Molecular Characterization of HIV-1 among Female Sex Workers (FSWs) in Five Departments of the Republic of Congo

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ABSTRACT

Introduction: HIV-1, an infectious agent with the greatest sequence diversity, including higher recombination rates. The objective of this study is to assess the genetic diversity of HIV-1 in FSWs in Congo.

Methods: A total of 39 plasma samples from FSWs ART-naive HIV-1-seropositive were collected from urban and semi-urban areas of the Republic of Congo. HIV-1 viral loads were determined by RT-PCR according the manufacturer's instructions of the Abbott® m2000 Real-Time System. Complementary DNA synthesis and PCR amplification of HIV reverse transcriptase gene was performed from extracts of samples with a viral load > 1000 copies/mL by Nested PCR and products was sequencing by using an ABI 3500XL Genetic Analyzer for determined genetic diversity of HIV strains and the antiretroviral resistance profile.

Results: Of the 39 samples extracted, only 14 had a viral load greater than or equal to 1000 copies/ml. We were able to sequence 12 samples. It emerges that five subtypes of HIV-1 (A, G, J, H, D) and a recombinant form, CRF02_AG circulate among FSWs. Subtype A was more predominant with 41% followed by subtype G (25%). Subtypes D, H, J and CRF02_AG had a proportion of 8.33% each. Two samples showed major G190GA mutations conferring resistance to INNRT like NVP, V106VA and M184MV to INRTs such as DOR, NVP FTC and 3TC. Minor mutations such as: K70N, T215TN, E138A and V179E causing intermediate resistances to: EFV, ABC, D4T, DDI, TDF.

Conclusions: This study showed a predominance of subtype A followed by G then J, H, D subtypes and recombinant form CR02_AG in FSWs. Despite the small study sample size, continued analysis of HIV-1 viral loads and sequences in this population of FSWs is of considerable interest to examine and monitor genetic variability.

Keywords: HIV-1 molecular characterization, HIV-1 drug resistance mutations, Female Sex Workers, Republic of Congo

INTRODUCTION

Human immunodeficiency virus (HIV) is the causative agent of Acquired Immune Deficiency Syndrome (AIDS) [1]. Infection with this virus is one of the greatest epidemiological challenges facing the whole world for over thirty years. Genetic diversity of HIV is an important factor influencing the epidemiological aspects of HIV infection worldwide, with a definite impact on diagnostic techniques, antiretroviral therapy and HIV vaccine research [2]. New HIV infections among adults and adolescents have declined by 30% since 2000, but despite many efforts, since 2010, around 1.8 million adults and children have been infected by HIV [3]. Regarding the distribution of new infections by type of population and by region, UNAIDS estimated at 47% in the world and 16% in West and Central Africa the contribution of key populations, designating the people most likely to contracting or transmitting HIV, in the occurrence of new infections in 2017 [3]. Among these populations most at risk of HIV infection, in most countries of sub-Saharan Africa, are Female Sex Workers (FSWs) [4]. FSWs are a priority population for HIV prevention programs, along with their clients who have also long been recognized as a possible epidemiological gateway to other populations [4]. Aware of the role played by this at-risk population in the occurrence of new infections, the Congo carried out in 2012 the first behavioral survey linked to HIV among FSWs, this study reports the seroprevalence in this population at 7.5% [4]. Molecular epidemiology of HIV-1 around the world shows that Western Europe and North America are dominated by subtype B; South Asia by CRF01_AE; Eastern Europe by CRF03_AB and South America by B and B/F and that all strains are found in sub-Saharan Africa, particularly in Central Africa, with an overwhelming majority of non-B strains [2,5].

In the Republic of Congo, several studies have demonstrated the genetic diversity of HIV-1 in the general population. In 2012, a study revealed a very large diversity of the virus with mostly undetermined recombinant forms (URF), followed by complex forms such as CRF37_cpx, CRF02_AG, subtypes G, A1, B [6]. In 2017, a similar study showed a predominance of subtype A followed by G and D with several recombinant forms such as: CRF01; CRF02; CRF18; CRF13; CRF37; CRF05 including other types such as: B; C; H; J [7]. However, to date, there are very few studies on the genetic diversity of HIV among FSWs in the Republic of Congo. Given that there is a great sexual promiscuity between FSWs and general population, we believe that the molecular profile of strains of HIV-1 circulating in FSWs would be similar to that observed in the general population in Congo. Thus, this study aimed to (i) evaluate the different strains of HIV-1 circulating in a population of FSWs by drawing a parallel with the variants of HIV-1 circulating in general population; (ii) identify the circulating strains and their recombinant forms and (iii) analyze the profile of primary resistance to antiretrovirals (ARVs) in Female Sex Workers.

