Molecular epidemiology of human immunodeficiency virus type 1 and therapeutic monitoring of patients treated in Kinshasa/Democratic Republic of the Congo

Erick Nytambwe Kamangu¹, Roger Di-Mosi Wumba², Hippolyte Nani-Tuma Situkibanza³, Prosper Ishiloba Lukusa⁴, Liévín kalala Kapendi⁵, Georges Ielo Mvumbi⁶, Marie-pierre Hayette⁶, Richard Lunganza Kalala¹

CONTENTS:
1. Introduction of Antiretroviral Drugs (ARVs) has reduced morbidity and mortality associated with the Human Immunodeficiency Virus (HIV) infection. But also at the other hand, the massive use of these molecules created the emergence of mutant strains resistant to the treatment.

OBJECTIVE: The objective set for this study was to determine the different variants of HIV-1 group M circulating in Kinshasa, the prevalence of mutations associated with resistance to antiretroviral treatment and their involvement in therapeutic monitoring of infected patients followed in different centers.

METHODS: A prospective cohort study conducted in collaboration with 8 centers in Kinshasa from August 2013 to October 2014. One hundred fifty-three (153) subjects diagnosed positive for HIV Type 1 by serology voluntarily and naïve of treatment participated in this controlled study. Five milliliters (5 ml) of blood were collected in a tube with anticoagulant EDTA. The DNA was extracted from 200 µl of Buffy Coat using the QIAamp DNA Blood Mini Kit QIAGEN® and RNA was extracted from 140 µl of plasma using the QIAamp RNA Mini Kit QIAGEN®. A Quantitative PCR was performed to determine the Viral Load for all samples. A Reverse Transcription PCR (RT-PCR) and Nested PCR were performed to amplify the regions of interest for the Protease and Reverse Transcriptase (RT) for sequencing.

RESULTS: One hundred and fifty-three (153) patients infected with HIV Type 1 were selected for this work. The population consisted of 61 (39.9%) men and 92 (60.1%) women. The median age was 37 years with extremes of 18 and 65 years. The median values of Viral Loads and rate of CD4 lymphocytes at baseline were respectively 5.68 log10 RNA copies/ml and 180 cells/ml. The subtype A is dominant with 35 cases (22.9%), followed by CRF02_AG (11.1%), C (9.8%), G (9.8%), K (9.8%), D (7.8%), H (7.8%) and J (5.0%). The most significant observed Major mutations were: L90M (2.0%), D30N (1.3%), V32I (1.3%), V82A (1.3%) and I84V (1.3%). The most frequent observed mutations for NRTI were: V75I/N/L/M (18.3%), K70E/N/R (9.8%), D67G/E/N (9.2%), M184V/L/K/R (9.2%) and T215F/N/L/R (9.2%). The most frequent mutations for NNRTI were: V179F/T/D (9.8%), K103N/I/R (8.5%), V106A/T (7.2%), Y181K/C (5.8%) and V90I/GIS (5.8%). At the 6th month of ART (M6), 138 patients (90.2%) including 81 women (59.4%) and 57 men (40.6%) returned to the Center. The median values of CD4 and VL of patients are respectively 480 cells/ml and 0.90 log10 RNA copies/ml. Thirty-four patients (24.6%) were in virological failure.

CONCLUSION: This study demonstrates a strong diversity of HIV-1 in Kinshasa, which is dominated by the subtype A and CRF02_AG, and several resistances associated with the treatment detected in patients naïve of treatment. The 6th month of treatment, the rate of virological treatment failure was of 24.6% which is highly correlated with transmitted mutations at baseline.

Key Words: HIV-1; Subtypes; Resistance; Antiretroviral Treatment; Kinshasa

ORIGINAL ARTICLE

Molecular epidemiology of human immunodeficiency virus type 1 and therapeutic monitoring of patients treated in Kinshasa/Democratic Republic of the Congo

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The Human Immunodeficiency Virus (HIV) is documented according to its types, groups, subtypes, sub-sub-types and Circulating Recombinant Forms (CRF) (1-3). The global epidemic of HIV is dominated by the Subtype C of the Group M of the Type 1 which alone accounts for nearly a quarter of infecting strains worldwide (2,3). In Sub-Saharan Africa, the HIV epidemic is very diverse and heterogeneous; it is dominated by subtypes A, C, G and several CRFs (4-5).

