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Genetic Diversity of HIV-1 Isolates and Anti-Retroviral Drug Resistance from Patients in Kirinyaga County, Kenya

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Abstract:
Background: Human Immunodeficiency Virus type 1 (HIV-1) is the prototype immunodeficiency virus; it is highly virulent and is the cause of AIDS in most parts of the world. AIDS-related deaths have drastically reduced with antiretroviral treatment, even though access to therapy is not universal especially in low-income countries. In recent times, HIV research has been augmented, but as yet, the prospects of an AIDS cure and an effective immunizing agent are still uncertain. There is a great diversity in HIV-1 strains circulating globally as evidenced by phylogenetic analyses. Detailed studies show an intricate pattern of recombination between subtypes with further proof of sub-subtypes in circulation. In Kenya, the prevalent subtype of HIV-1 in circulation is subtype A-1. With the devolution of the health docket from the national to the county government, there is the need to reorient HIV research to the county level to equip county medical staff with current and accurate data on the efficacy of antiretroviral therapy. The main focus of this research was to establish the genetic diversity and antiretroviral drug resistance of HIV-1 isolates from Kirinyaga County, Kenya. HIV-1 genetic diversity compounded with the problem of drug resistance have a great implication on treatment, spread, and HIV-1 viral evolution.
Methodology: A cross-sectional molecular epidemiological study using purposive sampling was used to select sixty HIV-positive blood samples from patients in Kerugoya County Hospital in Kirinyaga County. HIV-1 proviral DNA was extracted from genomic DNA and amplified using specific primers for the HIV genomic pol region encoding the enzyme reverse transcriptase, and the amplicons sequenced. Resistance mutations were inferred from the Stanford database on HIV drug resistance.
Results: Sequence analysis established that subtype A1 was the most predominant in the County accounting for 66.67%(22/33), followed by subtypes D at 12.12%(4/33) and C at 9.09%(3/33) each. Four of the samples were found to be recombinants accounting for 12.12% of all the samples sequenced. 12.12%(4/33) of the samples had major drug resistance mutations to reverse transcriptase inhibitor drugs. There were Minor mutations and polymorphisms in all the 33 isolates analyzed for subtypes and mutations.
Conclusions: The study has shown that HIV-1 subtype A1 dominates Kirinyaga County population while HIV drug resistance is significant (12.12%) in the population. Information on subtypes and DRMs generated from this study will help in updating the HIV surveillance data generated annually by the Kenya Medical Research Institute together with other Research Institutes carrying out HIV-1 research and monitoring. The data can also be useful in advising on policy for diagnosis and development of HIV-1 vaccines in the country.

Keywords: *HIV-1, subtypes, Anti-retroviral drug resistance, Kirinyaga*

1. Background

The control of infectious diseases largely depends on the understanding of the pathogens' genetic make-up, genetic diversity and their evolution over time. HIV-1 has four groups, M, N, O, and P, each of which has its origin from distinct zoonotic transmission events from Simian Immuno-Deficiency Virus (SIV) strains found in apes (Leoz, et al., 2015). HIV-1 group M is responsible for the pandemic and is currently divided into nine clades or subtype classes, namely, subtypes A, B, C, D, F, G, H, J, and K. Subtypes A and F are further sub-divided into distinct sub-clades known as A1 and A2, and F1 and F2 respectively (Worobey, et al., 2008; Leoz, et al., 2015). In addition, there are inter-subtype recombinants referred to as circulating recombinant forms (CRFs) and unique recombinant forms (URFs). The Los Alamos National Laboratory HIV database presently recognizes 72 CRFs together with numerous URFs (Tongo, et al., 2015). The most predominant subtype in Kenya is subtypes A with differences in the predominance of subtypes C, D, and CRFs (Dowling, et al., 2002; Lihana, et al., 2006; Khamadi, et al., 2009; Hue´, et al., 2012; Adungo, et al., 2014). A greater percentage of recombinants are A-containing recombinant strains such as A1C, A1D, A1G, A2D (Dowling, et al., 2002; Khamadi S. , 2005; Lihana, et al., 2006; Kageha, et al., 2012).

Highly active antiretroviral therapy (HAART) has considerably minimized the number of people dying from AIDS in recent years (Broder, 2010). However, this has also led to the emergence of drug resistant strains due to selective pressure of antiretroviral (ARV) drugs and the high mutation rate of HIV (Vandamme A. , 2009). The emergence of resistance-associated mutations remains a major cause for treatment failure (Jochmans, 2008)**.** Between 2003 and 2010, drug resistance prevalence in poor resource settings rose to reach a peak of 6.6 percent in the year 2009 (WHO, 2012). There is a clear indication that the levels of resistance to NNRTI in Africa is increasing. The prevalence of NNRTI resistance among recently infected persons reached 3.4% in 2009 (WHO, 2012; Hamers, Sigaloff, Kityo, Mugyenyi, & de Wit, 2013). There are three main mechanisms which HIV uses to generate genetic diversity namely, retroviral recombination, mutation, and hypermutation (Smyth, Davenporte, & Mak, 2012; Vandamme, et al., 2011). In retroviruses, introduction of most mutations occurs during reverse transcription. RT makes transcription errors without the ability to proofread (Smyth, Davenporte, & Mak, 2012). In every replication cycle, RT causes approximately one error per genome (Kerina, Babill, & Muller, 2013). Genetic recombination on the other hand occurs when there is dual infection with at least two genetically diverse HIV strains coexisting in the same cell arising from a co-infection or a superinfection (Kerina, Babill, & Muller, 2013; Redd, Quinn, & Tobian, 2013; Smyth, Davenporte, & Mak, 2012).Recombination shuffles mutations within a quasispecies with the result of rapidly assembling beneficial genetic combinations or effectively removing mutations that compromise viral fitness. Genetic recombination is an evolutionary strategy that enables survival of viruses with superior fitness in a dynamic environment(Smyth, Davenporte, & Mak, 2012; Kerina, Babill, & Muller, 2013)**.** Hypermutations are mediated by mutagenic host cell factors such as APOBEC3G. The APOBEC3G is a host cytidine deaminase incorporated by the virion that generates Guanine to Adenine hypermutations by deaminating cytosine to uracil. This hinders elongation by inhibiting reverse transcription in viruses with a defective Vif gene (Smyth, Davenporte, & Mak, 2012; Engelman & Cherepanov, 2012)**.** NRTIs are administered in an inactive form and must first be metabolised by host cellular kinases to their respective 5'-triphosphate active form (Sarafianos, et al., 2009). NRTIs block HIV by terminating reverse transcription. This is by mimicking natural dNTPs allowing them to be integrated by reverse transcriptase into the viral DNA. Since NRTIs lack the 3´-hydroxyl group, their incorporation into the chain terminates elongation(Engelman & Cherepanov, 2012). There are two mechanisms through which NRTI resistance develops. One way is through inhibition and consequently reduced NRTI incorporation for dNTPs, while the other involves increased elimination of the integrated nucleoside analogue monophosphate (NA-MP) by unblocking the primer thus terminating DNA chain extension as in the case of AZT (Shafer & Schapiro, HIV-1 Drug Resistance Mutations: An Updated Framework for the Second Decade of HAART, 2008). NNRTIs unlike NRTIs, non-competitively inhibit reverse transcription without interfering with dNTP binding. Unlike NRTIs, NNRTIs manifest their pharmacological action without intracellular stimulation (Sarafianos, et al., 2009). They do not bind to the reverse transcriptase enzyme's active site but instead bind to the allosteric hydrophobic site or nucleotide binding site which is about 10 Å away from the polymerase active site where they stop transcription by blocking the addition of more nucleotides and the polymerization of the double-stranded DNA (Grobler, et al., 2007; Sarafianos, et al., 2009; Das & Arnold, 2013). They normally lack the 3'-OH group or have an altered sugar moiety. They normally bind to the allosteric hydrophobic NNRTI-binding pocket (NBP) near the active site causing conformation changes. This causes a blockage that disrupts the substrate alignment in the formation the phosphodiester bond (Rittinger, Divita, & Goody, 1995).

Accurate and updated information on HIV subtypes distribution is crucial in the development of vaccines since vaccine immunogen sequences should have a close match to the sequences of the virus in circulation in the targeted population (Hemelaar, Gouws, Ghys, & Osmanov, 2011). *Pol* sequence data from genotypic resistance tests is used for HIV-1 subtyping and also to screen for drug resistance mutations (DRMs). This data may also be helpful in conducting phylogeographic analysis to enlighten more on the origin and distribution of the diverse HIV strains (Rusine, et al., 2012).

2. Methods

2.1. Participants

The study was carried out in Kirinyaga County in central Kenya and was based atthe County Hospital. The hospital serves patients from the entire County. Samples were collected from confirmed HIV positive clients attending the County Hospital and who were already on antiretroviral therapy. The clients were both male and female HIV patients of eighteen years and above voluntarily attending the comprehensive care clinic at the hospital and voluntarily submit blood samples for testing to the laboratory. This was a cross-sectional study where purposive sampling was used to get sixty (60) HIV positive blood samples using the formula; $N=Z^2P\ (1-$

P) D/d^2 (Fisher *et al*., 1998)**.** The amount of whole blood collected was 3 ml per person. The samples were tested by rapid HIV diagnostic kits and confirmed to be antibody positive for HIV before they were put in airtight EDTA tubes and transported to the research centre in cool boxes at room temperature.

