AIC**	**03_AB**	**URF**	**26C**	**13cpx**
NGONG	7	1	2	4	0	0	0	0	0	0	0	0	0
KANGEMI	6	0	2	3	0	0	0	0	0	0	0	0	0
KITENGELA	8	0	2		0	0	0	0	0	0	0	0	0
KASARANI	11	0	3	3	0	0	0	3	0	0	0	0	0
KAMITI	18	3	3	6	1	2	0	0	0	0	0	0	0
NAIROBI	62	0	22	6	0	4	1	1	1	1	1	1	1
TOTAL	112	1	34	20	1	6	1	4	1	1	1	1	1

4.1.3.1 HIV-1 subtypes detected from Kasarani

Alignment of sequences with reference sequences from Los Alamos database and Phylogenetic analysis revealed four main clusters; with HIV-1 subtypes; A1 (60%), D (5%), C (35%) prevalences (**Figure 4.1**). HIV-1 subtype A1 sequences clustered with reference sequences from Cameroon, Cuba, Kenya, USA, Argentina, and Uganda at 90% bootstrap values as first group. HIV-1 subtype A1 sequences clustered with reference sequences from Tanzania, Kenya, Uganda, and Democratic Republic of Congo at 72% bootstrap values. HIV subtype C sequences clustered with reference sequences from India, Cameroon, Italy, Democratic Republic Congo, Japan, Zambia and Kenya at 80% bootstrap values. HIV subtype D sequences clustered with reference sequences from Kenya, Uganda and Sweden at 70% bootstrap values (**Figure 4.1**).

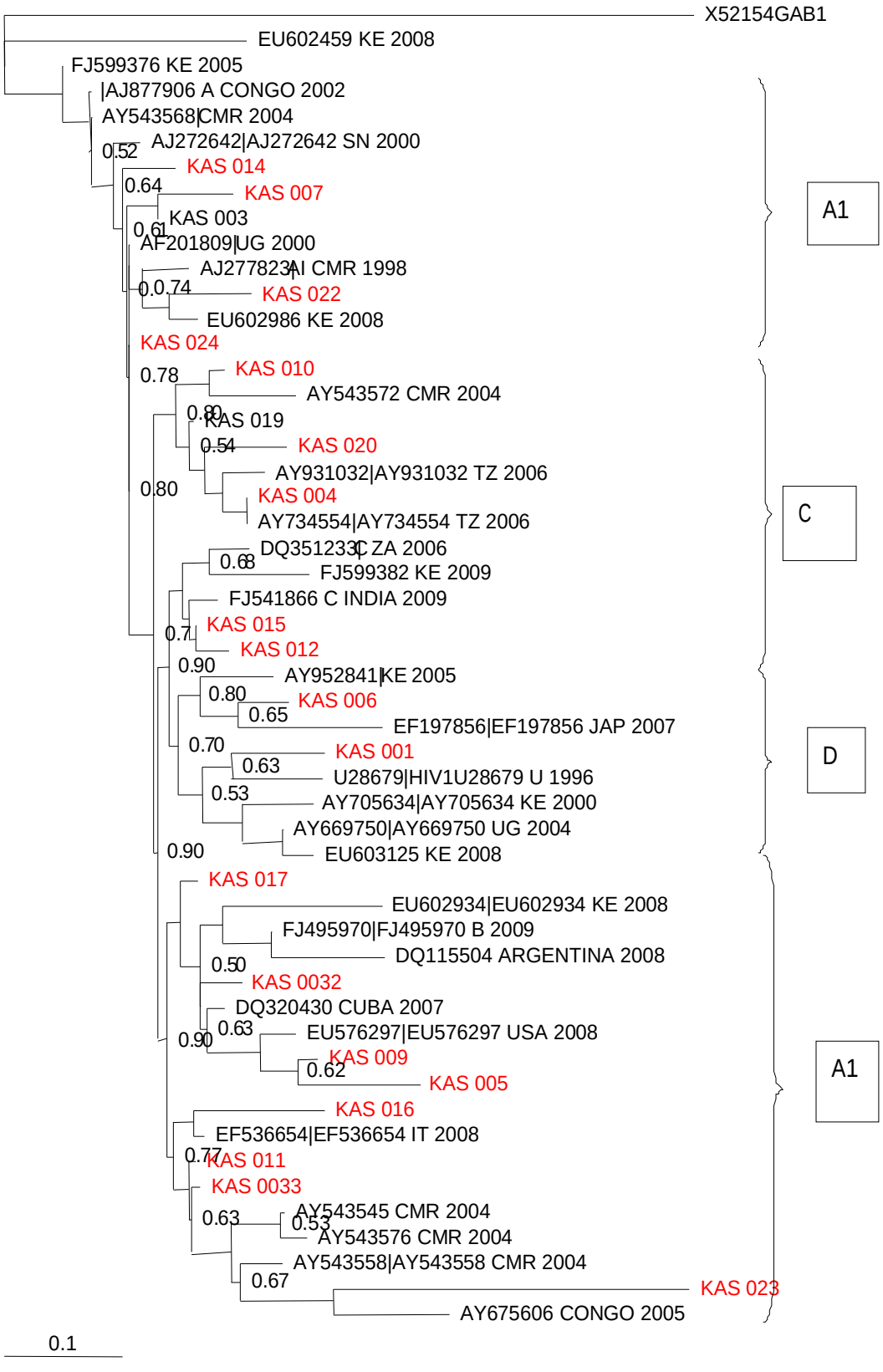

Figure 4.1 Phylogenetic analysis of *env C2V3* sequences generated from Kasarani site. **KEY:** The sequences from Kasarani are depicted in **red** and those in black are reference sequences

4.1.4. Analysis of samples from Kangemi, Ngong and Kitengela respondents

A total of thirty five samples collected from these sites were successfully sequenced cluster with *env C2V3* primers and analysed phylogenetically using ClustalW (Higgins *et al.,* 1994) and viewed using Treeview (Page, 1996). Three main clusters that is A1, C, and D HIV-1 subtypes were revealed with HIV-1 subtype A1 being the predominant HIV-1 subtype with a prevalence of 60%, this was followed by HIV-1 sub-subtype D with 20 %, HIV-1 subtype C with 3.4% and CRF 03_AB with 2.9%. Phylogenetic analysis therefore revealed that 85.6% were pure HIV-1 subtypes and 14.4% CRFs (**Figures; 4.1 and 4.2**).

HIV-1 subtype A1 sequences clustered with reference sequences from Tanzania, Kenya and Uganda at 88% bootstrap value. HIV-1 subtype C sequences clustered with sequences from Portugal, Zambia, Tanzania and India at 50% bootstrap values. HIV subtype D sequences clustered with reference sequences from Kenya at 70% bootstrap values. However, the outliers NNK 011 from Ngong site and KIT 006 were directly determined by BLAST analysis and found to be HIV subtype G at 70% bootstrap values (**Figure 4.2).**

HIV-1 subtype A1 clustered with sequences from Tanzania, Kenya, Cameroon, Democratic Republic Congo, Sweden and France with 76% bootstrap values. HIV-1 subtype C sequences clustered with sequences from Italy, India, Tanzania, Zambia, and Cameroon at 78% bootstrap values. HIV-1 subtype D strains clustered with reference sequences from Uganda and Kenya with bootstrap value of 98% and 78% (**Figure 4.3**).

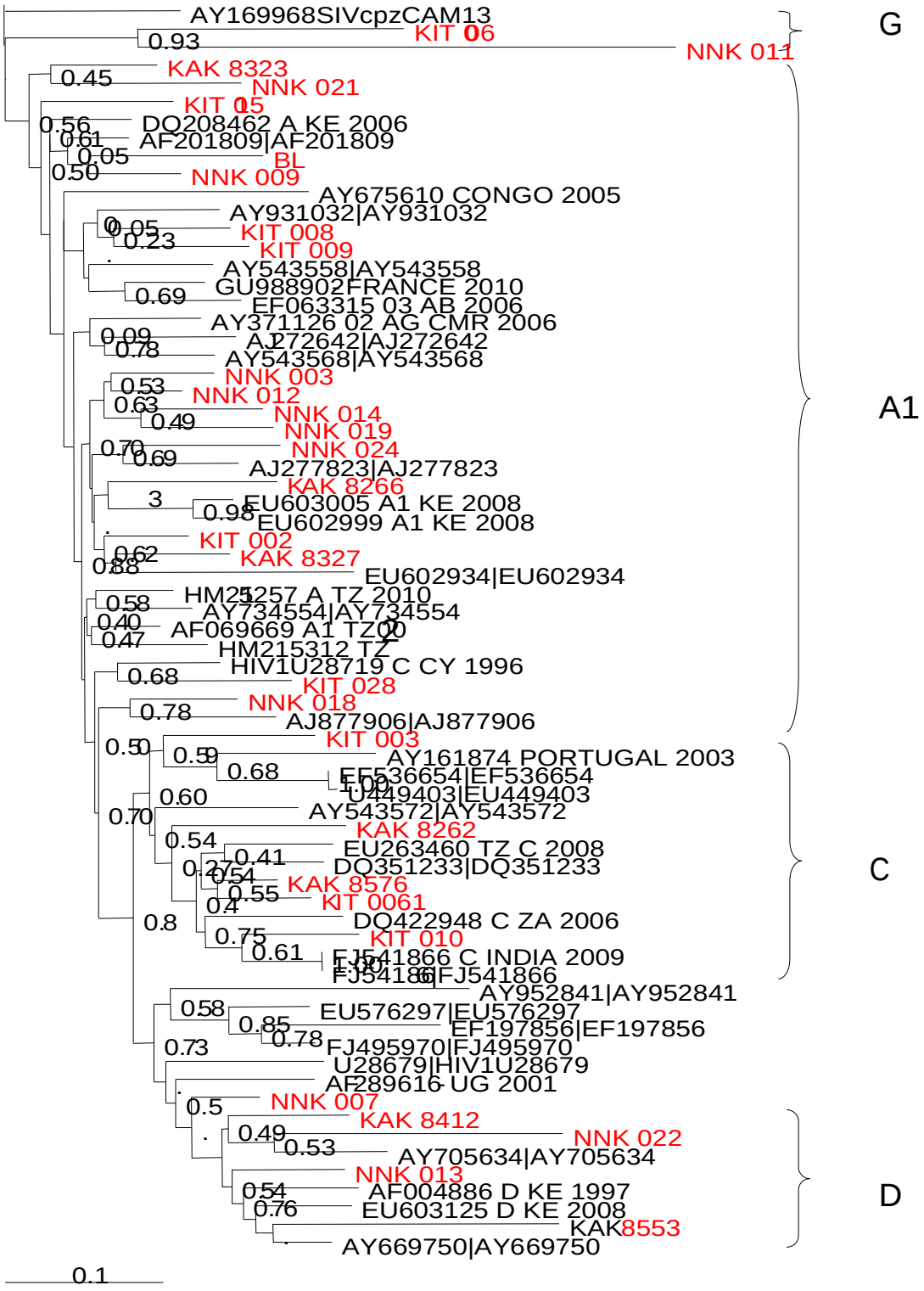

Figure 4.2 Phylogenetic analysis of *env C2V3* sequences generated from Kitengela, Ngong, Kangemi sites. **KEY:** The sequences from Kitengela (KIT), Ngong (NNK) and Kangemi (KAK) are depicted in **red** and those in black are reference sequences

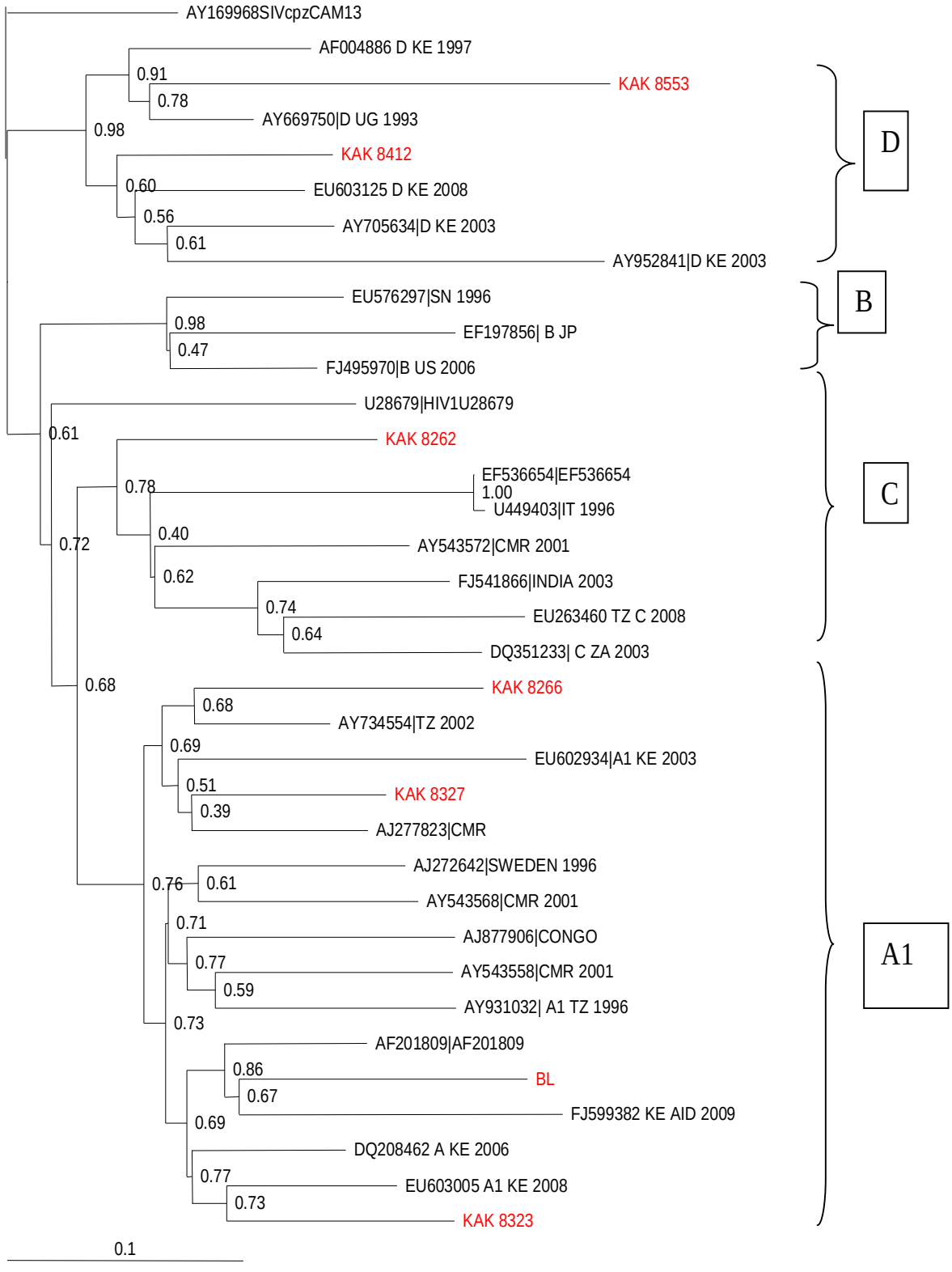

Figure 4.3 Phylogenetic analysis of *env C2V3* sequences generated from Kangemi sites. **KEY:** The sequences from Kangemi are depicted in **red** and those in black are reference sequences.

