HIV-1 Drug-Resistant Mutations in Relation to Virological Failure among Adults in Busia County, Kenya.

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Summary

INTRODUCTION

Busia County of Kenya with a Human Immune-deficiency Virus prevalence rate of 7.7% is two-fold higher than the Kenyan national prevalence at 4.9%. Antiretroviral (ARV) therapy is standard care for all HIV infected patients to mitigate viral loads. The current ARV therapy coverage in the county is 95% among adults. Recently there has been an observation of virologic failure due to viral drug resistance. Limited information on HIV-1 drug-resistance exists with the ARV therapy scale-up in the county. Therefore, this study was designed to investigate the existing HIV-1 drug-resistant mutations among adults in Busia County attributable to virological failure.

MATERIALS AND METHODOLOGY

The samples were analyzed for viral load and HIV-1 drug-resistance targeting the pol gene. Viral load was detected and quantified by real time PCR. Alignment of sequences was done using Recall software and HIV-1 drug-resistance was determined by Stanford University HIV database.

RESULTS

Of 50 samples, 37(74.0%) had mutations and 34(68.0%) had viral loads of 1000cp/ml and above. Generally, a total of 142 (82 in males and 60 in females) mutations were detected. M184V, K65R, K70R, D67N and T215F were the predominant mutations encoding resistance to nucleoside reverse transcriptase inhibitors. K103N, Y181C, G190A and K101E were predominant to non-nucleotide reverse transcriptase inhibitor mutations. L231, V82A, 150L, E138G, G48V, I501T, I54V and M461were the protease inhibitor mutations identified. About 30(60%) samples had mutations encoding for NRTIs, 34(68%) for NNRTIs, and 6(12%) for PIs ARVs. We observed an association between samples with mutations and viral load of 1000 cp/ml and above (Fisher's exact test of <0.05) and virological failure especially in males (Fisher's exact test of 0.013).

CONCLUSION

There is need for the change in the recombination of treatment regimens especially the NNRTIs and NRTIs as first line therapy. There is also need for more investigations as to why males are resisting drugs than females, this will inform intervention. Routine HIV drug-resistance testing is needed before the initiation of any ARVs. This will minimize resistance.

Keywords: ARVs, HIV-1, drug resistance, mutations, viral load, NNRTIs, NRTIs, PIs

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Introduction

Kenya is the 12th highest country with HIV epidemic worldwide where about 1.6 million people are living with the virus [1,2]. HIV prevalence among adults stands at 4.9% with 4.5% males and 5.2% females affected. 44,800 new HIV cases and 28,200 HIV related deaths

were observed in 2018. In the country, Busia is the fifth-largest county with HIV prevalence of 7.7% [3]. ARV treatment is recommended for all people infected with HIV because it reduces the HIV viral load and improves their immunity against other infections [4]. The number of people receiving ARV treatment nationally stands at 75% among adults. Consequently, Busia county has registered more coverage of individuals on antiretroviral therapy (ART) (95%) than that of the National [3]. Regardless of these milestones in the treatment of HIV, it has been observed that, viral loads in patients on ARVs is still high due to virological failure as a result of HIV drug-resistance especially in Busia.

ARV drugs interfere with functional activities of various enzymes, such as reverse transcriptase, integrase, and protease which are responsible for HIV replication [5]. HIV drug-resistance is caused by changes in the virus's genome leading to failure of many ARVs [6]. Such mutations occur randomly and frequently and have doubled edged-sword effect that can be beneficial to the host or deleterious leading to increase in susceptibility or resistance to the virion.
Mutations that develop confer resistance to specific classes of ARVs. In Kenya, there are three classes of ARVs available to treat HIV. These Drugs are non-nucleotide reverse transcriptase inhibitors (NNRTIs), nucleotide reverse transcriptase inhibitors (NRTIs) and Protease inhibitors (PIs) [7].

Studies conducted in the past have reported some mutations conferring resistance to these classes of drugs in Kenya. These mutations included M184V, K65R, D67N, K70R, K219Q, Q151M as NRTIs; K103N/S, Y181C/Y/I/V, G190A as NNRTIs[8,9,10,11,12] and M46I/L, D30N, M46I, V82F, L90M as PIs [13, 14, 15]. As much as ARVs are important in the fight against the HIV virus, mutations encoded by the virus against specific classes of ARVs may render them infective. Past studies in Kenya have identified mutations conferring resistance to specific classes of ARVs [15, 16, 17]. At the coastal region of Kenya, L90M, M46I and D30N mutations conferring resistance to PIs were identified among Intra-venous drug users [15].

