Drug Resistance Mutations Among Antiretroviral-Treated Female Sex Workers In Nairobi, Kenya.

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SUMMARY

Antiretroviral drug resistance remains an important and a growing challenge in HIV management as it almost always lead to treatment failure. Sex workers face persistent exposures to the different HIV-1 variants and in turn pose a risk of transmission to the general population and measuring drug resistance in this population may serve as a measure for the risk of transmission of these strains to drug naïve populations. The objective of this study was therefore to determine the pattern and prevalence of HIV-1 drug resistance mutations in a cohort of female sex workers in Nairobi, Kenya. Plasma from 60 female sex workers on antiretroviral treatment for over six months was analyzed by amplification and sequencing of the reverse transcriptase - *pol* region. Five samples (8.3%) showed antiretroviral resistance-associated mutations. One sample (1.7%) showed mutations conferring resistance only to the NNRTI class, 2 samples (3.3%) showed mutations conferring resistance only to the NRTI class while 2 samples (3.3%) showed mutations conferring resistance to both NRTI and NNRTI classes. Phylogenetic analysis revealed HIV-1 subtype A1 (96.7%) and HIV-1 subtype D (3.3%). The prevalence rate of 8.3% for reverse transcriptase-associated resistance mutations was observed. This is a lower rate than has been reported from studies among antiretroviral (ARV)-treated individuals in the country.

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Introduction

One of the major responses to the HIV/AIDS crisis in Kenya was the introduction of a national policy for infected persons to access antiretroviral therapy (ART), which led the Ministry of Health to develop a work programme and a national standard regimen[1]. With donor support, ART coverage rose progressively from under 5% in 2004, 12% in 2005, 28% in 2006 to nearly 40% by end of 2007[2]. By the end of September 2008, 229,700 people were on ART through programmes such as The President's Emergency Plan for AIDS Relief (PEPFAR) in Kenya[3]. Over 400,000 HIV/AIDS patients were receiving antiretroviral treatment by the end of 2010[4] and this number grew to 600,000 in 2012 [5]. The current standard regimen for first-line therapy in Kenya consists of two Nucleoside Reverse Transcriptase Inhibitors (NRTI) usually tenofovir disoproxil fumarate (TDF) or azidovudine (AZT), lamuvudine (3TC) and one Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) efevirenz (EFV) or nevirapine (NVP)[6]. Antiretroviral therapy is initiated following the current World Health Organization (WHO) recommendations for initiation of early treatment[4].

The recent expansion of ART in middle and low-income has resulted in increased development, transmission and prevalence of drug resistant HIV-1 strains[7] especially seen in countries that pioneered antiretroviral therapies[8, 9]. Data from treated prevention of mother to child transmission (PMTCT) and paediatric cohorts in Kenya have shown drug resistance rates as high as 30%[10, 11]. A study involving patients from the general population attending a Comprehensive HIV Care Centre (CCC) at a hospital in Mombasa, Kenya reported reverse transcriptase inhibitor (RTI) drug resistance in 87.5% of the population sampled [12].

The HIV-1 genome is highly variable ranging from 40% variation in the envelope gene to 8-10% variation in the pol, gag, and integrase genes. Exposure to antiretroviral drug pressure contributes to development of mutations and the number of mutational changes in the RT gene has been shown to increase from a median of 4 in untreated individuals to 14 in heavily treated individuals [13]. Outside of the mutations designated by the International AIDS Society (IAS), additional mutations known as accessory mutations have been associated with exposure to ARV [14] and may influence susceptibility to therapy and/or have either an inverse or a direct correlation to resistance [15] .

The bulk of new HIV infections in sub-Saharan Africa are attributable to unprotected heterosexual intercourse, including transactional sex. The greatest risk factor infection with HIV in the region is unprotected sex with multiple partners[16]. Also most at risk populations (MARPs) who include female sex workers, and their clients, men who have sex with men and injecting drug users play a significant role in driving the HIV epidemic in Kenya. It is estimated that 14% of new HIV infections in Kenya are linked to sex workers, their clients and other sex partners within the network[17]. However rates of drug resistance among sex workers in Kenya are unknown, yet this is an important population in HIV transmission and control dynamics such as antiretroviral resistance. We undertook this study to identify antiretroviral resistance genotypes in a cohort of highly exposed female sex workers in Nairobi, Kenya.

