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**Brief report**

**Primary Resistance to Anti-Retroviral drugs of HIV strains in Chad: a retrospective investigation by analysis of frozen Dried Blood Spot samples**

1362 Words

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1 **Abstract: (98 words)**

2 *Purpose:* No data concerning Anti-Retroviral drugs (ARV) primary resistance mutation rates  
3 in Chad are available.

4 *Methods:* We retrospectively analysed frozen-stored dried blood spot samples that were  
5 collected from 48 Chadian Human Immunodeficiency Virus (HIV)-1 seropositive patients  
6 naïve of ARV.

7 *Results:* HIV-1 protease and reverse transcriptase genes were successfully sequenced for 24  
8 (60.0%) of the 40 patients displaying a viral load >1000copies/ml. Seven (29.2%) displayed  
9 mutations conferring resistance against one or more classes of ARV.

10 *Conclusion:* We evidenced high levels of primary ARV resistance mutations in Chad,  
11 but lower than those observed in patients with failure to first-line ARV.

12

13

14 **Keywords:** HIV; Chad; Non-Nucleoside Reverse Transcriptase Inhibitor; primary resistance.

15

## 1 **Introduction:**

2           Access to Highly Active Antiretroviral drugs (ARV) has significantly reduced Human  
3 Immunodeficiency Virus (HIV) transmission in sub-Saharan African countries which still  
4 support the highest burden of the pandemic [1]. However in these countries, access to free  
5 but discontinuous first-line antiretroviral regimens consisting almost exclusively of  
6 Nucleoside Reverse Transcriptase Inhibitors (NRTI) combined with a Non Nucleoside  
7 Reverse Transcriptase Inhibitors (NNRTI) with low genetic barrier to resistance could rapidly  
8 lead to the emergence of antiretroviral drugs resistance among ARV-treated patients. In low-  
9 income countries of sub-Saharan Africa, monitoring of HIV plasma viral load that is  
10 recommended by World Health Organization (WHO) since 2013 and genotyping tests  
11 allowing the detection of HIV strains harbouring resistance mutations to antiretroviral drugs  
12 are not continuously available. Horizontal or vertical transmission levels of such HIV strains  
13 harbouring major resistance mutations to NNRTIs or NRTIs represent a major public health  
14 problem and may significantly impact subsequent choices of antiretroviral drugs regimen for  
15 newly HIV infected patients in sub-Saharan African countries.

16           Located in Central Africa, Chad had an estimated HIV seroprevalence of 1.3% among  
17 the sexually active population (15-49 years) and ranked fifth in terms of HIV mortality rate  
18 (71 per 1000) [2,3]. Moreover, Chad is partly bordered by Cameroon, where a wide diversity  
19 of HIV strains has been evidenced [4]. Thus, Chadian HIV strains could present a great viral  
20 genetic diversity including non M variants of HIV-1, group M non B subtypes or recombinant  
21 strains potentially carrying mutations conferring resistance to antiretroviral drugs used as first  
22 or second lines of treatment [5,6]. Only two previous investigations reported high rates of  
23 viral mutations conferring resistance to antiretroviral drugs in Chadian patients with  
24 virological failure [5,6]. To date, no published data concerning ARV primary resistance

1 mutation rates in Chad are available. In the present report, we retrospectively investigated  
2 frozen dried blood spot (DBS) samples from HIV-1 Chadian patients who were naïve of ARV.