Baseline characteristics of participants is as summarized in table1 below. Thirty-three samples out of the 60 collected were successfully amplified, sequenced and analysed. Of the 33 isolates, 19 were from females and 14 were from males. The Median CD4 countof all the subjects was 398 cells/µl (CD4 range 75 - 773 cells/ul) with that of females being 361cells/µl and that of males' 366.5cells/µl. The median age of all subjects was 39 years.

Characteristic	**Gender**		
	All N=33	**Female (n=19)**	**Male (n = 14)**
Age (Years) Mean (Range)	39(18-70)	37(18-70)	39.5(18-63)
CD4- T cell count (Cells/mm^3) Mean (Range)	398(75-773)	361(75-773)	366.5(87-696)
Range <300	10	6	4
301-400	7	4	3
401-500	4	2	2
>500	12	7	5

Table 1: Baseline Characteristics of participants

2.2. Proviral HIV DNA Extraction from PBMCs

Briefly, the pellet of PBMCs was dissolved by pipetting in five hundred microlitres of a genomic DNA extraction reagent called DNAzol (Gibco BRL®). Vortexing was not done as this shears and destroys the genomic DNA. The dissolved pellet was then mixed gently with two volumes of chilled (4°C) absolute ethanol without vortexing. Using chilled ethanol increases the efficacy of precipitation of DNA from the solution. The mixture was then spun for 15 minutes at 12000 rpm in a microfuge maintained at 4°C to obtain a pellet. 1000 µl of 70 percent ethanol was added to the pellet and thoroughly vortexed (the pelleted DNA can be vortexed at this stage without shearing). The 70% ethanol acts as a wash solution. The pellet was then isolated by spinning for 15 minutes at 12000 rpm in a microfuge maintained at 4°C. The pellet was then dried at room temperature in a safety cabinet. The DNA pellet was then dissolved in distilled DNAse and RNAse free water and Store at -30°C till further use (Khamadi S. , 2005).

2.3. Polymerase Chain Reaction (PCR)and Sequencing

The Protocol described by Lihana et al. (2009) was adopted. The starting template was proviral DNA extracted from PBMCs. A master mix was made containing 2 mM MgCl, 0.8mM dNTPs, 0.5 units Taq polymerase, 1x Buffer, 2ng of each primer and the DNA Template. A region of the pol-RT gene corresponding to nucleotides 2480 to 3180 of HIV-1 HXB2 was amplified by nested Polymerase Chain Reaction (PCR) using primer sets, RT18 (5'GGAAACCAAAAATGATAGGGGGAATTGGAGG-3') and KS104 (5'-TGACTTGCCCAATTTAGTTTTCCCACTAA-3') in the first round, and KS101 (5'GTAGGACCTACACCTGTTCAACATAATTGGAAG-3') and KS102 (5'-CCCATCCAAAGAAATGGAGGAGGTTCTTTCTGATG-3') in the second round. Amplification conditions were as follows; a hold at of 95 º C for 2 minutes, 35 cycles of 95 º C for 30 seconds, 55 º C for 30 seconds, and 72 º C for 1 minute and a final extension at 72 º C for 10 minute (Lihana, et al., 2009).

The PCR products were then sequenced using the Sanger (Dideoxynucleotide chain termination) method.

2.4. Data Analysis

The generated sequences were assembled, aligned and edited using Geneious v8.1. The National Centre for Biotechnology Information (NCBI) subtyping program (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi), the HIV Blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the REGA HIV-1 Subtyping Tool version 3.0 (http://hivdb.stanford.edu/RegaSubtyping/stanford-hiv/typingtool/ (Peña, et al., 2013), the Recombinant Identification Program (http://www.hiv.lanl.gov/cgi-bin/RIP3/RIP.cgi(Siepel, Halpern, Macken, & Korber, 1995), the jpHMM (jumping profile Hidden Markov Model online submission tool; http://jphmm.gobics.de/ and the SCUEAL Subtyping DataMonkey tool (http://www.datamonkey.org/dataupload_scueal.php (Pond, et al., 2009) were used for initial rapid subtype screening. Confirmation was done by phylogenetic analysis using reference sequences from The Los Alamos website (http://www.hiv.lanl.gov/cgi). The reference sequences were selected by a two-step procedure involving preliminary identification by HIV BLAST searches and verification using Neighbor-Joining tree analyses. Sequence alignment was done using ClustalW method. The Neighbor-Joining method (Saitou & Nei, 1987) was used to infer the evolutionary history of the isolates while computation of the evolutionary distances was done using the Kimura 2-parameter method (Kimura, 1980). Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis software version 7, MEGA7 (Kumar, Stecher, & Tamura, 2015) and Geneious v8.1 (http://www.geneious.com(Kearse, et al., 2012)

Reverse transcriptase nucleotide sequences generated (707bps) were translated into the respective amino acids (235 per sequence). The sequences were then submitted to the Stanford database on HIV drug resistance (http://hivdb.stanford.edu) for evaluation and identification of mutations as well as interpretation of resistance. The Stanford algorithm infers HIV drug resistance against 20 FDA-approved ARV drugs. These include four NNRTIs, seven NRTIs, one integrase inhibitor and eight protease inhibitors (PIs). Each HIV-1 DRM is given a drug penalty score and a remark by the HIV database system. Addition of the scores for each mutation associated with resistance to that drug gives the total score for the drug. The program then classifies HIV drug resistance at five levels based on the total drug score. The levels are susceptible, potential low-level, low-level, intermediate or high-level drug resistance. The 2013 International AIDS Society-USA (IAS-USA) guidelines were used to define antiretroviral resistance-associated mutations for Reverse Transcriptase Inhibitors (RTI) (Johnson, et al., 2013)

3. Results

3.1. Subtypes and Recombinants

There was general concordance between the four tools used in 25/33 (76%) of all sequences. The other 8/33 sequences showed subtype discordance with at least one of the tools. This was probably due to use of different reference sequences by the programs in subtyping. However, all the programs identified subtype A1 as the most common comprising 58% of samples by SCUEAL, 67% by REGA, 70% by jpHMM and 67% by RIP. The next subtypes in terms of occurrence were C and D with values ranging from 9 to 15%. SCUEAL, jpHMM and RIP tools were capable of determining recombinant subtypes while REGA classified the detected recombinants as complex. SCUEAL identified one case of subtype A3 and one case of recombinant CRF-19. These subtypes were ignored as artefacts since they were detected by only one tool and have previously not been reported in Kenya (Lihana, Ssemwanga, Abimiku, & Ndembi, 2012). CRF-19 is predominant in Cuba (Delatorre & Bello, 2013) and to a lesser extent, in Central and West Africa while subtype A3 is predominant in Central and West Africa (Lihana, Ssemwanga, Abimiku, & Ndembi, 2012). Inter-subtype recombinants detected by the various tools included A1-C, A1-D, A2-D, A1G, and CRF-19-like. Table 2 outlines the subtyping results of the four online tools used.

	SCUEAL[a]		REGA[b]		jpHMM[c]		RIP[d]	
Subtypes	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
A1	19	58	22	67	23	70	22	67
A3	1	3						
C	3	9	4	12	4	12	4	12
D	3	9	4	12	4	12	5	15
A1C	1	3						
A1D	2	6			2	6		
A2D	1	3					1	3
A1G	1	3					1	3
CRF19	1	3						
Complex	1	3	3	9				
Total (N)	33		33		33		33	

Table 2: Subtype Designation by SCUEAL, REGA, jpHMM and RIP Tools

a SCUEAL Subtyping DataMonkey tool (Pond, et al., 2009); http://www.datamonkey.org/dataupload_scueal.php). SCUEAL subtype designations of A or ancestral A were called A1, based on the phylogenetic clustering.
b REGA HIV-1 Subtyping Tool version 3.0 (Peña, et al., 2013); http://hivdb.stanford.edu/RegaSubtyping/stanford-hiv/typingtool/)
c jpHMM (jumping profile Hidden Markov Model online submission tool; (Schultz, et al., 2006); http://jphmm.gobics.de/;)
d Recombinant Identification Program (Siepel, Halpern, Macken, & Korber, 1995); http://www.hiv.lanl.gov/cgi-bin/RIP3/RIP.cgi)
The subtypes were then confirmed through phylogenetic analysis using MEGA7 software (Kumar, Stecher, & Tamura, 2015)and Geneious v8.1(http://www.geneious.com) (Kearse, et al., 2012). The two programs were used for comparison purposes. The phylogenetic trees drawn using the two programs were found to be essentially similar in their output. Reference sequences for subtypes A, C, D, G and recombinants A1C, A1D, A2D and A1G from the Los Alamos HIV sequence database (http://www.hiv.lanl.gov/) were used.All the pure subtypes detected clustered together with Kenyan reference strains. One recombinant (Ker80 subtype A1D) clustered with Ugandan strains. Samples that were found to be HIV-1 subtype A1 showed further diverse sub-type clustering in the Neighbour-Joining tree suggesting a divergence in the HIV-1 subtype A1 among the study participants. All the recombinants were related to subtype A (Figure 1 and 2)
In figure 1, evolutionary analyses were conducted in MEGA7 while inFigure 2, the analysis was done using Geneious v8.17. The Neighbor-Joining method (Saitou & Nei, 1987) was used to infer the evolutionary history and construct a phylogenetic tree. The reliability of the tree was estimated by 1000 bootstrap replications in both cases. Kimura two-parameter method was applied to compute the evolutionary distances (Kimura, 1980) and are in the units of the number of base substitutions per site. Both analyses involved 46 nucleotide sequences. The study strains have the prefix Ker. The sequences were gap stripped prior to tree construction (data not shown).