4.1.5. Analysis of samples from Kamiti respondents

Thirty three samples were successfully sequenced with *env* C2V3 primers and analysed phylogenetically (Figure 4.4 and Figure 4.5). The phylogenetic tree revealed five main clusters of sequences with HIV-1 subtype A1 being predominant with 54.5% prevalence. This was followed by HIV-1 subtype D with 18%, CRF02_AG 6%, HIV subtype C 3% and HIV subtype B 3%. HIV-1 subtype A1 sequences clustered with reference sequences from Kenya, Ghana and Tanzania at 82% bootstrap values. In HIV-1 subtype C, sequences clustered with reference sequences from Kenya, Uganda and Botswana at 79% bootstrap value in the first group and Kenya, and Tanzania at 94% bootstrap values. HIV subtype D sequences clustered with reference sequences from Belgium, Kenya, and Uganda at a lower 52% bootstrap value. HIV-1 circulating recombinant CRF02_AG sequences clustered with sequences from Cameroon and Rwanda at 54% bootstrap value. Samples KMT 0211 and KMT 016 were outlier that did not cluster with any reference sequences. They were therefore analysed directly by BLAST analysis and found to be circulating recombinant AID. Another group of outliers KMT 010 was found to be HIV-1 subtype B and KMT 015 and KMT012 to be HIV-1 subtype A (**Figure 4.4**).

Phylogenetic analysis of **Figure 4.5,** revealed three main clusters, HIV-1 subtypes A1, C and CRF02_AG. HIV subtype A1 sequences clustered with reference sequences from Kenya and Uganda at 70% bootstrap value. HIV subtype A sequences clustered with reference sequences from Cameroon, Rwanda, Kenya and Uganda at 50% bootstrap value. HIV subtype C sequences clustered with sequences from South Africa, Botswana, Kenya and Uganda at bootstrap 64%. However, KMT 029 and KMT 031 clustered together as well as KMT 025 and KMT 039 samples as well as samples KMT 077 and KMT 36 but without

clustering with any reference sequences. These outliers were directly BLASTed and found to be HIV-1 subtype C at 54%, 56% and 69% bootstrap values respectively. Samples KMT 024 and KMT 034 clustered with reference of HIV-1 circulating recombinant forms CRF02_AG sequences from Kenya at 70% bootstrap value (**Figure 4.5**).

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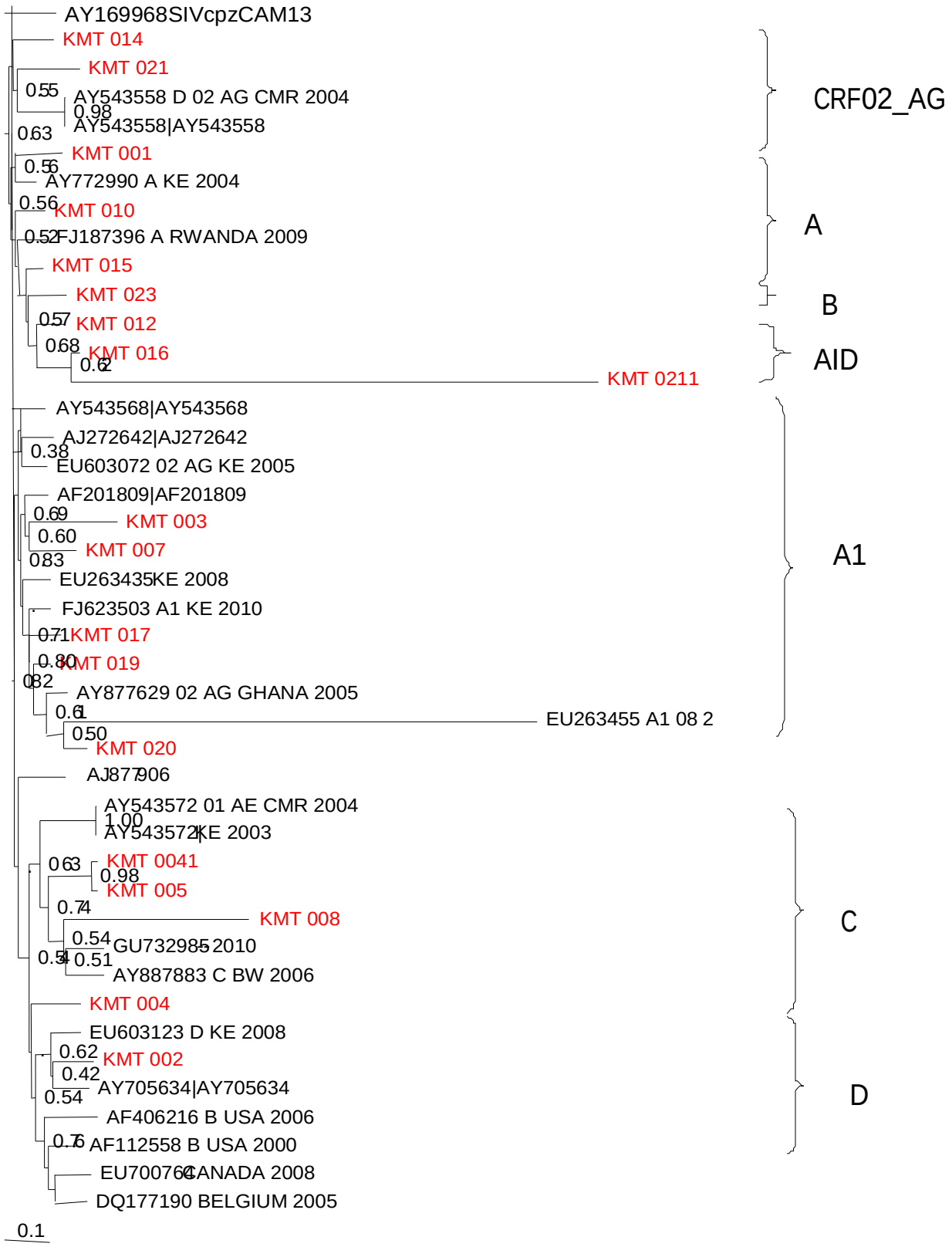

Figure 4.4 Phylogenetic analysis of *env C2V3* sequences generated from Kamiti site. **KEY:** The sequences from Kamiti are depicted in **red** and those in black are reference sequences.

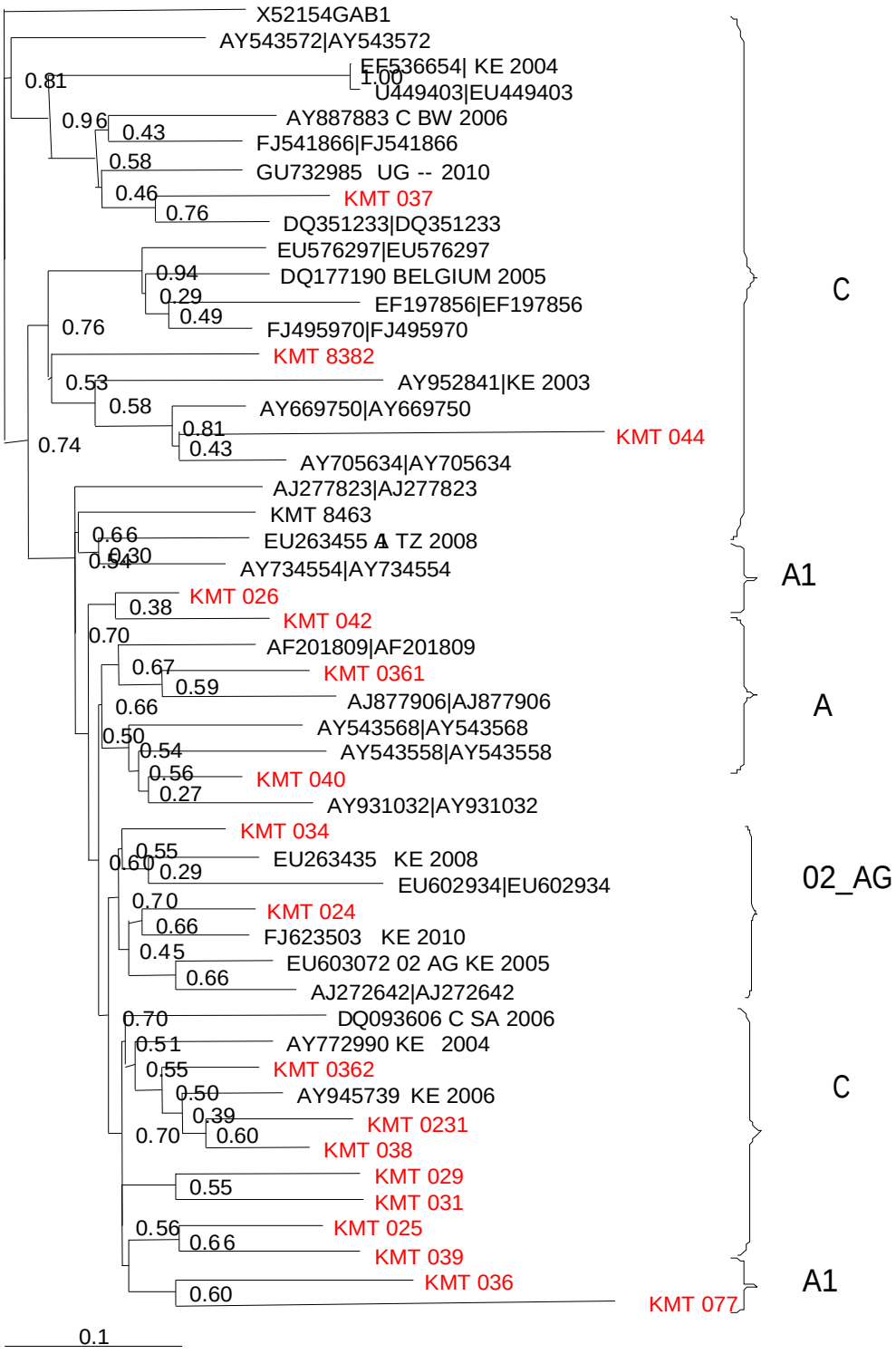

Figure 4.5: Phylogenetic analysis of *env C2V3* sequences generated from Kamiti site. **KEY:** The sequences from Kamiti are depicted in **red** and those in black are reference sequences.

4.1.6. Analysis of samples from Nairobi respondents

Ninety five samples were successfully sequenced with *env C2V3* primers and analysed phylogenetically. Due to large numbers of sequences, a single phylogenetic tree analysis could not be used to analyse all the sequences. It was therefore necessary to do four phylogenetic analyses resulting into; **Figure 4.6, Figure 4.7, Figure 4.8, and Figure 4.9**. The four phylogenetic trees revealed four groups of HIV-1 subtypes; HIV-1 subtype A1, C and D were revealed with HIV-1 subtype A1 being predominant with 44% prevalence. This was followed by HIV-1 subtype C (22%), HIV-1 subtype A (17%), HIV-1 subtype D (6%), CRF02_AG (4%) and 1% for HIV-1 subtypeA2, CRF01A1, HIV-1 subtype G, AIC, 26C, 13cpx and URF respectively.