Materials and methods

Adult women drawn from a sex worker cohort based in the Majengo slum, Nairobi and managed by the University of Nairobi/University of Manitoba collaborative HIV group were considered for enrolment into the study, after giving informed consent. A total of 84 participants who had been on ART for six months or more were enrolled for this cross-sectional study. The specimens that were analysed for this study were collected between December 2007 and March 2008. Clearance to carry out this study was sought from and granted by the KEMRI Scientific Steering Committee (Ref: ESACIPAC/SCC/2873) and the KEMRI Ethical Review Committee (Ref: KEMRI/RES/7/3/1).

Ten millilitres of blood was collected in tubes containing Ethylenediaminetetraacetic acid (EDTA) anticoagulant. Ribonucleic acid (RNA) was isolated from plasma using The QIAamp® Viral RNA Mini Kit (Qiagen Inc., Chatsworth, CA) following the manufacturer's protocol and complementary DNA (cDNA) was synthesised using The High Capacity cDNA Reverse Transcription kit (Applied Biosystems, foster City, CA) according to manufacturer's guidelines.

A region of the *pol*-RT gene corresponding to nt 2480– nt 3180 of HIV-1 HXB2 was amplified by nested Polymerase Chain Reaction (PCR) with primer sets, RT18 (5'GGAAACCAAAAATGATAGGGGGAATT GGAGG-3') and KS104 (5'-TGACTTGCCCAATTT AGTTTTCCCACTAA-3') in the first round, and KS101 (5'-GTAGGACCTACACCTGTTCAACATA ATTGGAAG-3') and KS102 (5'-CCCATCCAAAGA AATGGAGGAGGTTCTTTCTGATG-3') in the second round[18]. Amplification conditions were as follows; a hold at of 95 ° C for 2 minutes, 35 cycles of 95 ° C for 30 seconds, 55 ° C for 30 seconds, and 72 ° C for 1 minute and a final extension at 72 ° C for 10 minute. Agarose gel electrophoresis and ethidium bromide staining of samples was used to confirm PCR amplification. Amplicons were sequenced directly and analyzed using BigDye® v3.1 sequencing technology in an ABI 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA) as described previously[19].

Reverse transcriptase nucleotide sequences generated (697bps) were translated into the corresponding aminoacids and submitted to the Stanford HIV drug resistance database (http://hivdb.stanford.edu) and in the National Centre for Biotechnology Information- Genbank Database (http://www.ncbi.nlm.nih.gov/Genbank/)for evaluation and identification mutations as well as interpretation of resistance. The 2008 International AIDS Society-USA (IAS-USA) guidelines were used to define antiretroviral resistance-associated mutations for Reverse Transcriptase Inhibitors (RTI) [20].

The National Centre for Biotechnology Information (NCBI) subtyping program (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi), the HIV Blast tool (http://www.hiv.lanl.gov/content.hivdb/BASIC_BLAST/basic_blast.html) and the REGA HIV-1 Subtyping tool (http://www.bioafrica.net/subtypetool/) were used for initial rapid subtype screening before confirmation by phylogenetic analysis using reference sequences from The Los Alamos website (http://www.hiv.lanl.gov/content.hivdb/SUBTYPE_REF/align.html).

Sequence alignment was done using Clustal W and the phylogenetic tree generated and viewed in Treeview® by the Neighbour Joining (NJ) method based on the bootstrapped Kimura 2-parameter.

The sequences have been deposited in the GenBank database with accession numbers HM596786-HM596845

Results

Majority of participants were on first line therapy typically, nevirapine (NVP), lamivudine (3TC) and stavudine (d4T). One participant received combination protease inhibitor, Kaletra with tenofovir and zidivudine. The mean age of the participants was 30 yrs with mean of 6 years involvement in sex-work. The CD4 cell counts at sample collection ranged from 48-687 cells/ µl. The mean time of treatment was 88 weeks (Standard Deviation 42.3) (Table 1).

