

**MOLECULAR ANALYSIS OF HIV MUTATIONS AND DETERMINATION
OF SUBTYPES CIRCULATING IN GEM, WESTERN KENYA**

BY

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Abstract

As the HIV pandemic becomes increasingly complex and devastating in Africa, there is need to come up with better management strategies in terms of treatment, vaccine and better testing methods. However, this is getting hampered by the high diversity of Human Immunodeficiency Virus type 1 (HIV-1), which is brought about by high rate of replication and mutation. Such mutations may cause antiretroviral drug resistance if they occur in the gene regions coding for molecular drug targets. The high rate of mutation also leads to many viruses which are genetically related but distinguishable variants and subtypes. In this study, molecular analysis of the protease and reverse transcriptase gene sequences of the HIV-1 in plasma samples collected from Gem in western Kenya was done. Sequencing of these genes was done with the aim of identifying mutations and analyzing each for antiretroviral drug resistance. Also, determination of the subtypes based on these sequences was done. A total of 30 samples were taken for sequencing, however, 9 of them had primer failures and therefore did not successfully sequence, leaving only 21 samples for molecular analysis. The results showed several mutations in these gene sequences, and analysis of each of the mutation for drug resistance showed none to be causing resistance to any of the known classes of ARV drugs. For the phylogenetic analysis, 16 (76.2%) of the isolates were found to be subtype A, subtype D were 4 (19.0%), and the remaining 1 (4.8%) was circulating recombinant form, CRF_AD. To verify the results, control sample was analyzed by both TruGene and in-house methods and both gave the same results. Since this study revealed three different HIV-1 subtypes in Gem, it would be necessary to conduct a future study to find out the effect of these subtypes on the transmission, pathogenicity and the rate of HIV-1 disease progression in Gem, western Kenya.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background Information

Human Immunodeficiency Virus is a kind of virus that belongs to a group of viruses called retroviruses. This group of viruses has their genomic material in form of ribonucleic acid (RNA). HIV cannot replicate on its own, but instead needs a host cell to replicate. HIV is transmitted through sexual contact with an infected partner, transfusion of contaminated blood and use of contaminated medical instrument (Buve *et al.*, 2002). Mother to child transmission also occurs during breast feeding (Palasanthira *et al.*, 1993). During infection, the virus first gets into contact with the host cell through the receptors. It then empties its genetic material into the host lymphocyte, eventually taking control of the cell. This process leads to destruction of cells of the immune system eventually causing a condition called acquired immunodeficiency syndrome (AIDS). This condition can be salvaged by antiretroviral therapy; however, this is currently being hampered by viral mutations in the genes that encode the molecular targets of therapy.

HIV-1 has a high rate of mutation in its genome and this is necessitated by lack of 3' exonuclease proofreading activity by reverse transcriptase enzyme during replication. The high mutation rate together with the high replication rate enable the virus to exist in an individual as a complex mixture of genetically related but distinguishable variants referred to as quasispecies, which are distinguishable by single amino acid substitution (Najera *et al.*, 1995). Many of these viral mutations are mainly for improving viral fitness while some confer drug resistance. As recommended by international AIDS Society-USA Panel, drug resistance mutations should be distinguished from those that do not confer resistance for better management of HIV. So, with high HIV prevalence in western

Kenya (Moore and Hoggs, 2004), numerous mutations are expected to occur in the HIV circulating in Gem, western Kenya. Therefore it was important to determine these mutations in general then distinguish those that confer drug resistance from those that do not in this population, as was done in this study.

There are two major HIV viruses that cause AIDS, HIV-1 and HIV-2. HIV-1 is the most diverse and has evolved into three major groups, mainly, group O (Outlier), N (non M) and M (major) (Spiral *et al.*, 2003). M group is the most divergent group and has evolved into nine different subtypes, subtype A, B, C, D, F, G, H, J and K (Thomson *et al.*, 2002). Subtype distribution varies widely across the globe, but the greatest diversity has been reported in sub Saharan Africa, Central Africa having the greatest diversity and virtually all the known subtypes circulate in this region (Thomson *et al.*, 2002). In Eastern Africa, subtype C, A and D dominate, with Uganda having both A and C in high proportions (Butler *et al.*, 2007). Subtype C is dominant in Tanzania as opposed to subtype A which is the dominant subtype in Kenya (Jensens *et al.*, 1994). In western Kenya, subtype A still remains the dominant subtype (Yang *et al.*, 2004). However, studies on the HIV diversity in Kenya has been tilted to either antenatal clinic attendee or commercial sex workers in the urban centres thereby leaving out the general rural population such as Gem.

The HIV-1 subtype diversity has been shown in a study by Van *et al.*, (1997) to have influence on the pathogenicity, transmissibility and the rate of disease progression. However, a number of environmental factors such as viral load at the time of infection, presence of other sexually transmitted diseases, and sexual behavior also influence viral epidemic potential.

HIV genetic diversity also has significant clinical and public health consequences. HIV mutagenic potential and diverse assortment of antigenic epitope has hampered HIV

vaccine development effort. Many vaccine development attempts have concentrated on subtype B which is the dominant in the developed world which fund such vaccine development initiatives, raising the concern whether they will be effective in Africa where none subtype B dominate (Butler *et al.*, 2007).

The high genetic diversity also poses threat to testing and diagnosis of HIV. Most HIV tests detect the HIV antibodies, and originally, these tests relied on subtype B epitopes. For instance, when type O emerged in 1990s, it tested negative with kit manufactured based on subtype B model (Gashen *et al.*, 2002). Therefore, HIV diversity data is a very important tool in HIV and AIDS management and yet there is still relatively little information on the viral diversity in rural western Kenya. So it was necessary to carry out a study to determine the HIV subtypes circulating in Gem, western Kenya. The findings of this study will be useful for better management of HIV/AIDS in Gem western Kenya, and other parts of the country.

1.2 Problem Statement

HIV-1 mutates at a high rate (Coffin, 1995). Some of these mutations just improve the viral fitness while some occur in the gene region encoding the molecular targets of therapy, thereby conferring drug resistance (Yahi *et al.*, 1999). There is no study that has tried to identify these mutations in Gem western Kenya and the role they play, whether they just improve the viral fitness or cause ARV drug resistance in this population, and yet they are known to occur. Also, HIV genetic diversity has significant clinical and public health consequences. HIV-1 mutagenic potential of different types and assortment of different subtypes have hampered vaccine development efforts. Many vaccine attempts have focused on subtype B, with a different mutagenic potential, which is dominant in the developed countries (Butler *et al.*, 2007). Testing and diagnosis have also not been spared

by this phenomenon. Many testing and diagnosis methods are HIV antibody based, and with the diverse subtypes comes different antigenic sites which have the potential of eliciting specific antibodies (Butler *et al.*, 2007). Therefore, without standard test able to detect antibodies against all groups and subtypes, many cases are likely to go undiagnosed.

