Evolution of genetic diversity and drug resistance mutations in HIV-1 among untreated patients from Mali between 2005 and 2006

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Objectives: To describe HIV-1 variants circulating in Mali and to estimate the rate of transmission of HIV-1 drug resistance in 2006.

Patients and methods: Viral reverse transcriptase (RT) and protease (PR) genes from 198 antiretroviral (ARV)-naive patients diagnosed HIV-1 positive in May 2006 in Bamako and Segou were sequenced.

Results: Although CRF02_AG was always the predominant HIV-1 subtype observed (72%), a higher genetic diversity than that in 2005 was observed. The overall prevalence of primary resistance is 11.5% in Mali in 2006, according to the 2007 IAS-USA list of mutations [nucleoside RT inhibitor (NRTI): 1.5%, non-NRTI (NNRTI): 9% and PI: 1%], and 2.5% (NRTI: 1%, NNRTI: 1.5% and PI: 0%), according to the Stanford list of mutations. There was no significant difference between 2005 and 2006 in the overall primary resistance prevalence or in the prevalence of mutations in the different ARV classes. Resistance mutations found in RT and PR genes are in agreement with the highly active antiretroviral therapy regimen available in Mali, except for V90I, V106I and A98G mutations which are associated with etravirine resistance, but polymorphic in non-B subtypes.

Conclusions: HIV-1 genetic diversity seems increased in Mali, but the overall HIV-1 primary resistance prevalence remains low. This is consistent with the findings from other West African countries where prevalence rates are lower than 5%. However, considering the large scaling up of ARV use in this country, it is necessary to regularly monitor the development of primary resistance in Mali.

Keywords: antiretroviral drugs, primary resistance, HIV subtype, Africa

Introduction

HIV-1 is partly characterized by its extensive genetic diversity, caused by errors introduced during the synthesis of cDNA from RNA. HIV-1 is thus subdivided into three groups (M, N and O), group M being itself subdivided into nine subtypes (A, B, C, D, F, G, H, J and K).1 Genetic recombination events among different genetic subtypes of HIV-1 group M have been identified. Some of these mosaic HIV-1 genomes are unique, but others play a major role in the AIDS pandemic and are named circulating
recombinant forms (CRFs). To date, 37 CRFs have been identified (http://hiv.lanl.gov/content/index). All subtypes and most CRFs circulate in Africa. Most HIV-1 strains present in North America, Australia and Europe belong to subtype B. Therefore, the majority of resistance studies have been performed on subtype B viruses; however, subtype B only represents 10% of HIV-1 infections worldwide. Resistance mutation profiles for non-B subtypes—the major subtypes in developing countries—differ in certain aspects. The D30N mutation tends to be selected among subtype B strains in cases of nelfinavir failure, whereas N88S and L90M mutations are preferentially selected among non-B strains.\(^5\) The V106M mutation, associated with efavirenz and nevirapine resistance, is the most common substitution in subtype C viruses; whereas V106A, associated with nevirapine resistance only, predominates in subtype B.\(^6\) Clinical data also suggest that the secondary mutations M36I and L10I or L10V are associated with a faster decrease in drug susceptibility during treatment.\(^7\) Subtype G strains seem to be less susceptible in vitro to protease inhibitors (PIs).\(^8\) Many studies have also shown the existence of polymorphisms among non-B strains, particularly naturally occurring minor mutations in the protease gene and atypical substitutions in protease and reverse transcriptase (RT) proteins at positions associated with resistance in subtype B.\(^9\) Minor mutations may not result in a significant decrease in susceptibility, but may be associated with an increase in the resistance levels associated with major mutations and thus long-term failure of therapy.\(^10\) With the recent introduction of antiretroviral therapy (ART) in developing countries, RT and protease sequences of non-B HIV-1 subtypes need to be obtained from naive individuals. World Health Organization (WHO) recommends monitoring HIV drug resistance (HIVDR) in untreated patients for all countries involved in ART access programmes to elucidate factors determining ART drug resistance. Indeed, patients infected with transmitted drug-resistant viruses and beginning ART therapy have a higher risk of virological failure and a higher risk of developing resistance, despite the drugs in their regimen originally being fully active.\(^11,12\) We studied HIV-1 strains circulating in Mali, West Africa. Mali has a low prevalence of HIV estimated at 1.7%: this corresponds to approximately 146 000 people.\(^13\) The first HIV treatments were available in 1997. These were followed by large-scale use of highly active antiretroviral therapy (HAART) in 2004, with stavudine/lamivudine/nevirapine recommended for first-line therapy. The emergence of resistance to these ARVs must be closely monitored. Primary resistance did not exist in 2002, whereas its prevalence was 2% in 2005 (95% confidence interval (CI): 0–4.77).\(^14\) In this study, we estimate the transmission rate of HIV-1 drug resistance in treatment-naive patients in 2006 and describe HIV-1 variants circulating in Mali, at two different sites, Bamako and Segou.