The introduction of Antiretroviral Drugs (ARVs) has reduced morbidity and mortality associated with the HIV infection (6). But also at the other hand, the massive use of these molecules created the emergence of mutant strains resistant to the treatment. Thus, the World Health Organization (WHO) recommends epidemiological monitoring for newly HIV-infected patients (6). This includes monitoring the prevalence of different circulating strains and the search for mutations associated with treatment in the infected population. These mutations are often defined in terms of Major opposed to Minor, and Primary’s opposed to Secondary’s Resistance to treatment. Introduced in 2002 in the Democratic Republic of the Congo (DRC), the recommended therapy is a combination of 2 Nucleotide Reverse Transcriptase Inhibitors (NRTI) and 1 Non-Nucleotide Reverse Transcriptase Inhibitor (NNRTI) for first-line treatment (7). The most commonly used molecules for NRTI were: Abacavir (ABC), Didanosine (ddI), Lamivudine (3TC), Stavudine (d4T) and Zidovudine (ZDV); for NNRTI: Efavirenz (EFV) and Nevirapine (NVP) (7). Tenofovir (TDF), a Nucleoside Reverse Transcriptase Inhibitors (nRTI), was introduced in the first line in substitution to one NNRTI since 2013 (8).

In Kinshasa, the subtype A (34.3%) largely dominated by the epidemiology according to literature. In 2014, it was followed by CRF02_AG (11.1%), C (9.8%), G (9.8%), K (9.8%), D (7.8%), H (7.8%), J (5.9%), U (5.2%), F (3.9%), CRF01_AE (3.3%) and B (2%) (9). In 2012, the rate of severe antiretroviral treatment (ART) failure of 1st line in the same city was estimated at 17%. And the HIV type 1 Group M was dominant in the Democratic Republic of the Congo (DRC) (10).

The objective set for this study was to determine the different variants of HIV Type 1 group M circulating in Kinshasa, the prevalence of mutations...
associated with resistance to antiretroviral treatment and their involvement in therapeutic monitoring of infected patients followed in different centers.

**METHODOLOGY**

**Frame**

This study is a prospective cohort study conducted in collaboration with 8 treatment centers and support in Kinshasa. It was conducted in the period August 2013 to October 2014.

**Study population**

One hundred fifty-three (153) subjects diagnosed positive for HIV Type 1 by serology voluntarily participated in this controlled study. They were recruited in the different centers of Kinshasa respecting the demographics of the district and the heterogeneity of the population (11). Inclusion criteria for the subjects were: (i) be diagnosed positive HIV-1 according to national guidelines (8), (ii) be over 18 years to the date of inclusion, (iii) be eligible for the antiretroviral treatment (ART) in the monitoring center and (iv) be naïve to ART. All patients newly diagnosed in the center, eligible for ARV treatment (ART) according to the criteria of the center and not having started treatment in the given center, were considered naïve to ART for our study. Demographic, clinical and laboratory information of patient were recorded on pre-tested forms during the study. Participation in this study was voluntary. All patients gave written consent for participation in the study. The inclusions were conducted from August 2013 to February 2014. Each patient was put on first-line treatment in the center after blood sampling at the inclusion.

**Blood samples**

Five milliliters (5 ml) of blood were collected in a tube with anticoagulant EDTA from the vein of the elbow crease. The collected blood was centrifuged at 1000 x g for 10 minutes to obtain a clear separation in 3 phases. One milliliter of plasma (supernatant) was transferred to a microtube previously labeled and stored at -20°C in the laboratory of Molecular Biology of the Faculty of Medicine. Samples were aliquoted into 2 tubes of 500 µl each. One tube of each sample was used to determine the Viral Load (VL) of patients at baseline (12-14). The other tube was sent to the AIDS Reference Laboratory of the University Hospital of Liège (CHU LRS and Liège). The extracted samples were stored at -80 °C until use.

HIV positive patients were confirmed in the laboratory of Molecular Biology of the University of Kinshasa with the extracted DNA using a DNA Nested PCR previously described by Kamangu et al. (15).

After extracting RNA, in Kinshasa, a Quantitative PCR was performed to determine the Viral Load for all samples. In Liege, a Reverse Transcription PCR (RT-PCR) and Nested PCR were performed to amplify the regions of interest for the Protease and Reverse Transcriptase (RT) for sequencing. The PCR were performed in conditions and temperature cycles previously described by Steegen (Table 1) (18).

The primers used for these PCR assays are shown in Table 2. For the samples which were not amplified with the first primer (Normals), other PCR were performed with the primers Alternatives (ATL) previously described in literature (Table 2) (18).