HIV subtype A1 sequences clustered with reference sequences from Kenya, Sweden, Tanzania, Uganda and China at 80% bootstrap values **(Figure 4.6)**, Kenya, Uganda and Congo for HIV subtype A1 sequences at 72% bootstrap values, (**Figure 4.7**), Kenya, Iran and Tanzania at 89% bootstrap values (**Figure 4.8**) and Kenya, China, Cameroon, Tanzania, Sweden and Uganda at 80% bootstrap values in (**Figure 4.9**). HIV-1 subtype C sequences clustered with those from India and Democratic Republic of Congo at 72% bootstrap values (**Figure 4.6**), Zambia and Cameroon at 67% bootstrap values (**Figure 4.7),** from India and Zambia at 78% bootstrap values (**Figure 4.8**) and Democratic republic of Congo, India and Zambia at 79% bootstrap values (**Figure 4.9**). However HIV subtype G sequence clustered with a Nigerian sequence at 100% bootstrap values (**Figure 4.6)**. In addition samples NAR 4007 and NAR 8287 were found to be outliers and their HIV-1 subtype was determined directly by BLASTing and found to be HIV subtype G at 95% bootstrap values (**Figure 4.8**).

HIV-1 subtype D sequences clustered with reference sequences from Uganda and Kenya at 91% bootstrap values (**Figure 4.9**). In addition, there were outliers of NAR 015, NAR 0172 and NAR 017 whose HIV subtypes were directly determined and found to be HIV-1 subtype A1 at 57% bootstrap values (**Figure 4.6)**, outliers NAR 023, NAR 24, NAR 035, and NAR 042 that did not cluster with any reference sequences but their subtype was directly determined by BLAST analysis and found to be HIV subtype A1 at 54% bootstrap values while NAR 040 was directly determined by BLAST analysis and found to be probably CRF 03_AB. (**Figure 4.7**). However sequences NAR 8369, NAR 8442, NAR 8270 were outliers, their HIV-1 subtypes were directly determined by BLAST analysis and found to be CRF02_AG while NAR 8511 was CRF 01A1 at 86% bootstrap values each. (**Figure 4.8**). Samples NAR 2174 and NAR 2611 were outliers. They did not cluster with any reference but their HIV-1 subtype were directly determined by BLAST analysis and found to be HIV-1 subtype C at 55% bootstrap values (**Figure 4.9)**.

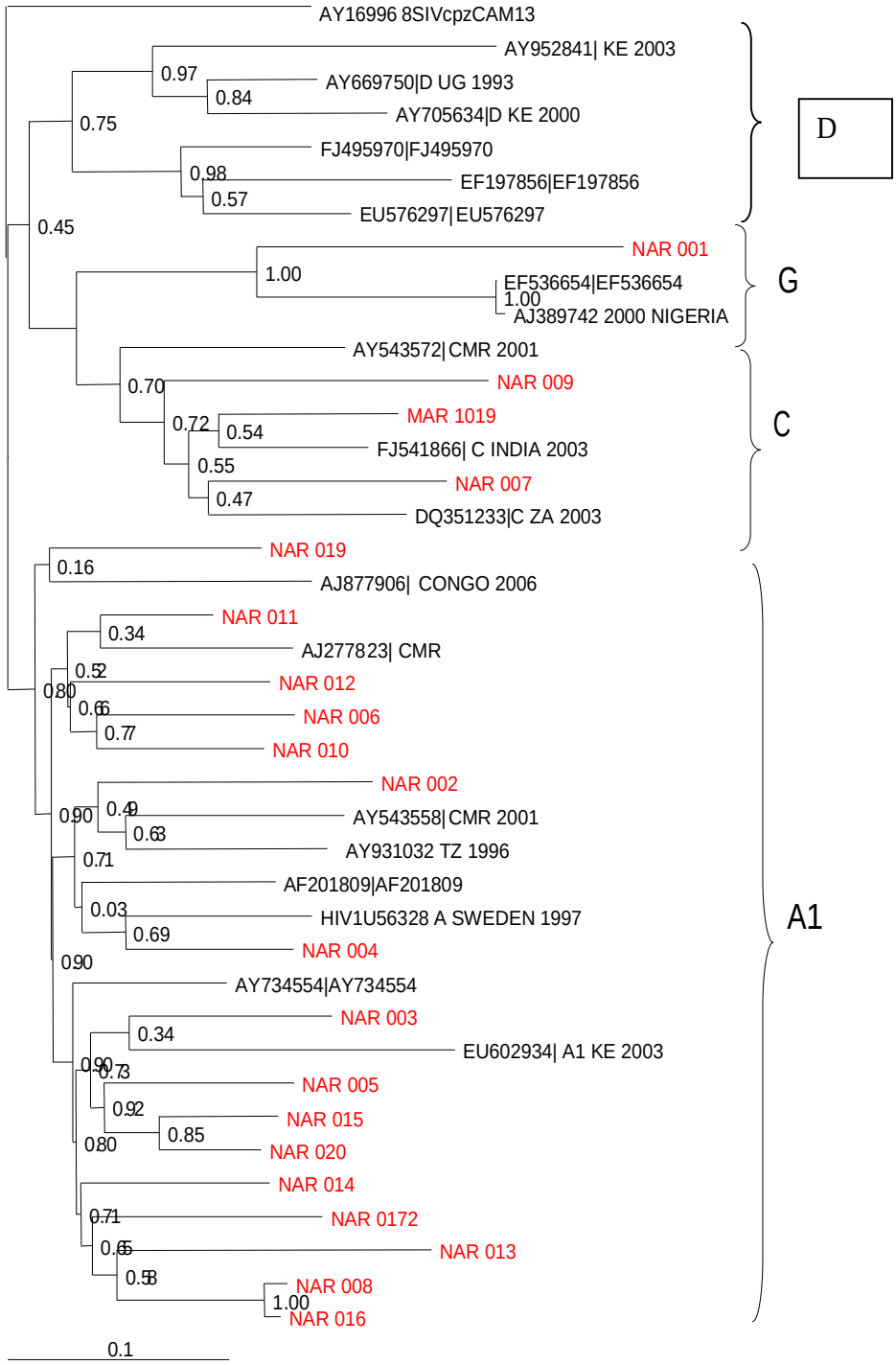

Figure 4.6 Phylogenetic analysis of *env C2V3* sequences generated from Nairobi site. **KEY:** The sequences from Nairobi are depicted in **red** and those in black are reference sequences.

Figure 4.7 Phylogenetic analysis of *env C2V3* sequences generated from Nairobi site. **KEY:** The sequences from Nairobi are depicted in **red** and those in black are reference sequences.

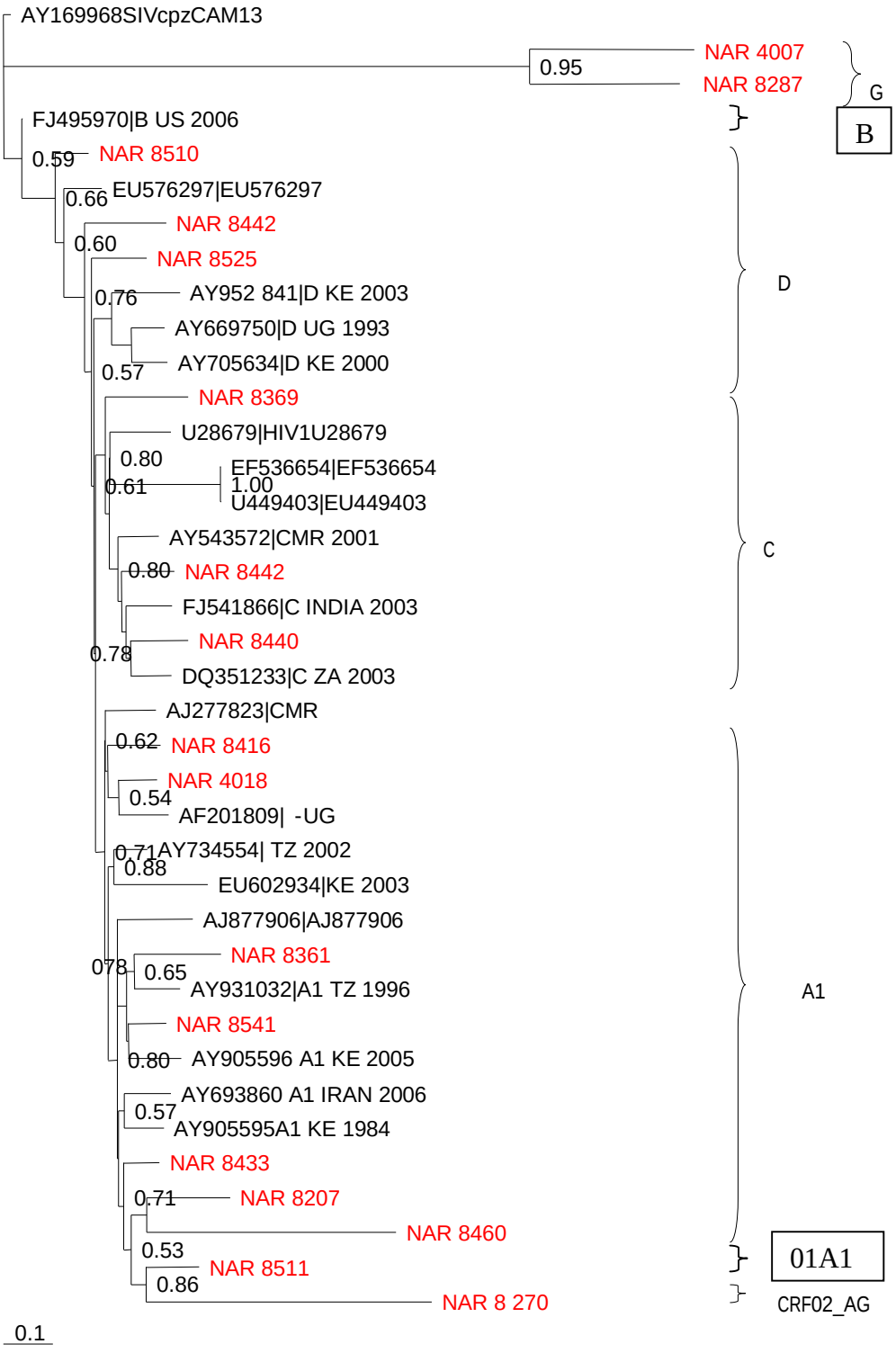

Figure 4.8 Phylogenetic analysis of *env C2V3* sequences generated from Nairobi site. **KEY:** The sequences from Nairobi are depicted in **red** and those in black are reference sequences.

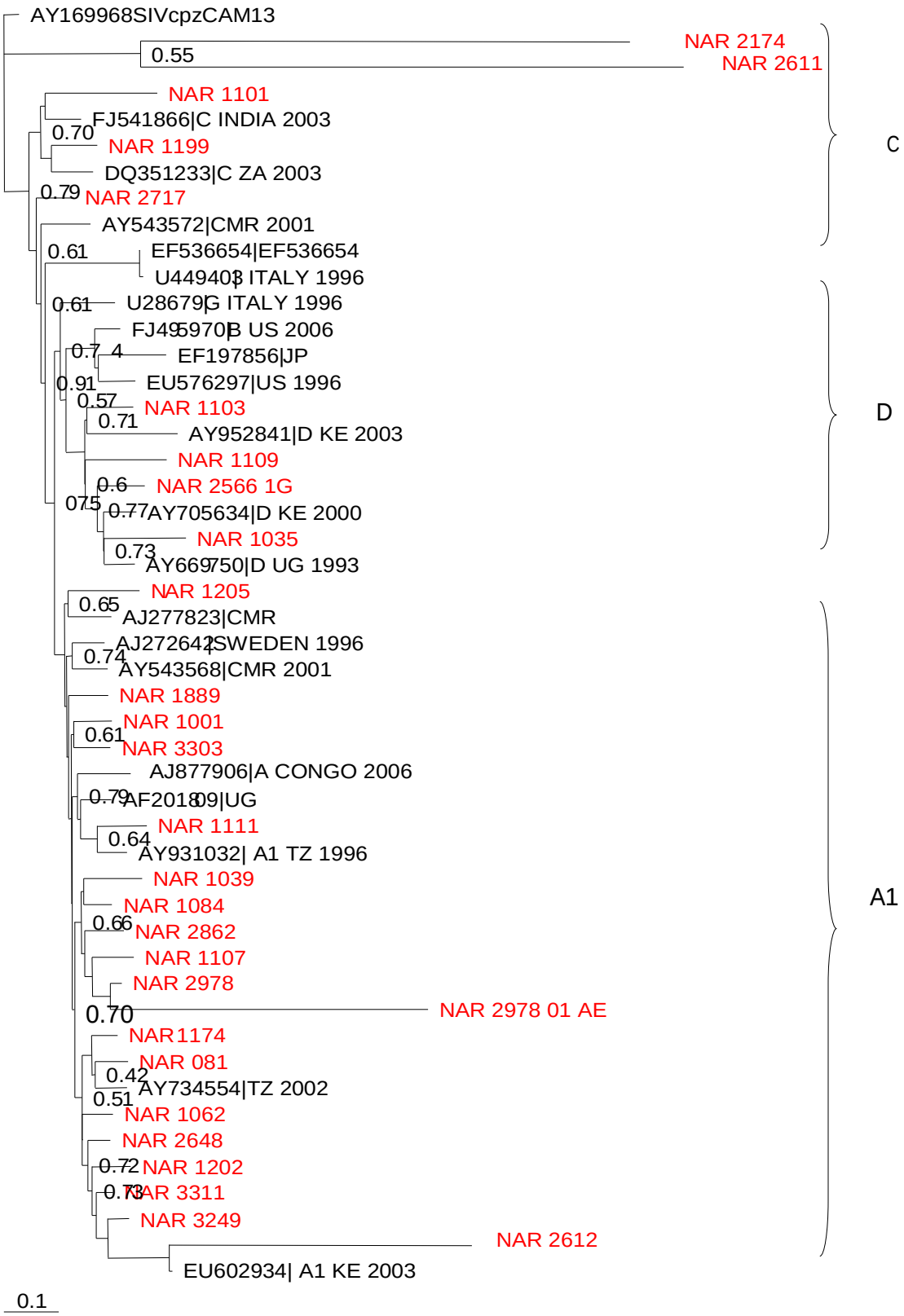

Figure 4.9 Phylogenetic analysis of *env C2V3* sequences generated from Nairobi site. **KEY:** The sequences from Nairobi are depicted in **red** and those in black are reference sequences.