MATERIALS & METHODS
Specimens Collection

This cross-sectional descriptive study was conducted from August to November 2018 in the Molecular Biology Unit of the National Public Health Laboratory in Brazzaville, Republic of Congo. A total of 39 plasma samples from FSWs treatment-naive HIV-1-seropositive were collected. These FSWs came from urban and semi-urban cities of the Republic of Congo such as: Brazzaville, Pointe-Noire, Dolisie, Pokola, Ouesso. Only plasma samples with a volume greater than or equal to 600 µl were included in this study; making a total of thirty-nine samples.

Molecular analysis
RNA isolation

Extraction and quantification of HIV viral RNA was performed according to the manufacturer's instructions of the Abbott® m2000 Real-Time System / Abbott Molecular kit (Abbott Park, Illinois, U.S.A).

HIV-1 viral loads were determined by real-time reverse transcription and polymerase chain reaction (RT-PCR).

Complementary DNA synthesis and PCR amplification of the HIV reverse transcriptase (RT) gene (a 1152 bp fragment) was performed from extracts of samples with a viral load > 1000 copies/mL by nested PCR. The supersciptIII one RT-PCR system kit (Invitrogen, Germany) and the external primers MJ3: 5'-AGTAGGACCTACACCTGTCA-3' (2480 to 2499) and MJ4: 5'CTGTTAGTGCTTTGGTTCCTCT-3' (3399 to 3420) were used during of the first round by RT-PCR. 50 µl of the reaction mix and the following thermal cycle: 1 cycle of 50°C for 3 min; 40 cycles of 94°C for 2 min, 55°C for 30 sec, 68°C for 30 sec, and 1 cycle of 68 °C for 5 min, 4 °C until infinity was used during this first PCR. The second round was performed on the products of the first round PCR using the internal primer pairs above: A (35): 5'TTGGTTGCACTTTAAATTTTCCCATT AGTCCTATT-3' (2530 to 2558); NE1 (35): 5'CCTACTAACTTCTGTATGTCATTGA CAGTCCAGCT-3' (3300 to 3334). The following thermal cycling allowed this amplification: 1 cycle of 94°C for 2 min, 4 cycles of PCR at 94°C for 30 sec, 55°C for 30 sec, 68°C for 1 min and 1 cycle of 68° C for 7 min with a final extension at 4°C to infinity.

Nested RT-PCR products were purified and sequenced using internal PCR primers, pureLinkm Quik PCR purification kits (Invitrogen, Germany), Big Dye Terminator v3.1 Cycle Sequencing (Applied Biosystems) and 5 X sequencing buffer using an ABI 3500XL Genetic Analyzer (Applied Biosystems). Electropherograms were assembled with SeqMan (DNAStar, Madison, USA) and edited manually.

Sequence alignment and analysis
The resulting sequences were visually inspected, manually edited and then assembled with Sequencer 5.1. Then, quality control procedures were implemented to remove sequences of potential contaminants from the online HIV sequence database. Then, the sequences were aligned, edited and analyzed by BioEdit software.

Virus subtyping and genotyping
After multiple alignment of the nucleotide sequences of the RT gene [including the reference sequences collected from the Stanford database, United States of America (USA)], the phylogenetic analysis was carried out with PhyML3.0 implemented using the software Geneious 8.1. 8. Samples were deposited in the Stanford (USA) database for subtyping and resistance testing against reverse transcription inhibitors.

STATISTICAL ANALYSIS
Field and laboratory data were entered in Excel version 2016 as well as the construction of graphs and tables. GraphPad software was used to perform descriptive statistics.