The amplified fragments obtained for the Protease are approximately 500 base pairs and that of the Reverse Transcriptase of 800 base pairs. These fragments were sequenced by the Sanger sequencing method. They were purified by the ExoSAP-IT technique to eliminate the residual products of the PCR, sequenced by amplifying the fragment purified, then subjected to a second purification of the product block, before reading the base sequences. The pairing of the resulting fragments (sense and antisense) was performed with the software Vector NTI Advance® 11.5 (Invitrogen, Life Technologies) and compared with the Stanford University database (www.hivdb.stanford.edu) (19), that of the French National Agency for Scientific Research (ANRS - www.hivfrenchresistance.org) and that of the Max Plank (www.gen2pheno.org) for identification of subtypes of HIV-1 and possible changes. Minor bases adjustments were performed if necessary to align the playback sequences.

**Statistical analysis**

The Friedman test was used to analyze the differences in the groups in terms of variants and mutations. The probability p value <0.05 was taken as significant to accommodate the exhaustive sampling of the study. Viral load is expressed in decimal logarithm RNA copies/ml.

**RESULTS**

One hundred and fifty-three (153) patients infected with HIV Type 1

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**TABLE 1**

**PCR cycles (Reverse Transcriptase PCR and nested PCR)**

<table>
<thead>
<tr>
<th>Stapes</th>
<th>RT-PCR Temperature</th>
<th>Nested Time</th>
<th>PCR Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcriptase</td>
<td>50°C</td>
<td>30'</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>2'</td>
<td>94°C</td>
<td>12'</td>
</tr>
<tr>
<td>Denaturation per cycle</td>
<td>94°C</td>
<td>30'</td>
<td>94°C</td>
<td>30'</td>
</tr>
<tr>
<td>Hybridation per cycle</td>
<td>55°C</td>
<td>30'</td>
<td>55°C</td>
<td>30'</td>
</tr>
<tr>
<td>Elongation per cycle</td>
<td>68°C</td>
<td>90'</td>
<td>72°C</td>
<td>120'</td>
</tr>
<tr>
<td>Final Elongation</td>
<td>N/A</td>
<td></td>
<td>72°C</td>
<td>7'</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>=</td>
<td>4°C</td>
<td>=</td>
</tr>
<tr>
<td>Number of Cycles</td>
<td>40</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N/A. Non-Applicable for this amplification.
were selected for this work. The population consisted of 61 (39.9%) men and 92 (60.1%) women; hence a sex-ratio of 1 man for 2 women. The median age was 37 years with extremes of 18 and 65 years (Table 3). The DNA Nested PCR was positive for all 153 patients included in the study. The median values of Viral Loads and rate of CD4 lymphocytes at baseline were respectively 5.68 log10 RNA copies/ml (481.927 RNA copies/ml) and 180 cells/ml (Table 3). Figure 1 shows the median values of VL and CD4 according to the different subtypes of HIV in the population.

Subtyping
Protease and Reverse Transcriptase (RT) were amplified and sequenced respectively for 130 (84.9%) and 145 (94.8%) patients. Hundred twenty-three (123) samples were amplified with the universal primers and 7 with alternative primers respectively for the region of the Protease region. One hundred twenty-five (125) samples were amplified with the universal primers and 20 respectively with the alternatives for the RT region. The subtype A is dominant with 35 cases (22.9%); followed by CRF02_AG (11.1%), C (9.8%) G (9.8%), K (9.8%), D (7.8%), H (7.8%) and J (5.0%) as described in Figure 2. The distribution of subtypes by gender and age is presented in Figure 3.

Mutations conferring resistance to Protease Inhibitors (PI)
The mutations associated with resistance to the PI can be classified into major mutations (one of which can cause resistance to a given treatment), minor (requiring another mutation to cause resistance to a given treatment), primary (transmitted) or secondary (acquired). In this study, several mutations (major and minor) associated with protease inhibitors have been observed. The most significant observed Major mutations were: L90M (2.0%), D30N (1.3%), V32I (1.3%), V82A (1.3%) and I84V (1.3%). The most observed Minor mutations were: K20I/RT/M (28.1%), L10I (27.5%), I47R/M (6.5%), V11I (5.2%), V32E/L (4.6%), G48R (3.3%) and A71T/V (2.0%).

Mutations conferring resistance to Reverse Transcriptase Inhibitors (RTIs)
Mutations associated with Nucleoside Reverse Transcriptase Inhibitors (NRTI) and Non-Nucleotide Reverse Transcriptase Inhibitors (NNRTI) were also observed. The most frequent observed mutations for NRTI were: V75I/N/L/M (18.3%), K70E/N/R (9.8%), D67G/E/N (9.2%), M184V/L/
K/R (9.2%), T215F/N/I/L (9.2%), Y115F (7.8%), M41L (7.2%), T69P/N (5.2%) and L74V/M/I (3.9%). The most frequent mutations for NNRTI were: V179F/T/D (9.8%), K103N/I/R (8.5%), V106I/A (7.2%), Y181K/C (5.8%), V90I/GIS (5.8%), A98G/GPR (5.2%), V108I/KN (5.2%), Y188C/L/D (4.6%) and F227C/L (4.6%).