A study on HIV type 1 drug-resistance patterns in Nairobi identified M184V, K65R, T215Y and K70R mutations conferring resistance to NRTIs and K103N, G190A, V106A, Y184V, A98G, Y181C mutations conferring resistance to NNRTIs [16]. The recently published study on HIV-1 drug resistance in Busia County focused on children aged between 6 weeks and 5 years [18] in which some mutations conferring resistance to NNRTIs and NRTIs were identified. However, data about resistance in individuals who are older than this group is missing. Therefore, this study was designed to look at HIV-1 drug-resistance in adults. In Kenya, many studies on the HIV drug-resistances were carried out in urban centers [19, 20, 21, 22]. However, reports on the general HIV drug-resistance is missing especially in rural settings with a longer history of ARV use. The findings from this study are therefore important to HIV infected individuals in rural settings, which comprise the majority of those affected with HIV especially in Busia County.

HIV drug-resistance remains a critical issue world-wide because people who started treatment in the early and mid-1990s are carrying multidrug-resistant viruses. To add on this, a substantial proportion of new HIV infections are caused by the transmission of already resistant strains. Furthermore, new infected individuals worldwide are being treated regardless of their CD4 counts. Inadequate virus suppression due to the development of drug-resistant mutations threatens the victory of future treatment regimens [23]. A study carried out in 2004 among Naive and -Experienced persons in South Korea discovered that 50% of patients with virological failure had strains with multiple resistant mutations and 10% had mutations to multi-class HIV drugs [24]. Therefore, this study was designed to identify the existence of multiple and multi-class HIV drug resistant mutations in a sample of adults in relation to viral suppression in Busia, Kenya. This information will advise drug regimen re-combination and development of novel effective drugs.

In Kenya, there are various types of ARVs available in the treatment of HIV. These include, Abacavir (ABC), Lamivudine (3TC), Tenofovir disoproxil fumarate (TDF), Azidovodine (AZT), Dolutegravir (DTG) which are grouped as NRTIs; Efevirenz (EFV), Nevirapine(NVP) which are grouped as NNRTIs; Atazanavir/ritonavir ATV/r and Lopinavir/ritonavir (LPV/r) which are grouped as PIs. Initially, some of these drugs were used as single dose in the treatment of HIV infections. Development of HIV drug-resistance triggered the combination of these drugs to meet treatment threshold. These combinations include: one NRTI with Lamivudine (NRTI) as a compulsory

drug and one NNRTI grouped as first line therapy. One NRTI with Lamivudine as compulsory drug and one Protease grouped as second line therapy.

RNA quantification is increasingly significant for monitoring of disease development, antiretroviral treatment start, and investigation of virological failure in HIV-infected persons [25]. In Kenya, highly active antiretroviral therapy treatment is considered where TDF + 3TC + EFV/NVP and AZT + 3TC + NVP/EFV are recommended as first-line antiretroviral regimens in treatment of inexperienced adults and teenagers while AZT + 3TC + LPV/r/ATV/r, TDF + 3TC + LPV/r/ ATV/r and TDF + 3TC + LPV/r/ATV/r are recommended as second-line antiretroviral regimens [26]. In expectant women, AZT based ART is preferred due to the long experience of AZT in gestation and its well-documented efficacy in preventing mother-to-child transmission of HIV. As much as possible, fixed-dose ARV drug combinations must be utilized to reduce the pill burden and encourage optimal adherence [26]. This study was designed to investigate the existing HIV-1 drug-resistant mutations among adults in Busia County attributable to virological failure.

Materials and Methods
Study Site and Population

Busia County referral hospital was considered for this study. The criteria for selecting this facility as a recruitment center was because it is a referral hospital in Busia county where the prevalence of HIV-1 (7.7%) is higher than the national. It is also strategically located at the border of Kenya and Uganda.

Study population involved were individuals who were ≤18 years, HIV-1 infected, on ARV therapy and consenting to participate in the study. All participants were Kenyans residing in Busia

Sample Collection

Filling a simple questionnaire on demographics (age and sex) and duration on ARVs were done. Approximately 5 ml of blood specimen was obtained using a sterile needle and syringe in EDTA tubes. Triple packaging of the samples was done and transported to Alupe KEMRI laboratories following the national sample shipment guideline for clinical specimens. Samples were removed from the cold environment and allowed to come to room temperature for at least one hour before processing. Samples were inspected for quality. Plasma was separated from the whole blood and stored at -80°C. Analysis of viral load was done in Alupe KEMRI. Plasma for HIV RNA extraction and subsequent sequencing was transported to KEMRI Nairobi for analysis.