1.3 Justification

HIV-1 has high rate of mutation and these mutations go on naturally in the virus. Some of these mutations are meant to improve viral fitness while some do confer the virus with drug resistance ability (Yahi *et al.*, 1999). It was therefore important to carry out a study to identify mutations the virus is undergoing in Gem, western Kenya and then analyze them to know if any could be conferring drug resistance to any of the currently known classes of ARV. Also, since, development of standard testing methods and vaccines rely on diversity information, and this data for western Kenya is limited. The little that is documented is for studies by Yang *et al.*, (2003, 2004), conducted in the urban centres and were specific for the antenatal clinic attendees. Therefore this called for the need to conduct this study among the general population of Gem to determine HIV subtypes circulating in this part of western Kenya.

1.4 General Objective of the Study

To determine mutations in the protease and reverse transcriptase genes of HIV and the subtypes circulating in Gem, western Kenya.

1.4.1 Specific Objectives

- a) To identify mutations on the protease and reverse transcriptase genes of HIV in Gem.
- b) To determine mutations causing drug resistance among this population
- c) To identify HIV subtypes circulating in Gem, western Kenya.
- d) To identify HIV recombinant forms circulating in Gem, western Kenya.

1.5 Hypothesis (Alternative)

There are mutations in the HIV subtypes circulating in Gem, western Kenya.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

Human Immunodeficiency virus (HIV) is a kind of virus that belongs to a group of viruses called retroviruses. It is believed that HIV originated in sub Saharan Africa during the twentieth century (Gao *et al.*, 1999), and it is now a pandemic, with an estimated 38.6 million people currently living with the HIV worldwide (UNAIDS, 2006). It has its genome in form of ribonucleic acid (RNA), and inside it are the three enzymes necessary for replication. These are surrounded by a coat of glycoprotein. This virus, like other pathogens needs a host cell to complete its life cycle. It starts its life cycle by first attaching to the host cell, usually the CD-4 T-lymphocyte cell, which is a cell of the immune system. After this attachment, it empties its content into the cell and with the help of its reverse transcriptase, it changes its genome, usually RNA into DNA, and with the help of its integrase enzyme, it intergrates its genome into the host cell DNA. Once intergrated, it is called a provirus.

When the host cell undergoes transcription it produces the viral messenger RNA (mRNA) which eventually get translated to viral protein which then gets chipped into sizes by protease enzymes into new viral particles which eventually burst the host cell. This process leads to killing of many of immune system cells thereby weakening the immune system, leading to a condition referred to as Acquired Immunodeficiency Syndrome (AIDS) (Marx, 1982). Once immune system is weakened, one remains prone to opportunistic infections and tumors. HIV is transmitted through direct contact of a mucous membrane or bloodstream with a bodily fluid containing HIV, such as blood, semen, vaginal fluid, preseminal fluid, and breast milk. This transmission can come in the

form of anal, vaginal or oral sex, blood transfusion, contaminated hypodermic needle, exchange between mother and baby during pregnancy, child birth, breastfeeding, or other exposure to one of the mentioned bodily fluids (CDC, 2003; Buve *et al.*, 2002). Currently, there is no known vaccine or medicine that cures HIV/AIDS, save for the antiretroviral drugs that inhibit action of viral enzymes that are necessary for viral replication. Vaccine development has been hampered by the high rate of viral mutations that occurs naturally, enabling HIV to exist in an individual as a complex mixture of genetically related but distinguishable variants (Najera *et al.*, 1995). If mutations occur in the gene region encoding molecular antiretroviral drug target, then this may cause ARV drug resistance (Yahi *et al.*, 1999).

2.2 HIV Prevalence

In African continent, HIV prevalence varies. For instance, in southern African countries, more than one in every 5 pregnant women are HIV infected; and in a few sub Saharan African countries, HIV prevalence in prenatal clinics in 2003 exceeded 10%, while in urban settings in southern Africa, antenatal sero-prevalence reached 40% (UNAIDS, 2002). In West Africa, HIV prevalence in pregnant women remain generally stable at low levels, though in some urban areas it exceeds 10 percent, while in rural areas the rates are generally low (UNAIDS, 2002).

In East Africa and parts of Central Africa, prevalence has fallen, for instance, in Addis Ababa, among 15-24 years old pregnant women, the prevalence fall to 11% in 2003 from 24% in 1995 (WHO/UNAIDS, 2003). Previous studies reveal that Asia is experiencing rapidly growing epidemics (UNAIDS/WHO, 2002). Likewise, eastern Europe is also experiencing rapid increase in prevalence, which is being attributed to rise in mother to child transmission (UNAIDS/WHO, 2005). The risk of mother to child infection increases if a breast feeding mother is newly infected, owing to the initially high

level of virus (Quinn *et al.*, 2000). It is therefore important that more attention should be directed to HIV/AIDS prevention and treatment.

2.3 HIV Diversity

There are two types of HIV responsible for infection, namely, HIV-1 and HIV-2. A major proportion worldwide is caused by HIV-1 virus which was first identified in 1983 (Gallo *et al.*, 1983). The HIV type 2 was first detected in West Africa and is significantly present in this region since the beginning of AIDS epidemics two decades ago (Larsen *et al.*, 1998). HIV-1 has evolved significantly varying from one geographical region to another (Spiral, 2003). HIV-1 is the most diverse and has evolved into three major groups, mainly, group O (Outlier), N (non M) and M (major) (Spiral *et al.*, 2003). M group is the most divergent group and has evolved into nine different subtypes, subtype A, B, C, D, F, G, H, J and K (Thomson *et al.*, 2002).

All the subtypes originated from central Africa (Thomson *et al.*, 2002). In addition, strains of HIV-1 recombinants forms have been identified to be in circulation (Thomson *et al.*, 2002). Other forms which have been recognized are, A to H with subtypes A and B being the most prevalent (Xiang *et al.*, 1997; Heredia *et al.*, 1998; Sarr *et al.*, 2000). A study conducted in South Africa indicated that the virus diversity influences transmission and pathogenicity, and it associated subtypes B with male homosexual transmission and C with heterosexual transmission (Van *et al.*, 1997). Other studies also associate subtype A and G with longer AIDS free survival period, which is opposed to other non B subtypes (Mahendale *et al.*, 2002).

