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Molecular Analysis of Part of Pol Gene of HIV-1 Isolates From ARV Naïve Patients of Gem Subcounty of Siaya County, West Kenya

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Abstract: **Despite advances being made in antiretroviral therapy that is revolutionizing HIV disease management, effective control of the HIV infection pandemic still remains elusive. This is attributed to high rate of HIV replication and the existence of a highly error-prone viral reverse transcriptase enzyme causing several mutations during replication. Studies have shown that some of these mutations enhance viral fitness whiles some may cause antiretroviral drug resistance especially, if they occur in the gene regions coding for molecular drug targets. 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 determining mutations and evaluating each for its ability to confer antiretroviral drug resistance. A total of 21 samples were sequenced and further analyzed. The results showed several mutations in these gene sequences. However, analysis of each of the mutation for drug resistance using Stanford HIV resistance data base revealed that they were secondary mutations which could not confer resistance on its own to any of the currently existing Protease inhibitor or Reverse Transcriptase inhibitor drugs. Since none was found to confer drug resistance, it would be necessary to conduct a future study to find out the effect of these mutations and any other that may arise in future because the virus is continuously undergoing mutation.**

Keywords: **HIV, mutation, antiretroviral drugs, Protease, Reverse transcriptase enzymes.**

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. [1] and it is now a pandemic, with an estimated 38.6 million people currently living with the HIV worldwide.[2] 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 intergrase enzyme, it integrates its genome into the host cell DNA. Once integrated, 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).[3]Once immune system is weakened, one remains prone to opportunistic infections and tumors. 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. [4] If mutations occur in the gene region encoding molecular antiretroviral drug target, then this may cause ARV drug resistance. [5]

In African continent, HIV prevalence varies. For instance, in southern African countries, more than one in every five pregnant women is HIV infected. [6] 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%.[6] 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.[6]

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. Previous studies reveal that Asia is experiencing rapidly growing epidemics. [7] Likewise, Eastern Europe is also experiencing rapid increase in prevalence, which is being attributed to rise in mother to child transmission. [8].The risk of mother to child infection increases if a breast feeding mother is newly infected, owing to the initially high level of virus. [9]. It is therefore important that more attention should be directed to HIV/AIDS prevention and treatment.

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.[10]] 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. [11] 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%.[12]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.[13] 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[14] [15], and with two or more drugs to about 7 percent at 6 weeks.[16] Prolonged breastfeeding has been reported to expose infants more to the risk of HIV transmission. [17] [18] The risk of the mother to child transmission has been associated with the maternal viral load in plasma. [19] A study 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. [20]

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.[21] An estimated 40 percent of overall transmission occurs in late pregnancy and during labour pain and delivery.[22] Peripartum antiretroviral prophylaxis reduces transmission risk in the period of and around delivery.[23] Studies have shown that antiretroviral therapy can reduce overall risk of HIV infection even in breastfeeding populations.[17]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.[24][25] Another study conducted revealed infant infection through wet nursing [26]

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. [27] 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. [28] HIV-1 has evolved significantly varying from one geographical region to another.[29] HIV-1 is the most diverse and has evolved into three major groups, mainly, group O (Outlier), N (non M) and M (major)[29] 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.[30] All the subtypes originated from central Africa.[30] In addition, strains of HIV-1 recombinants forms have been identified to be in circulation. [30] Other forms which have been recognized are, A to H with subtypes A and B being the most prevalent.[31][32][33] 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.[34] Other studies also associate subtype A and

G with longer AIDS free survival period, which is opposed to other non B subtypes.[35] All groups of HIV-1 are found in Africa; while group M is found all over the continent, group N and O are restricted to Central Africa.[36][37][38][39] Subtypes A, and D are prevalent in East Africa subtype A in West Africa, and C in South Africa, while recombinant subtypes viruses are found in Central and West Africa.[30] In West and Central Europe, America and Australia, subtype B is the most prevalent form.[40] Globally, subtype C is the predominant form causing much of heterosexual infections worldwide.[41] 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. [31][32][33].

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.[4]. Some of these mutations are meant to improve viral fitness while some do confer the virus with drug resistance ability. [5]. It is 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. [42] So far, There is no study that has tried to identify these mutations among drug naïve in Gem western Kenya and their clinical implication. It was therefore important to carry out a study to identify mutations among HIV isolate from drug naïve patients of 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.

2. MATERIALS AND METHODS

2.1 Study Area:

This study was conducted in Gem sub county of Siaya County in Kenya. It is an area of approximately 403.1 km^2, and about 42 kilometers northwest of Kisumu town. The sub county 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 were 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.

2.2 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[43]. so working with 14.9% which was the average adult HIV prevalence in Nyanza province [44], 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.