Evaluation at 6th month
At the 6th month of ART (M6), 138 patients (90.2%) including 81 women (58.7%) and 57 men (41.3%) returned to their control. Ten (10) cases of death (6.5%) were reported in M6 and 5 patients (3.3%) were lost to by community volunteers’ service in their respective centers. The median values of CD4 and VL of patients are respectively 480 cells/mm³ and 0.90 log10 RNA copies/ml. Thirty-four patients (24.6%) were virological failure with VL >2.30 log10 RNA copies/ml with 8 (23.5%) are minimal failure (2.30 log10 <VL <3.70 log10 RNA copies/ml), 23 (67.7%) in moderate failure (3.70 log10 <VL<4.48 log10 RNA copies/ml) and 3 (8.8%) in severe failure (VL> 4.48 log10 RNA copies/ml). According to the test of Pearson, the VLs at the 6th month were strongly correlated with that of baseline, to some mutations and to virological failure of treatment ($p<0.000$) (Table 4).

**Table 4**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment Success CV&lt;2,30 log10</td>
<td>104 (75.4%)</td>
</tr>
<tr>
<td>Treatment Failure CV&gt;2,30 log10</td>
<td>34 (24.6%)</td>
</tr>
<tr>
<td>Failure by age (year)</td>
<td></td>
</tr>
<tr>
<td>18-25</td>
<td>&gt;2,30 log10</td>
</tr>
<tr>
<td>26-35</td>
<td>18 (17.3%)</td>
</tr>
<tr>
<td>36-45</td>
<td>31 (29.8%)</td>
</tr>
<tr>
<td>46-55</td>
<td>31 (29.8%)</td>
</tr>
<tr>
<td>56-65</td>
<td>19 (18.3%)</td>
</tr>
<tr>
<td>Total 104 34</td>
<td>5 (4.8%)</td>
</tr>
</tbody>
</table>

**Type of Virological Failure**
- Minimal Failure (2,30<CV<3,70log10) | 8 (23.5%) |
- Moderate Failure (3,70<CV<4,48log10) | 23 (67.7%) |
- Severe Failure (CV>4,48 log10) | 3 (8.8%) |

**DISCUSSION**

The objective set for this study was to determine the different variants of HIV Type 1 group M circulating in Kinshasa, the prevalence of mutations associated with resistance to antiretroviral treatment and their involvement in therapeutic monitoring of infected patients followed in different centers. The population consisted of 61 (39.9%) men and 92 (60.1%) women; hence a sex ratio of 1 man 2 women. This difference is statistically significant ($p=0.02$). The most represented age group at baseline was that of 26 to 35 years with 42 patients (27.4%), followed by that of 36 to 45 years with 40 patients (26.3%) and of 18 to 25 years (20.2%) and that of 46 to 55 years (10.6%).
years (16.9%). These demographic data are found in several studies in our environment (20,21).

In a population of 153 treatment-naïve patients to Antiretroviral Treatment (ART), 130 samples (84.9%) were amplified on the protease region and 145 samples (94.8%) on the Reverse Transcriptase region. This gives an amplification rate of nearly 90% for the two regions. This amplification difference was also observed in different conditions; samples are more easily amplified on the Reverse Transcriptase than on the Protease because of the size of the first region of interest which is almost double the second (22,23).

Amplification failures can be explained by the high diversity of the variants site of HIV Type 1 that exists in Kinshasa (9). They can also be caused by the presence of lower Viral loads (VL) or near the limit of detection (18,22). Indeed, all 7 samples (4.6%) which had lower VL to 3.0 log10 did not give amplifications for the protease.

The subtype A is dominant in Kinshasa to 22.9% of the study population. The most observed Minor mutations were: K20I/RT/M (28.1%), L10I (2.0%), D30N (1.3%), V32I (1.3%), V82A (1.3%) and I84V (1.3%).