Sample Processing

Viral RNA Extraction

Plasma was obtained by centrifuging whole blood in purple top EDTA tubes at 1500rpm (use g) for 30 minutes at 4°C. Plasma samples were stored at -80°C until transported on dry ice to the KEMRI Laboratory for Molecular Biology in Nairobi. Plasma viral RNA was extracted using the Abbott m2000sp sample preparation system (Abbott Molecular Inc. USA). 0.2ml HIV-1 assay extraction protocol was used as per the manufacturer's instructions. RNA was extracted from 200ul of plasma samples using the Abbott RNA extraction kit according to the manufacturer's instructions [27].

Reverse Transcription-PCR

The Extracted RNA was transcribed into cDNA using an in-house RT-PCR protocol using Thermo Fisher Scientific's Genotyping kit. RT-PCR reaction mix which consisted of the RT-PCR master mix (normal) and SuperScript III enzyme was prepared for the required number of reactions. The extracted RNA was denatured in a thermocycler (Veriti™ 96-Well Thermal cycler) at 65°C for 10 minutes. 10 µl of the extracted RNA was added to 40 µl of the RT-PCR mix (39µl of the RT-PCR mix (normal) and 1 µl of the SuperScript III enzyme) and a total volume of 50 µl was reverse transcribed. The reverse transcription PCR was done under the following cycling conditions: 1 cycle of reverse transcription at 50°C for 45minutes, 1 cycle of enzyme inactivation at 94°C for 2 minutes, 40 cycles of denaturation at 94°C for 15 seconds, 40 cycles of annealing at 50°C for 20 seconds, 40 cycles of extension at 72°C for 2 minutes and 1 cycle of final extension at 72°C for 10 minutes. The sequence of the primers used for reverse transcriptase include reverse primer: 3' CGTCTCTTACTCTATCTCTC 5' and forward primer: 5' AAATATCTGGCTGAGTGTTT 3'. The pol gene of the HIV virus was amplified because it is a conserved region carrying reverse transcriptase, protease and integrase enzymes responsible for replication cycle of the virus.

Nested PCR

The Thermo Fisher Scientific's Genotyping kit was used in the Nested PCR process. The reverse transcription PCR product complementary deoxyribonucleic acid (cDNA) was amplified using nested PCR to amplify the region of interest. Sufficient nested PCR reaction mix (Nested PCR mix and AmpliTaq Gold LD DNA polymerase enzyme) was prepared for the required number of reactions. 2 µl of the RT-PCR products was added to 48µl of the nested PCR reaction mix (47.5 µl of Nested PCR mix, 0.5 µl AmpliTaq Gold LD DNA polymerase enzyme). A total volume of 50 µl was used. The Nested PCR was done under the following cycling conditions: 1 cycle of initial denaturation at 94^0C for 4 minutes, 40 cycles of denaturation at 94^0C for 15 seconds, 40 cycles of annealing at 550C for 20 seconds, 40 cycles of extension at 72^0C for 2 minutes and 1 cycle of final extension at 72^0C for 10 minutes. The pol gene of the HIV virus was amplified because it is a conserved region carrying reverse transcriptase, protease and integrase enzymes responsible for replication cycle of the virus. Forward and reverse primers were used

PCR Product Size Confirmation

Agarose gel (1%) was used to confirm the PCR amplified complementary DNA (cDNA), The gel was visualized on the imaging system and photographed (VisiDoc-It™ Imaging System, CA, and the USA). The PCR product was 1.1kb in size.

Purification of Nested PCR products

Purification of the PCR products was performed using Thermo Fisher Scientific's Clean Sweep Purification Reagent. Briefly 6.4 µl of ExoSAP IT™ PCR Cleanup Reagent product was added to 14 µl of each Nested PCR product. The plate was vortexed for 2-3 seconds, then centrifuged at 1000 x g for 5-10 seconds. The 20.4µl reaction was purified in a thermocycler under the following conditions: Digest at 370C for 15 minutes and heat deactivation at 800C for 15 minutes.

Cycle Sequencing

3µl of the purified Nested PCR products was added to 18 µ l of each of the 6 sequencing mixes (F1, F2, F3, R1, R2, and R3). 21µl of the PGEM sequencing control which contains primers, terminator and template were added to at least one well. The cycle sequencing conditions were set at 25 cycles of denaturation, annealing and extension at 96°C for 10secs, 50˚C for 5 seconds, and 60˚C for 4 minutes, respectively.