4.2.1 HIV-1 Circulating Recombinants determination

One hundred and eight eighty (188) samples were characterized by partial regions of *env* C2V3 region of HIV and intergrase genes. Out of the total 188 samples analysed, 6.9% were circulating recombinants. These were; CRF02_AG 4.3% and 0.5% for, the following; A1D, 03_AB, 26C, A1C, 13cpx, 01A1 and URFs (**Figure 4.10**).

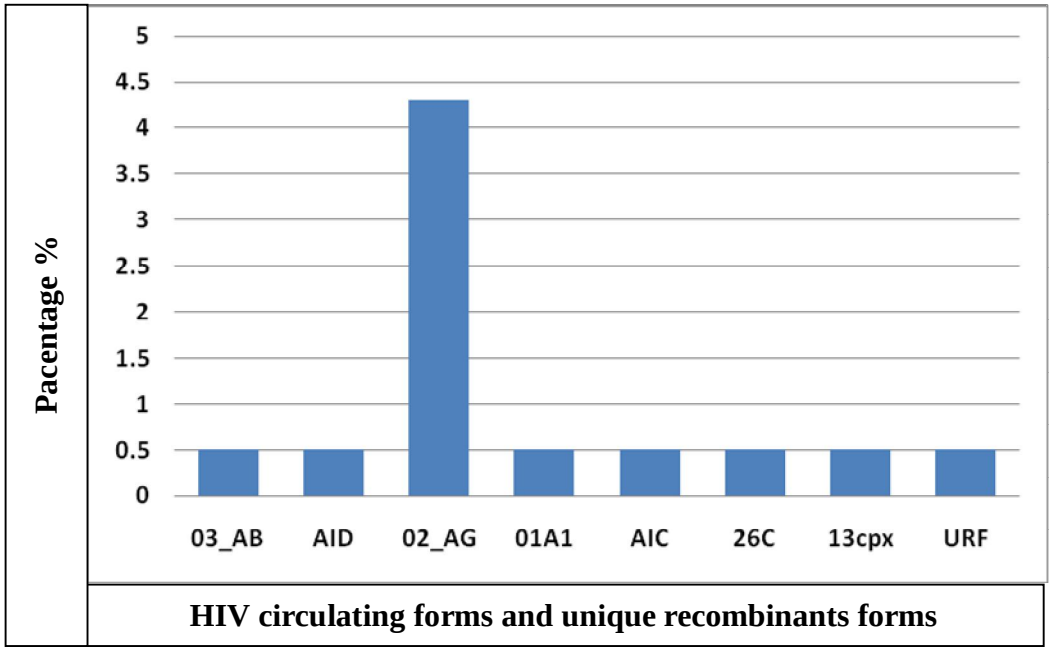

Figure 4.10 The prevalence of CRFs and URFs based on env gene

Based on integrase gene, 12.2% of the study subjects were found to be infected with HIV-1 circulating recombinant forms and unique recombinant forms. These were as follows: 2% for each of the following; AICD, A2D and BG, 4% for CRF02_AG and 6% URF prevalences (**Figure 4.11**).

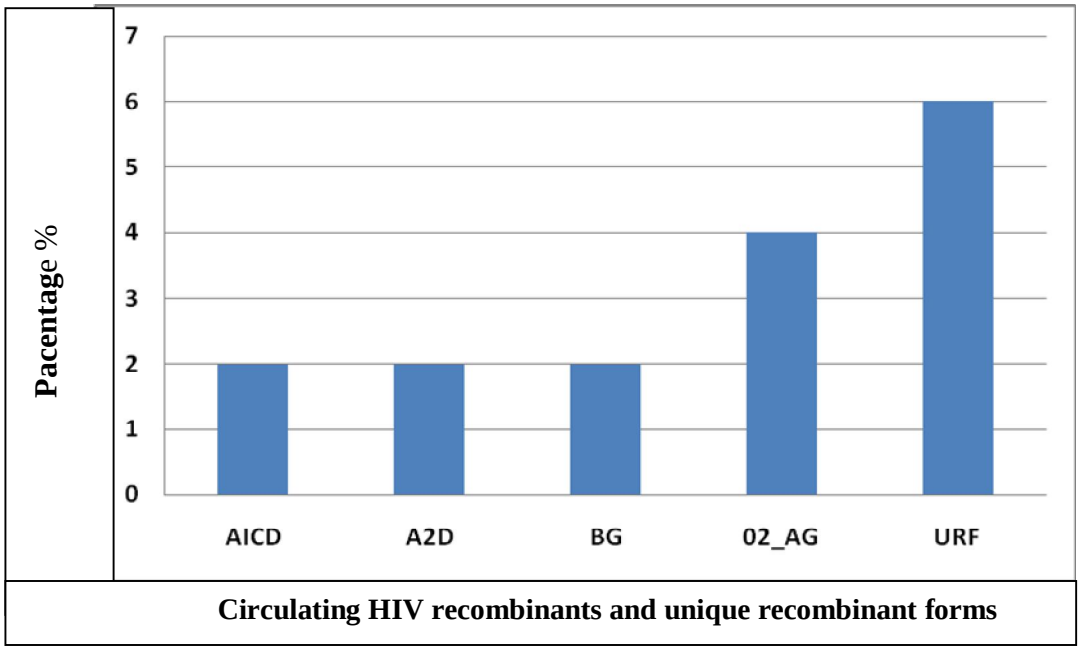

Figure 4.11 The prevalence of CRFs and URFs based on intergrase gene

4.3 HIV-1 co-receptor tropism

HIV-1 coreceptor tropism was analysed using online tools; WebPSSM, ds Kernel and Geno2pheno softwares. Out of 188 samples analysed, 69.6% were CCR5 tropic while 30.4% were CXCR4 tropic (**appendix 7**).

4.4 Drug resistance associated mutations to fusion inhibitor gp41

Envelope *gp*41 region was evaluated for drug associated mutations for Enfuvirtide (T20) that targets the viral fusion using **G**enotypic **R**esistance-**A**lgorithm **De**utschland (GRADE) online tool on the Stanford HIValg-Software at http://hiv.grade.de. The point mutations at; N42S was detected in 16.7% of all the samples, while N42D was detected in 4.2%, S138T/L 3.1%, L44M 2.1% and 1% in the following mutations; N43I and L45V drug resistance mutations. Therefore, 19.8% of the study subjects had mutations associated with drug resistance with 7.3% intermediate resistance to T20 (**Table 4.2**). In general, 33.3 % of total samples analysed

had other mutations that are not known to be associated with any drug resistance (**Appendix 8**). From the subjects that were detected with mutations, 53.8% were female with mean age of 37 years while 46.2% being men with a mean age of 30.1 years. In addition mean CD4 count of 323 among mutants was also detected.

Table 4.2 Prevalence of *env gp*41 drug resistance associated mutations

env gp*41 mutations	**Prevalence (%)**	**Drug**
N42S	19.8%	Enfuvirtide (T20)
N42D	4.2%	Enfuvirtide (T20)
S138T/L	3.1%	Enfuvirtide (T20)
L44M	2.1%,	Enfuvirtide (T20)
N43I	1%	Enfuvirtide (T20)
L45V	1%	Enfuvirtide (T20)

***** *env gp*41 mutations: frequencies of mutations at the *env*.gene identified in HIV-1 positive individuals

4.5 Co-receptor usage and CCR5 antagonist drug associated resistance mutations

HIV-1 co-receptor switch from CCR5 (R5) to CRCX4 (X4) was determined. In the total of 188 viral strains analysed; 69.6% were R5 strains and 30.4%, X4 strains based on the average online *in silica* tools.

4.5.1 glycoprotein 120 mutations (V3 loop)

From the *env* V3 loop sequence analysis using online tools; http://hivfrenchresistance.org/index.html, http://hivarca.net/hiv_resistance.asp, http://hiv_grade.de using international AIDS society consensus mutations online tools, natural polymorphisms were detected (**Table 4.3**). Mutations associated with Maraviroc resistance, A316T together with T320R that is associated with Vicriviroc resistance were the most prevalent. I323V and A316T+ I323V mutations that are associated with Maraviroc resistance were also detected though at low thresholds levels of between 1.1% and 2.6% prevalences. Combination mutations K305R+R315Q associated with Vicriviroc resistance

had a prevalence of 4.5%. Other detected mutations were K305R (10%), G321E (3.2%) and R315Q (35.1%) all associated with Vicriviroc resistance. In addition, combinations mutations that are associated with both Vicriviroc and Maraviroc were detected but at low thresholds levels of between 0.5% and 12.7%. These were; A316T+ K305R+ R315Q (1.6%), A316T+R315Q 12.7%, R3I5Q+A316T+I323V 3.2% and R315+A316T+G321E 0.5%.

Table 4.3 CCR5 drug resistance associated mutations

Antiretroviral	Observed prevalence for each Resistance mutation (%)
Maraviroc	
A316T	63.3
I323V	1.1
A316T+I323V	2.6
Vicriviroc	
K305R	10
G321E	3.2
R315Q	35.1
K305R+ R315Q	4.5
T320R	62.8
Maraviroc + Vicriviroc	
A316T+ K305R+ R315Q	1.6
A316T+R315Q	12.7
R3I5Q+A316T+I323V	3.2
R315+A316T+G321E	0.5

Key: Prevalences are given percentages; most of these mutations confer partial resistance; only A316T/I323V has been demonstrated to confer complete resistance to Maraviroc.

4.6 Intergrase associated drug resistance mutations

In this study, 2.4% of the study subjects had viral strains with a reduced susceptibility intergrase inhibitor Raltegravir with mutation at position T97A. However, 22.4% of other mutations were also detected. These included; F1S, L2E, D3K, I5K, D6E, A98APST, L101I, K103E, T112V, N117K, G123S, A124N, R127K, D167E, K188R, G190R, T93N, Q95A, E96G, I113V, D116ADGV, N117K, G123S, R127K, K188R, G190R, I191N, G192R, Y194N, S195L, A196L, G197L, E198*, R199I, I200H, V201I, D202K, I203L, I204R, A205S, S195L, S283CGRS, R284F, Q285Y, D286E/H, E287* and D288I/D.(**Table 4.4**). These were mutations that do not confer any drug resistance.

Table 4.4 Intergrase drug resistance associated mutations

Amino acids at specific codons in patient HIV-1 integrase gene	Frequency @ substitution
G163E,G197L,A205S,C280A,V281L,A282L,G4A,T97A,T93N,A98APST,K103E ,K111T,F100Y,S119T & G134	2.40%
I200H,V201I,D202K'L,I203L,I204N'R,S283L'C,R284F,Q285Y'I,D286E'H,E198*, R199I & K136R,D116ADGV & I135V	4.80%
A124N'T,E287D'S & E96G'V	7.10%
I113V,D167E,Y194L'N'H,S195K'L,L2E'V & D3K'G	9.50%
D288D'F'T'I & L101I	11.90%
Q95A'G & F1S'C'G'R	14.30%
T125A,N117K'D,E96G'V,K188R,G190R,I191N & G192R	16.70%
G123S & R127K	21.40%
T112V	23.80%

4.7 HIV evolutionary rate determination and co-receptor usage

From the fifty five subjects that were followed up to two and haly years, C2V3 sequences generated were analysed for viral tropism using *in silico* online tools; ds Kernel, Geno2Pheno and webPSSM softwares. In total of 55 samples, 94.5 % were X4 variants and 5.5% CCR5 (R5) strains (**Table 4.5**).

4.7.1 Accumulation rates of synonymous and nonsynonymous substitutions in HIV-1 envelope gene

The human system drives HIV-1 evolution by positively selecting variants with reduced sensitivity to cytotoxic T-lymphocytes (CTLs) and neutralizing antibodies that results into emergency of viral escape mutants that are associated with disease progression (Williamson, 2003). A change therefore of viral environment within the host imposes selection pressure on viral population, such that viral variants with genes best adapted to the new milieu are selected for. The HIV-1 *env* gene is one of the best-known examples of molecular adaptation (Yang, 2001). *Env* gene is targeted by immune system pressure and the virus adapts with development of mutations. Mutations are then used to estimate the rate of viral adaptations and evolution by determining the ratio of synonymous substitutions (*dN*) to nonsynonymous substitutions (*dS*). In this study, 12.5% of the study subjects had *dN*/*dS* ratio less than one suggestive of purifying selection while 87.5%, *dN*/*dS* ratio more than one suggestive of positive selection. No neutrality selection was detected from this study (**Table 4.5**).