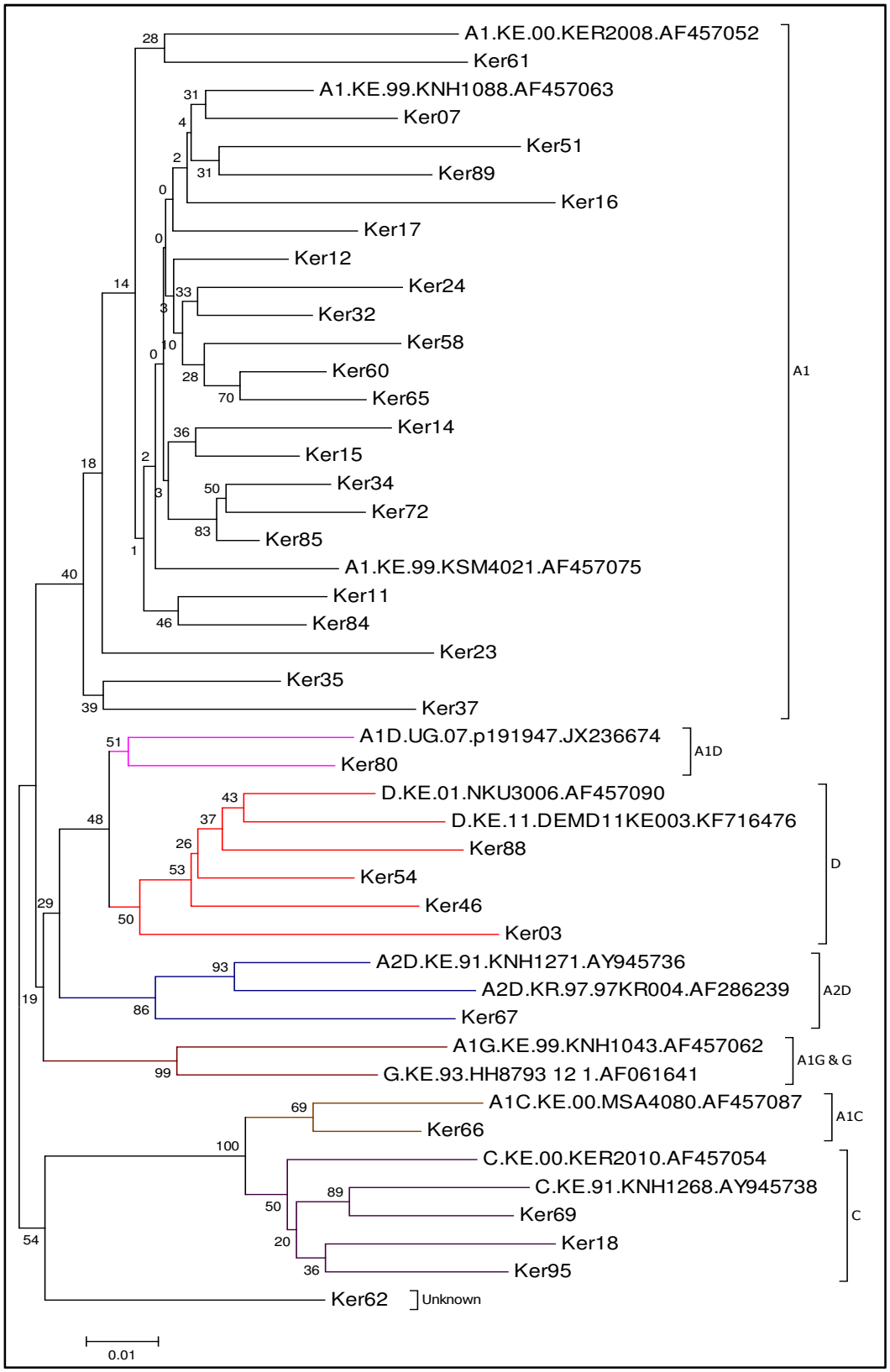

Figure 1: Evolutionary relationships of the study sequences together with 13 references. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2015)

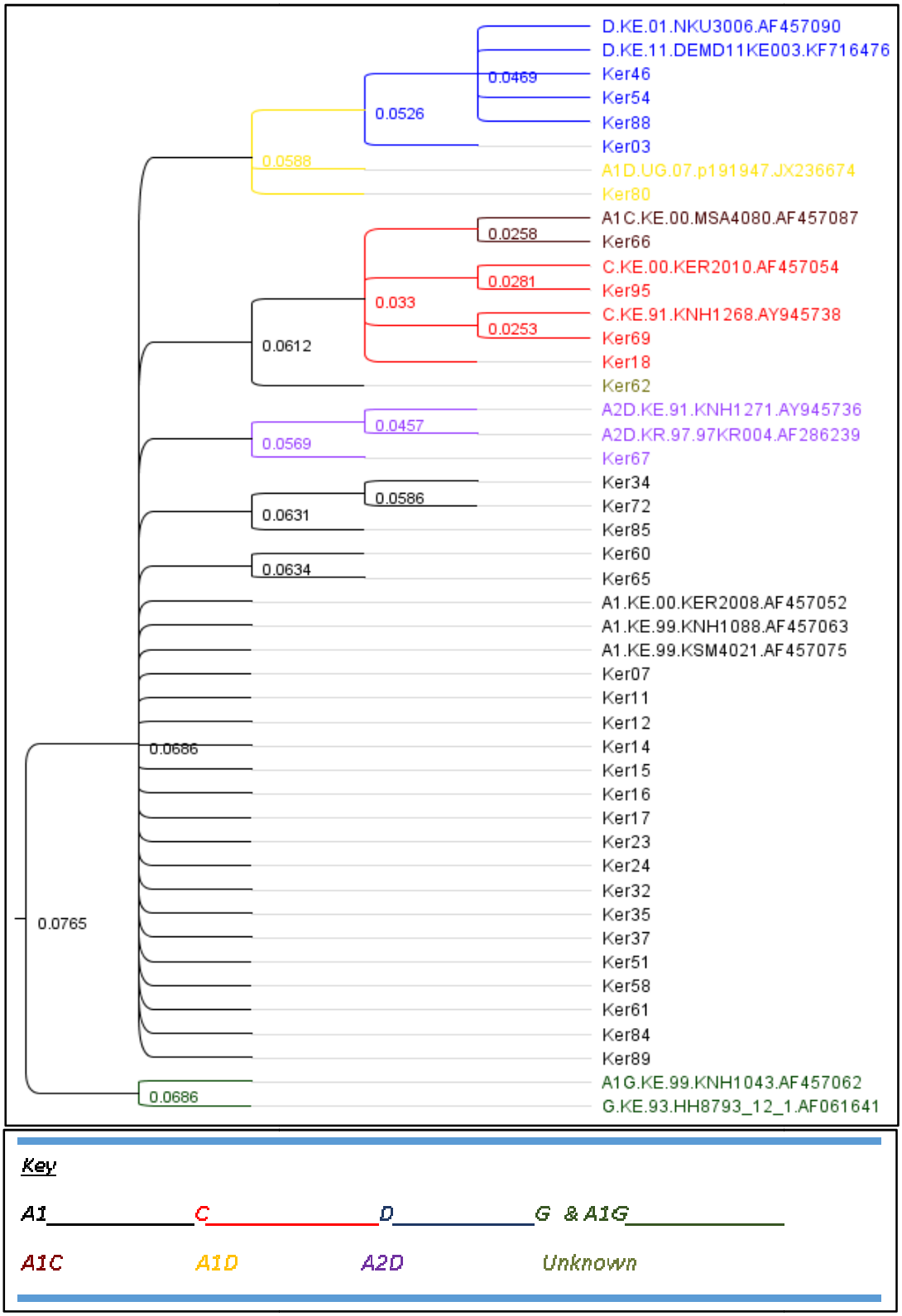

Figure 2: Phylogenetic Neighbor-Joining consensus tree diagram of the 33 sequences together with the reference sequences. Image created using Biomatter's Geneious version 8.1. (Available from http://www.geneious.com)(Kearse, et al., 2012)

Sequence alignment and phylogenetic analysis showed that a majority of the isolates belonged to subtype A1 (22/33, 66.67%), followed by subtypes D (4/33, 12.12%) and C (3/33, 9.09%). There were three cases each of recombinants A1D, A2D and A1C accounting for 3.03%of the samples in each case. One recombinant case could not be classified into any of the known circulating recombinant forms (table 3 and figure 3). Figure 4 shows the comparison of subtype prevalence in other parts of the country compared with the results of the present study.