3

#### 4 **Patients and methods**

5 From August to November 2012, 48 successive newly HIV-1 seropositive **adult** patients who  
6 were naïve of any ARV treatment (30 F/18 M, median age **32**years [18-52]) and who were  
7 attending to the chronic diseases centre of the hospital “le Bon Samaritain” (N’Djamena,  
8 Chad) were enrolled in the present investigation. **World Health Organization HIV disease**  
9 **Clinical stage has been defined elsewhere [7].** Median Lymphocytes T-CD4 cells count  
10 performed on FACS® count system was **273** cells per mm<sup>3</sup> [13 - 1049] for these 48 patients.  
11 An oral informed consent for medical research investigations was obtained from each study  
12 patient **and if needed from** their relative family members. **This study was approved by local**  
13 **ethics committee.** For each patient, blood samples were collected before ARV and sent abroad  
14 (“Université” Jean Monnet, Saint-Etienne, and “Université” Reims Champagne Ardennes,  
15 France) as dried blood spots (DBS) **after approximately one month of storage at room**  
16 **temperature** [8,9]. Each DBS sample was then stored at -80°C until performing HIV-RNA  
17 viral load and genotyping assays in France according to previously described protocols [8,9].  
18 **Protease gene and reverse transcriptase gene region were fully covered by sequencing**  
19 **analysis and obtained sequences were compared against referenced subtype B HBX-2 strain**  
20 **(Genbank accession number: K03455.1). All of our HIV-1 sequences were submitted to the**  
21 **Genbank and obtained an original accession number (Table 1).** Concerning drug resistance  
22 mutations, interpretation of sequencing results was performed according to the French  
23 National Agency for AIDS Research resistance algorithm [10]. HIV-1 subtypes were  
24 established using the Stanford HIV database **and Sierra version 1.1 HIVdb 8.1.1** [11].  
25 **Quantitative variables were compared using the Mann Whitney U-test and qualitative**

1 variables were compared using Pearson's Chi-square test. Statistical analyses were performed  
2 using Stat view 5.0 software (SAS institute).

### 3 **Results**

4 Following RNA extraction from DBS [8], median HIV-1 viral load was estimated to  
5 4.72 log copies/ml [3.24- 5.95] for 40 (83.3%) out of the 48 study patients with a detected  
6 viral load upper than 1000 HIV-RNA copies per ml. HIV-1 protease and reverse transcriptase  
7 genes were successfully amplified and sequenced for only 24 (60.0%) of the 40 patients with  
8 a detectable viral load (Table 1). Median HIV-1 viral load and median CD4 cells counts were  
9 respectively statistically higher and lower in patients who had successful sequences than in  
10 those that did not (Table 2).

11  
12 Interestingly, 7 (29.2%) of the 24 successfully sequenced DBS samples exhibited  
13 referenced nucleotide mutations conferring HIV-1-resistance against at least one antiretroviral  
14 drug. The most frequently detected mutation was the V106I (20.8%) described as a natural  
15 polymorphism in HIV-1 non-B subtypes and conferring resistance to etravirine when it is  
16 associated to at least one other mutation, according to French National Agency for AIDS  
17 Research resistance algorithm [9]. The K103N mutation that confers complete resistance to  
18 efavirenz and nevirapine was found in 2 subjects (8.3%) out of the 24. One K65E and one  
19 V179D were also detected in two distinct individuals conferring resistance to tenofovir and  
20 rilpivirine, respectively. The L90M major protease inhibitor resistance mutation was found in  
21 one patient (4.1%). Polymorphic mutations (L10I, G16E, K20I, M36I, I62V, V77I, L89M)  
22 were evidenced in 21 sequences (87.5%). HIV-1 protease sequences predicting phenotypic  
23 resistance to saquinavir and a combined resistance to nelfinavir, indinavir and atazanavir were  
24 evidenced in 3 patients (12.5%).

1           The most prevalent HIV-1 subtype was Circulating Recombinant Form (CRF)  
2 CRF11\_CPX subtype and was identified in 7 of the 24 samples (29,2%). Others previously  
3 described HIV recombinant forms **accounted** for 8 out of the 24 samples (33.3%) (Table 1).

4           All patients were treated by NRTI plus first generation NNRTI (such as efavirenz or  
5 nevirapine). Among these 24 patients, 4 died (16.6%) (None among those with mutations  
6 leading to resistance or possible resistance to NNRTI), and 14 (58.3%) were lost to follow-up  
7 which is consistent with the high attrition rates previously observed in Chad [12].