The transmitted mutations associated with the most NRTI found in our study population were: V75I/N/L/M (18.3%), K70E/N/R (9.8%), D67G/E/N (9.2%), M184V/L/K/R (6.5%), T215F/N/L (9.2%), Y115F (7.8%), M41L (7.2%), T69F/N (5.2%) and L74V/M (5.2%). For NNRTI, the most encountered mutations were: Y179F/V/D (9.8%), K103N/L/R (8.5%), Y181K/C (5.8%), V90I/GISV (5.8%), A98G/C/GPR (5.2%), V108I/K (5.2%), Y188C/L/D (4.6%) and F227C/L (4.6%). For NRTI, mutation T69F/N (5.2%) is associated with resistance to all NRTI; the K65R mutation (2.6%) is associated with resistance to Stavudine (d4T), Abacavir (ABC), Lamivudine (3TC), Didanosine (ddI) and Tenosifovir (TDF) which is a Nucleoside Inhibitor (nRTI); the K70E/N/R (9.8%) is associated with the TDF; the L14V/M (3.9%) to d4T and ABC; the V75I/N/L/M (18.3%) in the d4T; the Y115F (7.8%) in the ABC; the M184V/L/K/R (9.2%) to 3TC; and T215F/N/L (9.2%) to Zidovudine (ZDV) and d4T (29-31). For NNRTI, mutation A98G (5.2%) is associated with resistance to Nevirapine (NVP) while the E138A/K (3.3%) is incriminated for Efavirenz (EFV); the L10F/VA (2.6%), the K103N (8.5%), the V106A/M (7.2%), the Y181K/C (5.8%), the Y188C/L/D (4.6%), the G190R/A (3.9%) and M230L (1.3%) are all associated to the 2 molecules used in the DRC (30,33). At this level, over 15% of naive patients have a predisposition to first-line treatment failure as recommended for the DRC because of transmitted mutations.

For the most observed mutations in the literature for different resistances to the first line ART, there were varying prevalence among ART-naïve patients for NRTI and nRTI; V75I (18.3%), K70E (9.8%), D67N (9.2%), M184V (9.2%), T215F (9.2%), Y115F (7.8%), M41L (7.2%), T69F (5.2%) and L74F (3.9%); for NNRTI, mutation A98G (5.2%) is associated with resistance to Nevirapine (NVP) while the E138A/K (3.3%) is incriminated for Efavirenz (EFV). This brings us to the issue of monitoring of People Living with HIV/AIDS (PLHIV) in our environment (34,35). The median value of VLs of patients was 0.90 log10 RNA copies/ml. The minimum and maximum values of CD4 T lymphocytes rates were respectively of 98 and 1,050 cells/mm³. The median CD4 T cells count was of 560 cells/mm³ with 52 patients (46.02%) having CD4 count over 500 cells/mm³. It was not possible to determine the immunological failure based on CD4 count because of the irregularity of this parameter in patients. However, all patients who have the results at baseline and at 6th month, none were immunological failure because the CD4 values have evolved for all in a positive direction. Indeed, the differences in numbers could be the criteria of inclusion of the patients and the filtration criteria of treatment success compared to baseline was of 247.5 cells/mm³. This brings us to the issue of monitoring of People Living with HIV/AIDS (PLHIV) in our environment (34,35).

Several primary resistance were observed for Protease Inhibitors (PI); major and minor mutations. Genetic barrier is high for this class of drugs; only one major mutation for PI can lead to irreversible resistance to the specific PI, while it takes an accumulation of several minor mutations lead to irreversible resistance for an PI. The most significant observed Major mutations were: L90M (2.0%), D30N (1.3%), V32I (1.3%), V82A (1.3%) and 184V (1.3%). The most observed Minor mutations were: K201I/RT/M (28.1%), L10I (27.5%), H4RT/M (6.5%), V11I (5.2%), V32E/L (4.6%), G48R (3.3%) and A11IV (2.0%). PI are used in the DRC on the second line of treatment as recommended by the national program (8). These high frequencies of these transmitted mutations in naive patients are alarming especially since they can give patients a resistance to treatment intended for second line in case of regime change. In case of first line treatment failure, nearly 2.0% of patients will develop irreversible resistance to 2nd line ART that includes Lopinavir boosted by Ritonavir (LPV/r); thus involving a failure to this line of treatment.

CONCLUSION

This study demonstrates a strong diversity of HIV Type 1 in Kinshasa, which is dominated by the subtype A and CRF02_AG. Several major and minor resistances associated with Protease Inhibitors, as well as mutations associated with Reverse Transcriptase Inhibitors were detected in patients naïve of ARVs. The 6th month of treatment, the rate of virological treatment failure is of 24.6% for Kinshasa. These virological failures in the 6th month and transmitted mutations at baseline were highly correlated. These correlations reinforce the importance of the usefulness of genotyping tests and Viral Load at baseline to improve treatment and to adequate therapy.
CONFLICT OF INTEREST
None

REFERENCES