RESULT
Study population characteristics
39 plasma samples from female sex workers who tested positive for HIV-1 in the behavioral survey coupled with HIV serology among female sex workers in the Republic of Congo, were the subject of our study. These samples came from five departments of Congo: Brazzaville (n = 14), Pointe-Noire (n = 7), Niari (n = 10), Bouenza (n = 5), Ouesso (n = 3). Nearly half of female sex workers have reached at least secondary with a slightly higher literacy rate among female sex workers in large cities. The majority of them were single 32 (82.1%) and the median age was 29 [IQR, 26.5–32] years. Intravenous drug users were popular among prostitutes in Brazzaville and Pointe-Noire 9 (23.1%). Up to 34 (87.2%) FSWs began having sex when they were < 18 years old, while the remaining 5 (12.8%) were ≥ 18 years old. All FSWs were in mobile prostitution, most FSWs had 1-4 clients per day 21 (53.8%) while 18 (46.2%) had ≥ 5. Note that the record for the practice of prostitution was held by FSWs from

nonurban areas. In addition, all samples were obtained from antiretroviral (ARV) treatment naïve patients.

HIV Viral load

Of the 39 plasma samples of female sex workers naïve from antiretroviral (ARV) treatment, HIV viral load was 756 (IQR, 350 - 6184.75) Copies/ml, 7751.5 (IQR, 2315 - 10,269.75) Copies/ml, 2000 (IQR,265 - 17,344.5) Copies/ml, 403 (IQR, 250 - 500) Copies/ml, 100 (IQR, 100 - 125) Copies/ml and 500 (IQR, 215 - 8670) Copies/ml respectively for Brazzaville, Pointe-Noire, Dolisie, Pokola and Ouesso (Table 1).

Table 1: Sociodemographic and medical data of 39 Female Sex Workers recruited						
Variable	**Brazzaville** **n = 14**	**Pointe-Noire** **n = 7**	**Dolisie** **n = 10**	**Pokola** **n = 5**	**Ouesso** **n = 3**	**Total** **N = 39**
	No. (%)	**No. (%)**	**No. (%)**	**No. (%)**	**No. (%)**	**No. (%)**
Age, median (IQR), years	29.5 (27.25 - 32.75)	27 (25.5 -29)	29.9 (28 - 33.25)	32 (27 - 33)	29 (27.5 - 30)	29 (26.5 - 32)
Education Level						
None	2 (14.3)	1 (14.2)	2 (20)	4 (80)	1	10 (25.6)
Primary	5 (35.7)	3 (42.9)	3 (30)	1 (20)	0	12 (30.8)
Secondary	6 (42.9)	3 (42.9)	5 (50)	0	2	16 (41)
Superior	1 (7.1)	0	0	0	0	1 (2.6)
IDU						
Yes	5 (35.7)	4 (57.1)	0	0	0	9 (23.1)
No	9 (64.3)	3 (42.9)	10 (100)	5 (100)	3 (100)	30 (76.9)
Marital status						
Single	11 (78.6)	5 (71.4)	9 (90)	5 (100)	2 (66.7)	32 (82.1)
Married	3 (21.4)	2 (28.6)	1 (10)	0	1 (33.3)	7 (7.9)
Clients per day						
1 to 4	8 (57.1)	2 (28.6)	5 (50)	4 (80)	2 (66.7)	21 (53.8)
5 and above	6 (42.9)	5 (71.4)	5 (50)	1 (20)	1 (33.3)	18 (46.2)
Type of prostitution						
Mobile	14 (100)	7 (100)	10 (100)	5 (100)	3 (100)	39 (100)
Fixed						
Sex debut						
< 18	12 (85.7)	6 (85.7)	9 (90)	4 (80)	3 (100)	34 (87.2)
≥ 18	2 (14.3)	1 (14.3)	1 (10)	1 (20)	0	5 (12.8)
Duration as a sex worker in years						
1 to 3	9 (64.3)	7 (100)	4 (40)	2 (40)	0	22 (56.4)
> 4	5 (35.7)	0	6 (60)	3 (60)	3 (100)	17 (43.6)
Median HIV viral load, (IQR), Copies/ml	756 (350 - 6184.75)	7751.5 (2315 - 10,269.75)	2000 (265 - 17,344.5)	403 (250 - 500)	100 (100 - 125)	500 (215 - 8670)
IDU-intravenous drug users						