Purification of Cycle Sequence Reactions

For purification of cycle sequence reactions, Thermo Fisher Scientific's BigDye X-Terminator Purification Kit was used. The X-terminator solution stored at 4˚C was removed and allowed to equilibrate to room temperature. Vortexing was done at 10 seconds. A working solution of 90 µl of SAM solution and 20 µl of BigDye X-Terminator solution was prepared to purify 20 µl of the cycle sequencing products. 110 µl of SAM /Big Dye X-terminator solution was dispensed in each sample and mixed thoroughly. The plate was then sealed using microAmp clean adhesive Film. The plate was vortexed for 30 minutes at 7000 rpm and centrifuged at 1000x g for 2 minutes at room temperature.

Sequencing and Sequence Analysis

Sequencing was done in a Sanger sequencer. The Adhesive film was replaced with 96 well plate septa before loading on the 3730 XL DNA Analyzer (Applied Biosystems) for direct sequencing. Run module longSeq50-POP 7-1 and DyeSet Z-BigDye V3 were used. Sequencing was done to determine the order of nucleotide bases in the amplified products. Using the Applied Biosystems Sequencing Cycle kits version 3.1, fluorescently labeled dyes were attached to ACGT extension products in the cDNA sequencing reactions. The dyes were labeled as follows: red (labels Thymidine base), blue (Cytosine), black (Guanine) and green (Adenine). The dyes were incorporated using either 5'-dye label primers or 3'-dye label dideoxynucleotide terminators. Main technical parameters such as raw data, electropherograms and the quality of sequenced bases were evaluated by SeqScanner v.6 (Applied Biosystems Inc. USA). Alignment and editing of sequences were done using Recall software. The Stanford University HIV Drug Resistance Database (http://sierra2.stanford.edu/sierra/servlet/JSierra) was used to determine sub-types and possible mutations.

Data Analysis

Collected data were entered into Microsoft Excel. Cleaning of the data was done regularly in Microsoft excel. The analysis was done using SPSS version 20 (Armonk, NY: IBM Corp) Descriptive statistics were done to determine socio-demographic factors and the frequency of various HIV-1 drug-resistant mutations. Chi-square and post-hoc tests were used to analyze the associations of two qualitative variables.

Ethical Clearance and Considerations

Scientific and Ethical clearance for this study was sought from the ethical review committee at KEMRI and the protocol was given number (KEMRI/SERU/CIPDCR/008/3333). The study and its importance were explained to the participants. Samples were collected by qualified health officers from patients who had signed informed consent forms. Identity of study participants was concealed. Instead identifier sample codes were used to label the sample for confidentiality purposes.

Results

Patient's' Characteristics

A total of 50 (female, 62.0% and male, 38.0%) participants were considered in the present study. Their age was between 18 to 66 years old (median age 35; inter-quartile ranges 28 years). Many patients were taking AZT+3TC+NVP and TDF+3TC+EFV ARVs regimen combination. Also, many patients had taken ARVs for a period of 0 to 3 years and 6 to 9 years *(Table 1).*

A total of 34 participants with mutations had viral load of 1000cp/ml and above, only 3 participants with mutations had viral load below 1000cp/ml *(Table 2) next page.....*

Table 1: *Gender, ARVS Regimen Combination and Duration Participants were on ARVS, N=50*

Gender	ARVs Regimen									Total	Duration on ARVs(years)				Total
	ABC+ 3TC+ EFV	ABC+ 3TC+ LPV/r	AF5X-OTHER	AZT+ 3TC+ ATV/r	AZT+ 3TC+ NVP	TDF+ 3TC+ ATV/r	TDF+ 3TC+ DTG	TDF+ 3TC+ EFV	TDF+ 3TC+ LPV/r	TDF+ 3TC+ NVP		0.0-3.0	3.1-6.0	6.1-9.0	9.1-12.0	12.1-15.0	
Female	1	1	0	6	8	2	1	10	1	1	31	12	2	11	4	2	3
Male	1	0	1	2	3	2	2	7	0	1	19	7	5	6	0	1	1
Total	2	1	1	8	11	4	3	17	1	2	50	19	7	17	4	3	5

