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Age and CD4+ T cell counts are inversely associated with HIV drug resistance mutations in treatment naive female sex workers

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Abstract

The increasing prevalence of human immunodeficiency virus (HIV) drug resistance mutations (HIVDRM) in untreated seropositive persons has consequences for future treatment options. This is extremely important in key populations such as female sex workers (FSWs), where the prevalence of pretreatment drug resistance (PDR) and associated risk factors are unknown. In this study, we analyzed PDR and associated risk factors in recently diagnosed and treatment-naive FSWs in Nairobi, Kenya. In this cross-sectional study, we used 64 HIV-seropositive plasma samples collected from FSWs between November 2020 and April 2021. To identify HIVDRM, the *pol* gene was amplified and genotyped using sanger sequencing. The effects of age, tropism, CD4+ T cell count, subtype, and location on HIVDRM counts were examined using Poisson regression. Overall, the prevalence of PDR was 35.9% (95% CI: 24.3–48.9), which was strongly influenced by K103N and M184V mutations, which confer resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs) and nucleoside reverse transcriptase inhibitors (NRTI), respectively. Subtype A1 was predominant followed by subtype D with a notable increase in inter-subtype recombinants. We found statistically significant evidence that age was inversely related to HIVDRM. A FSW who is 1 year older had 12% less HIVDRM (incidence rate ratios [IRR]: 0.88; 95% CI: 0.82–0.95; $P < .001$), after adjusting for CD4+ T cell count, subtype, location, and tropism. Similarly, an increase in CD4+ T cell count by 1 unit, was associated with 0.4% fewer HIVDRM (IRR: 0.996; 95% CI: 0.994–0.998; $P = .001$), while controlling for the other variables. HIV-1 tropism was not associated with HIVDRM counts. In conclusion, our findings show a high prevalence of NNRTIs. Lower CD4+ T cell counts and younger age were significant risk factors that influenced HIVDRM loads. This finding underscores the relevance of targeted interventions and the importance of continuing to focus on FSWs as a way of addressing the HIV epidemic.

Abbreviations: ART = antiretroviral therapy, env = envelope gene, FSWs = female sex workers, HIV = human immunodeficiency virus, HIVDRM = human immunodeficiency virus drug resistance mutations, IRR = incidence rate ratio, NNRTIs = non-nucleoside reverse transcriptase inhibitors, NRTIs = nucleoside reverse transcriptase inhibitors, PDR = pretreatment drug resistance, PIs = protease inhibitors, SWOP = sex work outreach program.

Keywords: female sex workers, HIV drug resistance mutations, Kenya, pretreatment drug resistance (PDR), treatment naïve

1. Introduction

Female, male, and transgender sex workers are key populations driving the human immunodeficiency virus (HIV) pandemic.[1] Globally, female sex workers (FSWs) suffer a disproportionately heavy burden of HIV infection, with 2019 statistics showing FSWs were 30 times more likely to contract HIV than the general female population.[2] Notwithstanding decades of research and programs on this population, the factors behind the expansion HIV epidemic are not clear. This is may be due to the criminalization of sex work, which hinders access to health-care services, including effective HIV prevention and

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treatment. Furthermore, criminalization makes FSWs more vulnerable to violence perpetrated by various actors.[2,3] Sex work is common in Kenya, but it is illegal, making FSWs 30 times more vulnerable to acquiring HIV than the general female population.[2] Moreover, a study of ten Sub-Saharan African nations indicated that the odds of living with HIV were 7.17 times greater for a sex worker in a country that criminalizes sex work compared to a country that partially legalized sex work.[4]

Over the last decade, antiretroviral therapy (ART) has been scaled up at an unprecedented rate: by the end of December 2020, 27.5 million people worldwide were receiving ART.[5] Increasing pretreatment drug resistance (PDR) levels have been observed in regions with increasing ART coverage, based mainly on fixed-dose combinations consisting of 2 nucleoside reverse transcriptase inhibitors (NRTI) plus a non-nucleoside reverse transcriptase inhibitor (NNRTI).[6] NNRTI-based regimens have a low genetic barrier to resistance, which results in treatment failure in up to 30% patients per year in low-/middle-income countries.[7] Kenya has a test and treat policy, which implies viral load and genotypic testing are not required at the start of ART, and regimens are determined at the population level based on drug efficacy, cost, and logistics.[8] Viral load testing is performed 6 months after initiating treatment and annually thereafter to assess ART effectiveness in virological suppression, whereas genotypic resistance testing is performed if a protease inhibitors (PI)-based or second-line regimen has failed. This policy is partly responsible for increasing drug resistance and needless toxicity and pill burden that could be avoided if resistance testing was performed prior to initiating therapy.