2.4 Geographical Distribution of HIV Subtypes

All groups of HIV-1 are found in Africa (Figure.1.0). While group M is found all over the continent, group N and O are restricted to Central Africa (Tatt *et al.*, 2003; Simon *et al.* 1998; Vidal *et al.*, 2000; Yamaguchi *et al.*, 2003). Subtypes A, and D are prevalent in East Africa (figure.1.0), subtype A in West Africa, and C in South Africa, while recombinant subtype viruses are found in Central and West Africa (Thomson *et al.*, 2002). In West and Central Europe, America and Australia, subtype B is the most prevalent form (Osmanov *et al.*, 2000). Globally, subtype C is the predominant form causing much of heterosexual infections worldwide (Walter *et al.*, 2009). Unlike HIV-1, HIV-2 does not vary so much in geographical distribution, and almost all types of HIV-2 are found in West Africa and majority of HIV-2 characterized belong to the group A reported in West Africa region (Xiang *et al.*, 1997; Heredia *et al.*, 1998; Sarr *et al.*, 2000).

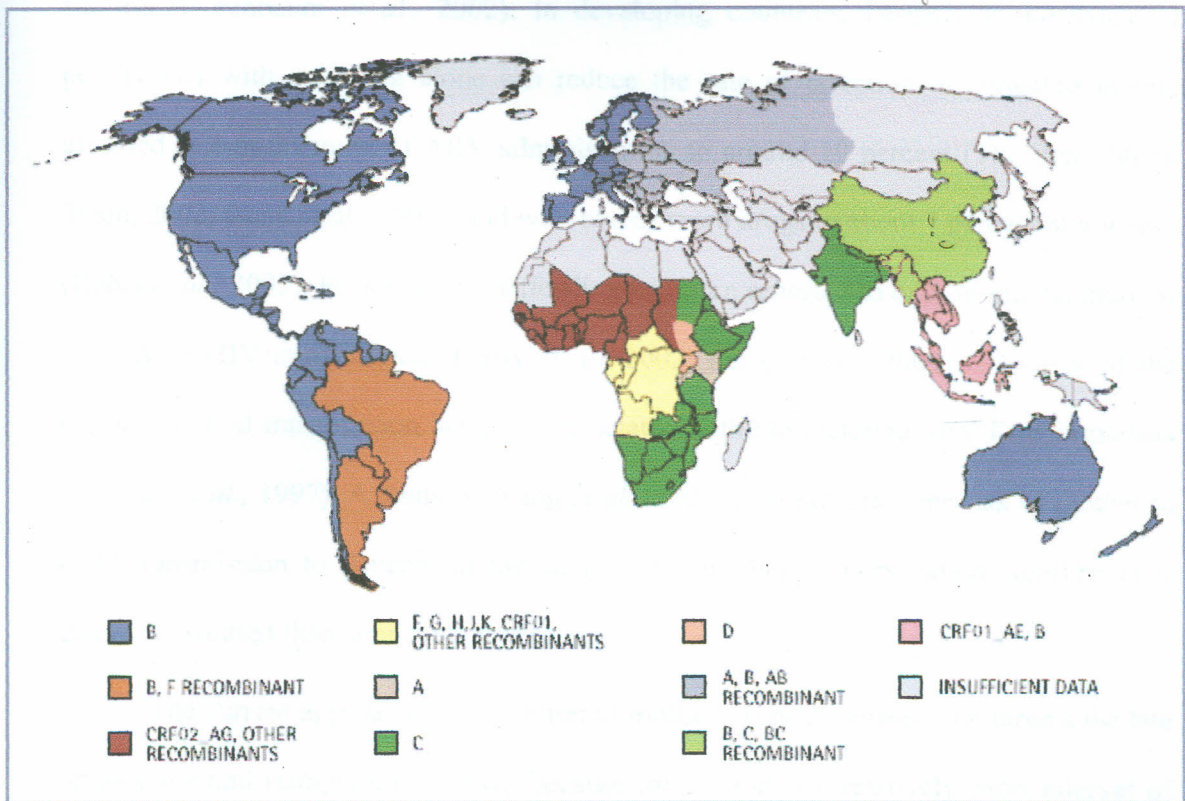


Figure 1: Current global distributions of HIV-1 strains (map adapted from international AIDS Vaccine Initiative-IAVI)

2.5 HIV Transmission

HIV infection is most commonly due to unprotected sex with infected partner, but can also occur from receiving contaminated blood during transfusion or exposure to non sterile instrument or medical procedure (Buve *et al.*, 2002). Most infected children under 15 years have contracted the virus by transmission from their mothers, an indication of the prevalence of infection in women of child bearing age. Mother to child transmission of HIV can occur before, during or after delivery, but only in rare cases does it occur during early stages of pregnancy (Senise *et al.*, 2003). In Europe and America, the estimated rate of mother to child HIV transmission ranges from 14-25% while in developing countries, it ranges from 13- 42% (Msellati *et al.*, 1995). In developed countries, antiretroviral therapy, elective caesarean section, and refraining from breastfeeding have been used to reduce the rate of mother to child transmission with some

success (Dorenbaum *et al.*, 2002). In developing countries, peripartum antiretroviral prophylaxis with one drug alone can reduce the rate of infection in breastfed infants assessed at 2 or 3 month of ARV administration to around 10 percent (The Petra Study Team, 2002; Guay *et al.*, 1999), and with two or more drugs to about 7 percent at 6 weeks (Dabis *et al.*, 2005). Prolonged breastfeeding has been reported to expose infants more to the risk of HIV transmission (Leroy *et al.*, 2002; Leroy *et al.*, 2003). The risk of the mother to child transmission has been associated with the maternal viral load in plasma (Mayaux *et al.*, 1997). A study by Yang *et al.*, (2003) also indicated the risk of mother to child transmission to depend on the subtype the mother harbors, where subtype D is easily transmitted than subtype A and C.

The current approach to prevention of mother to child transmission targets the late intrauterine and intrapartum periods, because this period is a relatively short interval of relatively high risk (Hofemeyr and McIntyre, 1997). An estimated 40 percent of overall transmission occurs in late pregnancy and during labour pain and delivery (Oxtoby, 1990). Peripartum antiretroviral prophylaxis reduces transmission risk in the period of and around delivery (Dabis *et al.*, 1999). Studies have shown that antiretroviral therapy can reduce overall risk of HIV infection even in breastfeeding populations (Leroy *et al.*, 2002). Transmission through breastfeeding has been well documented with the initial reports indicating the possibility of transmission through breast milk in breastfed infants being of women who had been infected postnatally through blood transfusion or through heterosexual exposure (Palansanthiran *et al.*, 1993; Van de Perre *et al.*, 1991). Another study conducted revealed infant infection through wet nursing (Nduati *et al.*, 1994).