Table 1: Baseline characteristics of participants

Characteristic	*N=60*
Age (mean years)	30 years
Time engaged in sex work (mean years)	6 years
Time on treatment (mean weeks)	88 weeks (SD 42.3)
$CD4^+$ T cell count (Range cells/mm^3)	48-687 cells/mm^3

Sixty samples out of the 84 collected were successfully amplified sequenced and analysed. Sequence alignment and phylogenetic analysis showed that 58 isolates (96.7%) were HIV-1 subtype A1 while 2 isolates (3.3%) were HIV-1 subtype D. Samples that were found to be HIV-1 subtype AI showed further diverse sub-clustering in the Neighbour-Joining tree (Fig. 1) suggesting divergence in the HIV-1 subtype A1 among the study subjects.

Of the 60 samples analysed, 55 (91.6%) were wildtype while 5 (8.3%) showed mutations conferring resistance to reverse transcriptase inhibitors indicating an HIV-1 drug resistance prevalence rate of 8.3%. Out of the 5 samples showing RTI resistance - associated mutations, 3 (5%) were infected with HIV-1 subtype A1 while 2 (3.3%), were infected with HIV-1 subtype D (Table 2a).

Resistance to NRTIs, most commonly lamivudine was identified in four samples while resistance to NNRTIs, most commonly nevirapine was identified in three samples. Complete class resistance to NNRTI was identified in 2 samples, (3.3%), both, HIV-1 subtype D. Complete class resistance was not observed for the NRTI class. Two samples (3.3%), both HIV-1 subtype A1, showed susceptibility to all drugs in the NNRTI class while 1 sample (1.7%) was susceptible to all NRTI. There was no statistically significant difference in time of exposure to ART between the participants who developed resistance and those that did not (mean 64 weeks versus 90 weeks, p= 0. 192) (Table 2a).

The reverse transcriptase inhibitor-associated major mutations G190A, M184V, Y181C, T215S, V118I, K101E, D67N and D67A were detected. G190A, Y181C and K101E compromise efficacy to NNRTI while T215S, V118I, D67N, D67A and M184V compromise efficacy to NRTI. G190A, M184V and Y181C appeared most frequently (40%) in the 5 samples (Table 2a). The three mutations occurred together (in combination with others) in isolate ML2572 while two of these mutations, M184V and Y181C occurred together in isolate ML1935 (Table 2b).

Minor mutations and polymorphisms were detected in all the 60 isolates that were analysed. V60I was present in all (100%) of the samples. K11T, K20R, V21I, V35T, T39R/I, V60I, K122E, D123S, K173A/V, Q174K, V179I, G196E, T200V, Q207A/E/L/D and R211S/H/K were present in over 50% of the isolates.

Others present in lower frequencies were IL2, P4H, E6Q/A/G, K22N, Q23L, E28K, E40D, K23R, K49R, I50M, R78G, D86G, L92I, H96P, P97R, K102R, T107P, D113E, T131P, P133L, S134G, I135T, N136T, E138G, T146S, S162G, E169D, F171L, D177E, I178M/V, R199T, I202V, E203G, E204G, R206N, H208R, L209A, L210X, W212V, F214X/L/I and T215X.

Table 2a: Important characteristics of samples showing RTI resistance-associated mutations