The rising prevalence of HIVDRM in untreated key populations is disturbing since it has implications for both HIV transmission as well as treatment options. A recent study on the oldest FSW cohort, Pumwani, found a high level of PDR (38%) across all ART classes.[9] A systematic review of 332 datasets with 63,111 subjects focusing on key populations found that the pooled prevalence estimate of any PDR was high among men who have sex with men at 13.0% (95% CI: 11.0–14.0), and sex workers at 17.0% (95% CI: 6.0–32.0), but lower among injecting drug users at 7.0% (95% CI: 5.0–10.0).[10] Of concern is the rise in PDR in the general population to non-nucleoside reverse transcriptase inhibitors (NNRTIs) after the ART scale-up, with substantial increases in resistance to NNRTI in East Africa (36%, 95% CI: 21–52) and southern Africa (23% 95% CI: 7–42).[7] In context of a statistic indicating a higher prevalence of PDR in key populations and in Sub-Saharan Africa, we assessed the prevalence of PDR in recently infected FSWs. In addition, we used multivariable poisson regression modeling to identify predictors of the observed human immunodeficiency virus drug resistance mutations (HIVDRM).

2. Methods

2.1. Research ethics

This study was approved by the Kenyatta National Hospital - University of Nairobi Ethics and Research Committee granted ethical approval to collect HIV-positive plasma for the HIV prevention and care program (KNH-UON-ERC: P258/09/2008). Participants in the study provided informed consent for the storage and use of their samples in subsequent HIV-related research. Thus a waiver of consent was sought and granted for conducting this HIV drug resistance study (KNH-UON-ERC: P556/07/2019).

2.2. Study design and participants

This was a cross-sectional study that utilized 157 plasma samples obtained from HIV-1 seropositive and treatment-naïve FSWs between November 2020 and April 2021. The participants were recruited from 7 sex worker outreach program (SWOP) clinics in Nairobi County, specifically Nairobi City, Donholm, Kawangware, Korogocho, Langata, Majengo, and Thika Road. The study comprised subjects who satisfied the following criteria: 18 years old; HIV-1 seropositive; treatment naive; located in Nairobi county during the enrollment period (November 2020 - April 2021); and engaging in sex work, defined as an adult who consents to sexual exchange for monetary gain. Among the exclusion criteria were: treatment experienced subjects; subjects under the age of 18; and subjects not participating in sex work as indicated above.

2.3. $CD4^+$ T cells profiling

$CD4^+$ T cell count/% were determined using BD FACSPrestoTM as per manufacturer instructions. Briefly, blood samples were added to the inlet ports on the BD FACSPrestoTM cartridge using pipets provided. The cartridges were incubated on the workstation for 18 minutes at room temperature before the tear strips were removed and the sample IDs matching to the cartridges were inserted in the machine and read.

2.4. HIV-1 viral RNA extraction

HIV-1 RNA extraction from plasma samples was carried out using the Invitrogen PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA) as per manufacturer instructions. Briefly 500 µL of plasma was lysed using 500 µL viral Lysis buffer and 62.5 µL Proteinase K. The lysate was transferred to sterile spin column, washed twice with 500 µL of wash buffer, centrifuged at 6800g for 1 minute and then 17,000g for 1 minute, and finally eluted in 20 µL of sterile, RNAase-free water. RNA was stored at –80°C.