2.3 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.

2.4 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.

2.5 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.

2.6 Sample Collection and Processing:

All the prospective study participants were requested to give 5 mililitres 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 mililitres tubes (an extra tube as a back-up) and kept in a - 80^0C freezer awaiting RNA extraction and nucleic acid analysis.

2.6.1 Qiagen™ RNA extraction:

During extraction, one tube of plasma of each sample was drawn from the freezer and thawed in ice (4^0C) in the biosafety cabinet. An aliquot of 500 microlitres was made into another 1.5mililitre tube. This was then centrifuged for one hour at 16400 revolutions per minute at 4^0C in a refrigerated centrifuge (micro centrifuge, Eppendorf, USA). After spinning, 360 microlitres of the supernatant was pippeted 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 votexing. 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 span for 1 minute at 8000 revolutions per minute and at 25^0C 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 25^0C. Elution of the membrane trapped RNA was finally done with 60 microlitres of diethylpyrocarbonate (DEPC-treated) water in a separate 1.5 mililitres sterile RNase free tube. In cases where the RT-PCR reaction was not to be set immediately, the RNA extract was stored at -80^0C.

2.6.2 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 65^0C for 10 minutes, and 50^0C for 45 minutes, respectively; denaturation of cDNA at 94^0C for 2 minutes, 40 cycles at 94^0C for 15 seconds, 55^0C for 20 seconds, and 72^0C for 2 minutes, and final extension of 72^0C for 10 minutes. Deionized water was used as negative control for the reaction.

2.6.3 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 94^0C for 4 minutes followed 40 cycles at 94^0C for 15 seconds, 55^0C for 20 seconds, and 72^0C for 2 minutes with a final extension step at 72^0C for 10 minutes. Deionized water was used as negative control for the reaction.

2.6.4 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.

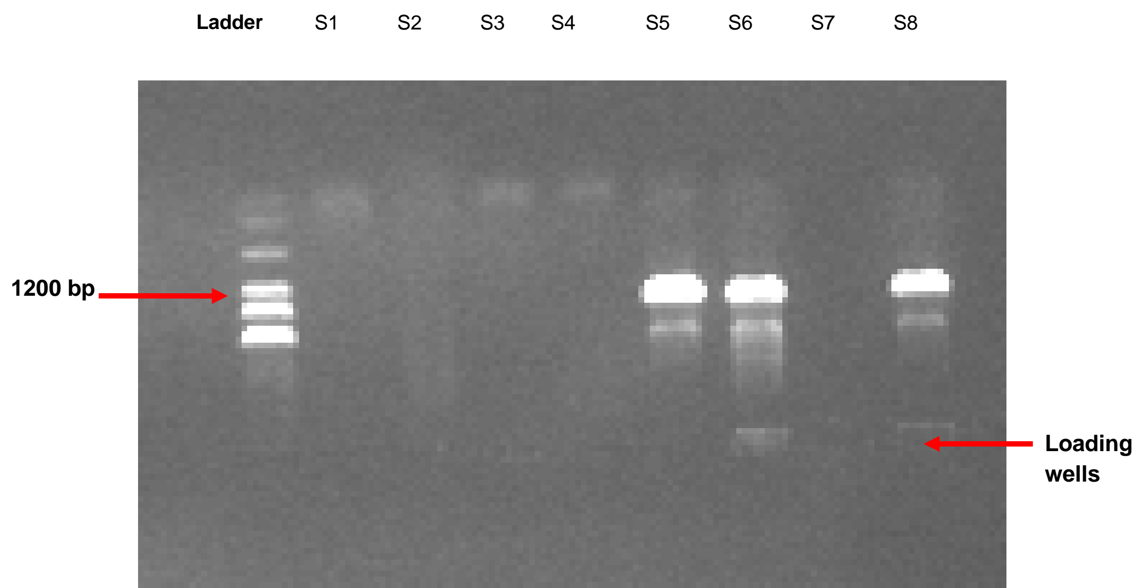

Fig 1: Picture of a gel showing positive and negative samples.

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.

2.6.5 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.

2.6.6 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.

2.6.7 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.

2.6.8 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 Columns^{TM} (Applied Biosystems, USA). The Centri-sep Column^{TM} 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 Column™. 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.

2.6.9 Speed vac drying of the sequenced:

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

2.6.10 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.

2.7 Sequence Analysis:

The sequencing products were run on ABI 3100™ Genetic Analyzer (Applied Biosystems), and then assembled by Sequencher™ software (Gene Code, Ann Arbor MI). The Protease and Reverse Transcriptase 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. This was done by comparing amino acid sequences of both PRO and RT with the subtype B consensus sequence (Stanford University HIV database Version.4.1.7). (http:givdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivalgo&action=showSequenceForm).