	Occurrence	Frequency
Subtype	**n=33**	**%**
A1	22	66.66667
D	4	12.12121
C	3	9.090909
A1D	1	3.030303
A2D	1	3.030303
A1C	1	3.030303
Unknown Recombinant (URF)	1	3.030303

Table 3: HIV Subtypes Distribution in the Study Population

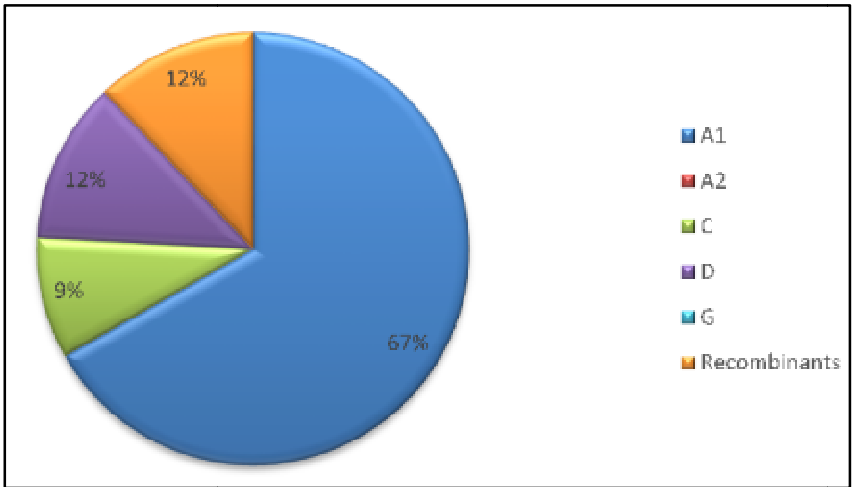

Figure 3: Pie chart of HIV-1 group M subtypes isolated from the study participants

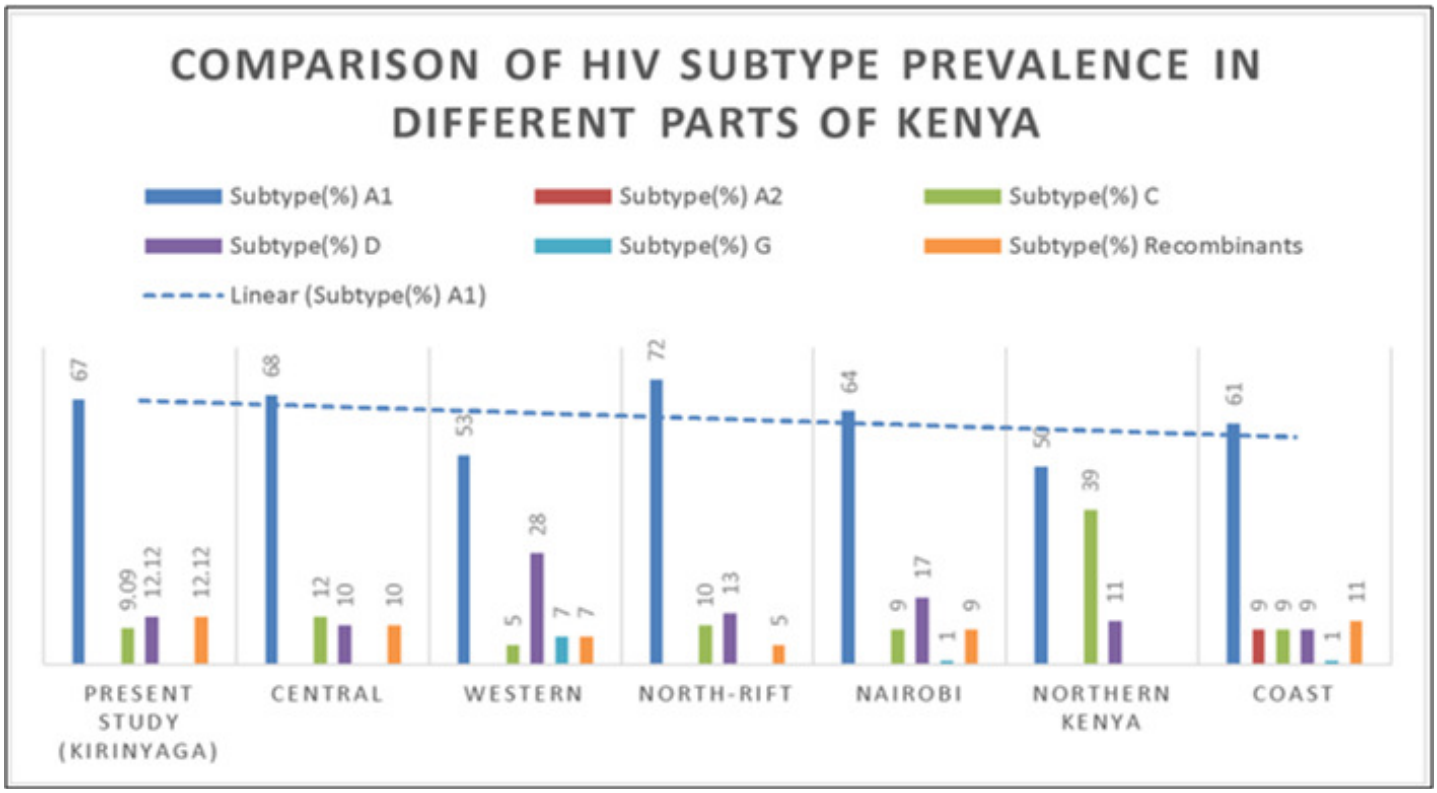

Figure 4: Comparison of HIV subtype prevalence in different parts of Kenya. The data used was obtained from the following publications; Central (Kageha, et al., 2012), Western (Adungo, et al., 2014), North-Rift (Kiptoo, et al., 2008), Nairobi (Lihana, et al., 2006), Northern Kenya (Khamadi S. , 2005) and Coast (Hue´, et al., 2012).

3.2. Drug Resistance Mutations

Of the 33 samples analysed, 4(12.12%) showed mutations conferring resistance to reverse transcriptase inhibitors (RTIs) indicating an HIV-1 drug resistance occurrence rate of 12.12% among the samples sequenced and analysed. The samples showing RTI resistance - associated mutations, were all infected with HIV- 1 subtype A1 and were all female (Table 4). The fact that they were all female may not be significant due to the small sample size. The reverse transcriptase inhibitor-associated major mutations M184I, K70R, A98G, V106I, E138A, and G190A, were detected. M184I and K70R conferred resistance to NRTI while A98G, V106I, E138A and G190A conferred resistance to NNRTI (Table 4). A98G and V106I mutations occurred together in isolate Ker61 while K70R and G190A mutations occurred together in isolate Ker24 (Table 4).

High-level resistance to NRTIs, emtricitabine (FTC) and lamivudine (3TC) was identified in one isolate with intermediate to low level resistance to zidovudine (AZT) and stavudine (d4T) as a result of M184I mutation (Table 4). In the other case of NRTI resistance, mutation K70R conferred resistance to zidovudine (AZT) and stavudine (d4T) but susceptible to lamivudine (3TC) (Table 4). Mutation G190A in isolate Ker24 offered high-level resistance to NNRTI nevirapine (NVP) and low to intermediate resistance to etravirine (ETR) rilpivirine (RPV) and efavirenz (EFV) drugs (Table 4). The other mutations against NNRTI drugs; A98G, V106I, and E138A offered low level to intermediate resistance to NNRTI (Table 4).

Minor mutations and polymorphisms were detected in all the 33 isolates that were analysed (Table 5). K122E and V35T were present in all (100%) of the samples. V60I, I135T, Q174K, Q207A, T200A, R211S, V179I and D177E occurred in over 50% of the isolates (Table 5). Other polymorphisms observed in lower frequencies included D121H, K173S, D123N, D123S, R211K, Q207E, K173E, K173L, S48T, E40D, K11T, K20R, K49R, D123G, F214L, G196E, K173T, S162C, T39A, T39E, E36A, I142V, S162Y, D113E, D121C, D121Y, E204D, E6K, I202V, I5V, K104Q, K11Q, K166Q, Q207D, T39I, V118I and V21I (Table 5).

Evidence of Apobec 3G/F Hypermutation was found in two samples, Ker16 (G51R, G93R and G213R) and Ker51 (G155R and G45R).

Patient ID	**HIV-1 Subtype (Sex)**	**ART at point of study**	**Major DRMs detected**		**Drug Resistance inference**			
			NRTI	NNRTI	Against NRTI		Against NNRTI	
					High Level	Intermediate/Low Level	High Level	Intermediate/Low Level
Ker16	A1(F)	NVP/d4T/3TC	M184I		**3TC** FTC	ABC ddI		
Ker61	A1(F)	NVP/d4T/3TC		A98G V106I				EFV, ETR **NVP,** RPV
Ker17	A1(F)	NVP/d4T/3TC		E138A				ETR RPV
Ker24	A1(F)	NVP/d4T/3TC	K70R	G190A		ABC, AZT **d4T**, ddI, TDF	**NVP**	EFV ETR RPV

Table 4: Major Drug Resistant Mutations Detected Among the HIV Isolates

Drugs whose efficacy was compromised and were part of patient's regimen are highlighted in bold type.