2.6 Antiretroviral Drug Resistance

Antiretroviral drug resistance refers to the presence of viral mutations that reduce drug susceptibility compared with the wild-type viruses (Clavel and Hance, 2004). Certain factors contribute to development of antiretroviral drugs resistance. Some of these factors relate to the viral life cycle. The high rate of viral replication and lack of 3' exonuclease activity by the reverse transcriptase enzyme leads to high rate of mutation (Yahi *et al.*, 1999). These mutations may confer ARV drug resistance when they occur in the gene region encoding the molecular targets of drug therapy. High mutation rate together with high replication rate leads to many variants which may have different sensitivities for drug, a factor that may complicate selection for a drug (Zdanowicz, 2006). Therefore, development of antiretroviral drug resistance may occur independent of drug pressure as has been confirmed by studies by Yerly *et al.*, (1999) and Boden *et al.*, (1999). However, factors such as poor patient compliance leading to subtherapeutic blood levels of the drug may also enhance development of drug resistance (Zdanowicz, 2006).

2.7 Convention for Describing Mutations

There is a standard numbering system for HIV protease and reverse transcriptase regions based on the amino acids sequence. The most commonly used wild type reference is the subtype B consensus sequence which was originally derived from the alignment in the HIV sequence data base at Los Alamos natural laboratory (Kuiken *et al.*, 1999) and can also be found on HIV reverse transcriptase and protease sequence database (Rhee *et al.*, 2003). Mutations are typically described using shorthand notations in which a letter indicating the consensus B wild type amino acid is followed by the amino acid residue number, followed by a letter indicating mutation. For instance, in M184V, M is code for amino acid in the wild type which in this case is methionine, 184 is codon position in the sequence, and V is code for changed amino acid which in this case

is valine (Clavel and Hance, 2004). If there is a mixture of more than one amino acid at a position, it is indicated as M184M/V which denotes that the sequence has a mixture of amino acid in the wild type and a mutant one at position 215 in the sequence (Antonarakis, 1998), which in this case is a mixture of methionine which exist in the wild type and valine which is the mutant amino acid and at the same position in the sequence.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Gem, western Kenya, within Nyanza province. It is an area of approximately 403.1 km², and about 42 kilometers northwest of Kisumu town. Gem lies between latitude 0° 26' to 0° 12' north of Equator and longitude 33° 58' east and 34° 05' west of Prime meridian. It is an area where cultural practices, which fuel HIV transmission, such as wife inheritance and polygamy are common. It lies along the Kenya - Uganda highway, with trading centres along the highway.

3.2 Study Population and Sampling Scheme

According to 2007 Kenya AIDS Indicator Survey (KAIS), 7.1% adult (aged 18 years and above) Kenyans (representing 1,417,000) were HIV infected; Nyanza province in which Gem, western Kenya falls under had an average of 14.9% adult infection. Gem population was estimated at approximately 75,000 (van Eijk *et al.*, 2008), so working with 14.9% which was the average adult HIV prevalence in Nyanza province (NASCO, 2009), an estimated 11,175 adults were HIV infected in Gem, western Kenya, from which the study sample of 45 was drawn. The study sample of 45 tested HIV positive by Unigold. Using the software Power and Sample Size program, 45 HIV positive adults were randomly selected to be able to reject the null hypothesis with a power of 0.95, given a standard deviation of 1.96 and type I error probability of 0.05.

3.3 Inclusion Criteria

To be included in the study, one had to be an adult aged 18 years and above and tested HIV positive with Unigold™, and also, must have been resident of Gem for the last 6 months and willing to participate in the study.

3.4 Exclusion Criteria

Any persons below 18 years of age, or testing HIV negative with Unigold™ was excluded from the study. All persons not having been living in Gem in the previous 6 months, regardless of age and/or HIV status, were also excluded from the study.

3.5 Ethical Considerations

Informed consent was obtained from those individuals meeting inclusion criteria. Approval for this study was obtained from the ethical review committee at Kenya Medical Research Institute (KEMRI) and Human Investigational Review Board (HIRB) at Centre for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. Since the process of drawing blood samples exposed the participants to some risk of pain, the process was carried out professionally and in a sterile manner to minimize the risk of infections.

3.6 Sample Collection and Processing

All the prospective study participants were requested to give 5 millilitres of blood and this was taken through venipuncture, put into anticoagulant tubes (EDTA tubes) and then put in cooler box and transported to the main KEMRI/CDC- HIV research laboratory for plasma separation. The amount chosen was sufficient for an extra aliquot to be stored as back-up during the experiments. The samples were centrifuged to separate plasma

from whole blood and the plasma aliquoted in two 1.5 millilitres tubes (an extra tube as a back-up) and kept in a -80°C freezer awaiting RNA extraction and nucleic acid analysis.

3.6.1 Qiagen™ RNA extraction

During extraction, one tube of plasma of each sample was drawn from the freezer and thawed in ice (4°C) in the biosafety cabinet. An aliquot of 500 microlitres was made into another 1.5 millilitre tube. This was then centrifuged for one hour at 16400 revolutions per minute at 4°C in a refrigerated centrifuge (microcentrifuge, Eppendorf, USA). After spinning, 360 microlitres of the supernatant was pipetted out leaving 140 microlitre as the pellet. Then 560 microlitres of lysis buffer (in the Qiagen™ RNA extraction kit) was added to the pellet, mixed by pulse vortexing and then briefly centrifuged. This was then incubated at room temperature for 1 hour to allow for maximum lysis of viral particles to occur. This was then followed by brief spinning and pulse vortexing. Exactly 560 microlitres of absolute ethanol was added and mixed by pulse vortexing followed by brief spinning to coagulate the viral nucleic acid. Then 630 microlitres of this sample was then added to the spin column, put in a 2-millilitres collection tube, and then spun for 1 minute at 8000 revolutions per minute and at 25°C and the filtrate discarded. (The membrane in the spin column is meant to trap the viral nucleic acid as the filtrate passes.) This step was repeated with the remaining sample. Then 500 microlitres of wash buffer-1 in the kit, was added to the spin column to wash the already membrane trapped RNA. Same procedure was repeated with wash buffer-2 in the kit, and spinning at 8000 revolutions per minute done for 3 minutes at 25°C . Elution of the membrane trapped RNA was finally done with 60 microlitres of diethylpyrocarbonate (DEPC-treated) water in a separate 1.5 millilitres sterile RNase free tube. In cases where the RT-PCR reaction was not to be set immediately, the RNA extract was stored at -80°C .