Table 4.5 Genetic diversity and non-synonymous/synonymous substitution ratios

Followed Patient	HIV Genetic period (months)	tropism diversity		dN/dS
SHC 2052-001A	34	A1	X4	0.9361
SHC 5139-110B	28	A1	X4	1.2929
SHC 1444-208C	24	D	X4	2.1109
SHC 3555-107G	29	A1	X4	1.3069
SHC 3938-220K	30	C	X4	1.0098
SHC 2929-057L	32	C	X4	1.2791
SHC 399-142V	35	A1	X4	1.3069
SHC 1460-209M	31	A1	X4	2.1109

Key: ds/dn >1 Negative selection, ds/dn <1 positive selection and ds/dn ≠ 1 Neutral selection

CHAPTER FIVE
DISCUSSION

5.1 Circulating HIV-1 subtypes

HIV-1 subtype surveillance studies have been carried out in different parts of Kenya mainly in the Coastal, Northern parts of Kenya, Central, Western parts of the country and Nairobi City (Dowling *et al.,* 2000; Songok *et al.,* 2003; Khamadi *et al.,* 2005; Lihana *et al,* 2006, 2009). Kenya's HIV/AIDS epidemic has grown with the rest of the world and diverse HIV-1 subtypes and recombinants have been reported (Janssen *et al.,* 1994; Dowling *et al.,* 2002). In Kenya, like the rest of the other East Africa countries of Uganda and Tanzania, HIV-1 subtypes A1, C and D are the main subtypes of HIV-1 circulating, occurring in different proportions in different populations (Hoelscher *et al.,* 2001).

From this study, samples, studied were found to harbour diverse HIV-1 subtypes with majority of the sequences analyzed belonging to HIV-1 subtype A1 (**Figures 4.1-4.9**). 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; Lwembe *et al.,* 2007; Khoja *et al.,* 2009; Steegen *et al.,* 2009; Lihana *et al.,* 2009).

Phylogenetic analysis revealed that 93.1% were pure HIV-1 subtypes and 6.9% CRFs. 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; Papathanasopolos *et al.,* 2003; Lihana *et al.,* 2009) where 92.8% pure subtypes and .2.4% CRFs; 71% pure subtypes and 29% CRFs respectively. In this study, the prevalence of recombinants was at low levels probably due to settled populations and

therefore less human migrations or movement of people compared to work carried out by Khamadi *et al.,* (2005) who observed high recombinations in the northern part of Kenya. However, these populations tend to move alot as pastoralist migration in search for food and water, and cross border movements due to political upheavals in neighbouring countries.

5.1.1 Analysis of Sequences from Kangemi, Kitengela and Ngong

Phylogenetic analysis of sequences generated from samples collected from Kangemi, Ngong and Kitengela respondents showed that these viruses were of divergent origin. Ngong, Kitengela and Kangemi are areas most frequented by foreigners who could easily have transmitted these varying HIV-1 subtypes from neighbouring countries (Bello *et al.*, 2008; de Oliveira *et al.,* 2010). Kitengela being on the highway to Namanga border and Kangemi, serve as HIV/AIDS hotspots where commercial sex workers are in high numbers as truck drivers stop over on transit to Eastern Africa countries of Uganda, Zambia, Tanzania and Congo. In addition, Mombasa being an area with high numbers of tourists makes it an ideal route of transmission from Europe and other parts of the world, to Mombasa then to the rest of East Africa. Results from this study support this theory based on relationship of HIV sequences and the countries mentioned above.

The results also revealed that the HIV-1 subtype D sequences were of East African origin suggesting transmission being within East Africa countries (**Figure 4.2** and **4.3**).HIV subtype G continues to be detected in Kenya though at low prevalences. HIV-1 subtype G is predominant in West Africa (Bikandou *et al.*, 2000; Piot *et al.,* 2002) but has moved to Kenya but at low prevalences. In this study, 2.1% prevalence was seen that concurs with

previous reports (Neilson *et al.,* 1999; Yang *et al.,* 2004; Khoja *et al.,* 2008; Lihana *et al.,* 2009) who also recorded low levels in prevalences of between 1% and 4%.

5.1.2 Analysis of Sequences from Kasarani

The circulating HIV strains among the Kasarani respondents were found to be more divergent. The viral strains from Kasarani suggested that they could likely have originated from their respective countries and spread among Kasarani residents (**Figure 4.6**). However, Kasarani like the rest of the Nairobi districts is frequented by migrants from other countries, either on trade or due to political instability from their countries, transmitting the viral strains to Kasarani residents. However, samples from Kangemi, the sequences were likely to be of East Africa origin suggestive of possible transmission from truck drivers to Uganda, Tanzania and Democratic Republic of Congo or refugees from these countries. In addition, the urban setting of Kangemi compared to that of Kasarani is of low economic status hence not able to support tourist. Nevertheless, the viral strains from Kangemi were likely to be of East Africa origin, suggesting that transmission could be among people within countries that of East Africa.

The epidemiology of HIV-1 among the Kamiti respondents showed HIV-1 A1 strains likely to be of East African origin while HIV-1 subtypes C of divergent origin. The clustering of HIV-1 subtype C sequences with sub-Saharan Africa countries where HIV-1 subtype C is predominant suggested its possible origin. HIV-1 circulating recombinant CRF02_AG sequences clustered with reference sequences from Cameroon and Rwanda (**Figure 4.4**). These results implied that this recombinant may have originated from West Africa countries where it is most dominant even though the clustering was not significant (Konings *et al.,*

2006; Njai *et al.,* 2006). The CRF02_AG results from viral recombination of HIV-1 subtype A and G but with high transmission and replication fitness (Konings *et al.,* 2006) than parental strains. In addition, the outliers detected KMT 016 and KMT 0211(**Figure 4.4)**, were found to be probably circulating recombinant AID. This suggests that foreign viral strains or evolving circulating strains may have recombined with the circulating HIV-1 subtypes among the population leading to viral forms that do not cluster with any reference sequences. Alternatively, these could be new infections by viruses that have been circulating and recombined in other persons within the population who may be dually infected (Lihana *et al.,* 2009). It implies therefore that there could be an increasing number of infections due to recombinant forms of the virus (Wainberger *et al,* 2004).

5.1.3 Analysis of Sequences from Kamiti

The HIV-1 genetic diversity findings from the Kamiti respondents, concurred with previous studies on HIV-1 genetic diversity in the Kenya (Songok *et al.,* 2003; 2004; Yang *et al.,* 2004; Lihana *et al.,* 2006; 2009; Lwembe *et al.,* 2007; Allison *et al.,* 2008; Khoja *et al.,* 2009; Steegen *et al.,* 2009). The clustering of samples from Kamiti respondents with countries within East Africa and surrounding regions, implied possible prior exposure to HIV-1 subtypes from the endemic areas where HIV-1 subtype C is predominant like Zambia, South Africa and Rwanda.

5.1.4 General Picture of HIV-1 subtypes in Nairobi

Phylogenetic analysis of samples from Nairobi respondents showed that HIV-1 subtype A1 was the dominant virus with prevalence of 44%. These results were in agreement with previous molecular epidemiological studies conducted in Kenya (Dowling *et al.,* 2000; Poss

et al., 2001; Songok *et al.,* 2003; Yang *et al.,* 2004). 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).

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). In this study, HIV-1 subtype C had increasing prevalence (18%) that surpassed HIV-1 subtype D (11.7%). These findings did not concur 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). However, a similar situation was observed in the northern part of Kenya where the cross border effect from Ethiopians where HIV-1 subtype C is most predominant (Khamadi *et al.,* 2005). Robbins *et al.,* (1996) also reported similar results although the sampling numbers were relatively small. The increased number of HIV-1 subtype C virus strains identified could indicate an increasing prevalence of subtype C HIV-1 strains in Kenya (Robbins *et al.,* 1996). This study confirms a report by Zachar *et al.* (1996) of a subtype C virus in Kenya was not spurious and that HIV-1subtype C viruses are being transmitted in this region (Zachar *et al.* 1996; Robbins *et al.,* 1999; Khamadi *et al.,* 2005). Phylogenetic analyses of HIV-1 subtype C strains were divergent, suggesting that they could have been transmitted from the respective countries of Democratic Republic of Congo, Zambia, and India.

In phylogenetic analysis of HIV-1 subtype D and A1 strains among the Nairobi respondents revealed that these strains were of East Africa origin while those of HIV-1 subtype G were likely to be of West African origin. This suggested that transmission of these strains occurs within Kenya and Uganda. However, it is difficult to tell whether these viruses originate from here or are from other parts of continent or world. Nevertheless, these HIV-1 subtypes are commonly circulating in East Africa (Gray *et al.,* 2009). In this study, presence of outliers as well as high circulating recombinant forms were detected. These findings suggest that these outliers may have resulted from viral mixing and dual infection hence viral recombination of existing HIV-1 subtypes with newly introduced ones. Similar findings were reported by Lihana *et al.,* (2006) in a Nairobi cohort study where he reported an evolving HIV-1 epidemic where more virulent and complex subtypes are evolving through transmission of complex recombinants as a result of viral mixing of existing strains of HIV-1 subtype A1, D and C with newly introduced ones. The consequence of recombination has been found to result into high HIV diversity (Lihana *et al.,* 2009).

In phylogenetic analysis of samples from this cohort, majority of sequences analysed belonged to HIV-1 subtype A1, though the clustering with reference HIV-1 subtypes was not ideally significant from the low bootstrap values. This was followed by HIV-1 subtype C, subsubtypes A, then D, and G. This is in agreement with previous reports on evolution of HIV-1 subtype A to form sub-subtypes which have been reported in different continents (Gao *et al.,* 2001; Lwembe *et al.,* 2007; Allison *et al.,* 2008,Khoja *et al.,* 2008; Lihana *et al.,* 2009; Steegen *et al.,* 2009).

A major finding from this study was the recorded high prevalence of HIV-1 subtype C (18.1%) surpassing HIV-1 subtype D at (11.7%) in contrast to previous studies. All the previous studies done in Nairobi have reported a higher prevalence for HIV-1 subtype D compared to HIV-1 subtype C (Songok *et al.,* 2003; 2004; Yang *et al.*, 2004; Allison *et al.,* 2008; Lihana *et al.,* 2006; 2009; Lwembe *et al.,* 2007; Khoja *et al.,* 2008; Steegen *et al.,* 2009). These results confirm the continued evolution of HIV-1 subtype C from countries where it is predominant and its continued spread to other countries (de Oliveira *et al.,* 2010). The study population had an average age of 36 years. This age group is made up of persons who are active in society and widely travelling (Buvé *et al.,* 2002). It is also the group that is most affected by HIV-1 (UNAIDS, 2009). These results concur with UNAIDs, (2009) that showed that age group 15-49 years as the most affected with HIV epidemics in Kenya and other countries of the world (UNAIDS, 2009).

This study showed that the HIV-1 genetic diversity in Nairobi is greater than what has been previously published (Lihana *et al.,* 2009). This broad genetic diversity may be attributed to several factors; Firstly, Nairobi is a cosmopolitan city that harbours people from all over Africa as well as the rest of the world. These people contribute greatly to the diverse viral strains that are present here (Lihana *et al.,* 2009). Secondly, Nairobi is a connecting city to other major cities of Africa as major trade routes pass through here. Thirdly, due to tourism which is a major earner of Kenya's economy, high numbers of tourists frequent this country including those infected with HIV-1 who may end up transmitting it to the locals. Fourthly, the political instability in many of the countries bordering Kenya, including Central Africa, Somali, Sudan, Democratic Republic of Congo, Ethiopia and Rwanda has resulted in an influx of refugees into Kenya from these countries. Lastly, the movement of people along

Africa's coast as well as mainland trafficking of merchandise has been well documented (Buvé *et al.,* 2002)

As such, these increased human interactions, could lead to high diversity and dual infections. Since the HIV-1 genes regions analyzed are limited, it is possible that sequencing of larger gene fragments and more samples could shed more light on subtype diversity and recombination in this population and in Kenya (Paraskevis *et al.*, 2001; Khamadi *et al.*, 2006; Lihana *et al,* 2009). In the current study, the diverse HIV-1 subtypes, CRFs and URFs being contributed by viral mixing from diverse in and out of the country due to migration may be higher than previously published 9% (Wainberger *et al,* 2004); 18.7% (Yang *et al.,* 2004) 9% (Lihana *et al.,* 2009) and 29% (Lihana *et al.,* 2009) prevalences, therefore require frequent monitoring (Allison *et al.,* 2008).

5.2 HIV-1 Circulating Recombinants

From the total of 188 sequences analyzed based on partial regions of *env* gene C2V3 region, 6.9% were classified as circulating recombinants and the rest as pure subtypes. The following recombinants were detected; 4.3% CRF02_AG, 0.5% in each of the following A1D, 03_AB, 01A1, AIC, 26C, 13cpx and URFs (**Figure 4.10**). The results show that CRF02_AG was most prevalent with 4.3% prevalence. These findings concurred with a recent study in which analysis of *env* gene sequences from 54 persons were identified to be 7% in CRF02_AG (Lihana *et al.,* 2009) suggestive of an increase in CRF02_AG.

Based on the integrase gene, a total of eight strains comprising 12.2% of the circulating recombinant forms and unique recombinant forms as follows; 2% for each of the following; AICD, A2D and BG, 4% for CRF02_AG and 6% URF, were evidenced. These results show

that CRF02_AG and URFs are the most prevalent in this cohort. A unique recombinant form (URF) is one that has been identified in one to a few patients in an isolated geographic region. When a unique recombinant form becomes more established in a population, it becomes known as an inter-subtype recombinant (ISR). If an inter-subtype recombinant is transmitted from one patient to others and becomes one of the circulating forms of the epidemic in different geographic regions, it becomes known as a Circulating Recombinant Form (CRF) that are numbered rather than lettered in order of their identification.