Table legend: *ARVs= Antiretrovirals; ABC= Abacavir; 3TC= Lamivudine; EFV= Efevirenz; LPV/r= Lopinavir/ritonavir; AZT= Azidovodine; ATV/r= Atazanavir/ ritonavir; NVP= Nevirapine; TDF= Tenofovir disoproxil fumarate; DTG= Dolutegravir*

Table 2: *Patient's Samples with Mutations and Viral Load, N=37*

Gender	Patient's ID	Age	Regimen	Duration on ARVS	Viral load	Mutations identified		
						NRTIs	NNRTIs	PIs
	1	36	TDF+3TC+EFV	1.5	56348	None	K103N	L23I
Males	2	53	TDF+3TC+EFV	6	130049	D67N, K70R, M184V, T215F,K219Q	V106A,F227L	None
	3	19	ABC+3TC+EFV	2	48010	M184V	K103N,M230L	None
	4	56	AZT+3TC+NVP	4	250406	None	K103N	None
	5	53	TDF+3TC+ATV/r	9	8000	None	K103N	None
	6	50	TDF+3TC+ATV/r	9	374000	None	K103N	None
	7	19	AZT+3TC+ATV/r	3	341658	M41L, D67N, K70R,	M184V,T215Y,K219Q	K103N,V179T
	8	42	TDF+3TC+EFV	4	31109	M184V	Y181C,H221Y,M230MI	None
	9	26	TDF+3TC+EFV	13	17146	M184V	V108I,Y181C,H221Y	None
	10	25	TDF+3TC+EFV	8	14564	K65R, L74V, Y115F,	K101P,K103S,G190A	
	11	42	TDF+3TC+DTG	9	54000	K70E,M184I	K101E,Y181C	None
	12	48	TDF+3TC+NVP	2	651800	T69G,K70R,L74I	K101E, E138A	None
	13	58	AZT+3TC+NVP	5	4456	V75M,M184V	K103N,P225H, K238T	None
	14	19	AZT+3TC+ATV/r	1	4938	V75I,M184V	Y181C,H221Y	None
	15	48	TDF+3TC+DTG	2	12000	K65R,M184V	K101E,Y181C	None
	16	18	AF5X-OTHER	9	90000	D67N, M184V, T215F K219E	G190A	G48V, I54V, V82A
	17	44	AZT+3TC+NVP	7	38727	K65R,M184V	K103N	None
Females	18	35	AZT+3TC+NVP	8	13572	D67Y,K70N	None	None
	19	20	TDF+3TC+EFV	10	41119	K65R,M184V,K219E	V108I,Y181C,G190A	None
	20	21	ABC+3TC+EFV	2	897	K65R	K103N	None
	21	27	AZT+3TC+NVP	8	7686	M184V	K103N	None
	22	35	TDF+3TC+EFV	7	14237	M184L	None	None
	23	26	TDF+3TC+EFV	0.5	98630	M184V	K101E,K103N,V106I	E138G,G190A

Females	24	30	AZT+3TC+ATV/r	9	869	None	K103N	None
	25	34	TDF+3TC+ATV/r	9	933	M41L, D67N, M184V, K103N,V179T	E44D,L210W,T215Y	None
	26	42	TDF+3TC+EFV	2	8084	L74V,Y115F,M184G	K101P,K103S,G190A	None
	27	28	AZT+3TC+NVP	3	62609	M184M/V	E138A	None
	28	18	TDF+3TC+EFV	7	12076	M41L,M184V,T215F	Y188C	None
	29	41	AZT+3TC+NVP	8	3630	K65R	K103N	None
	30	42	AZT+3TC+NVP	14	46907	None	K103N	None
	31	50	AZT+3TC+ATV/r	3	3265	K65R,L74L/V	L1001,K103N	I501T
	32	18	TDF+3TC+EFV	0	26001	K65R,V75I	K103N	None
	33	34	AZT+3TC+NVP	4	32452	None	K103N	None
	34	32	TDF+3TC+ATV/r	12	14000	K70R,M184V,K219Q	K103N,P225H	None
	35	28	TDF+3TC+LPV/r	3	1800	V106A,Y181V,M184V	None	None
	36	18	ABC+3TC+LPV/r	3	164000	M184V	K101P	None
	37	19	AZT+3TC+ATV/r	13	262000	L74I,M184V	A98G,K103N,P225H	None

In this study viral load versus presence of mutations among participants was determined statistically. 34(68%) participants had viral load of 1000 and above and all had mutations. 16(32%) had viral load of less than1000 and only 3(6.0%) had mutations. Patients with viral load of more than 1000 were associated with mutations and virological failure (Table 3) and especially in males (Table 4) with fisher's exact test and chi-square of < 0.05 for both. HIV Virological failure is defined as plasma viral load above 1000 copies/ ml based on two consecutive viral load measurements after 3 months28