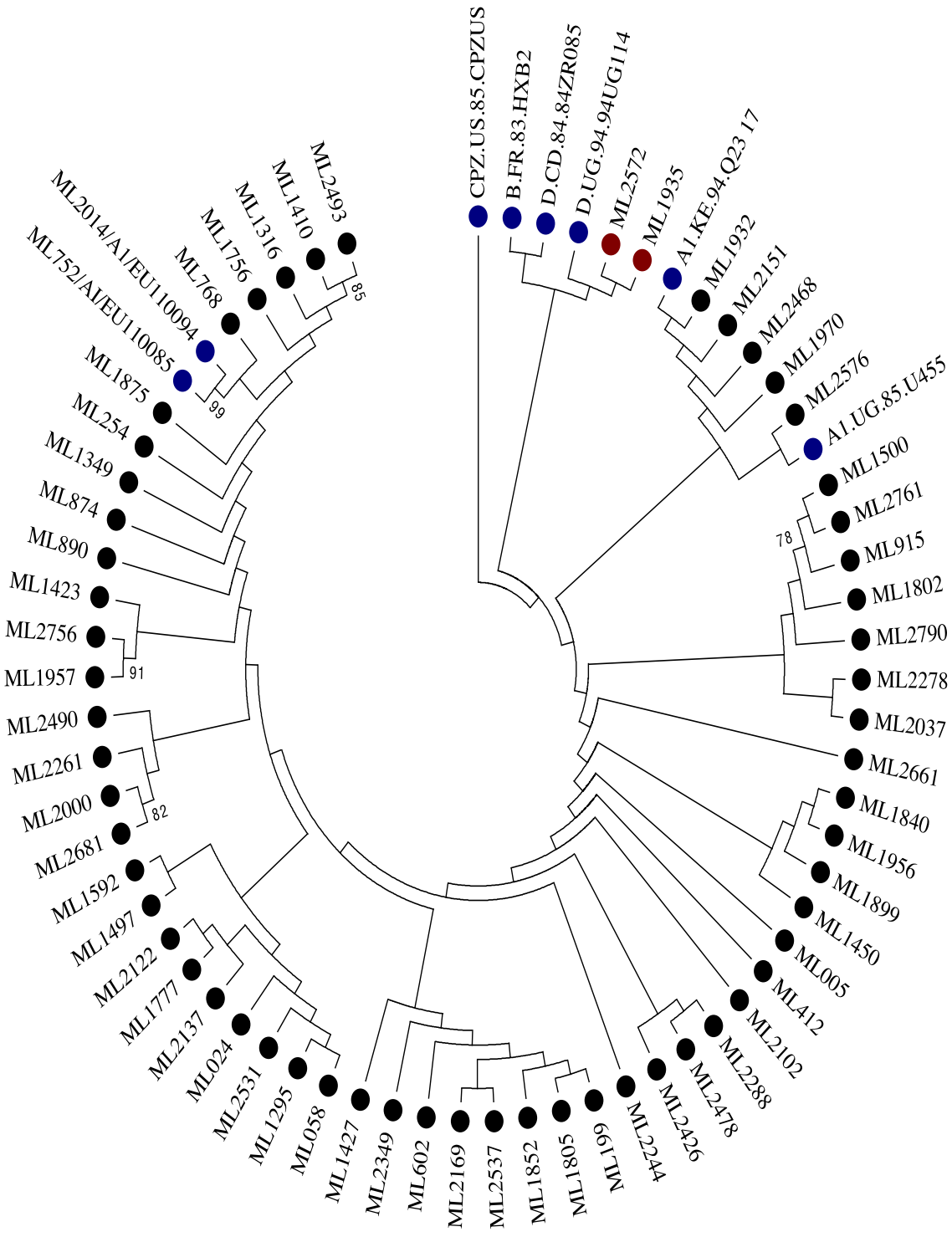
Patient ID	**ART at study point**	**Length of time on ART (wks)**	**HIV-1 Subtype**	**RTI resistance mutations**	**Class: NRTI**	**Class: NNRTI**
ML1756	NVP/d4T 30/3TC	34	A1	T215S	ABC, AZT, **d4T***, ddI, TDF	None
ML1932	NVP/d4T 40/3TC	141	A1	G190A	None	**NVP***, ETR, EFV
ML1935	NVP/d4T 40/3TC	60	D	M184V, Y181C	**3TC***, ABC, FTC	**All*** (DLV, EFV, ETR, NVP)
ML2572	TDF/KALETRA/ AZT	51	D	D67N, V118I, M184V, K101E, Y181C, G190A	3TC, ABC, **AZT***, d4T, ddI, FTC	**All*** (DLV, EFV, ETR, NVP)
ML2661	NVP/d4T 30/ 3TC	34	A1	D67A	**d4T***, **3TC***, AZT	None

* – Drugs for which the mutations shown compromise efficacy

Table 2b: Mutation map for samples showing RTI resistance-associated mutations

		Nucleoside RT Inhibitor (NRTI) Resistance Mutations													Non-Nucleoside RT Inhibitor (NNRTI) Resistance Mutations									
Patient ID	$CD4^+$ T cell Count	41 M	65 K	67 D	69 T	70 K	74 L	75 V	77 F	115 Y	116 F	184 M	210 L	215 T	219 K	100 L	101 K	103 K	106 V	179 V	181 Y	188 Y	190 G	225 P	230 M
ML 1756	276													S											
ML 1932	407																						A		
ML 1935	103											V													
ML 2572	157			N								V					E				C		A		
ML 2661	319			N																					