Median HIV viral load was, 500 (IQR, 215 - 8670) Copies/ml. In 10 of the FSWs plasma samples, the HIV viral load was undetectable (Figure 1).
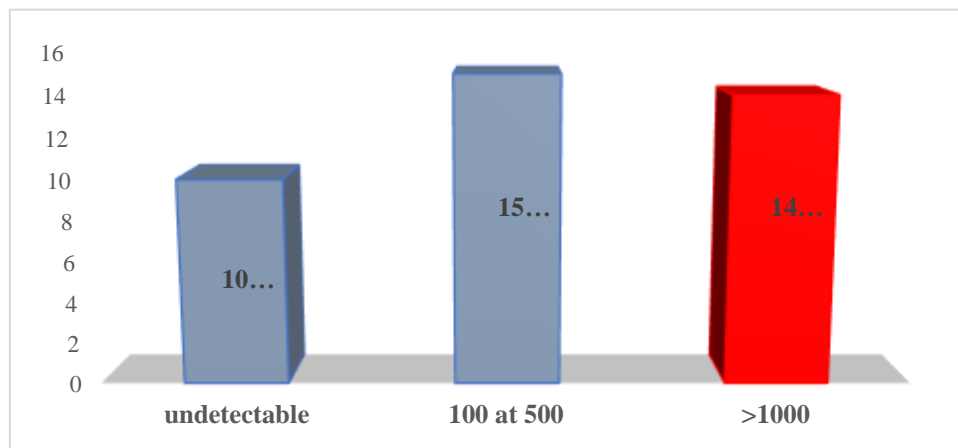

Figure 1: distribution of the plasma viral load according to the number of copies

HIV subtypes and recombinant strains circulating in Congo

Of the 39 persons eligible for enrollment in this study, 14 with viral load ≥ 1000 copies/ml were selected for subtyping and drug resistance genotyping analysis. We were able to sequence 12 samples among which the subtype A was the most represented strain, n = 5 (41.68%) followed by G subtype 3 (25%). The CRF02_AG, D, H and J subtypes were 1 (8.33%) respectively

(Figure 2). The report of primary ARV resistance mutations generated from the Stanford HIV database allowed us to note the presence of several minor retrotranscriptase mutations contributing less to the appearance of ARV resistance and cases of major mutations that can lead to resistance to ARVs. The relationship between subtypes and mutations of resistance to INNRT and INRT is shown in Tableau 2.
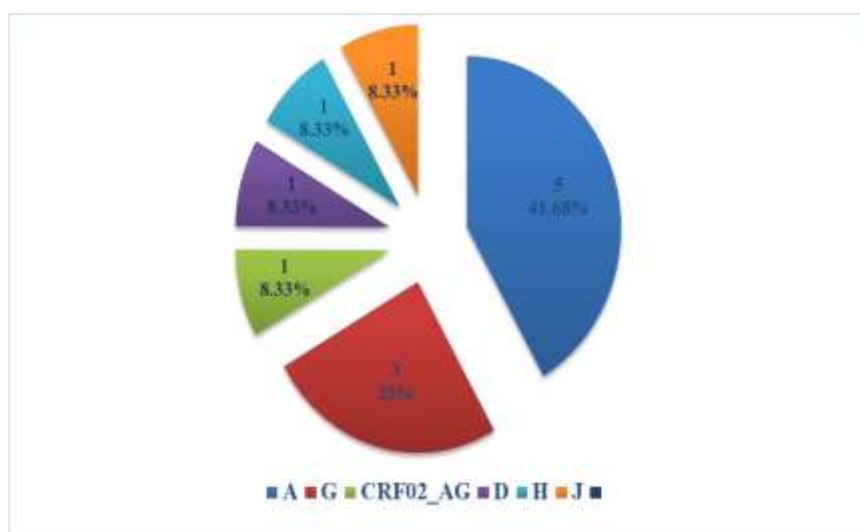

Figure 2: Distribution of PCR results according to subtypes at the RT level

Table 2: Mutations associated with resistance to INNRT and INRT, major mutations are shown in bold

Subtype	Mutation	INNRT resistance profile	INRT resistance profile
G (1)	K70N	None	ABC; D4T; DDI; TDF
G (1)	None	None	None
D (1)	G190A; T215N	NVP; EFV; RVP	AZT; D4T
A (5)	None	None	None
J (1)	E138A	RVP	None
H (1)	V106A; M184V; T215S	DOR; NVP; EFV	FTC; 3TC
CRF02_AG (1)	None	None	None

Resistance-associated mutations in RT genes

For the RT inhibitor susceptibility test, we classified the samples into 3 categories: Resistant, Intermédiate and sensitive. Two patients presented resistance to FTC, 3TC, DOR, NVP. Five of the intermediate resistances to EFV, ABC, D4T, DDI, TDF and others susceptible to all retrotranscriptase inhibitors.