2.5. The pol gene amplification

Complementary DNA was generated using Superscript III 1-step RT-PCR Kit as per the manufacturers' instructions. Briefly, 1 µL of RNA was incubated at 65°C for 10 minutes and mixed with 39 µL RT-PCR Master Mix. The cycling conditions were as follows: reverse transcription at 50°C for 45 minutes, enzyme inactivation at 94°C for 2 minutes, PCR initial denaturation at 94°C for 2 minutes, 40 cycles (94°C for 15 seconds 50°C for 20 seconds , 72°C for 2 minutes), and a final 10 minutes extension at 72°C. For nested PCR, 2 µL of PCR products were amplified in a 50 µL reaction with AmpliTaq Gold LD DNA polymerase (Thermo Fischer Scientific Inc., Massachusetts, USA) as follows: initial denaturation 94°C for 4 minutes, 40 cycles (94°C for 15 seconds, 55°C for 20 seconds, 72°C for 2 minutes), and a final 10 minutes step at 72°C. The amplified PCR fragment was purified using ExoSAP-IT PCR Product Clean-up Reagent as follows, 10 µL of nested PCR product was mixed with 4 µL ExoSAP-IT reagent and incubated at 37°C for 15 minutes, 80°C for 15 minutes incubation, and cooling at 4°C. All cycling conditions were performed on Veriti Thermal Cycler (Thermo Fisher Scientific, San Francisco, USA). The amplified product (1.08kb) was verified by 1% agarose gel electrophoresis and visualized on UVITEC Gel Documentation System (Cleaver Scientific, Cambridge, UK).

2.6. Sanger sequencing

Thermo Fisher Scientific HIV Genotyping kit was used for the *pol* gene sequencing, and includes 6 overlapping primers. 2 µL of PCR product was mixed together with 18 µL of sequencing mix. The sequencing conditions were as follows: 25 cycles of 10

Table 1

Demographic and clinical characteristics of 64 study subjects included in the analysis.

Characteristic	DRM, N = 23	No DRM, N = 41
Tropism, n (%)		
CCR5	15 (65)	28 (68)
CXCR4	8 (35)	13 (32)
Age (yr), median (IQR)	30 (28–34)	38 (34–44))
CD4 %, median (IQR)	18 (12–28)	21 (12–26)
CD4 count (copies/mm^3), median (IQR)	291 (159–499)	355 (197–579)
HIV-1 Subtype (*pol* gene), n (%)		
A1	17 (74)	24 (59)
A2	0 (0)	2 (4.9)
B	1 (4.3)	0 (0)
C	1 (4.3)	0 (10.6)
D	1 (4.3)	8 (20)
A1/C	0 (0)	1 (2.4)
A1/D	3 (13)	4 (9.8)
C/D	0 (0)	1 (2.4)
SWOP clinic n (%)		
Thika road	2 (8.7)	14 (34)
City	4 (17)	6 (15)
Kawangware	6 (26)	13 (32)
Donholm	1 (4.3)	1 (2.4)
Korogocho	7 (30)	1 (2.4)
Langata	0 (0)	3 (7.3)
Majengo	3 (13)	3 (7.3)

Results are summarized by median (IQR) for continuous data and frequency (%) for categorical data.

CCR5 = C-C motif chemokine receptor 5, CXCR4 = CXCR4 C-X-C motif chemokine receptor 4, DRM = drug resistance mutations, SWOP = sex worker outreach program.

seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C and were carried out on an Applied Biosystems 3500xL DX genetic analyzer using the BigDye XTerminator kit (Applied Biosystems, Foster City, USA). The Stanford HIV Drug Resistance Database genotyping algorithm was used to identify drug resistance mutations from sequencing files that were automatically interpreted by RECall.[11] The *pol* sequences have been archived in the DDBJ Nucleotide Database [LC723952-LC724015].

2.7. HIV-1 subtyping

HIV-1 subtyping was carried out by submitting raw *pol* sequences to REGA HIV-1 subtyping tool REGA,[12] which predicted the HIV-1 subtype and associated bootstrap support level. The greater the bootstrap support level, the more precise the prediction.

2.8. HIV-1 tropism determination

HIV-1 tropism was determined using third hypervariable region in the viral envelope glycoprotein gpl20 (V3) loop sequences as described by Abisi and colleagues.[13] Briefly, trimmed V3 loop sequences were translated to the corresponding amino acid sequences, aligned, and analyzed for coreceptor usage using various in silico genotypic tools. There were tropism data for 58/64 subjects. The remaining 6 were imputed using "mice," an R package.