Figure 1



Phylogenetic analysis of HIV-1 RT (corresponding to nt 2480– nt 3180 of HIV-1 HXB2) sequences from ARV-treated female sex workers in Nairobi Kenya. A blue circle marks the reference sequences, red circle marks sequences that are subtype D and a black circle marks sequences that are subtype A1. An ML number indicates study samples

Discussion

This study found the prevalence of RTI-associated mutations to be 8.3% in a population of ARV treated female sex workers sampled in 2008. This is the first study to identify antiretroviral resistance genotypes in a population engaged in sex work in Kenya. This prevalence is low relative to previous studies on treated individuals attending CCC and PMTCT clinics within Kenya [11]. More recent studies in Kenyan cohorts have however reported an even higher risk for development of antiretroviral resistance mutations even in populations with short-term treatment [12, 21]. Antiretroviral therapy was introduced into the Majengo cohort less than two years prior to sampling for this study and this could account for the much lower prevalence observed. The samples studied were collected at different times in the treatment courses of individual patients, ranging from 34 weeks to 149 weeks on treatment. The length of time on treatment however did not appear to have an influence on the number of antiretroviral resistance mutations identified. This is despite findings that show that prevalence of drug resistance is directly proportional to the length of time of exposure to ART[22-25].

HIV-1 subtype A1 was predominant in this population at 96.7%, while HIV-1 subtype D was observed in 3.3% of the women. This is consistent with previous studies that have shown HIV-1 subtype A to be the predominantly circulating subtype in various regions in Kenya [26-29]. The few studies on HIV-1 subtype distribution that have been carried out within the Majengo cohort show that HIV-1 subtype A predominates, followed by subtype D and a significant proportion of intersubtype recombinants [30, 31].

A total of seven mutation points (at codons 67, 101 118, 181, 184, 190 and 215) were identified in the 5 samples with RTI-associated resistance mutations. Presence of the revertant mutation I/S at position 215 suggests suboptimal therapeutic regimens that are unable to prevent development of resistance [32]. All participants with RTI-associated mutations showed evidence of resistance to at least one drug in their treatment regimen. Complete class resistance to NNRTI was observed in 40% of participants. NNRTI resistance occurs most often at lower levels of adherence to ART [33]. This, coupled with the occurrence of mutations that have been associated with suboptimal therapeutic regimens as mentioned above, suggests that adherence to ART in this population needs be monitored closely.

V60I, which was detected in all 60 isolates that were analyzed, has been identified as a polymorphic amino acid residue specifically in HIV-1 subtypes A and D. Q207A and R211S have been identified in HIV-1 subtype A and K49R in HIV-1 subtype D [34]. Mutations at positions K20, T39, V35, K122, D123, K173, Q174, V179, T200, Q207 and R211 have been have been shown to have a possible association with a decrease in phenotypic susceptibility to RTI. Mutations at positions K20 and K43 are associated with previous use of RTI with a correlation between the presence of K20R and the use of lamivudine while T39R was associated with the previous use of AZT and with the development of thymidine analog resistance [13, 15]. Fifty-nine of 60 participants in this study were on lamivudine and 28 were treated with AZT.

A limitation to this study is that while these findings show the level of RTI-associated resistance in this population, the cause for the development of this

resistance cannot be conclusively identified. Development of antiretroviral resistance may be as a result of singular factors or interactions of factors such as levels of adherence to ART [35, 36] and levels of HIV RNA suppression [37, 38]. In this study, we were unable to collected data for both ART adherence and HIV RNA load.

Conclusion

The findings of this study corroborate those in earlier studies on subtype distribution in Kenya. Recombination analyses would be useful in extending the dataset on the Majengo cohort, in any follow-up studies. The drug resistance rate observed in this study though low for a population of treated individuals is of great public health significance because the sex worker population play an important role in transmission of antiretroviral resistant HIV-1 genotypes.

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