## 8 9 **Discussion**

10           In the present report we retrospectively analysed frozen-stored DBS sampled from a  
11 series of HIV-1 seropositive patients who were naïve of Highly Active Antiretroviral drugs  
12 (ARV) and were living in Chad (Ndjamena), 2012. All of these patients were newly HIV-1-  
13 diagnosed and were sampled before initiation of ARV consisting of NRTI and NNRTI  
14 combination. We evidenced a CRF11\_CPX HIV subtype predominance in accordance with a  
15 previously published investigation on Chadian HIV-1 infected patients [5]. Because this  
16 previous study focused only patients with detectable viral loads after 6 months of ARV, the  
17 reported rates of mutations conferring resistance to at least one antiretroviral drug was high  
18 and estimated to 64% [5]. In 2018 Keita et al. observed in plasma samples from newly HIV-  
19 diagnosed Malian patients a significant increase of the ARV primary resistance mutation rates  
20 evolving from 7.8% in 2010 to 17.5% in 2014, especially mutations conferring resistance to  
21 NNRTI like K103N mutation or other natural polymorphic mutations conferring resistance or  
22 potential resistance to etravirine, another NNRTI drug not yet currently used in Mali [8]. In  
23 the present retrospective monocentric study including a limited series of patients, we showed  
24 for the first time high rates of similar ARV primary resistance mutations (29.2%) in Chad,

1 whose levels were lower than those previously observed in Chadian patients (36-64%)  
2 displaying a failure to first-line ARV regimen [5,6].

3  
4 Our HIV genotyping results could be due to the combined presence of viral RNA and  
5 DNA in DBS and the detection of archived mutations currently not detectable by classical  
6 genotypic test on plasma RNA. It has been shown that high levels of HIV-1 DNA can induce  
7 falsely positive results for the detection of HIV-RNA resistance mutations in about 35% of  
8 cases on DBS [13]. However, **even if genotyping assays efficiency declined after DBS storage**  
9 **at room temperature [14]**, the use of DBS offers the advantage of a stable and easy to be  
10 transported samples with a minimal biohazard risk. Moreover, sending collected blood  
11 samples abroad as DBS for further genotyping analysis could be the most reliable way to  
12 monitor resistance to antiretroviral drugs because of recurrent blackouts and material/reagent  
13 stockouts in low-income countries such as Chad [5,6]. Samples to send abroad could be  
14 randomly selected in case of global monitoring of circulation of strains harbouring resistance  
15 or individually selected in suspected virological failure cases. In the absence of available  
16 HIV-1 RNA load monitoring for each patient, absence of rise of lymphocyte T-CD4 count  
17 under ARV is commonly used as a proxy for virological failure in central Africa. In this  
18 situation, first line ARV using NRTI plus NNRTI combination is empirically switched to a  
19 second line treatment with Protease Inhibitor without waiting for of HIV RNA load results or  
20 genotyping analysis performed abroad.

21 Taking into account the few previous published Sahelian reports and our present  
22 original results, we suggest that free-access to new cheap antiretroviral drugs with high  
23 genetic barrier to resistance should be prioritized over any implementation strategy of HIV  
24 genotyping assays in low-income central Africa countries such as Mali or Chad. Dolutegravir  
25 containing regimen could be a good candidate for new first-line ARV regimen in these low-

1 income countries, it could be used in association with rifampin and first line anti-tuberculous  
2 agents as well as in pregnant women [15]. Dolutegravir containing regimen is now  
3 recommended by WHO [15], but to date this ARV drug is not yet in full free-access for the  
4 population of Chad.

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15 France).

16 **Conflicts of interest/Competing interests:** none to declare

17 **Ethics approval:** not required in this non interventional study

18 **Consent to participate:** all patients were informed of the study and gave oral consent for the  
19 analysis of their samples abroad.