2.9. Statistical analyses

The main outcome (dependent variable) of the study was HIVDRM counts, and the predictor variables were age, CD4$^+$ T cell count and CD4$^+$ T percentage, location of the SWOP clinic, HIV-1 subtype, and tropism (independent variables). To compare categorical variables and continuous variables, respectively, Fisher exact test and Wilcoxon rank sum test were utilized, with 2-sided *P* values reported in all cases. Prevalence and associated 95% confidence intervals were computed using epiR package. A Poisson regression was used to calculate incidence rate ratios (IRR) and the associated 95% confidence intervals. Except for tropism, which was included in the final model regardless of statistical significance, all other variables were only included if they had a $P < .25$ at univariate analysis as they were deemed to influence the outcome. There was missing data for 6 subjects whose envelope gene (*env*) gene could not be amplified for tropism determination, which was imputed using the R package "mice." Statistical analyses were conducted with the R statistical package (R version 4.1.0).

3. Results

3.1. Demographic, clinical and virological characteristics of 64 treatment naïve FSWs

Table 1 shows a summary of the 64 study participants' demographic and clinical characteristics. There was 6 subjects missing tropism data which was addressed through multiple imputation. Prior to conducting the imputation, we determined that the data was not missing completely at random (MCAR), (P = .0160), and thus multiple imputation where several imputed datasets are generated and combined into a pooled result was appropriate. HIVDRM were found in 36% of the subjects (n = 23). The overall median age was 36 years (IQR, 30–40), with subjects with HIVDRM being on average 8 years younger 30 years (IQR, 28–36), when compared to those without HIVDRM 38 years (IQR, 34–44; P = .001) (Fig. 1). The median CD4$^+$ T cell count among subjects with HIVDRM was 291 cells/mm^3 (IQR, 159–499), compared to 355 cells/mm^3 (IQR, 197–579) among those without HIVDRM. There was a lower proportion of CXCR4 C-X-C motif chemokine receptor 4-tropic viruses (33%) (n = 18), but there was no significant difference related to HIVDRM (Fig. 1). HIV-Subtype A1 largely dominated at 64% (n = 41), followed by subtype D at 14% (n = 9). There were 14% (n = 9) inter-subtype recombinants, distributed as follows: A1/D, 11% (n = 7); A1/C, 1.6% (n = 1); and CD, 1.6% (n = 1). There were differences in the predicted *pol* and *env* gene subtypes in 28% (n = 16), indicating the possibility of dual infection or superinfection.

3.2. Pretreatment drug resistance

The HIVDRM counts followed a poisson distribution and ranged from 0 to 8 (Fig. 1). The complete HIVDRM and PDR profiles for all 64 viral strains are shown in Supplementary Table 1, http://links.lww.com/MD/J147. The overall prevalence was 35.9% (95%CI: 24.3–48.9), which was strongly influenced by NNRTI prevalence 32.8% (95%CI: 21.6–45.7). The high resistance to NNRTIs was conferred mainly by the K103N 19% (n = 12) mutation, alongside P225H 3.1% (n = 2), V179E/L/T 4.7% (n = 3), G190A 1.6% (n = 1), Y181C 1.6%(n = 1), V108I 1.6% (n = 1), and V106I 1.6% (n = 1) (Table 2). NNRTIs have low genetic resistance, and a single NNRTI-related mutation confers high-level resistance to all 3 NNRTIs (NVP, EFV, and ETV). The prevalence of NRTI mutations was low, at 6.3% (95% CI: 1.7–15.2), similar to the prevalence of PI mutations, which was 4.7% (95% CI: 1.1–13.1). Resistance to NRTIs was conferred by M184V 6.2% (n = 4), K65R 3.1% (n = 2), L74I 1.6% (n = 1), and Y115F 1.6% (n = 1) mutations, together with thymidine analog mutations M41L 1.6% (n = 1) and K70E 1.6% (n = 1). M184V/I confers high-level resistance to 3TC and ABC while also increasing susceptibility to AZT and TDF, whereas K65R mutations confer intermediate resistance to TDF. Notably, 4.7% (n = 3) of the subjects had high-level multi-drug resistance to NRTI and NNRTI drug classes. A 1-of-a-kind case involved