Table 5 shows the minor mutations detected per isolate while Table 6 gives the frequency of the minor mutations detected among the 33 isolates.

Ker03	V35T, T39A, E40D, V60I, K104Q, V118I, D121C, K122E, I135T, I142V, T165V, G196E, T200A, E204D, Q207E, R211K, F214L
Ker07	V35T, T39L, D123N, V60I, I135T, K122E, I142V, K173S, D177E, Q174K, Q207A, T200A, V179I, R211S
Ker11	Q207A, K122E, V35T, T39I, S48T, D121H, V60I, I135T, D123S, S162Y, K173S, R211S, Q207A, T200A, Q174K
Ker12	V60I, Q174K, R211S, Q207A, V179I, K223Q, K122E, K173L, I135T, V35T, D123N, D177E, T200A
Ker14	V179I, V60I, I5V, V35T, I135T, D123S, Q174K, K173A, , R211S, Q207A, K122E, D177E,
Ker15	K173T, Q174K, V35T, E36D, K122E, Q207A, D123N, V60I, I135T, V179I, D177E, R211S, K49R
Ker16	V35T, T39M, I50V, G51R, V60I, D86N, G93R, G112E, K122E, I135T, D123N, K173S, Q174K, V179I, G196E, Q207A, T200A, G213R
Ker17	V35T, S68G, K122E, D123N, T39A, E40D, V60I, I135T, K173S, D177E, Q174K, I178V, V179I, G196E, Q207A, T200A, F214L
Ker18	K122E, D123G, Q207E, A158S, E36A, T39E, V35T, S48T, K173A, R211K
Ker23	V60I, V35T, K102Q, D123S, K122E, S162C, K173Q, D177E, Q207A, R211K
Ker24	E6K, K32T, V35T, N57T, K122E, V60I, I135T, K166Q, K173T, D177E, Q174K, I178L, T200A, V179I, R211S, Q207A
Ker32	T200A,V60I, D121Y, K11Q, , R211S, K122E, V35T, D123G, K173L, I135T, D177E, Q174K, Q207A, V179I
Ker34	T200A, D121H, I5V, K20R, V60I, V35T, I135T, D123S, K173S, D177E, Q174K, V179I, R211S, Q207A, K122E
Ker35	V35T, I135T, K173T, T39I, K122E, D123G, , Q174K, Q207A, E224A, V179I, D177E, T200A,
Ker37	V35T, K43M, V60M, K104N, S105L, D113E, V118F, D123S, K122E, S162C, D177E, Q174K, Q207A, T200A, V179I, F214L
Ker46	E6K, V35T, E40D, K49R, K122E, V60I, D123E, I135T, K166Q, D177E, Q174K, I178M, T200I, Q207D, R211S
Ker51	T165I, K173S, T200A, I202V, Q207A, K122E, V60I, V35T, V21I, G45R, I135T, D123N, G155R, V179I, D177E,
Ker54	V179I, D177E, V35T, D123N, Q174E, , Q207E, R211K, K122E, V60I
Ker58	R211S, Q207A, K11Q, T39A, K122E, D121H, V60I, K173S, D177E, V35T, K20R, I135T, T139A
Ker60	K11T, V35T, T39A, V60I, V118I, D177E, D121H, R211S, Q207A, K173L, I135T, Q174K, K122E
Ker61	, D177E, I202V V35T, V21I, T200A, V179I, K20R, T39R, Q207N, R211S K43E, V60I, S162Y,D121Y, K122E, K173S, Q174K
Ker62	V35T, K20R, E36D, V60I, V118L, K122E, D121H, D123S, S162C, L168F, K173A, D177E, Q174K, G196E, T200A, Q207D, R211N
Ker65	K11T, V35T, K43R, S48E, K122E, D121H, V60I, I135T, I142V, K173L, D177E, Q174K, T200A, R211S, Q207A, F214L
Ker66	E36A, V35T, T39E, S48T, K122E, D123N, K173A, D177E, T200A, Q207E, R211K
Ker67	K11T, V35T, T39N, K122E, D121H, I135T, S162C, Q174R, V179I, D177E, Q207E, R211K
Ker69	K20R, E36A, V35T, T39E, S48T, K122E, V60I, D123G, I135V, K166R, K173T, D177E, T200A, Q207K, R211K
Ker72	V60I, V35T, D113E, K122E, D121H, I135T, D123N, K173S, D177E, Q174K, R211S, Q207A, K220T
Ker80	V35T, K49R, V60I, K104Q, K122E, K173L, D177E, Q174K, R211S, Q207A
Ker84	K11T, V35T, K43N, D121H, V60I, D123S, K122E, I135T, S162Y, K173L, D177E, Q174K, T200A, V179I, R211S, Q207A
Ker85	V60I, V35T, K122E, D121H, I135T, D123S, K173S, D177E, Q174K, V179I, R211S, Q207A
Ker88	E6D, K32I, V35T, T39K, K49R, V60I, R83K, D121C, K122E, K173E, Q207E, R211K
Ker89	E6Q, K11T, V35T, S48T, K49R, K122E, D121H, V60I, I135T, E169D, K173S, D177E, Q174K, T200A, V179I, R211S, Q207A
Ker95	V35T, T39E, S48T, V90F, K104R, K122E, K173A, T200A, I202M, Q207E

Table 5: Minor Mutations Detected Per Isolate

Mutation	Frequency	Percentage	Mutation	Frequency	Percentage
K122E	33	100	E6D	1	3.030303
V35T	33	100	E6Q	1	3.030303
V60I	27	81.81818	G112E	1	3.030303
I135T	22	66.66667	G155R	1	3.030303
Q174K	22	66.66667	G213R	1	3.030303
Q207A	22	66.66667	G45R	1	3.030303
T200A	20	60.60606	G51R	1	3.030303
R211S	18	54.54545	G93R	1	3.030303
V179I	18	54.54545	I135V	1	3.030303
D177E	17	51.51515	I178L	1	3.030303
D121H	11	33.33333	I178M	1	3.030303
K173S	11	33.33333	I178V	1	3.030303
D123N	9	27.27273	I202M	1	3.030303
D123S	8	24.24242	I50V	1	3.030303
R211K	8	24.24242	K102Q	1	3.030303
Q207E	7	21.21212	K104N	1	3.030303
K173E	6	18.18182	K104R	1	3.030303
K173L	6	18.18182	K166R	1	3.030303
S48T	6	18.18182	K173Q	1	3.030303
E40D	5	15.15152	K220T	1	3.030303
K11T	5	15.15152	K223Q	1	3.030303
K20R	5	15.15152	K32I	1	3.030303
K49R	5	15.15152	K32T	1	3.030303
D123G	4	12.12121	K43E	1	3.030303
F214L	4	12.12121	K43M	1	3.030303
G196E	4	12.12121	K43N	1	3.030303
K173T	4	12.12121	K43R	1	3.030303
S162C	4	12.12121	L168F	1	3.030303
T39A	4	12.12121	N57T	1	3.030303
T39E	4	12.12121	Q174E	1	3.030303
E36A	3	9.090909	Q174R	1	3.030303
I142V	3	9.090909	Q207K	1	3.030303
S162Y	3	9.090909	Q207N	1	3.030303
D113E	2	6.060606	R211N	1	3.030303
D121C	2	6.060606	R83K	1	3.030303
D121Y	2	6.060606	S105L	1	3.030303
E204D	2	6.060606	S48E	1	3.030303
E6K	2	6.060606	S68G	1	3.030303
I202V	2	6.060606	T139A	1	3.030303
I5V	2	6.060606	T165I	1	3.030303
K104Q	2	6.060606	T165V	1	3.030303
K11Q	2	6.060606	T200I	1	3.030303
K166Q	2	6.060606	T39K	1	3.030303
Q207D	2	6.060606	T39L	1	3.030303
T39I	2	6.060606	T39M	1	3.030303
V118I	2	6.060606	T39N	1	3.030303
V21I	2	6.060606	T39R	1	3.030303
A158S	1	3.030303	V118F	1	3.030303
D123E	1	3.030303	V118L	1	3.030303
D86N	1	3.030303	V60M	1	3.030303
E224A	1	3.030303	V90F	1	3.030303

Table 6: Frequency of the Minor Mutations Detected Among the Isolates

4.Discussion

4.1. HIV Subtype Diversity

HIV-1 subtype A1 was found to be the most dominant virus in circulation within Kirinyaga County with 66.67% occurrence followed by subtype D (12.12%) and C (9.09%). Even though this was a smaller sample size, it conforms well with findings in a study conducted in central Kenya, where 68% of the samples analysed were subtype A1, 10% were subtype C, 12% subtype D, while the remaining 10% were circulating recombinant forms (Kageha, et al., 2012). This is noteworthy since Kirinyaga County is found within the wider former Central Province of Kenya. In another study conducted in North Rift Kenya on the prevalence of nevirapine-associated resistance mutations among antenatal clinic attendees, subtype A1 accounted for 71.8 % of total samples, D accounted for 12.8%, C for 10.3%, A2 for 2.6%, and G accounted for 2.6% of total samples (Kiptoo, et al., 2008). In the coastal region in Kilifi County, a predominance of subtype A1 was reported with a lower prevalence of subtypes A2, C, D, G and a number of recombinants(Hue´, et al., 2012). Lihana *et al.,* (2012) documents HIV-1 subtype A1 as the most predominant in Kenya. He recognizes other pure subtypes circulating in Kenya as A2, D, C, and G(Lihana, Ssemwanga, Abimiku, & Ndembi, 2012).