Phylogenetic analysis, showed existence of diverse circulating recombinants. The presence of recombinant strains identified in this study indicates circulation of parental strains, especially those containing subtype A, which have been known to exist in Kenya for a long time (Lihana *et al.,* 2006). The continuous emergence and increase of CRF02_AG and A2D are indicators of an evolving epidemic among the study population in this region. This has implications for laboratory diagnosis, treatment and considerable multiple obstacles for the development for HIV-1 vaccines (Delwart *et al*., 1998). CRF02_AG has been found to be one of the most prevalent HIV-1 lineages worldwide (Konings *et al.,* 2006) with interesting features of its high transmission and replication fitness than parenteral strains (Carr *et al.,* 1999; Konings *et al.,* 2006). CRF02_AG, recombinant of HIV-1 subtype A and HIV-1 subtype G lineage, is most predominant in West Africa (Carr *et al.,* 1999; Konings *et al.,* 2006). With continued introduction of new HIV-1 subtypes into Nairobi and Kenya as a whole, end up mixing with existing viral strains leading to development of diverse circulating recombinant forms (Takebe *et al.*, 2003)

Nairobi being a diverse population that is on high migration movements and foreign visitors has led to acquisition of diverse subtypes hence a high viral recombination. Unique recombinant forms (URF), exist in the regions where several subtypes co-exist (Butler *et al.*, 2007).The current study confirms previous studies on continued increase in CRF02_AG (Yang *et al.*, 2004; Lihana *et al.*, 2006; 2009). Since CRF02-AG has become predominant in major cities in Cameroon and Thailand over time (Nkangasong *et al.*, 1994; Morison *et al.*, 2001; Songok *et al.*, 2003; Lihana *et al.*, 2009) it will be important to determine in a broader spectrum (prospective study) which recombinants will become predominant infecting strains in Nairobi (Lihana *et al.*, 2009).

Unique recombinant forms were detected with 28% prevalence based on intergrase gene and 7% on *env* gene. These results concurred with previous studies on levels of HIV-1 recombination based on single genome analysis that have recorded up to a maximum up 25% (Neilson *et al.* 1999; Songok *et al.*, 2003; Yang *et al.*, 2004; Lihana *et al.*, 2006). Presently, the AID recombinant that is predominant in Kenya, but had low prevalence compared to CRF02_AG. This data suggests there is possible increasing diverse recombinants' originating from the already existing pure subtypes circulating in Kenya. To date, all Kenyan epidemiologic surveys have found that more than half of the HIV-1 strains are clade A1, with a minority of clade D and clade C, and only an occasionally identified clade G (Songok *et al.*, 2004). HIV-1 intersubtype recombinants are diverse and on the rise (Tee *et al.*, 2005), a trend likey to end up with population being infected with circulating recombinant forms as evidenced in Thailand (Tee *et al.*, 2005).

5.3 HIV-1 co-receptor tropism

HIV-1 is one of the fastest evolving pathogens, and is distinguished by geographic and genetic variants that have been classified into different subtypes and circulating recombinant forms (CRFs) (Esbjörnsson *et al.,* 2010). In early stages of infection, the primary coreceptor is CCR5, but as the infection progresses, the virus switches to CXCR4 and as the diseases progresses towards AIDS it ends up with X4 viral stain HIV-1 populations (Scarlatti *et al.,* 1997; Lihana *et al.,* 2009). This has been correlated with accelerated disease progression (Lihana *et al.,* 2009; Esbjörnsson *et al.,* 2010; Neogi *et al.,* 2010).

The viral envelope glycoprotein *gp*120 is organized in five hypervariable regions (V1-V5), interspersed within five conserved regions (C1-C5). The major viral determinants of the interaction between *gp*120 and the co-receptors CCR5 or CXCR4 are located in the V3 region, even though other regions, such as the V1/V2 and the C4 regions have been shown to influence coreceptor use (Pastore *et al.,* 2006).

HIV-1 co-receptor use in this study was predicted based on the amino acid composition of the V3 loop. Though this genotypic assay is less sensitive and needs validation, it is relatively fast and less expensive especially in an African and resource-poor setting. A more sensitive and reliable phenotyping assay would be ideal to elucidate HIV-1 coreceptor usage (Lihana *et al.,* 2009). Basic knowledge of HIV-1 coreceptor evolution has become increasingly important due to the recent introduction of CCR5 antagonists as part of antiretroviral therapy against HIV-1 (Sax, 2007).

In this study, the data showed that majority of the study cohort had R5-using viruses (69.6%), variants that are most found to be predominant on early stages of HIV infection in non B

HIV-1 subtypes, suggesting that in this population, most of the infection is in early stages, less than 5 years (Koot *et al.,* 1993). The finding that most of the viruses are R5 viruses suggests that CCR5 antagonist Maraviroc that targets viral infection using CCR5 chemokines co-receptor could be used in management of these patients. In addition, CCR5 inhibitors may thus be used before salvage therapy and before significant CD4 + T cell depletion (Hunt *et al.,* 2006). In addition, it often found that the R5 strains were detected in HIV-1 patients that had an average of 435 CD4 count and most of them (67%) being drug naive. These results were consistent with previous studies on frequency of R5 strains on predictive use of new class of fusions inhibitors (Hunt *et al.,* 2006; Lihana *et al.,* 2009; 73.7% in Kenya (Lihana *et al.,* 2009; 76.9% in, China (Zhang *et al.,* 2009) while 96 % in Indian (Neogi *et al.,* 2010). However, even in patients with a dominant non-X4 virus, minorities of X4 variants exist (Hunt *et al.,* 2006).

In this study, X4 variants were also detected accounting for 30.4% of the viral sequences analysed. Persons with these type of viruses had an average CD4 count of 258, suggesting that a majority of these persons are in the AIDS stage of infection. Less than half of the study subjects were at the late stage of their disease, where previous studies have confirmed R5X4 or X4 populations emerge (Koot *et al.,* 1993). It could be inferred that these patients harbored viral strains already predicted to be resistant to new class of fusion inhibitors that is CCR5 antagonist Maraviroc or Vicriviroc.

The X4 variants populations in the current study had prevalence of 30.4% that was found to be consisted with previous studies where it X4 populations are thought to appear in approximately 50% of the patients infected with HIV-1 subtype B (Peeters *et al.,* 1999;

Lihana *et al.,* 2009; Zhang *et al.,* 2009; Neogi *et al.,* 2010). These results on X4 strains suggested that these strains may be resistant to CCR5 antagonist (Connell *et al.,* 2008)

The coreceptor switch from R5 to X4 is closely associated with the progression to AIDS (Zhang *et al.,* 2009). Since CCR5 antagonist drugs have no effect on X4 populations HIV-1 coreceptor tropism must be identified before the initiation of treatment (Westby *et al.,* 2007). With 30.4% of study subjects harbouring X4 variants, being detected, it is of clinical value (Huang *et al.,* 2005) as well as 69.6% being predicted susceptible for Maraviroc or Vicriviroc use. The implementation of antiretroviral therapy (ART) in resource-limited settings requires use of standard first- and second-line therapies (Neogi *et al.,* 2010). CCR5 receptor antagonists such as Maraviroc, is a potential future option for second-line therapy in populations where R5-tropic strains predominate (Castonguay *et al.,* 2003; Soriano *et al.,* 2009).

5.4 HIV-1 anti-retroviral drug resistance-associated mutations

The addition of integrase inhibitors and fusion inhibitors to HAART is a considerable advance in the management of patients infected with HIV-1 (Low *et al.,* 2009). Sequence analysis of the integrase, *gp*41 and C2V3 coding regions in viruses from 188 cohort 100 drug naive and 88 experienced subjects were done (Low *et al.,* 2009). The results showed 2.4% of the studied subjects had mutations that confer reduced susceptibility to intergrase inhibitor Raltegravir in Nairobi cohort similar to those seen in other studies conducted in UK and Germany in 2009 among drug naive (Sichtig *et al.,* 2009; Garrido *et al.,* 2009). However, it should be noted that Raltegravir is currently not in use in Kenya.

Previous studies among drug naive populations of Kenya showed drug resistance levels to be at 7.5% (Lihana *et al.,* 2009), similar to levels detected in Cameroon and Mozambique in

2004, which showed 9.8% and 5.9% respectively (Koizum *et al.,* 2006; Bartolo *et al.,* 2009). However, other studies in Africa have shown HIV-1 drug resistance levels in drug naïve populations to be at less than 5% (Ndembi *et al.,* 2008; Somi *et al.,* 2008; Kamato *et al.,* 2008).

5.4.1 Intergrase inhibitors; Raltegravir

In reference to intergrase inhibitors, 2.4% of the studied subjects were found to harbour strains with reduced susceptibility to Raltegravir. This indicates no exception to drug resistance, limitations to the new class of ARVs. A T97A mutation that is associated with reduced susceptibility to intergrase inhibitors, Raltegravir was detected. This results suggest that there exits intergrase drug resistance but at low thresholds but the levels could be high or yet to be with intergrase exposures. Though reported low thresholds (2.4%) drug resistance, the problem could be more serious than reported here especially with continued scaling up of intergrase together with rest of the HAART in Kenya. Studies have shown that in patients failing Raltegravir, the virus develops primary signature mutations against Raltegravir on (Y143R, Q148H/K/R, N155H) or elvitegravir (T66I, E92Q, S147G, Q148H/K/R, N155H) as primary mutations, as well as on (H51Y, T66A/K, E138K, G140S/A/C, Y143C/H, K160N, R166S, E170A, S230R, D232N, R263K) as secondary mutations.

In addition this study, no primary mutations were detected towards the drug. These findings concur with previous studies conducted in US (Low *et al.,* 2009). Nevertheless secondary mutations associated with resistance to RAL, EGV and/or other inhibitors V201 and I203L, were detected in more than 4.8% of samples. In addition, 26.2% of other mutations were detected, that are not associated with any drug resistance (**Table 4.2**). The mutations detected

on intergrase gene, indicated possible evolution due to immune pressure or other class of antiretroviral drugs adaptations that could favour drug resistance.

Despite substantial numbers of polymorphisms in the integrase coding regions, similar to that seen in other analyses (Malet *et al.,* 2008; Myers and Pillay, 2008; Low *et al.,* 2009), no amino acid substitutions associated with high level resistance to the clinically relevant inhibitors, raltegravir and elvitegravir was discovered (Low *et al.,* 2009). The mutations suggest that there is already an evolution of the viruses in circulation against some drugs, though this could be occurring as a chance event and hence the low levels of resistance evidenced. These mutations detected on intergrase gene were not associated with any drug resistance. However, it was also shown that 26.2% who had been detected with other mutations had also the following polymorphism under development; Y143-, Q148-, N155-, T66-, E92-, S147-, Q148-, N155-, H51-, T66-, E138-, G140-, Y143-, K160-, R166-, E170-, S230-, D232- and R263- mutations. This indicated that with possible actual pressure with these drugs the virus could develop resistance since 26.2% of the viral strains have already developed mutations on the same known positions linked to drug resistance though the actual amino acids substitutions has not taken place (Greenberg *et al.,* 2002; Rodes *et al.,* 2002).

These mutations occurred as natural polymorphism due to immune pressure and other ARVs targeting RT region. Q148H and G140S mutations known to establish a high resistance to Raltegravir was not detected among the study subjects. These findings concurred with those from other regions of the world where intergrase inhibitors have not been introduced or have low use (Myers and Pillay., 2008; Eshleman *et al.,* 2009; Low *et al.,* 2009; Garrido *et al.,* 2010; Silberstein *et al.,* 2010). From a therapeutic perspective, viral entry is one of the most

attractive points for intervention in the viral life cycle, since drug activity is independent of intracellular access (Greenberg *et al.,* 2004).

5.4.2 Fusion inhibitor, Enfuvirtide (T 20)

Development of Enfuvirtide (T20) and other agents that target viral fusion or entry is a welcome despite for the growing population of HIV-infected patients with virus that is resistant to some or all of the other available drug classes and could herald the advent of an entirely new therapeutic approach (Poveda *et al.,* 2002). In this study, the HIV-1 *gp*41 region that determines resistance to the drug Enfuvirtide at codon T-1249 was analysed. It was found that, 4.2% of the study subjects were infected with viral strains that had mutations associated with intermediate drug resistance to Enfuvirtide (T 20) while 22.9 % had mutations associated with Enfuvirtide resistance. However, 10.4 % of the study subjects had other mutations that have not been associated with any drug resistance. It indicated the possibility of development towards drug resistance through viral evolution within the host. These findings concur with Zollner *et al.* (2001) and Lataillade *et al.* (2006), which showed that no primary genotypic resistance to Enfuvirtide among Enfuvirtide-naive patients (Zollner *et al.,* 2001). This could hold true since the drug has not yet been introduced to Kenya populations. However, there was a wide range of susceptibility to Enfuvirtide among primary isolates derived from these patients but this does not appear to have any clinical relevance (Eckert, *et al.,* 1999). Nevertheless, in 33.3 % of the study subjects who harboured viral strains that had mutations, 19.8 % of them had N42S mutations. Mutations at N42S in combination with other mutations often lead to drug resistance to Enfuvirtide (T20).