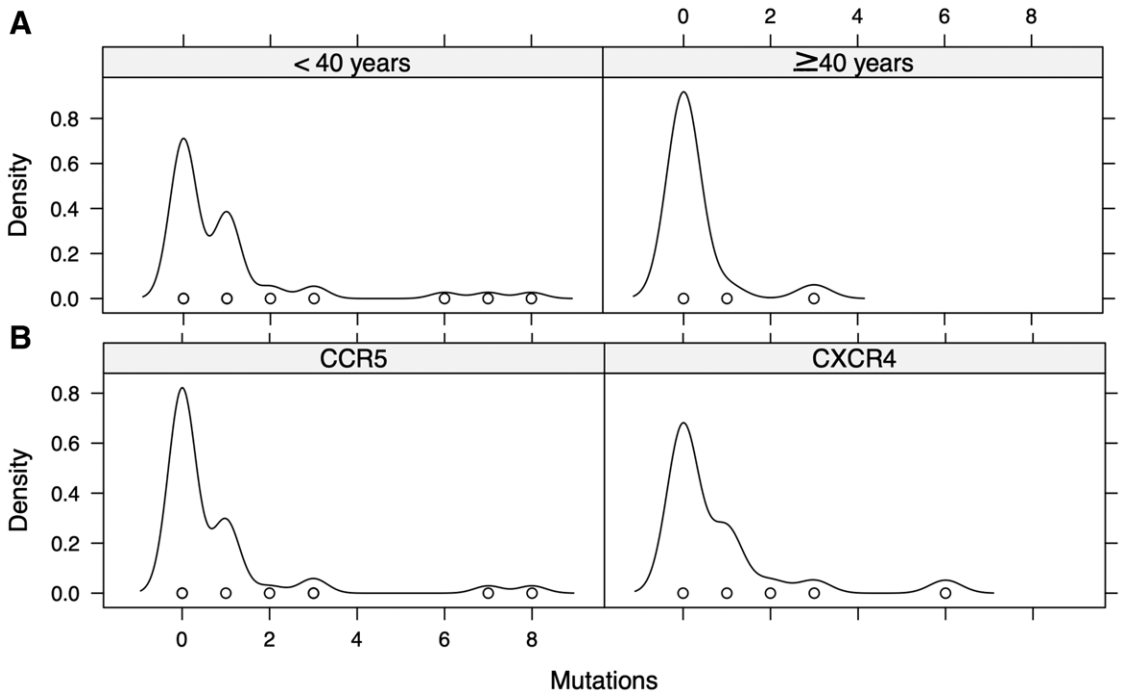

Figure 1. Distribution of HIV-1 drug resistance mutations in the study subjects stratified by age and tropism. This density plot shows a right skewed distribution with the circles depicting the location of the mutations while the curve shows the frequency of that mutation. In (A), subjects who are <40 yr have more mutations, range (0–8) compared to subjects who are 40 yr or older, range (0–4), and the difference is statistically significant (P = .001). In (B), subjects infected with CCR5-tropic viruses had a range of (0–8) compared to (0–6) for CXCR4-tropic viruses, and the difference was not statistically significant (P = .80). CCR5 = C-C motif chemokine receptor 5, CXCR4 = CXCR4 C-X-C motif chemokine receptor 4.

a subject who had drug resistance mutations across 3 classes, implying a case of acquired HIV drug resistance through previous ARV drug exposure.

3.3. Predictors of PDR

The strongest predictors of HIVDRM counts were age, CD4⁺ T cell counts and % (Table 3). Due to multicollinearity between CD4⁺ T cell counts and CD4⁺ T cell %, only CD4⁺ T cell counts, alongside subtype, geographical location, and tropism, were included in the final model. Chi-square goodness-of-fit test for poisson assumption had a P value of .33 indicating good model fit. A FSW who is 1 year older had 12% less HIVDRM (IRR 0.88; 95% CI: 0.82–0.95; P < .001), controlling for CD4⁺ T cell count, subtype, location and tropism. Similarly, while controlling for model variables, an increase in CD4⁺ T cell count by 1 unit (1 cell/mm^3) was associated with 0.4% fewer HIVDRM (IRR 0.996; 95% CI: 0.994–0.998; P = .001). When CD4⁺ strata (200 cells/mm^3, 200–349 cells/mm^3, 350–500 cells/mm^3, >500 cells/mm^3) are used in lieu of CD4⁺ T cell count as a continuous variable, we see that subjects with >500 cells/mm^3 had 83% lesser HIVDRM counts than those with 200 cells/mm^3 (reference group). In this study, subtype A1 predominated (64%) and was used as the reference. While controlling for the other variables, subjects infected with subtype C had 15.2 times higher HIVDRM (IRR 15.2; 95% CI: 1.48–156; P = .022) than subtype A1 (reference). Subjects infected with subtype D, on the other contrary, had a lower risk of HIVDRM, with an IRR of 0.10 (95% CI: 0.01–0.92; P = .042) after controlling for the other variables. The geographic location of a subject also had an impact on HIVDRM counts. Given that all other independent variables are equal, an FSW recruited from the city SWOP clinic is 32.1 times more likely to have higher HIVDRM than an FSW recruited from the Thika road clinic (reference) (IRR = 32.1; 95% CI: 3.67, 281; P = .002). The 95% CIs were quite wide due to the uneven distribution of patients between the HIV subtype and SWOP clinic groups, but this did not appear to be a concern given that the computed coefficients' standard errors were reasonable. Tropism was not found to be a significant predictor of HIVDRM counts.