In this study, three inter-subtype recombinants were found, namely, A1/D, A2/D, and A1/C accounting for 9.09% of the samples analysed. Kageha *et al.,* (2012) in her study in central Kenya had found three recombinant types, namely; A1/D (6%), A1/G (2%) and C/D (1%)(Kageha, et al., 2012). In a study in Nairobi region, six diverse recombinants were documented; A1/C, A1/D, G/A1, A1/A2, C/A1, D/A1 and D/A2 accounting for 24.5% of the samples studied(Lihana, et al., 2006). Subtypes A1/C and A1/D were also found in Western Kenya(Adungo, et al., 2014). From these studies, it is evident that a high proportion of the recombinations revolve around the most prevalent subtype, A1. The HIV subtype's prevalence in different parts of the country as discussed above is represented in Figure 4 above.

Knowledge of subtypes can be used in determining the best drug regimens for use in the treatment of HIV. Seventy to eighty percent of individuals with subtype C have been reported to have NVP resistant mutations such as K103N and Y181C against only 42% of people with subtype A (Brenner, 2007). In this study, however, all the mutations detected were from clients with subtype A. Subtyping may also be important in deciding the best stage to initiate therapy in future since some studies have determined that the rate of disease progression may vary with subtypes. People infected with subtype A virus tend to progress to AIDS at a slower rate than those infected with subtype D viruses in East Africa (Kaleebu, et al., 2002; Kiwanuka, et al., 2008). Patients with subtype C viruses have a higher risk of disease than those with subtype B viruses (Wainberg M. A., 2015).

4.2. HIV Drug Resistance Mutations

Mutations that confer RTI resistance occurred in 12.12% of the patients. Of the drug resistant mutations detected in this study, M184I and K70R compromised efficacy to NRTI while A98G, V106I, E138A and G190A compromised efficacy to NNRTI. The most common non-B HIV transmitted drug resistance mutations (TDRM), Y188C/H/L, Y181C/I, and K103N were not detected in this study (Singh, et al., 2014). In a study published in 2012, eight years after the start of HAART in East Africa, the estimated prevalence of HIV-1 drug resistance had been approximated at 7.4% (4.3-12.7) (Gupta, et al., 2012). The same study reported that East Africa had the highest rate of increase at approximately 29% per annum (95%CI; p=0.0001). Four cases or 7.5% of transcriptase inhibitor (RTI) resistance-associated mutations were reported in three patients having NRTI resistance mutations, such as D67N, K65R, K70R, M184V, and K219Q in a study in Nairobi. The same study also reported the detection of NNRTI resistance mutations, Y181C in one patient and K103N in three patients (Lihana, et al., 2009).

The HIV-1 RTlacks proofreading ability and therefore is extremely error-prone resulting to many polymorphisms. Of all the polymorphisms detected, two were notable. V179I is a polymorphic mutation that occurred in 18 samples (54.54%). It is frequently selected in patients receiving RPVandETR with little or no effect on NNRTI susceptibility. The other is V118I found only in two cases (6.06%). This is a polymorphic accessory NRTI-resistance mutation that often occurs together with multiple TAMs (Thymidine analogue mutations) such as those occurring at RT positions 41, 67, 70, 210, 215, and 219. TAMs have been shown to confer clinically significant resistance to each of the NRTIs except 3TC (Shafer, Rationale and Uses of a Public HIV Drug-Resistance Database, 2006; Marcelin, 2006). Additionally, other mutations not on the International AIDS Society (IAS) list have been found to be linked to ARV drugs exposure (Cane, Green, Fearnhill, & Dunn, 2007). Populations with many minor mutations that may have influence or no influence on the main/major ART-associated mutations do occur among both drug naive and ARV-treated populations and may have either an inverse or a direct correlation to resistance (Saracino, et al., 2006).

Minor mutations such as E36A, S48T, K49R, V60I, T200A, V179I, Q207A/E/D, R211S/K, and F214L have been identified as naturally occurring subtype B-associated polymorphisms (Nyombi, Holm-Hansen, Kristiansen, Bjune, & Müller, 2008). The same author has documented polymorphic amino acid residues in consensus sequences of subtype A such as R211S, Q207A, and V60I, subtype C; T200A, S48T, and E36A and subtype D; V60I and K49R in RTI drug positions in Kenyan isolates as also noted in this study.

The major reasons for selection of acquired drug resistance (ADR) in patients under treatment are suboptimal treatments and non-adherence to medication. Acquired drug resistance is then transmitted from both untreated and treated patients carrying transmitted drug resistance (TDR) mutations (so-called onward transmission) to ART-naive individuals (De Luca & Zazzi, 2015). The prevalence of TDR associated with NNRTIs is on the rise especially in east and southern Africa where NNRTI regimens are very common(Siliciano & Siliciano, 2013). The build-up of these mutations during first-line therapy is preventable through early testing and a well-timed switch to second-line ART (Hamers, Sigaloff, Kityo, Mugyenyi, & de Wit, 2013). Strengthened adherence support

should also be prioritised and emphasised before virologic treatment failure and treatment switch. In the case where virologic failure is detected and confirmed, unnecessary/premature switches can be prevented by targeted HIVDR testing (Hassan, et al., 2014).

Patient-directed treatment should be guided by constant HIV-1 genotyping and assessment of drug resistance at the start and throughout therapy. This eventually will help to decrease cases of therapy failure in the face of rising HIV-1 diversity and limited ART options (Kiptoo, et al., 2013; Johnson, et al., 2013). Genotypic testing for drug resistance is beneficial in designing the best treatment regimen for individual patients. The data generated can additionally be used to enrich a local HIV database that is more focused on local strains of the virus providing information on HIV resistance mutations. This is especially important due to a relatively lower availability of banked sequences from non-B subtype predominant areas (Wainberg & Brenner, 2010). Generating a database on HIV drug-resistance using Kenyan strains can be of major benefit in future HIV/AIDS clinical management(Kebira & Khamadi, 2011). Due to the high cost of genotypic resistance testing and the lack of technical capacity in low and middle income countries such as Kenya, it is viable to invest in surveillance and monitoring of drug resistance in order to minimize the overall negative impact of the HIV pandemic.

The growing number of resistance mutations and the high viral diversity has made the interpretation of HIV-1 sequence data more complex (Booth, et al., 2007). This makes it rather problematic in certain instances to distinguish between transmitted drug resistance mutations from natural polymorphisms. In this study, sampling was done after ART commencement. Hence it could not be determined whether the mutations were primary or secondary. This is important in drug resistance surveillance to determine whether mutations are occurring naturally or due to selective drug pressure. This information is also crucial in understanding the rate at which the virus is evolving. Treatment histories were not entirely available for the current data set. Such information would have provided additional insights such as how treatment history affected the genotypic variation within a phenotypic cluster.

This study has had its limitations primarily since it was self-sponsored. There were many challenges in the acquisition of reagents which sometimes involved ordering from outside the country. The staff at KEMRI however, were most helpful assisting where they could to ensure the completion of this study. The other major problem faced was the failure of DNA amplification probably due to contamination of reagents or machine failure. This problem was alleviated by ordering for new primers and PCR kit, though still, the success rate was modest. This accounts for the relatively low sample size than anticipated at the beginning of the study.Proviral DNA was used instead of plasma RNA in this study. Although proviral DNA may not be the best representative of the circulating viral genotype as would be the case from RNA in people who had relatively longer infection times, it has been shown that resistance mutations on the proviral DNA can be similar to the mutations on the plasma viral sequences at the time of virological failure (Boucher, et al., 2005). Archived viruses can become active when the ART selective pressure compromises the current plasma population (Joos, et al., 2008). It is, therefore, important to document the proviral population as one way of monitoring the emergence of drug resistance.

The fact that all the mutations noted in this study were all from female clients who also happened to be subtype HIV-1A could be merely coincidental, although it is known that HIV prevalence rate is higher in women than in men in Kenya.

5. Conclusion

(i) This study has determined that HIV-1 subtype A1 is the most predominant subtype in Kirinyaga County accounting for 66.67%, followed by subtypes C and D at 12.12% each.

(ii) Recombinants were also detected within the County with an occurrence of9.09% of all the samples with one recombinant each for A1D, A2D, and A1C.

(iii) (ii) Despite the fact that the sample size may not have been large enough to shed more light on the prevalence trend of the various HIV-1 subtypes, the figures are clearly consistent with findings in other parts of the country.

(iv) Four out of thirty-three or 12.12% of the samples had major mutations conferring resistance to reverse transcriptase inhibitors indicating an HIV-1 drug resistance occurrence of 12.12%. One isolate (Ker 16) had a high-level resistance to NRTIs, lamivudine (3TC) and emtricitabine (FTC) while another (Ker24) had a mutation associated with high-level resistance to NVP. Minor mutations and polymorphisms were detected in all the 33 isolates analysed.