3.6.2 RT-PCR

The protease and the RT regions from each patient sample were first amplified using RT-PCR. During this reaction, 10 microlitres of the RNA extract was added to the PCR reaction mix containing 1µl of supper script III one step enzyme (Gibco Chemicals, USA), 2 microlitres of 10 milimolar dNTP (Applied Biosystems USA), 10microlitres of 5X PCR buffer (Applied Biosystems, USA.), 0.5 microlitres of RNase inhibitor (Applied Biosystems, USA), 23.5 microlitres of DEPC treated water (Sigma Chemicals, USA) and a set of the following 1.5 microlitres of 8 micromolar of each primers: RT gen 4R, 5'-TAATTTTTTAGGGAAGATCTGGCCTCC-3' bp2082-2108 and pro-out 5'-CTGTTAGTGCTTTGGTTCCTCT-3' bp 3399-3420 (CDC, HIVR Laboratory, Atlanta, Geogia, USA). The reaction was performed under the following conditions: initial denaturation and subsequent reverse transcription at 65⁰C for 10 minutes, and 50⁰C for 45 minutes, respectively; denaturation of cDNA at 94⁰C for 2 minutes, 40 cycles at 94⁰C for 15 seconds, 55⁰C for 20 seconds, and 72⁰C for 2 minutes, and final extension of 72⁰C for 10 minutes. Deionized water was used as negative control for the reaction.

3.6.3 Nested PCR

Since RT-PCR ended up with a little bit larger fragments, it was therefore necessary to do nested PCR to narrow the amplification to the specific fragment of interest. This second round of amplification was carried out in a 50 microlitres reaction mixture comprising of 10 microlitres of the RT-PCR product, 1 microlitres of 2.5 units of Taq polymerase (Applied Biosystems, USA), 2 microlitres of 10 milimolar dNTP (Applied Biosystems, USA), 10 microlitres of 5x PCR buffer (Applied Biosystems USA) and 24.5 microlitres of DEPC treated water (Sigma Chemical, USA) and a set of the following 1.5 microlitres of 8 micromolar of each primer: 215/219, 5'

TCAGAGCAGACCAGAGCCAACAGCCCC-3' bp 2136-2163 and PAF-4
5'CCTTACTAACTTCTGTATGTCATTGACAAGTCCAGCT 3' bp 3300-3334 (CDC,
HIVR Laboratory, Atlanta, Georgia, USA), and the reaction performed with the cycling
parameters of 94⁰C for 4 minutes followed 40 cycles at 94⁰C for 15 seconds, 55⁰C for 20
seconds, and 72⁰C for 2 minutes with a final extension step at 72⁰C for 10 minutes.
Deionized water was used as negative control for the reaction.

3.6.4 Gel electrophoresis

Gel electrophoresis was done by mixing 5 microlitres of the nested PCR product
with 2 microlitres of the loading dye and loaded onto a 1% ethidium bromide stained
agarose gel. This was followed with electrophoresis at 130V for 20 minutes. The loaded
well that did not show any band as shown in Figure 2.0 were considered negative for
HIV-1. Those that did show band as shown in Figure 2.0 at position 1200 basepairs as
indicated by mass ladder control, were considered to be positive for HIV-1, and so were
taken for purification prior to sequencing. At this stage, out of the 45 samples initially
taken for amplification, only 30 samples amplified.

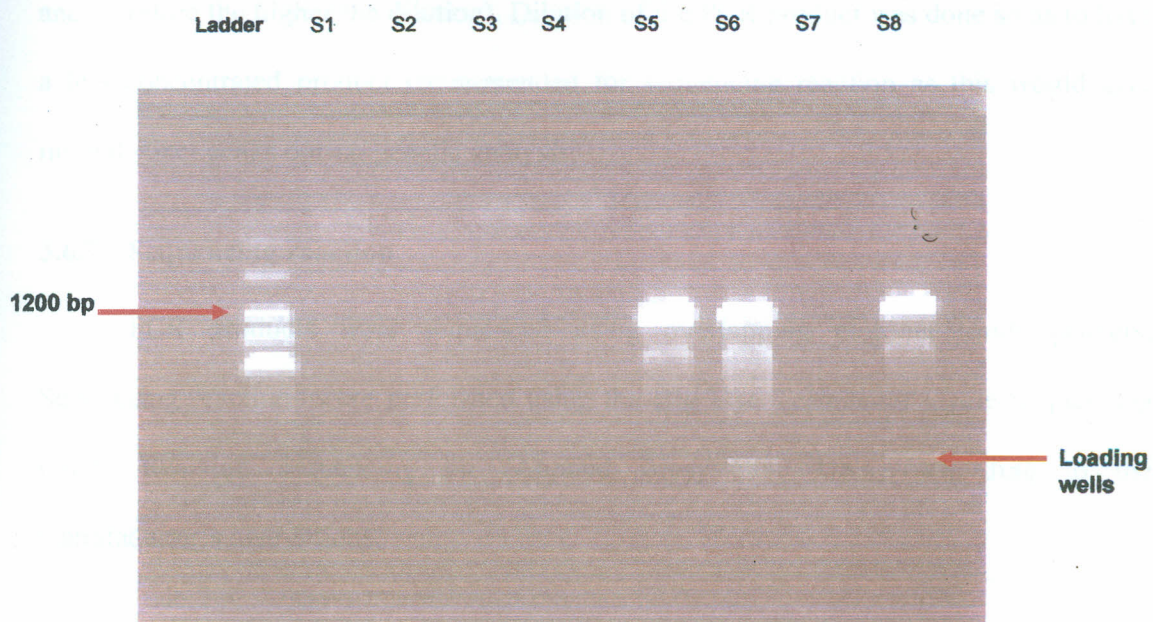


Figure 2: Picture of a gel showing positive and negative samples.

A picture of a gel taken after electrophoresis of nested PCR product to identify the samples where gene amplification occurred. Each sample marked as S1-S8 was loaded into its well and electric current applied at a voltage of 130v for 20 minutes. Since the region amplified was 1200 basepairs in size, the samples in which gene amplification occurred; S5, S6 and S8 had bands corresponding to a 1200 basepair band in the ladder (control) as shown.

3.6.5 QIAquick purification of PCR product

Due to the presence of nonspecific bands on some samples, the PCR products were purified with the QIAquick gel extraction kit (Qiagen, USA) as per the manufacturer's instructions.

3.6.6 Dilution of PCR products

For the samples that showed bigger bands, which meant high concentration of PCR product, dilution with DEPC-treated water was done either 1 in 4 or 1 in 10 depending on the size of the band (the bigger the band the more the DNA in the sample

and therefore the higher the dilution). Dilution of the PCR product was done so as to have a less concentrated product recommended for sequencing reaction as this would give more distinct peaks during genetic analysis.

3.6.7 Sequencing reaction

PCR products were sequenced using overlapping oligonucleotide primers. Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Ready Reaction sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions.