4. Discussion

In this study, we investigated PDR in recently infected treatment naïve FSWs. pretreatment HIV drug resistance can either transmitted or acquired resistance or both. Thus, for the purpose of this study, the drug resistant viruses are assumed to have been transmitted at the time of infection (transmitted HIV drug resistance), since the subjects have a history of follow-up from 1 of the 7 SWOP clinics, where sex workers have access to HIV prevention and treatment services.[14,15] The rationale for establishing the SWOP clinics stems from the fact that sex workers face stigma associated with sex work, and if they seroconvert, this underlying stigma restricts their access to essential HIV services. Six subjects had missing tropism data, which was addressed using multiple imputation, which protected against statistical power loss at the expense of adding noise or extra variance, which can cause bias. Failure to impute, and thus deletion of values with missing values, would have resulted in bias as well as a loss of statistical power.

High levels of transmitted drug resistance have been described for antiretroviral drugs with low genetic barriers of resistance, after long-term usage. These include M184I/V, linked with NRTI resistance, and K103N/S, Y181C/I, and G190A/S, associated with resistance to 1st NNRTIs.[16] In this study, the prevalence of HIVDRM conferring resistance to NRTI was (6.3%), PI (4.7%), and NNRTI (32.8%). The high levels of NNRTI resistance were due to the K103N, G190A, and Y181C mutations, which confer resistance to NVP, EFV and to some extent ETR. This is not surprising as NNRTIs have a low genetic barrier to resistance.[17] This is in line with findings that show significant increases in NNRTI resistance in east Africa (36%) and

Table 2

Pretreatment drug resistance mutations found in 21 subjects and their clinical implications.

ID	Age	Site	CD4	NRTI mutations	3TC	ABC	ddI	FTC	TDF	NNRTI mutations	EFV	NVP	RPV	ETR	DOR	PI mutations	SQV	LPV/r
1	37	DONHOLM	504	M184V,K65R, M41L	HR	HR	HR	HR	IR	K103N, Y181C	HR	HR	IR	IR	LR	I54M, L90M	HR	IR
2	33	CITY	246	M184V,K70E,K219R	HR		IR	HR	LR	K103N,E138A,K238T,V179L,P225H	HR	HR	IR	LR	IR			
3	29	KOROGOCHO	23	M184V, L74I, Y115F	HR	HR	HR	HR	IR	K103N,E138A,K238T	HR	HR	LR	LR				
4	30	KAWANGWARE	355	M184V	HR			HR										
5	34	TKA RD	494							K101E,E138A,G190A	HR	HR	HR	IR	LR			
6	30	KAWANGWARE	210							K103N,P225H,F227L	HR	HR			HR			
7	24	MAJENGO	49							K103N,K238T	HR	HR						
8	42	CITY	291							K103N	HR	HR						
9	28	KAWANGWARE	587							K103N	HR	HR						
10	40	KAWANGWARE	143							K103N, E138A, V179E	HR	HR	LR	IR				
11	38	TKA RD	583							K103N	HR	HR						
12	36	KOROGOCHO	40							K103N	HR	HR						
13	25	MAJENGO	57							K103N	HR	HR						
14	22	KOROGOCHO	343							K103N	HR	HR	HR					
15	35	KOROGOCHO	175							K103N	HR	HR						
16	27	CITY	824							E138A			LR	LR		K43T		
17	30	KOROGOCHO	85							E138A			LR	LR				
18	37	KOROGOCHO	273							V106I			LR	LR	LR			
19	24	KAWANGWARE	283							E138A			LR	LR				
20	29	CITY	698							V108I					LR			
21	28	MAJENGO	406							E138A			LR	LR				