20

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16

1 **Table caption:**

2 **Table 1: Virological data obtained from frozen-stored dried blood spot samples of 24**

3 **Chadian HIV-1 seropositive patients naïve of Highly Active Antiretroviral Drugs (ARV).**

4

<b>Patient number</b>	<b>Viral load (log/mL)</b>	<b>HIV-1 subtype</b>	<b>Protease gene mutations</b>	<b>Reverse transcriptase gene mutations</b>	<b>Drug resistance</b>	<b>Possible Drug resistance</b>	<b>Genbank reference</b>
<b>1</b>	4.57	<b>CRF11_CPX</b>	I62V, V77I				MW250374
<b>2</b>	4.44	<b>B</b>		K65E, V75E, I135V, M164L, I178M		<b>TDF</b>	MW250369
<b>3</b>	3.82	<b>CRF11_CPX</b>	G16E, V77I				MW250377
<b>4</b>	4.71	<b>G</b>		K73EQ, K122P, I142V, K173T, Q174K, D177E, E174D, V292I, D324E, I329V			MW250370
<b>5</b>	4.28	<b>CRF45_CPX</b>	K20I, L24F, M36I, L89M	L74H, V179D, K238N, V179D		<b>RPV,</b> <b>EFV,</b> <b>NVP</b>	MW250372 (P) MW250373 (RT)
<b>6</b>	4.37	<b>A1</b>	L90M, L10I, M36I, L89M		<b>NFV</b>	<b>SQV,</b> <b>IDV, ATV</b>	MW250375
<b>7</b>	4.02	<b>CRF13_CPX</b>	K20I, M36I, V77I, I50N				MW250376
<b>8</b>	5.26	<b>CRF11_CPX</b>	L10I, M36I, V77I, L89M				MW250371
<b>9</b>	4.78	<b>CRF11_CPX</b>	L10I, M36I, V77I, L89M				MW250353
<b>10</b>	3.63	<b>D</b>	K20R, M36I	V106I			MW250354
<b>11</b>	4.61	<b>CRF45_CPX</b>	K20X,				MW250356

			M36I, L89M				
<b>12</b>	5.36	<b>D</b>	K20I, M36I, V77I, L89M	V106I			MW250355
<b>13</b>	4.73	<b>CRF45_CPX</b>	L10I, K20I, M36I, L89M			<b>SQV</b>	MW250357
<b>14</b>	4.99	<b>CRF13_CPX</b>	K20I, M36I, L89M	V106I			MW250358
<b>15</b>	4.97	<b>D</b>	M36I, I62V	V106I			MW250359
<b>16</b>	5.79	<b>D</b>	K20R, M6I	V106I			MW250360
<b>17</b>	5.70	<b>D.G</b>	K20I, M36I, I62V, L89M			<b>SQV</b>	MW250361
<b>18</b>	5.43	<b>G</b>					MW250368
<b>19</b>	5.75	<b>CRF11_CPX</b>	I62V, V77I, L89I				MW250362
<b>20</b>	5.56	<b>CRF02_AG</b>	K20I, M36I, L89M	K103N	<b>EFV,</b> <b>NVP</b>		MW250363
<b>21</b>	5.47	<b>CRF11_CPX</b>	I62V, V77I				MW250364
<b>22</b>	4.38	<b>CRF13_CPX</b>	K20I, M36I, V77I				MW250365
<b>23</b>	5.49	<b>CRF02_AG</b>	K20I, M36I, L63P, L89M				MW250366
<b>24</b>	5.95	<b>CRF11_CPX</b>	I62V, V77I	K103N	<b>EFV,</b> <b>NVP</b>		MW250367

**Table 2 : Differences between patients with successfully amplified sequences than those without.** \* The 8 patients with indetectable viral load were considered as missing data among the viral loads of patients without successfully amplified sequences.

	Patients with successfully amplified sequences (n=24)	Patients without successfully amplified sequences (n=24)	P
Male sex (n%)	11 (45.80)	7 (29.10)	0.23
Median Age [range] (years)	33 [20-45]	30 [18-52]	0.23
Median World Health Organization HIV disease Clinical stage [range]	3 [1-4]	2 [1-4]	0.15
Median CD4 Cells counts [range] (/mm <sup>3</sup> )	230 [22-1049]	415 [13-918]	0.01
Median viral load [range] (log copies/ml)	4.87 [3.63-5.95]	4.03 [3.24-4.99]*	0.003