3.6.8 Purification of the sequenced product

This step was carried out to get rid of excess dDNTPs from the sequenced product and the purification at this step was done using Centri-sep ColumnsTM (Applied Biosystems, USA). The Centri-sep ColumnTM was first prepared by tapping it for the gel in it to settle at the bottom, the top was then removed. 0.8 ml of distilled or deionized water was added to the column and the top taken back, and this was allowed to settle for 1 hour at room temperature so as to fully wet the gel in the Centri-sep ColumnTM. The column was then gently tilted on one side and tapped to remove any air bubble that could be trapped in the gel. Then, the top of the column was removed followed by the bottom seal and the column put in collection tube to collect the filtrate while spinning for 2 minutes at 300rpm. After this the filtrate was discarded and columns put in sterile 1.5ml tubes and the sample i.e the sequenced product, added by gently pipetting from the top of the gel in the column and then briefly spinning to get out the purified sample as the filtrate.

3.6.9 Speed vac drying of the sequenced product

The samples were then arranged in the Speed Vac™ (Applied biosystems, USA) for drying, and drying manually done at medium speed for 30 minutes.

3.6.10 Denaturation

The dried samples were then resuspended in the 20 microlitres of formamide and pulse vortexed and left to settle for 30 minutes after which the samples were put back to the optical tubes and loaded to the thermocycler set at 94°C for 2 minutes for the denaturation before sequence analysis.

3.7 Sequence Analysis

The sequencing products were run on ABI 3100™ (Applied Biosystems) Genetic Analyzer and then assembled by Sequencher™ software (Gene Code, Ann Arbor MI). The PRO and RT gene sequences generated were analyzed for antiretroviral drug resistance mutations at the positions known to be associated with drug resistance according to drug resistance interpretation algorithm (Stanford University Database Version 4.1.7), (http://givdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivalgo&action=showSequenceForm). The same sequences were then aligned with consensus sequences obtained from Los Alamos HIV Sequence database (http://hiv_web.lanl.gov) representing HIV-1 subtypes including A1, A2, B, C, D, G, F1, F2, K, H, J, and the circulating recombinant forms. Phylogenetic analysis was performed using PAUP™, Version 4.0, and maximum likelihood tree constructed and confidence of analysis tested using bootstrap values. The gene sequences were further analyzed by similarity analysis using SimPlot™ to determine whether they were pure subtypes or recombinant forms. Similarity plot using SimPlot™ version 2.5 Software (S.Ray,

<http://www.med.jhu.edu/deptmed/sray>) determined the percent similarity between Gem sequence and a selected group of reference sequences by moving a window of 400 basepairs fragment with 20 basepairs increment along the alignment. Similarity values were plotted at the midpoint of 400 basepairs fragment and similarity curve generated.

3.8 Limitations of the Study.

The study used in-house PCR method, which had just been developed by CDC/KEMRI HIVR Laboratory, Kisian, so was still not yet well refined and therefore could affect the PCR result. (It could amplify only upto 2000 viral copies per milliliters of plasma). The study was carried out under very strict management conditions that at times did not allow for repeat of certain assays that failed, like when some primers failed to anneal during sequencing reaction leading to failure of some samples to sequence.

4.0 RESULTS

4.1. Mutations in the Protease and Reverse Transcriptase Genes

There were several mutations in the protease gene as indicated in Table 1. Some mutations such as E35D, M36I, R57K, H69K and L89M were most frequent, occurring in all the 21 sequences, while R41K occurred in 20 out of 21 sequences. R57R/K occurred in 9 sequences followed by M36I/V in 7 sequences, then T12S in five, and finally I13V which was the least frequent occurred in only 3 sequences out of 21 sequences. However, when each mutation was analyzed for protease inhibitor drug resistance, none was found to cause resistance to any protease inhibitor drugs as shown in Table 1.

Table 1: Protease mutations, their frequency and protease inhibitor drug resistance analysis results

Mutations	Frequency (n=21)	Protease inhibitor drug resistance
T12S	5	None
I13V	3	None
E35D	21	None
M36I	21	None
M36I/V	7	None
R41K	20	None
R57K	21	None
R57R/K	9	None
H69K	21	None
L89M	21	None

In the Reverse transcriptase gene region, mutations occurred at different positions of the sequence as shown in table 2. V35T, V60I, and K122E were the most frequent mutations occurring in all the 21 sequences. E40D mutation occurred in 5 sequences followed by D123S which occurred in 4 sequences. The least frequent mutations were I135T and E169D, which occurred in only 2 sequences each out of 21. Analysis of each of these mutations for reverse transcriptase inhibitor drug resistance found none to be causing drug resistance to any of the currently known reverse transcriptase inhibitors drugs as shown in Table 2.

Table 2: Reverse transcriptase mutations, their frequency and reverse transcriptase inhibitor drug resistance analysis results

Mutations	Frequency (n=21)	RT inhibitor drug resistance
V35T	21	None
E40D	5	None
V60I	21	None
K122E	21	None
D123S	4	None
I135T	2	None
E169D	2	None

4.2 Phylogenetic Analysis

Phylogenetic analysis was done using PAUP and phylogenetic tree constructed as shown in Figure 3. Many of the sequences marked 'Gem' clustered next to subtype A reference sequences, confirming them as subtype A sequences. Some clustered next to subtype D reference sequences confirming them as of subtype D, while one sequence, Gem.3001670 was next to subtype A, but with a very short branch as compared to other subtype A. Other analysis (similarity analysis) revealed it as a recombinant form (CRF_AD). The phylogenetic analysis result was extracted from the tree and tabulated as shown in Table 3, so as to show the subtype of each individual sequence analyzed phylogenetically. Out of 21 samples analyzed, 16 (76.2%) were subtype A, subtype D were 4 (19.0%), while circulating recombinant form (CRF_AD) was 1 (4.8%), as summarized in Table 4.