3TC = lamivudine, ABC = abacavir, ddI = didanosine, EFV = efavirenz, ETR = etravirine, FTC = emtricitabine, HR = high-level resistance, ID = unique subject identifier, IR = intermediate resistance, LPV/r = lopinavir/ritonavir, LR = low-level resistance, NNRTI = non-nucleoside reverse transcriptase inhibitor, NRTI = nucleoside reverse transcriptase inhibitor, NVP = nevirapine, PI = protease inhibitor, RPV = ralpivirine, SQV = sasquinavir; TDF = tenofovir.

Table 3

Factors associated with HIV drug resistance mutation counts in 64 HIV-1 infected treatment naïve female sex workers.

	Univariable			Multivariable		
Characteristic	**IRR**	**95% CI**	***P* value**	**aIRR**	**95% CI**	***P* value**
Age	0.93	0.90,0.97	**<.001**	0.88	0.82,0.95	**<.001**
CD4 count	0.998	0.997, 1.000	.067	0.996	0.994, 0.998	**.001**
HIV-1 subtype						
A1	—	—		—	—	
A1/D	0.52	0.16, 1.68	.27	0.18	0.02, 1.66	.13
B	8.44	3.74, 19.0	**<.001**	∞	0.00, ∞	>.99
C	1.81	0.56, 5.89	.33	15.2	1.48, 156	**.022**
D	0.27	0.06, 1.12	.070	0.10	0.01, 0.92	**.042**
SWOP clinic						
Thika Road	—	—		—	—	
Kawangware	2.11	0.66, 6.71	.21	7.04	0.89, 56.0	.065
City	4.80	1.55, 14.9	**.007**	32.1	3.67, 281	**.002**
Donholm	14.0	4.1, 47.8	**<.001**	0.00	0.00, ∞	>.99
Korogocho	6.00	1.94, 18.6	**.002**	5.91	0.76, 45.8	.089
Langata	0.00	0.00, ∞	>.99	0.00	0.00, ∞	>.99
Majengo	2.67	0.67, 10.7	.17	17.7	0.88, 359	.061
Tropism	—	—		—	—	
CCR5						
CXCR4	0.99	0.53, 1.77	.98	1.24	0.59, 2.63	.57

Bold value indicates $P < .05$. *P* values from χ^2 test for categorical variables or Wilcoxon rank sum test for continuous variables

aIRR = adjusted incidence rate ratio, CCR5 = C-C motif chemokine receptor 5, CI = confidence interval, CXCR4 = CXCR4 C-X-C motif chemokine receptor 4, IRR = unadjusted Incidence Rate Ratio, SWOP = sex work outreach program.

southern Africa (23%), with no increases for any other drug classes in any region.[7] This could be explained by factors such as the pharmacokinetics of these drug classes, as well as prior use of single-dose nevirapine in the prevention of HIV transmission from mother to child (PMTCT).[18,19] M184V/I was found in 6.2% of the sequences and confers high-level resistance to 3TC and ABC while also increasing susceptibility to AZT and TDF.[20] The fact that 3TC is a backbone for most Kenyan ART-regimens isn't a cause for alarm as in a triple combination, virological suppression can be accomplished by 2 active drugs. PI-resistant viruses were found in a small percentage of subjects with no other resistant viruses except for the unique case with multidrug resistance described previously. Except for the unique case of 8 drug resistance mutations, the remainder of the subjects should be virally suppressed by the current first-line regimens. This is in recognition of the fact that many developing countries have limited access to routine viral genotyping prior to ART initiation, and that routine viral load monitoring is done 6 months after ART initiation and then on a yearly basis to ascertain ART efficacy.