6. Abbreviations

- 3TC : Lamivudine
- ABC : Abacavir
- ARV : Anti-retroviral
- AZT : Zidovudine
- CRF : Circulating Recombinant Forms
- d4T : Stavudine
- ddI : Didanosine
- dNTPs : Deoxynucleoside triphosphates
- DRMs : Drug Resistant Mutations
- EFV : Efavirenz
- ETR : Etravirine
- HAART : Highly active antiretroviral therapy
- HIVDR : HIV Drug Resistance
- NNRTI :Non-Nucleoside Reverse-Transcriptase Inhibitor
- NRTI : Nucleoside Reverse-Transcriptase Inhibitor
- NVP : Nevirapine
- ORF : Open Reading Frames
- PBMCs : Peripheral Blood Mononuclear Cells
- RPV : Rilpivirine
- RT : Reverse Transcriptase
- TAMs : Thymidine Analogue Mutations)
- TDF : Tenofovir
- TDR : Transmitted Drug Resistance
- URFs : Unique Recombinant Forms

7. Ethics Consent and Permissions

Permission was sought from the Ethical Review Committee at Kenyatta University under the application PKU/250/I 250 "Genetic Diversity of Hiv-1 Isolates and Anti-Retroviral Drug Resistance from Patients in Kirinyaga County, Kenya". Permission also was sought from the medical board of Kerugoya County Hospital where the sample aliquots was sourced. This study was a laboratory analysis one using positive HIV blood samples collected from Kerugoya County hospital, Kenya. Only positive samples were collected from Participants who were required to give consent to voluntarily participate in this research. The samples were given codes and not person names to conceal the identities of their source. Whole blood samples were drawn by technical staff at the HIV testing laboratory by venipuncture using standard sterile techniques. This was a once off sample collection during routine clinical check-up and no additional samples were required from the participants. There was no follow up for patients from whom samples were collected.

Blood samples collected did not have names of the source individuals but were given codes. All the information concerning the project was explained in detail and clearly to the head of the hospital and subsequently to the study participants. A form was signed by the County Chief medical officer giving authorization for this exercise to be done. A consent form was also signed by the participants.

8. Availability of Data and Materials

The sequences have been deposited in the GenBank database with accession numbers KT721738 - KT721770.

9. Competing Interests

The authors declare no conflicting interests.

10. Funding

There was no external funding for this project. It was purely self-funded.

11. Authors' Contributions

CMN, MMG, SAK and RL conceived and designed the experiments, CMN, JM and SK carried out the laboratory work, CMN and JM analyzed the data, CMN wrote the paper and SAK and MMG approved the final manuscript.

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13. References

i. Adungo, F. O., Gicheru, M. M., Adungo, N. I., Matilu, M. M., Lihana, R. W., & Khamadi, S. A. (2014). Diversity of Human Immunodeficiency Virus Type-1 Subtypes in Western Kenya. World Journal of AIDS, 4, 365-372. doi:10.4236/wja.2014.44043

ii. Booth, C. L., Garcia-Diaz, A. M., Youle, M. S., Johnson, M. A., Phillips, A., & Geretti, A. M. (2007). Prevalence and predictors of antiretroviral drug resistance in newly diagnosed HIV-1 infection. Journal of Antimicrobial Chemotherapy, 59, 517–524.

iii. Boucher, S., Recordon-Pinson, P., Neau, D., Ragnaud, J. M., Titier, K., Faure, M., . . . Masquelier, B. (2005). Clonal Analysis of HIV-1 Variants in Proviral DNA During Treatment Interruption in Patients With Multiple Therapy Failures. J Clin Virol, 34, 288-294.

iv. Brenner, B. G. (2007). Resistance and Viral Subtypes: How Important are the Differences and Why do They Occur? Curr Opin HIV AIDS, 2, 94-102.

v. Broder, S. (2010). The Development of Antiretroviral Therapy and its Impact on the HIV-1/AIDS Pandemic. Antiviral Res, 85, 1-18.

vi. Cane, P., Green, H., Fearnhill, E., & Dunn, D. (2007). Resistance Identification of Accessory Mutations Associated With High-Level Resistance in HIV-1 Reverse Transcriptase. AIDS, 21, 447–455.

vii. Das, K., & Arnold, E. (2013). Hiv-1 reverse transcriptase and antiviral drug resistance. Curr. Opin. Virol., 3. part1, 111–118.

viii. De Luca, A., & Zazzi, M. (2015). Interplay Between Transmitted and Acquired HIV Type 1 Drug Resistance: Reasons for a Disconnect. The Journal of Infectious Diseases , 212, 5-7. doi:10.1093/infdis/jiv008

ix. Delatorre, E., & Bello, G. (2013). Phylodynamics of the HIV-1 Epidermic in Cuba. PLoS ONE, 8(9), e72448.

x. Dowling, W. E., Kim, B., Mason, C. J., Wasunna, K. M., Alam, U., Elson, L., . . . J, C. K. (2002). Forty-One near Full-Length HIV-1 Sequences from Kenya Reveal an Epidemic of Subtype A and A-Containing Recombinants. AIDS, 16(13), 1809-1820.

xi. Engelman, A., & Cherepanov, P. (2012, April). (2012). The structural biology of HIV-1: mechanistic and therapeutic insights. Nature Reviews Microbiology, 10, 279-290.

xii. Grobler, J. A., Dornadula, G., Rice, M. R., Simcoe, A. L., Hazuda, D. J., & Miller, M. D. (2007). HIV-1 Reverse Transcriptase Plus-Strand Initiation Exhibits Preferential Sensitivity to Non-Nucleoside Reverse Transcriptase Inhibitors in Vitro. J Biol Chem, 282, 8005-8010.

xiii. Gupta, R. K., Jordan, M. R., Sultan, B. J., Hill, A., Davis, D. H., Gregson, J., . . . Bertagnolio, S. (2012). Global trends in antiretroviral resistance in treatment-naive individuals with HIV after rollout of antiretroviral treatment in resource-limited settings: a global collaborative study and meta-regression analysis. Lancet 2012, 380, 1250–58.

xiv. Hamers, R. L., Sigaloff, K. C., Kityo, C., Mugyenyi, P., & de Wit, T. F. (2013). Emerging HIV-1 drug resistance after roll-out of antiretroviral therapy in sub-Saharan Africa . Curr Opin HIV AIDS, 8, 19–26.

xv. Hassan, A. S., Nabwera, H. M., Mwaringa, S. M., Obonyo, C. A., Sanders, E. J., Rinke de Wit, T. F., . . . Berkley, J. A. (2014). HIV-1 virologic failure and acquired drug resistance among first-line antiretroviral experienced adults at a rural HIV clinic in coastal Kenya: a cross-sectional study. AIDS Res Ther, 11(9). doi:10.1186/1742-6405-11-9

xvi. Hemelaar, J., Gouws, E., Ghys, P. D., & Osmanov, S. (2011). Global trends in molecular epidemiology of HIV-1 during 2000-2007. AIDS, 25, 679–689.

xvii. Hue´, S., Hassan, A. S., Nabwera, H., Sanders, E. J., Pillay, D., Berkley, J. A., & Cane, P. A. (2012). HIV Type 1 in a Rural Coastal Town in Kenya Shows Multiple Introductions with Many Subtypes and Much Recombination . AIDS Research and Human Retroviruses , 28(2).

xviii. Jochmans, D. (2008). Novel HIV-1 Reverse Transcriptase Inhibitors. J. Virus Research, 134, 171–185.

xix. Johnson, V. A., Calvez, V., Günthard, H. F., Parades, R., Pillay, D., Shaffer, R. W., . . . Richman, D. D. (2013, Feb-Mar). Update of the drug resistance mutations in HIV-1. Top Antivir Med., 21(1), 6-14.

xx. Joos, B., Fischer, M., Kuster, H., Pillai, S. K., Wong, J. K., Boni, J., & et al. (2008). HIV rebounds from latently infected cells, rather than from continuing low-level replication. . Proc Natl Acad Sci USA, 105, 16725-16730.

xxi. Kageha, S., Lihana, R. W., Okoth, V., Mwau, M., Okoth, F. A., Songok, E. M., . . . Khamadi, S. A. (2012, Feb). HIV type 1 subtype surveillance in central Kenya. AIDS Res Hum Retroviruses, 28(2), 228-231.

xxii. Kaleebu, P., French, N., Mahe, C., Yirrell, D., Watera, C., Lyagoba, F., . . . Whitworth, J. (2002). Effect of human immunodeficiency virus (HIV) type 1 envelope subtypes A and D on disease progression in a large cohort of HIV-1-positive persons in Uganda. J Infect Dis, 185, 1244–1250.

xxiii. Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., . . . Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647-1649.

xxiv. Kebira, N. A., & Khamadi, A. S. (2011). HIV-1 drug resistance among untreated patients in Kenya: Current status. Journal of Applied Biosciences, 43, 2958 – 2966.

xxv. Kerina, D., Babill, S., & Muller, F. (2013). HIV Diversity and Classification, Role in Transmission. Advances in Infectious Diseases, 3, 146-156. doi:http://dx.doi.org/10.4236/aid.2013.32022

xxvi. Khamadi, S. (2005). HIV Type 1 Subtypes in Circulation in Northern Kenya. AIDS RESEARCH AND HUMAN RETROVIRUSES, 21(9), 810–814.