NJ

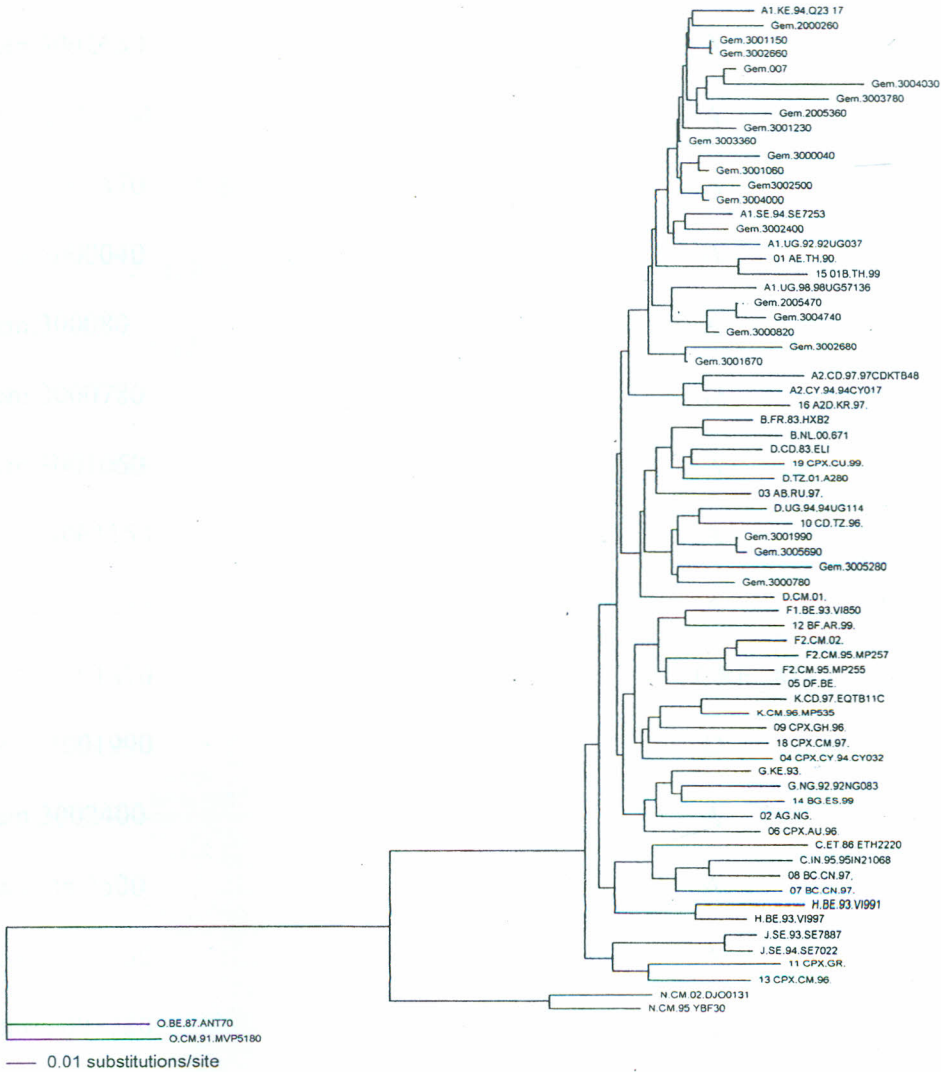


Figure 3: A phylogenetic tree

The tree estimates the evolutionary past based on gene sequences. It consists of branches and nodes. The tree consists of both internal and terminal nodes as shown. The internal node corresponds to hypothetical last common ancestor of gene sequences arising from it, while the terminal node corresponds to the sequences from which the tree was derived. The length of the branches corresponds to amount of evolution (percent sequence difference) between the two nodes they connect. Thus the longer the branch, the more divergent the sequences attached to it. In Figure 3, the sequences analyzed are marked in the tree as 'Gem' in the branches, and amongst them are other sequences that act as reference sequences in the tree.

Table 3: Distribution of HIV-1 subtypes for individual Gem sequences.

Sample codes	Subtypes
Gem. 007 (control sample)	A
Gem.2000260	A
Gem.3002680	A
Gem. 2005360	A
Gem.2005470	A
Gem.3000040	A
Gem.300080	A
Gem.3000780	D
Gem.3001060	A
Gem. 3001150	A
Gem. 3001230	A
Gem. 3001670	CRF_AD
Gem. 3001990	D
Gem 3002400	A
Gem.3002500	A
Gem. 3003360	A
Gem. 3003780	A
Gem. 3004000	A
Gem. 3004030	A
Gem.3004740	A
Gem. 3005280	D
Gem.3005690	D

Table 4: Summary of HIV-1 subtypes circulating in Gem, western Kenya.

Subtype A	Subtype D	CRF_AD
16 (76.2%)	4 (19.0%)	1 (4.8%)

4.3 Recombination Analysis

Similarity curve showing sequence Gem 2000260 as subtype A is shown in Figure 4, while Figure 5 shows a similarity curve for sequence, Gem 3005280, which was a representative of subtype D. Figure 6 shows similarity curve for sequence, Gem 3001670, which was found to be a recombinant form (CRF_AD). So the similarity analysis which was meant to confirm recombination among the subtypes showed only one sequence, Gem 3001670 (Figure 5) as the only recombinant form.

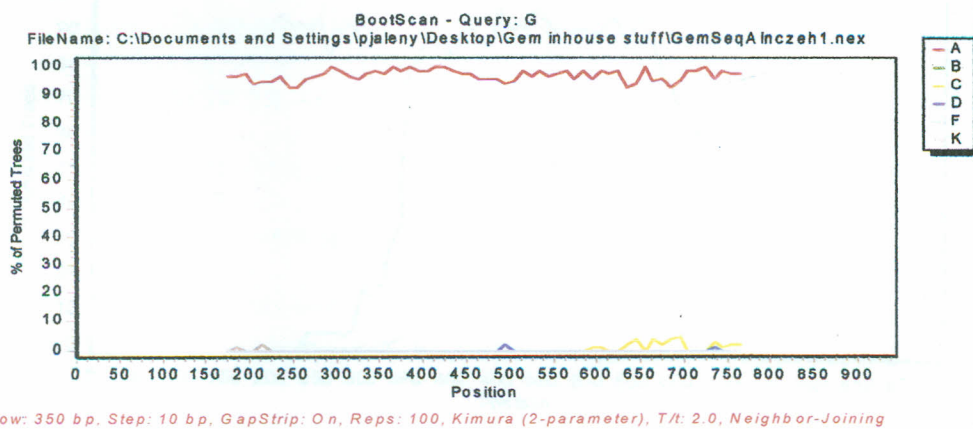
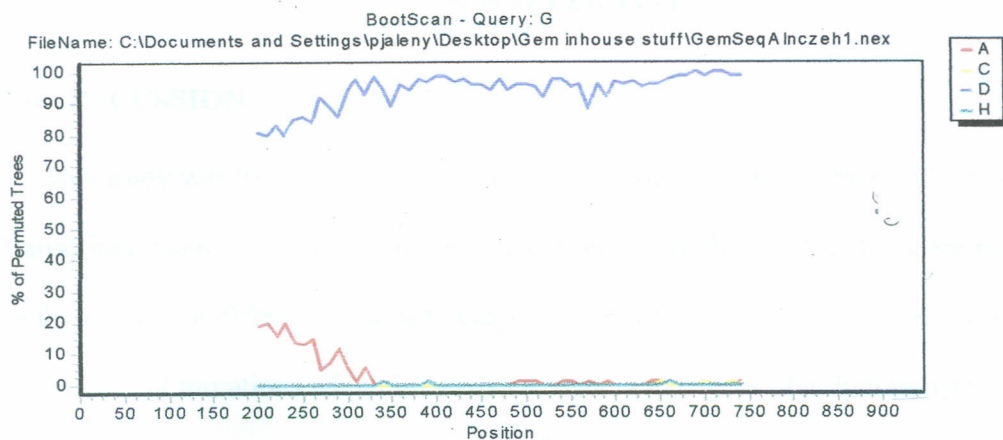