HIV-1 subtype A1 was the most common subtype observed, followed by subtype D with inter-subtype recombinants, particularly A1/D, in line with past findings.[21–23] A significant increase in inter-subtype recombinants among recent infections, as seen with A1/C and C/D recombinants, suggests an epidemic shift. This expansion of subtype D epidemic is likely to have negative consequences due to the observed high virulence and faster disease progression of the D strain by itself or via recombinants.[24,25] Evidence of dual infection or superinfection was found in 28% of cases where the predicted subtype for the *env* and *pol* genes differed for the same sample.[13] This could be attributed to reverse transcriptase enzyme which has a very high error rate, producing approximately 2 mutations per 10^5 nucleotides.[26] The other plausible explanation is template switching, which offers a significant mechanism for HIV recombination at a rate of 3 to 30 template switches per single genome replication.[27] Moreover, superinfection with HIV is presumed to occur frequently because of the existence of interclade recombinants, and has been observed more frequently in high-risk compared to general populations.[28,29] Although unlikely, it is possible that the subjects described were infected with a strain that had different *env* and *pol* regions but the fitness of such a viral strain will be highly compromised.

To better understand how different factors influenced HIVDRM counts, we used a multivariable poisson regression model to predict HIVDRM counts based on clinical, viral, and sociodemographic factors. We found a strong association between younger age and higher HIVDRM counts ($P < .001$), which is in line with previous studies.[30] This observation could be explained by the fact that younger FSWs are deemed more "attractive," and thus more likely to attract more clients, increasing the likelihood of infection by different variants.[31–33] This is exacerbated further by gender power dynamics, which may contribute to younger women being less likely to negotiate condom use. We also found a statistically significant inverse relationship between $CD4^+$ T cell count and HIVDRM ($P = .001$), which was also associated with tropism in the same samples.[13] $CD4^+$ T cell number is an immunological marker for HIV disease diagnosis, hence why it was previously used to determine the priority for initiating ART before the current test and treat policy.[34] The low $CD4^+$ T cell counts in the treatment naïve subjects might be as a consequence of untreated HIV replication causes progressive $CD4^+$ T cell loss, or a faster decline in $CD4^+$ T cell counts in persons infected with transmitted drug resistance strains than in persons infected with wild-type virus during the first year, but not in subsequent years.[35] However, contradictory findings were observed where transmitted drug resistance viruses had lower fitness and pathogenicity and thus had a higher initial $CD4^+$ T cell count than persons with wild-type virus, which tended to disappear over time, possibly coinciding with the emergence of fitter variants.[36]

In a previous study using the same samples, we found that the Kawangware SWOP clinic was statistically associated with CXCR4 C-X-C motif chemokine receptor 4-tropic viruses.[13] However, in this study, tropism was not associated with HIVDRM ($P = .57$), nor was the Kawangware clinic, but rather the Nairobi City clinic. Contrary to what was found in the multivariable analysis, the result was different in the univariable analysis, where, in addition to the Nairobi city clinic, Donholm and Korogocho were statistically associated with higher HIVDRM counts. This is an ideal illustration of Simpson paradox, in which results from whole data analysis contradict

findings from subgroups of the same data.[37] This highlights the uniqueness of each clinic, though we cannot rule out the possibility of a sex worker visiting a clinic away from their work location due to the stigma associated with sex work.[38]

Certain limitations should be considered when interpreting our findings. First, we examined mutations conferring resistance to NRTI, NNRTI, and PI, but not mutations that confer INSTI resistance. This is relevant because Kenya switched from using NVP- to dolutegravir-based regimens to treat adults and children with virologic failure due to dolutegravir high genetic barrier to resistance.[39] Second, there was no data on viral load, which was due in part to the test and treat policy, which specifies that viral load monitoring is not required at the commencement of treatment but is performed 6 months later to assess ART effectiveness in virological suppression. As observed in our previous study, viral load is an important predictor variable factor in the development of HIVDRM.[40] Finally, due to a poor amplification rate, the study sample size was small (64/157), with 6 samples imputed for tropism. Therefore, we cannot rule out the possibility of low study power as the cause of contradictory findings.

In conclusion, we present proof of the existence of PDR mutations in our FSW cohort. Alarmingly, 1 subject had a triple class drug resistance which is a cause for concern since from the onset the subject had very limited treatment options. The K103N mutation, which conferred resistance to NNRTIs such as nevirapine and efavirenz, was a leading cause of PDR, while $CD4^+$ T cell counts and age were significant predictors of HIVDRM counts. This underscores the importance of targeted interventions for this vulnerable key population.

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