Table 3: *Viral Loads and Mutations*

Viral load (cp/ml)	Mutation		Total	Fisher's exact test	Chi-square
	Mutations present	No mutations			
1000 and above	34	0	34		
below 1000	3	13	16	< 0.05	< 0.05
Total	37	13	50		

Table 4: *Viral Loads and Gender*

Gender	viral load(cp/ml)		Total	Fisher's exact test	Chi-square
	1000 and above	1000 and below			
F	17	14	31		
M	17	2	19	0.013	0.011
Total	34	16	50		

A total of 142 mutations (82 in males; 60 in females). The most predominant NRTIs mutations are M184V and K65R and NNRTIs mutations are K103N and Y181C in both genders. Males were more associated with NNRTIs mutations than female with a chi-square p-value of 0.043 and Fisher's Exact Test of 0.021. *(Table 5).*

Table 5: *Total Mutations Identifie, N=142*

Mutations encoding resistance to NRTIs	Gender		Total	P Value
	Number of mutations in females	Number of mutations in males		
D67N	1	3	4	
D67Y	1	0	1	
E44D	1	0	1	
K219E	1	2	3	
K219Q	1	2	3	
K65R	5	4	9	
K70E	0	1	1	
K70N	1	0	1	
K70R	1	3	4	
L210W	1	0	1	>0.05
L74I	1	1	2	
L74L/V	1	0	1	
L74V	0	2	2	
M184G	0	1	1	
M184I	0	1	1	
M184L	1	0	1	
M184M/V	1	0	1	
M184V	9	11	20	
M41L	2	1	3	
T215F	1	3	4	
T215Y	1	0	1	
T69G	0	1	1	
V106A	1	0	1	
V75I	1	1	2	
V75M	0	1	1	
Y115F	0	2	1	
Y181V	1	0	1	

Table 2 :continued

NNRTIs		Gender		Total	P Value	
		Female	male			
	A98G	1	0	1		
	E138A	1	1	2		
	F227L	0	1	1		
	G190A	2	3	5		
	H221Y	0	3	3		
	K101E	1	3	4		
	K101P	1	2	3		
	K103N	11	8	19		
	K103S	0	2	2		
	K238T	0	1	1	0.043 and 0.021	
	L1001	1	0	1		
	M230L	0	1	1		
	M230MI	0	1	1		
	P225H	2	1	3		
	V1061	1	0	1		
	V106A	0	1	1		
	V108I	1	1	2		
	V179T	1	1	2		
	Y181C	1	5	6		
	Y188C	1	0	1		
			PIs			
PIs	L23I	0	1	1		
	150L	0	1	1		
	E138G	1	0	1		
	G48V	0	1	1		
	I501T	1	0	1	>0.05	
	I54V	0	1	1		
	M461	0	1	1		
	V82A	0	2	2		
	Total	60	82	142		

In Busia County referral hospital, 50 samples from equivalent number of participants were sequenced successfully of which 30(60%) had mutations encoding for NRTIs, 34(68%) for NNRTIs, and 6(12%) for PIs. Out of the 30 samples with NRTIs mutations, 10(33.3%) samples had multiple mutations: 10(33.3%) had dual mutations and 10(33.3%)had single mutations. Out of 34 samples with NNRTIs mutations, 8(23.5%) samples had multiple mutations, 10(29.4%) had dual mutations and 16(47.1%) had single mutations *(Table 6).*

Table 6: *Mutations Identified Conferring Resistance to NRTIs*

Mutations Conferring Resistance to NNRTIs	Number of Participants	%
D67N,M184V,T215F,K219E	1	2.0
D67Y, K70N	1	2.0
K65R	2	4.0
K65R, L74L/V	1	2.0
K65R,L74V,Y115F,M184V	1	2.0
K65R, M184V	3	4.0
K65R, M184V, K219E	1	2.0
K65R, V75I	1	2.0
K70E, M184I	1	2.0
K70R, M184V, K219Q	1	2.0
L74I, M184V	1	2.0
L74V,Y115F,M184G	1	2.0
M184L	1	2.0
M184M/V	1	2.0
M184V	6	12.0
M41L,D67N,K70R,M184V,T215Y,K219Q	1	2.0
M41L,D67N,M184V,E44D,L210W,T215Y	1	2.0
M41L,M184V,T215F	1	2.0
None	20	40.0
T69G, K70R, L74I	1	2.0
V106A,Y181V,M184V	1	2.0
V75I, M184V	1	2.0
V75M, M184V	1	2.0
Total	50	100.0

Continued.....