In addition, 4.2 % were N42D mutations that cause intermediate drug resistance to Enfuvirtide. This occurrence of drug resistance even before the introduction of Enfuvirtide and other fusion inhibitors in Kenya population poses a challenge in management of HIV patients. Other drug resistance associated mutations included; S138T/L with 3.1%, and L44M 2.1% and 1% in the following mutations; N43I and L45V drug resistance mutations (**Table 4.2**). However, other nmutations that are not yet associated with any drug resistance were detected (**Appendix7**), suggesting that these mutations occurrence might be due to natural polymorphism (Isarangkura-na-ayuthaya *et al.,* 2010). According to these mutations, not much has been confirmed in relations to drug resistance to Enfuvirtide.

In this study, most mutations occurred on outlier positions, an occurrence that could be probably due to immune pressure towards development of mutations associated with Enfuvirtide resistance (Greenberg *et al.,* 2004). This study, showed that, with continued selective drug pressure to the virus, the high replication rate of HIV and the low fidelity of the HIV reverse transcriptase enzyme could lead to the development of resistance to ARVs which includes the occurrence of resistance to Enfuvirtide (Poveda *et al.,* 2002).

5.4.3 CCR5 anatagonists; Maraviroc and Vicriviroc

Maraviroc and Vicriviroc, the new CCR5 antagonists even before being introduced in most developing countries, Kenya included, different mutations associated with drug resistance have already been detected. The switch from CCR5 to CXCR4 receptor often indicates CCR5 antagonist Maraviroc or Vicriviroc will not be effective to these classes of drugs. CCR5 antagonist work by inhibiting HIV-1 binding to the CCR5 seven –transmembrane G protein- coupled receptor. Maraviroc binds to a pocket formed by the transmembrane helices,

while the HIV-1 gp120 interacts with the N-terminus and the second extracellular loop of CCR5. HIV-1 may escape from Maraviroc treatment by utilizing CXCR4 (Westby *et al.,* 2007) or acquiring mutations in the env gene that interferes with CCR5 binding (Westby *et al.,* 2007). With confirmed studies showing that, up to 50% of the HIV-1 subtypes B often switch from R5 to X4 co-receptor usage in the course of disease progression, will limit treatment with CCR5 antagonist. In this study, 30.4 % of the study subjects were infected with R5 variants hence not eligible for treatment with CCR5 antagonist. Co-receptor usage varies across the subtypes with others like HIV-1 subtype C mainly use CCR5 with very few CXCR4 or duo tropic viruses (Colliers *et al.,* 2003). Therefore, the use of these compounds could significantly improve the therapy outcome of individuals infected with subtype C viruses if no drug resistance associated mutations (DRAMs) are present (Gonzalez *et al.,* 2010). However, the susceptibility and the development of resistance to these inhibitors by non B subtype viruses are not known (Tsibris *et al.,* 2008).

For Maraviroc, the mutations A316T and I323V have been reported to confer partial resistance for subtype B viruses. Complete resistance to this entry inhibitor occurs when both mutations (A316T/I323V) are present (Westby *et al.,* 2007b). Surprisingly, some of these mutations were already present in *env* sequences from our Nairobi cohort 2.6% had both mutations A316T/I323V. However, in single point mutations 63.3% had A316T and 1.1% I323V **(Table 4.4**). The mutation A316T was prevalent in 63.3% of sequences and it concurs with previous studies in Zambia (Gonzales *et al.,* 2010). The high prevalence of this mutation suggests that A316T is a natural polymorphism. However 1.1% prevalence I323V observed that is also observed in combination with A316T mutations, this indicates that these individuals also harbour viruses that are resistance to Maraviroc. To analyze the presence of

resistance against Vicriviroc, mutations recently reported from an in vitro study and from a clinical trial were included in the analysis (Tabris *et al.,* 2008; Ogert *et al.,* 2001) with the positions of each of these mutant amino acids based on the HXB2 reference strain (Gonzalez *et al.,* 2010).

Mutations associated resistance to Vicriviroc was also evaluated even though this drug is still under clinical trial. The prevalence rate of 62.8% T320R was detected equally high compared to A316T that was got in occurrence as natural polymorphism in Maraviroc. Other mutations detected that are associated with Vicriviroc resistance included; 35.1% R315Q, 10% K305R, 3.2% G321E and 4.5% K305R+ R315Q combination. The high prevalence in T320R indicates a possible natural polymorphism. The different combinations of mutations associated with resistance to both Maraviroc and Vicriviroc were also analyzed. The different combinations of mutations associated with resistance to both Maraviroc and Vicriviroc were also analyzed. The four combinations observed were A316T (Maraviroc) + K305/R315Q (Vicriviroc), A316T (Maraviroc) + R315Q (Vicriviroc), A316T/I323V (Maraviroc) + R315Q (Vicriviroc), and A316T (Maraviroc) + R315Q/G321E (Vicriviroc). Both the Maraviroc mutation A316T and the Vicriviroc mutation R315Q were present in each of these combinations and the prevalence of the A316T+R315Q combination was 12.7%. On the other hand, the other three combinations were observed at a prevalence range of 0–3.2%.

This study concurs with other similar studies in Zambia among drug naïve (Gonzales *et al.,* 2010) and in vitriol studies done in the United Kingdom (UK) (Westby *et al.,* 2007). Most of HIV-1 strains *env* harbour polymorphism at the cost of viral adaptations against entry inhibitors. These findings are significant because the high prevalence of these mutations in

the population may affect the efficiency of CCR5 entry inhibitors for the treatment of Kenyan patients infected with HIV-1.

5.5 HIV evolutionary rate

Evolution of the C2V3 region within a cohort was conducted and three sequences obtained from each patient in one and half years of follow up analysed. In this study, 12.5% of the study subjects had dN/dS ratio less than one suggestive of purifying selection while 87.5%, dN/dS ratio more than one suggestive of positive selection. No neutrality selection was detected from this study (**Table 4.5**). *d*s and *d*n mutations ratios greater than one implied that there is positive selection for change in the C2 to V3 regions. In addition, majority of the studied subjects had viral strains that were adapting from immune pressure resulting to a virus developing mutations. However, in this study, 12.5% of the study subjects had dN/dS ratios less than one a factor that was suggestive of negative selection.

The negative selection indicated that, the viral strains were under immune pressure with poor adaptation mechanisms. In addition, this study confirms previous reports that have shown positive selection predominating across the study subjects suggestive of genes striving to be conserved so that the proteins they code for maintain their structure and function (Buckee *et al.*, 2004; Goodreau *et al.*, 2006; Gordo *et al.*, 2007;). However, in positive (diversifying) selection, this implied that the observed mutations occurred to change of an HIV-1 protein to ensure survival of the virus. Most of the substitutions here were also deleterious. However, some would result in an advantageous change in the protein and would, therefore, be fixed in the viral population. This is often reflected in the higher frequency of non-synonymous substitutions, and consequently, higher genetic variability. This was confirmed in our study. Certain regions of HIV-1 *env* gene, such as the V3 region that was used in this study, are

highly variable and under strong positive selection pressure, since they are targeted by immune system and in need of constant change (Wolfs *et al.,* 1990; Yamaguchi *et al.,* 1997). HIV-1 *env* gene is usually targeted by immune cells and tends to adapt the prevailing immune pressure with development of favourable mutations (Levy *et al.,* 1993; Lemey *et al.,* 2007). Just like the *pol* gene that ends up with development drug resistance, the *env* gene evades the immune recognition; viral tropisms switch from R5 to X4; as well as development drug resistance to new class of fusion inhibitors (Gonzales *et al.,* 2010). In this study, a switch from R5 to x4 occurred in 95% with only 5% still using R5 coreceptor on infection. These results implied that the HIV-1 was at high evolving rate. Similar studies, have studied with confirmed high rates of positive selections indicative of HIV-1 adapting to immune pressure and no low or less change on the viral gene (Ganeshan *et al.,* 1997; Zhang *et al.,* 2005; Lemey *et al.,* 2007). These results suggest that HIV-1 within host tries to adapt to immune pressure and drug pressures for those under ARVs by development of mutations. Theses mutations have implications to either development of drug resistance or reduced replication fitness of the virus.

5.6. GENERAL CONCLUSION AND RECOMMENDATION

- This study has shown that HIV-1 subtypes in Nairobi province and its surrounding regions of Ngong, Kamiti and Kitengela of Kenya were diverse with HIV-1 subtype A1 as predominantly subtype. In addition, there was higher proportions of HIV-1 subtype C that surpassed HIV-1 subtype D in prevalence.
- With continued people migrations and increasing Nairobi populations, there is need for a constant monitoring of HIV-1 subtypes.

- Intersubtype HIV-1 recombinations exist in Nairobi with CRF02-AG as the most predominant. CRFs and theres seems to be rise in intersubtype recombination in Nairobi.
- Information on up to date 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.
- Results from this study showed that most of studied subjects were infected with CCR5 tropic strains that are targeted by Maraviroc.
- The CCR5 antgonists could be used in Kenya population and therefore determination of HIV-1 co-receptor usage prior to management of patients using entry inhibitors is very important.
- This study shows that drug resistance among drug naïve is still at low thresholds; 2.4% against intergrase inhibitors, 4.2% to Fusion inhibitors Enfuvirtide, 4.5% Vivicrovir and 1.6% maraviroc and Vivicrovir combinations.
- This implied that these drugs are effective according to WHO guidelines on drug resistance.
- Results from this study, recommends Enfuvirtide , use for salvage therapy in individuals with advancing immunosuppression who are considered unlikely to achieve sustained virus suppression with PIs and NRTIs alone while Maraviroc, Vivriviroc, Raltegravir and Elvitegravir could be used as thirdline drugs in patients failuring second line drugs.
- This study recommends constant monitoring of drug resistance levels among the population and implementation of early warining indicators in HIV drug resistance

development in Kenya population according to WHO 2011 guideline in preveting increase in drug resistance in poor resource settings like Kenya.

- The virus under study among the followed study subjects were under positive selection due to their adaptation mechanisms of development of mutations due to immune pressure that resulted into drug resistance, co-receptor switch from R5 to X4 and immune escape variants.
- Therefore, the adaptive mechanism of the virus has therefore been shown to have high implications on disease progression and antiretroviral management.

5.7 Further studies

This study was limited in analysis of many samples due to financial constrain. Further analysis on large numbers from more parts of the country should be carried out. The low sample size used in the study out of the large infected population is just a baseline study hence is not a very good representative. This study was able to show drug resistance levels using population DNA sequencing, however, there is need to study minor population viral quasi-species by pyrosequencing sequencing to asses drug resistance that could not be captured.

The short sequences generated from portions of the *pol* and *env* genes of the HIV-1 genome to make conclusions on recombinations, require a to get samples that showed aspects of recombinations and perform full-length sequencing to determine the exact recombination profiles of these sequences.

5.8 Application of generated results

The results from drug resistance level, gives a guide in the way forward on the implementing of the WHO 2011 antiretroviral treatment guideline on possible alternatives for management of patients failing second and thirdline drugs and status on effectiveness on the first-line drugs being implemented. It also guides the status on our first line drugs drugs based ton the drug resistance levels amng drug naive. In addition, feasibility study on yet drugs to be introduced in the Kenyan population. Information of CCR5 antaggonists and fusion inhibitors could be used in designing microbicides agent.

The current updates on circulating HIV-1 subtypes and CRFs enables the monitoring of HIV epidemics, behavioural changes towards HIV transmission among the population, and way forward on prevention strategoies.

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APPENDICES

Appendix 1

ETHICAL CONSIDERATIONS

Evaluation of HIV evolution and its role on development of antiretroviral drug resistance in a Cohort study

Purpose of the study

This is a research study designed to establish the HIV-1 evolution and its role on development of antiretroviral drug resistance in a Cohort study

Sample collection

a) Plasma to be used will be primarily acquired under study protocol SCC No. 1394 from consenting participants.

b) Full informed consent will be sought from participants

Benefits

The study subjects will not receive direct benefit from participating from in the study but viral susceptibility status results will be released to them. However, 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, the study subject may feel discomfort from blood draw and sometimes bruises forms, which go away in about 3-5 days.

INFORM CONSENT FORM.

Study title

Evaluation of HIV evolution and its role on development of antiretroviral drug resistance in a Cohort study

Purpose of the study

This is a research study designed to establish the HIV-1 evolution and its role on development of antiretroviral drug resistance in a Cohort study

Inclusion criteria

Women patients attending comprehensive HIV clinics and will be considered eligible. Those who will give informed consent will be recruited to participate in the study for a period of 3 years.

Exclusion criteria

Women patients attending comprehensive HIV clinics and not will to participate in the study for a period of 3 years will not considered eligible. Those who will not give informed consent will not be recruited to participate in the study

Benefits

You will not receive direct benefit from participating from in the study but viral susceptibility results will be released to you. However, information gained from the study will be used to develop more effective management services.

Subject's right to confidentiality

The results of study may be published, released to a funding agency or presented in a scholarly fashion. The confidentiality of the study subjects will be protected and they will not be identified in ay away.

Risk to subject

There are no known major physical, psychological or social associated with participating in the study. However, the study subject may feel discomfort from blood draw and sometimes bruises forms, which go away in about 3-5 days.

Subject right to refuse to participate or withdraw

The study subject may refuse to participate or withdraw room the study at any time.