DISCUSSION

The general objective of our study was to study the genetic diversity of HIV-1 among sex workers, by identifying the circulating

strains within this population, in order to establish a parallel with the variants found in the general population. This first work on the molecular characterization of HIV-1 in sex workers shows that out of 39 samples extracted, only 14 had a viral load greater than or equal to 1000 copies/ml. We were able to sequence 12 samples, 2 were not amplified for unclear reasons. It emerges those five subtypes of HIV-1: A, G, J, H, D and a recombinant, CRF02_AG have been reported. Subtype A was more predominant with 41.68% followed by subtype G (25%). D, H, J and CRF02_AG had a proportion of 8.33% each.

These data seem to be similar to those reported in 2012 by *Sscemwanga et al.* in Uganda which reported a predominance of subtypes A among sex workers [8]. It appears from our study that there was no URF and that CRF02_AG was the only circulating recombinant in this population contrary to the observations of *Saeng-Aroon et al.* in Thailand (2016) who reported a predominance of forms recombinant CRF01_AE, an indeterminate recombinant form (URF) and new inter-subtype forms such as: C/B; AE/B/C; AE/B with different recombination breakpoints [9], this difference may be due to the fact that they were able to sequence all 3 regions of HIV-1 including gag (p24), pol (prot-RT) and env (C2/V3), and the genetic subtypes were determined in 159 plasma samples. *Sanders-Buell et al.* in Afghanistan in 2010 reported a predominance of CRF01_AE [10], study population was made up not only of sex workers but also of injecting drug users and analysis of the recombinants was carried out by boot scanning and manual alignment of the sequences, always with the gag and env regions unlike our study. Furthermore, in 2017 *Deng et al.* reported in China a predominance of CRF01_AE followed by CRF08_BC and CRF07_BC within the PS population [11].

The observation of A, G, D, J, Het CRF02_AG subtypes in our study may or may not be reflective of all circulating subtypes among female sex workers due to our modest sample size. of study. This may be why we were only able to identify CRF02_AG as a recombinant form.

The differences observed may result from the gene analyzed. Indeed, by analyzing a single gene, that of RT, it seems possible to detect only a few variants of HIV-1, which may have been the case in our study. Analysis of the env and gag genes which is the most used to study genetic diversity, if we had been able to sequence the env and gag genes we could have better assessed the dynamics of the viral strains in this population.

All the subtypes identified in our study, in particular: A, G, D, J, H and CRF02_AG are subtypes already circulating in the country. Studies conducted in the general population by *Niama et al.* in 2006, showed the predominance of subtypes A and G followed by subtype *Pischer et al.* reported the presence of subtypes A, C, D, G and H and that the G subtype was more than 20% dominant [12]. Another study conducted by *Niama et al.* in 2017 reported a predominance of subtype A followed by G and D with several recombinant forms among them CRF02_AG, including other types such as: B; C; H; J. [7].

This shows that there is a great deal of sexual promiscuity between sex workers and the general population. The molecular HIV epidemic among FSWs in Congo therefore seems to confirm the character of sex workers in Congo as a key population key of HIV transmission. We found major resistance mutations to INNRTs and INRTs. These are transmitted resistances or Primary Transmitted Drug Resistance (PTDR), resulting from the circulation in the general population of resistant strains carrying these mutations. This phenomenon has also been observed in other studies conducted among ARV-naïve sex workers by *Do et al.* in Vietnam (2017) [13] and *Coetzee et al.* in South Africa (2017) [14].

Our study has some limitations: (i) the fact that it is retrospective, conducted on laboratory samples without allowing us to question sex workers in order to collect exhaustive and extensive sociodemographic characteristics, (ii) the choice of a single gene for sequencing, (iii) we were not able to sequence all of our samples. In conclusion, this first study on the molecular characterization of HIV-1 strains in sex workers in the Republic of Congo showed a predominance of subtype A in 41.66 % followed by G in the whole of the population. In only 12 sequenced samples we were able to observe a great genetic diversity within this population, including major resistance mutations observed. These results therefore show that there should be rapid and effective interventions in order to reduce the possible

weight of SWs in the dynamics of HIV in the country.

CONCLUSION

this study on the molecular characterization of HIV-1 strains among sex workers in the Republic of Congo showed a predominance subtype A in 41.66% followed by G in the general population. In just 12 sequenced samples, we were able to observe a great genetic diversity within this population, including major resistance mutations observed. These results show therefore that it is desirable that there be rapid and effective interventions in order to reduce the possible burden of sex workers in the dynamics of HIV in the country.

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