Table 6: Continued

Mutations Conferring Resistance to NNRTIs	Number of Participants	%
A98G,K103N,P225H	1	2.0
E138A	1	2.0
G190A	1	2.0
K101E, E138A	1	2.0
K101E, K103N, V106I	1	2.0
K101E, Y181C	2	4.0
K101P	1	2.0
K101P,K103S,G190A	2	4.0
K103N	12	24.0
K103N, M230L	1	2.0
K103N, P225H	1	2.0
K103N, P225H, K238T	1	2.0
K103N, V179T	2	4.0
L1001, K103N	1	2.0
None	16	32.0
V106A, F227L	1	2.0
V108I,Y181C,G190A	1	2.0
V108I,Y181C,H221Y	1	2.0
Y181C, H221Y	1	2.0
Y181C,H221Y,M230MI	1	2.0
Y188C	1	2.0
Total	50	100.0
G48V, I54V, V82A	1	2.0
I501T	1	2.0
L23I	2	4.0
M461, I50L,V82A	1	2.0
E138G, G190A	1	2.0
None	44	88.0
Total	50	100.0

Table legend: %=percentage of mutations identified; %= percentage of participants; NRTIs= nucleotide reverse transcriptase inhibitors; NNRTIs= non-nucleotide reverse transcriptase inhibitor; PI=protease inhibitors

Discussion

The benefits of ARV treatment to HIV infected individuals is enormous as it prolongs their lives, improves their quality of life and reduces the morbidity and mortality rates [29]. ARV treatment is thus the standard care for all HIV infected people in Kenya [4]. However, there is a challenge due to HIV drug-resistance which makes the drugs not work effectively. Consequently, HIV-1 drug-resistance testing is important in decision making regarding the management of HIV infected patients [30] although HIV-1 drug-resistance tests are not routinely available in the country and are only carried out in a few research laboratories in Kenya.

The study involved adults of 18 years and above infected with HIV and on ART. These groups of people may harbor HIV viruses that are resistant to one or more ARV regimens. The study was designed to investigate the existence and effect of HIV-1 mutations in relation to viral suppression among adults in Busia County. More HIV infection was frequently observed in females than males confirming earlier studies [8,13]that the prevalence of HIV among females is more than that of males in the country and that males are poor health seekers compared to females. Additional analyses demonstrated that the ratio of females to males was similar, giving a platform of gender comparison as it is reported with National AIDS and STI Control Program (NASCOP) that slightly more females are infected with HIV than males [3,31,32].

In general, the current study determined drug-resistant mutations in samples of HIV-1 infected patients on ARVs. Specific mutations encoding for resistance to various classes/types of ARVs were analyzed. The frequency of the mutations in the samples was also recorded. The study also identified the current drug regimen combinations which the patients were taking. The viral load for the three consecutive visits was also determined. The current study recorded higher prevalence of HIV drug-resistance compared to prevalence recorded in the previous studies [9,10,22,33] of similar kind by a bigger margin. The World Health Organization (WHO) guidelines that all persons infected with HIV to be started on ARVs treatment irrespective of CD4 tally [29] contributed to this higher prevalence compared to prevalence in previous studies before this recommendation. Besides, the ART coverage (95%) in Busia County which is higher than the National (75%) [32] might have also contributed to this raised prevalence of HIV drug-resistance. Further analyses demonstrated that males are associated with mutation more than female due to virological failure. The fact that women open up to speak and share their social problems with others might have influenced their medication uptake appropriately than men who don't share their matters thus affecting their adherence to ARVs [4] with results of developing resistance more than females. Other studies have reported that more women than men take up dynamic responsibility to seek ARV treatment [34,35,36,37] thus escalating their adherence effectively which in turn minimizes drug resistance.