xxvii. Khamadi, S. A., Lihana, R. W., Osman, S., Mwangi, J., Muriuki, J., Lagat, N., . . . Okoth, F. A. (2009). Genetic diversity of HIV type 1 along the coastal strip of Kenya. AIDS Res Hum Retroviruses, 25, 919-923.

xxviii. Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution, 16, 111-120.

xxix. Kiptoo, M., Brooks, J., Lihana, R. W., Sandstrom, P., Ng'ang'a, Z., Kinyua, J., . . . Songok, E. M. (2013). HIV-1 drug resistance-associated mutations among HIV-1 infected drug-naïve antenatal clinic attendees in rural Kenya. BMC Infectious Diseases , 13, 517. doi:10.1186/1471-2334-13-517

xxx. Kiptoo, M., Ichimura, H., Wembe, R., Ng'Ang'a, Z., Mueke, J., Kinyua, J., . . . Songok, E. (2008). Prevalence of Nevirapine-Associated Resistance Mutations after Single Dose Prophylactic Treatment among Antenatal Clinic Attendees in North Rift Kenya. Aids Research and Human Retroviruses, 24(12).

xxxi. Kiwanuka, N., Laeyendecker, O., Robb, M., Kigozi, G., Arroyo, M., McCutchan, F., . . . Gray, R. (2008). Effect of human immunodeficiency virus type 1 (HIV-1) subtype on disease progression in persons from Rakai, Uganda, with incident HIV-1 infection. J Infect Dis, 197, 707–713.

xxxii. Kumar, S., Stecher, G., & Tamura, K. (2015). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets . Molecular Biology and Evolution, 33(7), 1870-1874.

xxxiii. Leoz, M., Feyertag, F., Kfutwah, A., Mauclère, P., Lachenal, G., Damond, F., . . . Plantier, J. (2015). The Two-Phase Emergence of Non Pandemic HIV-1 Group O in Cameroon. PLoS Pathog, 11(8), e1005029. doi:doi:10.1371/journal.ppat.1005029

xxxiv. Lihana, R. W., Khamadi, S. A., Kiptoo, M. K., Kinyua, J. G., Lagat, N., Magoma, G. N., . . . Songok, E. M. (2006). HIV type 1 subtypes among STI patients in Nairobi: A genotypic study based on partial pol gene sequencing. AIDS Res Hum Retroviruses, 22, 1172–1177.

xxxv. Lihana, R. W., Khamadi, S. A., Lubano, K., Lwembe, R., Kiptoo, K. M., Lagat, N., . . . Ichimura, H. (2009). HIV Type1 Subtype Diversity and Drug Resistance among HIV Type 1-Infected Kenyan Patients Initiating Antiretroviral Therapy. AIDS Res. Hum. Retroviruses, 25(12), 1211- 1217. doi:10.1089=aid.2009.0007

xxxvi. Lihana, R. W., Ssemwanga, D., Abimiku, A., & Ndembi, N. (2012). Update on HIV-1 Diversity in Africa: A Decade in Review. AJDS Rev., 14, 83-103.

xxxvii. Marcelin, A. G. (2006). Resistance to nucleoside reverse transcriptase inhibitors. In A. M. Geretti (Ed.), Antiretroviral Resistance in Clinical Practice. London: Mediscript.

xxxviii. Nyombi, B. M., Holm-Hansen, C., Kristiansen, K. I., Bjune, G., & Müller, F. (2008). Prevalence of reverse transcriptase and protease mutations associated with antiretroviral drug resistance among drug-naïve HIV-1 infected pregnant women in Kagera and Kilimanjaro regions, Tanzania. AIDS Research and Therapy, 5, 13. doi:10.1186/1742-6405-5-13

xxxix. Peña, A., Faria, N., Imbrechts, S., Libin, P., Abecasis, A., Deforche, K., . . . Vandamme, A. (2013). Automated subtyping of HIV-1 genetic sequences for clinical and surveillance purposes: Performance evaluation of the new REGA version 3 and seven other tools. Infectious Genetics and Evolution, 19, 337-348. doi:10.1016/j.meegid.2013.04.032

xl. Pond, S., Posada, D., Stawiski, E., Chappey, C., Poon, A., Hughes, G., . . . Frost, S. (2009). An Evolutionary Model-Based Algorithm for Accurate Phylogenetic Breakpoint Mapping and Subtype Prediction in HIV-1. PLoS Computational Biology, 5(11), e1000581.

xli. Redd, A., Quinn, T., & Tobian, A. (2013). Frequency and implications of HIV superinfection. Lancet Infect Dis, 13, 622–628. doi:10.1016/S1473-3099(13)70066-5

xlii. Rittinger, K., Divita, G., & Goody, R. (1995). Human immunodeficiency virus reverse transcriptase substrate-induced conformational changes and the mechanism of inhibition by nonnucleoside inhibitors. Proc. Natl. Acad. Sci. USA , 92, 8046–8049.

xliii. Rusine, J., Jurriaans, S., van de Wijgert, J., Cornelissen, M., Kateera, B., Boer, K., . . . Ondoa, P. (2012). Molecular and phylogeographic analysis of human immuno-deficiency virus type 1 strains infecting treatment-naive patients from Kigali, Rwanda. PLoS One, 7(e42557).

xliv. Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4, 406-425.

xlv. Saracino, A., Monno, L., Scudeller, L., Cibelli, D., Tartaglia, A., Punzi, G., . . . Angarano, G. (2006). Impact of Unreported HIV-1 Reverse Transcriptase Mutations on Phenotypic Resistance to Nucleoside and Non-Nucleoside Inhibitors. J Med Virol, 78, 9–17.

xlvi. Sarafianos, S., Marchand, B., Das, K., Himmel, D., Parniak, M., Hughes, S., & Arnold, E. (2009). Structure and Function of HIV-1 Reverse Transcriptase: Molecular Mechanisms of Polymerization and Inhibition. J. Mol. Biol., 385, 693–713.

xlvii. Schultz, A. K., Zhang, M., Leitner, T., Kuiken, C., Korber, B., Morgenstern, B., & M, S. (2006). A jumping profile Hidden Markov Model and applications to recombination sites in HIV and HCV genomes. BMC Bioinformatics, 7, 265.

xlviii. Shafer, R. W. (2006). Rationale and Uses of a Public HIV Drug-Resistance Database. Journal of Infectious Diseases, 194(Suppl 1), S51-8.

xlix. Shafer, R. W., & Schapiro, J. M. (2008). HIV-1 Drug Resistance Mutations: An Updated Framework for the Second Decade of HAART. AIDS Rev., 10(2), 67–84.

l. Siepel, A., Halpern, A., Macken, C., & Korber, B. (1995, Nov). A computer program designed to screen rapidly for HIV type 1 intersubtype recombinant sequences. AIDS Res Hum Retroviruses. 1995 Nov;11(11):1413-6., 11(11), 1413-6.

li. Siliciano, J. D., & Siliciano, R. F. (2013). Recent trends in hiv-1 drug resistance. Curr. Opin. Virol., 3, 487–494.

lii. Singh, K., Flores, J. A., Kirby, K. A., Neogi, U., Sonnerborg, A., Hachiya, A., . . . Sarafianos, S. G. (2014). Drug Resistance in Non-B Subtype HIV-1: Impact of HIV-1 Reverse Transcriptase Inhibitors. Viruses, 6, 3535-3562. doi:10.3390/v6093535

liii. Smyth, R. P., Davenporte, M. P., & Mak, J. (2012). The origin of genetic diversity in HIV-1. Virus Research, 169, 415–429.

liv. Tongo, M., Dorfman, J. R., Abrahams, M. R., Mpoudi-Ngole, E., Burgers, W. A., & Martin, D. P. (2015). Near Full-length HIV type 1M genomic sequences from Cameroon. Evidence of early diverging undersampled lineages in the country. Evolution, Medicine, and Public Health, 254–265. doi:10.1093/emph/eov022

lv. Vandamme, A. (2009). Basic Concepts of Molecular Evolution. (P. Lemey, M. Salemi, & A. M. Vandamme, Eds.) The Phylogenetic Handbook, pp. 3-29.

lvi. Vandamme, A. M., Camacho, R. J., Ceccherini-Silberstien, F., de Luca, A., Palmisano, L., Paraskevis, D., . . . Panel, E. H. (2011). European Recommendations for the Clinical Use of HIV Drug Resistance Testing: 2011 Update. AIDS Reviews, 13, 77-108.

lvii. Wainberg, M. A. (2015). Clinical Significance of HIV Subtype Variability in Regard to Treatment Outcome. Clinical Infectious Diseases, 60(10), 1550–1.

lviii. Wainberg, M. A., & Brenner, B. G. (2010). Role of HIV Subtype Diversity in the Development of Resistance to Antiviral Drugs. Viruses, 2, 2493-2508;. doi:10.3390/v2112493

lix. WHO. (2012). WHO HIV Drug Resistance Report. HIV/AIDS Programme. Geneva: WHO Press, World Health Organization.

lx. Worobey, M., Gemmel, M., Teuwen, D. E., Haselkorn, T., Kunstman, K., Bunce, M., . . . Wolinsky, S. M. (2008). Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. Nature, 455, 661–664.