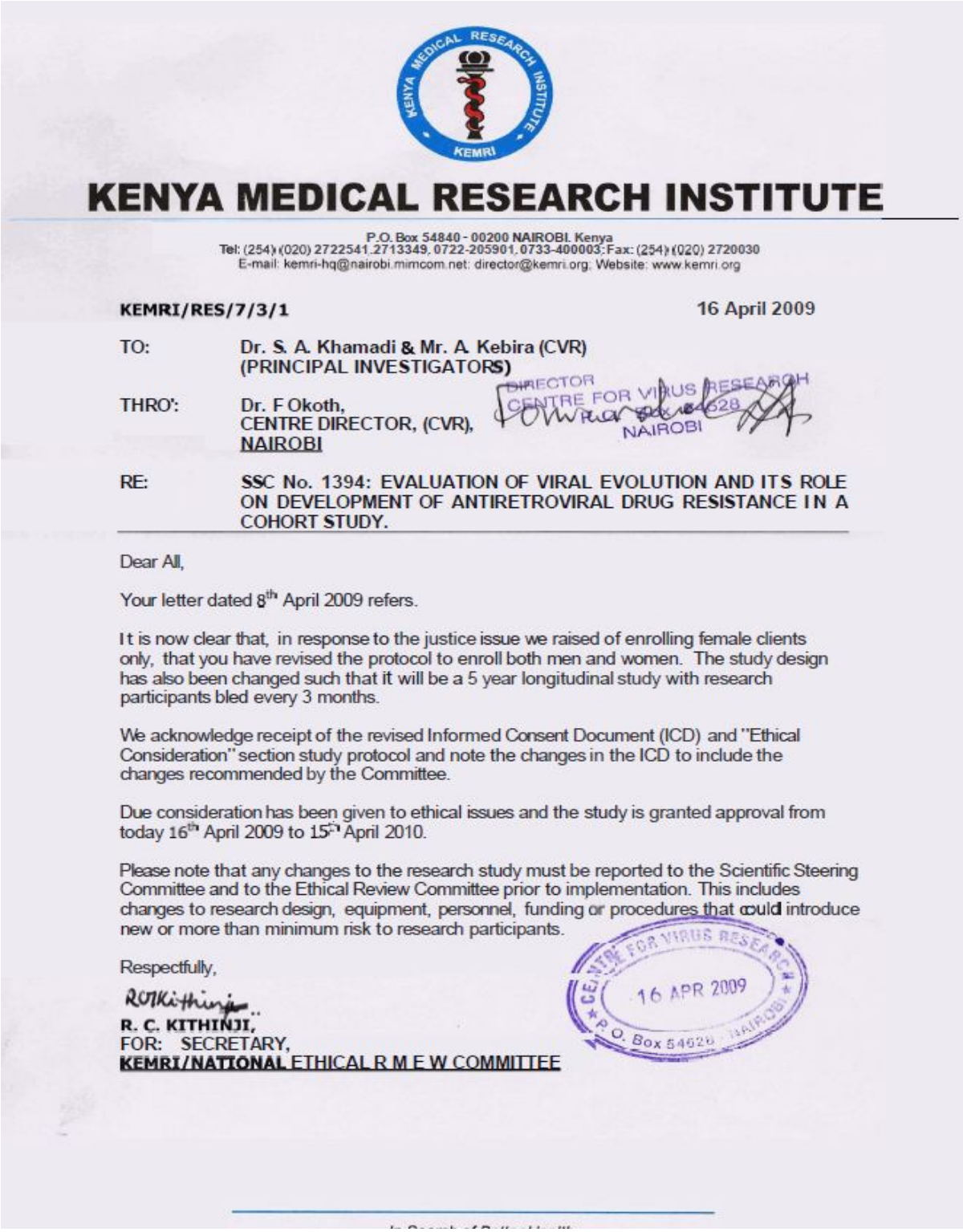
Signatures

The study has been discussed with me and all my questions have been answered. I understand that additional questions regarding the study should be directed to the investigators. I agree with terms above and acknowledge that I have accepted voluntarily.

……	**……**
Signature of participant	**date**
……..	**…….**
Signatures of witness	**date**
……..	**……**
Signature of investigator	**date**

Appendix 2

Ethical approval letter



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KEMRI/RES/7/3/1 16 April 2009

TO: **Dr. S. A. Khamadi & Mr. A. Kebira (CVR)**
(PRINCIPAL INVESTIGATORS)

THRO': Dr. F Okoth,
CENTRE DIRECTOR, (CVR),
NAIROBI

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

RE: **SSC No. 1394: EVALUATION OF VIRAL EVOLUTION AND ITS ROLE ON DEVELOPMENT OF ANTIRETROVIRAL DRUG RESISTANCE IN A COHORT STUDY.**

Dear All,

Your letter dated 8th April 2009 refers.

It is now clear that, in response to the justice issue we raised of enrolling female clients only, that you have revised the protocol to enroll both men and women. The study design has also been changed such that it will be a 5 year longitudinal study with research participants bled every 3 months.

We acknowledge receipt of the revised Informed Consent Document (ICD) and "Ethical Consideration" section study protocol and note the changes in the ICD to include the changes recommended by the Committee.

Due consideration has been given to ethical issues and the study is granted approval from today 16th April 2009 to 15th April 2010.

Please note that any changes to the research study must be reported to the Scientific Steering Committee and to the Ethical Review Committee prior to implementation. This includes changes to research design, equipment, personnel, funding or procedures that could introduce new or more than minimum risk to research participants.

CENTRE FOR VIRUS RESEARCH
16 APR 2009
P.O. Box 54628 NAIROBI

Respectfully,

R.C. Kithinji

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICAL R M E W COMMITTEE

Appendix3

Sample size Determination

A simple random sampling technique was used in prospective cohort study design. This was applied in general population regardless of age, sex or race. Using a HIV-1 prevalence of 10% in Kenya (Khamadi *et al.*, 2005), and the sample size was derived at using the formualr below by (Fischer *et al.*, 1922; Zodpey *et al.*, 2004) as shown below:

$$N = \frac{Z^2\ 1\text{-}ap\ (1\text{-}P)}{d^2}$$

Where:

N= minimum sample size required

Z =1.96 (STD)

A= level of significant at 5%

P =0.10 (7.4%) HIV-1 infection prevalence rate by 2008

D =0.05 (5% absolute precision)

Therefore:

$$N=\frac{1.96^2(1-(0.05x0.1)\ (1-0.1)}{0.05^2} = \textbf{138 samples}$$

Therefore, the working samples size was **138,** However, the used sample size of 188 was used to exclude occlusion errors.

Appendix 4

Primer sequences

HIV-1 Region	***Annealing temp °C***	***Primer IDs***	**5' - 3' primer**	**Region of amplificatio**
env gp 41	*45*	*gp* 40 F1	TCTTAGGAGCAGCAGGAAGCACTATGGG	7789–7816
		gp 41 R1	AACGACAAAGGTGAGTATCCCTGCCTAA	8347–8374
		gp 46 F2	ACAATTATTGTCTGGTATAGTGCAACAGCA	7850–7879
		gp 47 R2	TTAAACCTATCAAGCCTCCTACTATCATTA	8281–8310
Pol	60	Unipol 5F	TGGGTACCAGCACACAAAGGAATAGGAGGAA	3434–3765
		Unipol 6R	ACCACAGCTGATCTCTGCCTCTCTGTAATAGA	4483–4516
		Unipol 1F	CCAGTGGATTCATAGAAGCAGAAGT	4052–4074
		Unipol 2R	CCCCTATTCCTTCCCCTTCTTTTAAAA	4363–4388
C2V3	55	M5 F1	CCAATTCCCATACATTATTGTGCCCCAGCTGG	6451-6482
		M10 R 1	CCAATTGTCCCTCATATCTCCTCCTCCAGG	7225-7254
		M3 F2	GTCAGCACAGTACAATGACACATGG	6541-6566
		M8 R2	TCCTTCCATGGGAGGGGCATACATTGC	7114-7140

Appendix 5

General generated sequences

The generated sequences were deposited in the genebank with the following accession numbers.

env C2V3 sequences

integrase sequences

The *pol intergrase* sequences were deposited at the Genebank under accession numbers HQ177046-HQ177063, HQ177068-69, HQ177071- HQ177088, HQ177090 and HQ177093

Appendix 6

A representative samples of the HIV-1 coreceptor prediction among cohort isolates

ID	Pred	Geno	WebPSSM Tropism	ds Kernel	Geno2pheno
NAR 029	1	GK	X4	R5	X4
NAR 019	0	SD	R5	R5	R5
NAR 001	0	S-	R5	R5	R5
NAR 028	0	SE	R5	R5	R5
NAR 003	0	SD	R5	R5	R5
NAR 021	1	GR	X4	R5&X4	R5
NAR 014	1	ID	X4	R5	R5
NAR 016	1	ID	R5	R5	R5
NAR 017	1	GK	X4	X4	R5
NAR 026	0	GE	R5	R5	R5
NAR 009	0	SD	R5	X4&R5	R5
NAR 010	0	SE	R5	R5	R5
NAR 012	0	DT	R5	X4	R5
NAR 024	0	GN	R5	R5	R5
NAR 030	1	RS	X4	R5	R5
NAR 002	0	SD	R5	X4	R5
NAR 014	0	GS	R5	R5	R5
NAR 016	1	RA	X4	R5	R5
NAR 033	1	RA	X4	R5	R5
NAR 007	1	RA	X4	R5	R5
NAR 032	1	RA	X4	R5	R5
NAR 010	0	SQ	R5	R5	R5
NAR 020	0	SD	R5	R5	R5
NAR 011	0	SE	R5	X4&R5	X4
NAR 012	0	SG	R5	R5	R5
NNK 009	0	SD	R5	R5	R5
NNK 013	0	SD	R5	R5	R5
KAS 007	0	SA	R5	R5	R5
KAS 017	0	S-	R5	R5	R5
KAS 016	1	RV	X4	X4	R5
KAS 014	0	SD	R5	R5	R5
KAS 022	0	SD	R5	R5	R5
KAS 023	1	GK	X4	X4&R5	X4
KAS 015	1	GK	X4	R5	R5
KIT 008	0	SD	R5	X4&R5	X4
KIT 006	0	SS	X4	R5	R5
KIT 010	0	SE	R5	R5	R5
KMT 025	0	SD	R5	R5	R5
KMT 024	0	SD	R5	R5	R5
KMT 008	1	SG	X4	X4&R5	X4
KAS 003	0	SD	R5	R5	R5
KMT 023	0	SN	R5	R5	R5

Key: In C-PSSM a 1: CXCR4 use predicted; 0: CCR5 use only predicted

Appendix 7

Prevalence of other *env gp*41 mutations

***gp* 41 other mutations**	**Prevalence**
K154Q, L130A, I135P, I171L, I171N, I171T, I173M , I84L, K144N, V72I, E151T, E143D, F162V, N43I, W167M, L152F, Q141P, W155, W165Q, W117X, W120C, S102T, S157Q, S32A, S129K, S157G, Y170*, S104S_K, S129H, N166E, Q139X, N166X, N160T, N166I, L152S, N105N_N,K77Q, N160E, K77S, K77V, M118F, L108H, L108K, L130F, L130V, L130Y, l135L, L150*, L150I, L152D, L158P, L168M, L33, L34V, L44, L45V, L54V,L57I, L81F,L81R, L91X, E151P, E123A, E123X, E151H,E151K, E136G, E151, E137I, E148A, E137K, E137R, E143X, G61X, G86X,G89X, H53X, L130K, 13X, F162C, D113H, L108M, E121X, D163*, D163G, I164L, I84M, I84V, I92L, K106I, K144I, K144L, K144T, K63X, K77H, D78X, E119K, E119D, D125A, D125E, D125H, Q141L, L108V, A156Q, L108I, L108K, L150, L91V, K172S, Q51H, S107T,A101T,K154S, K172E, K172P, T94A, W161L, W85, W167, W159, W117L, V72M, V72I, V28P, Q142X, T94P, T165X, T165L, T165X, Y170, T165Q, T133E, T128S, T165F, Q29P, S35T, Q56X, Q80R, Q80X, R122Q, R31K, R31K, R74N, R46I, D109K, F162Y, D109H, A108Q, D109F, D10E, D109G, C93F, A156X, A47X and A156T	1%
D163Y, T133D, Q66K, M118Q, S107T, L34M, T133V, L152M, V72L, T133R, T133G, S35A, L151A, L149X, K106R, Q79X, P98R, M118R, N166Q, E137Q, D109Q, E148X, F162S, I124V, A67T, A30S, A156E, A101N, L108Y, K172R, K154E, L108Y, D163Y, T133D	2.1%
N160S, N166K, D113E, D113G, D109S , W167L, N166S, R46K, N140I, Q141X, L34I, I171, H53Q, E137D, D109N, D113N_S, D113E, D113G, D109S, A96S, D125N, T133Q, L108F, L168C, L34F, W169X, D163S and W167L	3%
E119Q, K90R, E110D, D163N, E151Q, E151X, S129D, R74S, I173L, K90R, N140T, Q147K, N166R and W169V	4.2%
S129N, S129Q, S129E, W167C and L130T	5.2%
I135L and I171M	6.3%
W167X, L91H and T133N	7.3%
L130I, L54M, D125S and K77R	8.3%
K77R, L108Q, Q56K and S129G	9.4%
T165S, T133S and F162X	10.4%
D109E	11.5%
Q56R	12.5%
A96T, V69I	13.3%
S157N and E151A	13.5%
E148D	13.6%
M118L	16.7%
A96N,E119Q	17.7%
R122K and E121D	18.8%
A101S	24%