2.8 Limitations of the Study:

The study used in-house PCR method, which had just been developed by KEMRI/ CDC HIVR Laboratory, Kisumu, so was still not yet well refined and therefore could affect the PCR result.

3. RESULTS

3.1. Mutations in the Protease Genes:

On comparing amino acid sequences of Protease with the subtype B consensus sequence, none of the primary mutations were detected. However, secondary mutations were found in all the strains at different codons: 12, 13, 35,36, 41, 57,69 and 89 (Table 1). The most frequent mutations encountered were E35 D , M36I, R57K, H69K and L89M which occurred in all the PR sequences of 21 samples. They were followed by R41K (95.2%) R57R/K (42.8%), then M36I/V (33.3%) T12S (23.8%) and I13V (14.23%). Previous studies suggested that M36I represents a molecular signature for non-B strains (Duman *et al.,* 2002; Tanuri *et al.,* 1999). However, in this study mutation M36I occurred with very high frequency.

Table 1: Amino acid substitutions in the protease gene sequences of HIV strains among ARV naïve patients of Gem western Kenya

Codon Position in PRO	SubtypeB consensus amino acid	Amino acid substitution	Frequency(n=21)	Percentage
12	T	S	5	23.8
13	I	V	3	14.2
35	E	D	21	100
36	M	I	21	100
		I/V	7	33.3
41	R	K	20	95.2
57	R	K	21	100
		R/K	9	42.8
69	H	K	21	100
89	L	M	21	100

3.2. Mutations in the Protease Genes:

In the Reverse transcriptase gene region, secondary mutations were found in all the strains at different codons: 35, 40, 60, 122,123,135 and 169. (Table 2). The most frequent mutation in the RT gene were V35T, V60I, and K122E which occurring in all the 21 isolates. E40D mutation occurred in 5 (23.8%) isolates followed by D123S which occurred in 4 (19.0%) isolates. The least frequent mutations were I135T and E169D, which occurred in only 2 (9.5%) each. These mutation are only enhancing polymorphism. However, studies by polymorphism at codon 122 leads to development of thyamine associated mutations which eventually leads to NRTI resistance. Moreover, an extensive cross-sectional study by Gariga, *et al.,* (2009) has demonstrated that some HIV-1 RT polymorphisms strongly correlate with virological failure of NRTI-based treatments.[45]

Table 2: Amino acid substitutions in the RT gene sequences of HIV strains among ARV naïve patients of Gem western Kenya

Codon Position in the RT	Consensus Subtype B amino acid	Amino acid substitution	Frequency(n=21)	Percentage
35	V	T	21	100
40	E	D	5	23.8
60	V	I	21	100
122	K	E	21	100
123	D	S	4	19.0
135	I	T	2	9.5
169	E	D	2	9.5

4. 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. The protease gene appears to be less conserved than the RT gene. No primary resistance mutations were found, but at least, every isolate analyzed was harboring accessory mutations. The M36I mutation in the protease gene, which according to Paraschiv *et al.,* (2007) [46] is associated with resistance to ritonavir and nelfinavir in B subtype viruses was found in all isolates. This finding supports the suggestion by Paraschiv *et al.,* (2007, that Methionine in this position may represent a polymorphism specific to the B subtype.[46] Substitutions were also observed in other protease gene codons: 12, 13, 35, 36, 41, 57,69 and 89, some of them with rather high frequency:

A study by Paraschiv *et al.,* (2007) indicates that accessory mutations may not result in a significant decrease of sensitivity to ARV drugs, but associated with increased viral fitness in viruses with primary mutations.[46]. However, selection of primary mutations under selective pressure could precipitate the development of highly resistant HIV variants during ARV treatment in viruses where one or more secondary mutations are already present [47], [46] (Kantor et al., 2005; Paraschiv *et al.,* 2007). The study findings on the polymorphism at the protease gene region concur with the findings of another study by Kantor et al., (2005).[47] The high degree of polymorphism of the protease gene observed in this study also concurs with another study by Konzal *et al.,* (1996)[48] even though a slightly higher frequency of protease polymorphisms which can be likened to the higher homogeneity of the isolates. Also, in this study codon 36 showed the highest amino acid variation which is consistent with a study by Handema *et al.*, (2003).[49]

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.[50] 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.

4.1 Conclusions and Recommendation:

No major mutations associated with ARV drug resistance were detected in the isolates, but many accessory amino acid substitutions were found as natural variants. It is not yet clear if the high frequency of accessory protease and Reverse transcriptase mutations may contribute to a more rapid ARV drug resistance emergence. Sequential follow up of these patients would be necessary, especially with the onset of antiretroviral therapy, to evaluate the clinical significance of these minor PR mutations.

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