Figure 4: Similarity curve for sequence Gem 2000260 (subtype A)

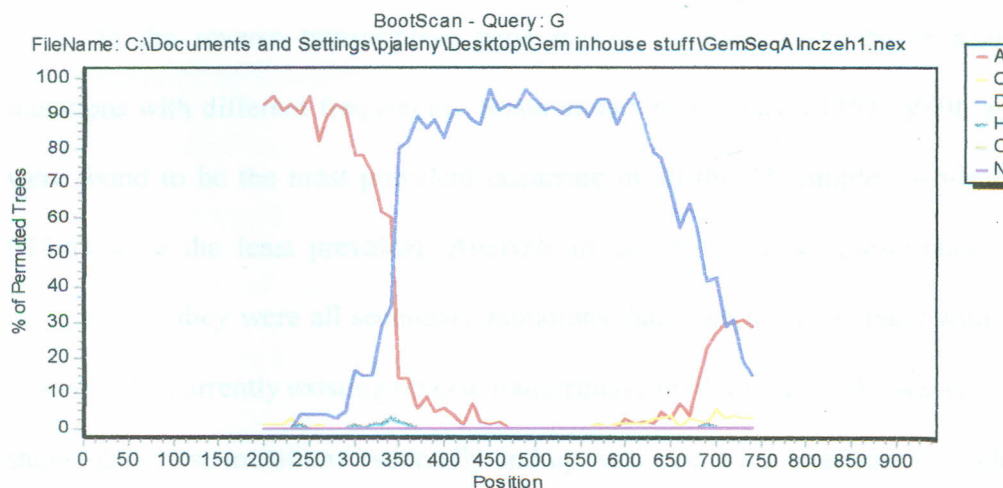
Similarity plot to determine percentage similarity between sequence, **Gem 2000260** and a group of selected reference sequences for subtype A, B, C, D, F, and K by moving a window of 350 basepairs fragments with steps of 10 basepairs increments along the alignment and similarity values plotted at the midpoint of 350 basepairs fragment.



indow: 400 bp, Step: 10 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2.0, Neighbor-Joining

Figure 5: Similarity curve for sequence Gem 3005280 (subtype D)

Similarity plot to determine percentage similarity between sequence, **Gem 3005280** and a group of selected reference sequences for subtype A, C, D, and H, by moving a window of 400 basepairs fragments with steps of 10 basepairs increments along the alignment and similarity values plotted at the midpoint of 400 basepairs fragment.



indow: 400 bp, Step: 10 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2.0, Neighbor-Joining

Figure 6: Similarity curve for sequence Gem 3001670 (recombinant CRF_AD)

Similarity plot to determine percentage similarity between sequence, **Gem 3001670** and a group of selected reference sequences for subtype A, C, D,H, O, and N by moving a window of 400 basepairs fragments with steps of 10 basepairs increment along the the alignment and similarity values plotted at the midpoint of 400 basepairs fragment.

CHAPTER FIVE

5.0. DISCUSSION

This study was based on the molecular analysis of the HIV protease and reverse transcriptase gene sequences. The findings of this study showed that there are mutations in these genes at different codons in Gem. In the protease gene region, there was high prevalence of mutations which is consistent with high degree of polymorphism of the protease gene observed in other study by Konzal *et al.*, (1996). In the protease, the most prevalent mutations were E35D, M36I, R57K, H69K, and L89M and the least prevalent was found at codon 12, with T12S as the amino acid substitution. Codon 36 showed the highest amino acid variation and is consistent with a study by Handema *et al.*, (2003). Analysis of these mutations revealed that they were all secondary mutations that were only improving viral fitness and therefore did not confer any drug resistance to any of the currently existing protease inhibitor drugs.

In the reverse transcriptase gene region, all the 21 sequences analyzed had mutations with different frequencies. Some mutations such as V135T, V60I, and K122E were found to be the most prevalent occurring in all the 21 samples, while I135T and E169D were the least prevalent. Analysis of all the Reverse transcriptase mutations revealed that they were all secondary mutations that were not associated with resistance to any of the currently existing reverse transcriptase inhibitor drugs. However, a study has shown that these mutations eventually end up with numerous quasispecies with different antiretroviral drug sensitivities which may finally cause drug resistance among some quasispecies (Zdanowicz, 2006). The mutations shown in this study were found to vary slightly with other similar studies by Kozal *et al.*, (1996) and Handema *et al.*, (2003). The slight difference was seen in the different amino acid substitution at different codons. The variation could be attributed to the difference in the sample origin.

Phylogenetic analysis revealed that HIV epidemics in Gem was driven by subtype A, which was 76.2%, followed by subtype D which was 19.0%. There was also a small percentage of recombinant form (CRF_AD), 4.8%. The subtypes found in this study were similar to those found in another study by Yang *et al.*, (2004), except that it did not find subtype C and G which were found in the previous study (Yang *et al.*, 2004). The finding of this study were also in line with another HIV-1 diversity study by Lihana *et al.*, (2009) which was conducted in Kenya and found HIV-1 subtype A to be the dominant followed by subtype D. As has been observed, all the HIV-1 recombinant forms in Kenya have a portion of HIV-1 subtype A (Yang *et al.*, 2004), so the recombinant form found in this study too was a combination of subtype A and subtype D. The existence of HIV recombinant form in Gem may confirm a possibility of co-infection by HIV-1 subtype A and subtype D in Gem, western Kenya as was confirmed in a study by Cornellisen *et al.*, (1997).

5.1 Conclusions and Recommendation.

The study confirmed presence of mutations in the protease and reverse transcriptase genes. However, these mutations were secondary in that they do not confer resistance to any of the currently existing protease inhibitor or reverse transcriptase inhibitor drugs. The study also revealed that in Gem, HIV-1 diversity is constituted by two subtype A and subtype D, with subtype A being the dominant. The result also showed that recombinant form (CRF_AD) also exists in small proportion. The findings of this study are in consistence with other studies that are so far conducted in Kenya save for the slight variations which may be attributed to sample origin.

Since studies conducted in another region in Africa by Van *et al.*, 1997, has shown that HIV diversity has influence on the transmission, rate of disease progression

and pathogenicity, it would be advisable to carry out a future study to determine the effect of subtypes found in this study on the transmission, rate of HIV disease progression, and pathogenicity in Gem, western Kenya.

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