Further analysis showed no statistically significant difference between patients' samples with mutations conferring resistance to first line and second line antiretroviral treatment with $p > .05$. These preliminary findings in the study suggests resistance to second line ARVs may rise soon thus posing a threat to the entire ARVs treatment. The study identified major mutations encoding for all the three classes of ARVs that are commonly prescribed. The largest number of samples had mutations conferring resistance to NNRTIs, this was followed by NRTIs then PIs the least. This result could be because NNRTIs and NRTIs as first-line ARVs have been in existence longer than PIs which are second-line ART treatment drugs [38]. This observation is similar to a study by Ochieng et al., 2015 which showed more HIV-1 infected Kenyan individuals are on NVP with a substantial percentage as high as 35% failing treatment and demonstrating poor adherence [39]. Higher existence of HIV-1 drug-resistant mutations among NNRTIs and NRTIs could also be explained by the above-stated reason. Identification of multiple mutations in majority of our samples in our study is of great concern in that this may contribute to very slow suppression of the virus or complete no suppression of the virus which is evidenced by our findings on viral load and mutations *(Table 2)*.

The findings reveal more than 50 major mutations conferring resistance to NRTIs with M184V, K65R, K70R, D67N, T215F being prevalent. More than 40 major mutations conferring resistance to NNRTIs with K103N, Y181C, G190A, K101E P225H, H221Y, being prevalent. Only nine PIs major mutations were identified with L23I, V82A being prevalent. Some of these mutations have been identified in other similar studies in Kenya with K103N and M184V being

predominant [9,10,11,18,22,40] a scenario observed in the current study. However, more than 100 mutations identified in only 50 samples analyzed is alarming in that these may put pressure on the existing ARVs not to work effectively. Further analysis of results revealed that many samples had resistant mutations to both NRTIs and NNRTIs, this could be the fact that the two classes of ARVs are the first line ARVs and also many HIV infected patients are started on these classes of ARVs before they are switched to second line ARVs that consist of PIs [7] a case seen in our study where many patients were taking first line classes of drugs.

In this study, participants were taking 10 different regimens of ARVs that are commonly available. Further analysis showed that many participants were taking TDF+3TC+EFV and AZT+3TC+NVP as first line HIV-1 treatment because the former regimen is substitute first line ART regimen and the latter is ideal first line ART regimen in pregnancy [41,42]. These outcomes concur with the fact that many women were put on these two drugs than other regimens of ARVs. This study did not investigate pregnancy history. AZT+3TC+ATV/r was being taken by many participants compared to other second line drugs, perhaps due to the guidelines that after failure on the above ideal and substitute of first line drugs, the patient be switched to AZT+3TC+ATV/r as a second-line drug[43], evidenced by our study that more patients were on the two first line drugs compared to the rest.

In this study, multiple mutations have rendered most of these two or all the three-dose drug regimen combinations infective, a situation that has never been reported before. Out of the total patient samples with mutations, substantial samples are carrying viral mutations that render ineffective all or two of the three-dose drug regimen combinations. This situation is likely to rise if proper intervention is not put. This is evidenced by the fact that some individuals have been taking ARVs for only three and fewer years and are already carrying viruses that are already resistant to all or two of the three-dose drug regimen combination.

All the patient's samples with mutations have indicated virological failure, this is evidenced by the fact that more than 1000 copies per microliter of blood were recorded in all the three consecutive visits. However, lower suppression or complete to no suppression of the virus was observed in samples with multiple mutations. The study reveals higher viral load with significantly lower suppression of the viruses in the samples with multiple resistant mutations. Generally, all the samples with multiple resistant mutations have shown to render two and/or all the three-dose drug regimen ineffective.

Conclusion

Existences of resistant mutations in individuals slow down suppression or do not completely suppress the virus thus rendering drugs infective. There is need change in the recombination of treatment regimens especially the NNRTIs and NRTIs as first line therapy. There is also need for more investigations as to why more males are resisting drugs than females. This will initiate intervention. Routine HIV drug-resistance testing is needed before the initiation of any ARVs to minimize resistance.

Limitation of the Study

- The sample size was too small to conclude whether mutations are associated with the dose drug regimen combination and duration on ARVs.

- The study did not investigate HIV drug-resistance in pregnancy as much as some drugs are selectively prescribed for them.

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Authors Contributions

OM, Conceptualization, data curation, formal analysis, Investigation, Methodology, validation, writing – original draft, Writing – review & editing; DM- Supervision, Conceptualization, Data curation, Methodology, writing – original draft, Writing – review & editing; MM- Supervision, Validation, Visualization, Writing – review & editing; JM, Conceptualization, Supervision, Writing – review & editing;TM-Investigation, Methodology, Validation, Visualization,

Writing – original draft; MA- Investigation, Methodology, Validation, Visualization, Writing – original draft; LO- Investigation, Methodology, Writing – original draft

Conflict of interest

The authors declare that they have no conflict of interests

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