Materials and methods

Patients

One hundred and ninety-eight ARV drug-naive HIV-1-infected patients were studied. Plasma samples were collected from patients who were consecutively diagnosed as HIV-positive in May 2006, at two sites in Mali: a clinical centre (CESAC, Centre d’Écoute, de Soins, d’Animation et de Conseil) in Bamako, capital of Mali, and the regional hospital of Segou, a rural city, north of Bamako. This study was approved by the National Ethics Committee of Mali.

Dating infection by detuned assay

Recent HIV-1 infection was identified using an immunoassay based on an indirect ELISA to quantify antibodies specific for two HIV-1 antigens: consensus V3 peptides (A to E subtypes) and consensus peptides of the immunodominant epitope gp41 (consensus among subtypes + D subtype). This technique allows HIV-1 infection to be dated to either more or less than 6 months, as described previously.\(^15\)

RNA extraction, PCR and sequencing

Plasma HIV-1 RNA was used for the sequence analysis of the genes encoding RT (codons 1–240) and protease (PR) (codons 1–99). Viral RNA was isolated from plasma with the COBAS AmpliPrep Total Nucleic Acid Isolation kit (Roche). HIV-1 RNA was amplified by a one-step reverse transcription-PCR using the TITAN One Tube Reverse Transcription PCR kit (Roche), with outer primers 5’T1 and 3’P1 for PR\(^16\) and RTAG1 (5’-CCTACACC TTGCAAATAATTGGAC-3’) and RTAG2 (5’-CCATTCTAATT CTGTTTCTCCAGTC-3’) for RT. These two primers were designed specifically to amplify non-B subtypes. Nested PCR was then performed using AmpliTaq Polymerase (Applied Biosystems), with inner primers 5’P2 and 3’P2 for PR and RTAG3 (5’-CCAGT AAAATTAAGCCAGAATTG-3’) and RTAG4 (5’-TTCTGT ATATCATTGACAGCCAGCAG-3’) for RT. Amplified fragments were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) on an ABI 3100 Genetic Analyzer (PE Applied Biosystems). Sequences were analysed using the Sequence Navigator software (Applied Biosystems), comparing the sense and antisense strands of each fragment with the wild-type virus HXB2 sequence.

Phylogenetic and sequences analyses

Genetic subtypes were determined by phylogenetic tree analysis. The new PR and RT sequences were aligned with sequences from reference strains representing all subtypes and CRFs with the CLUSTAL W program.\(^17\) Phylogenetic trees were constructed using the neighbour-joining method and the Kimura two-parameter model. We evaluated 100 replicates by phylogenetic analysis. Recombination breakpoints and subtypes involved in the recombination of unclassified viruses sequences were determined by the bootscanning method of SimPlot software.

PR and RT amino acid sequences were compared with a subtype B consensus reference (HXB2) and analysed for resistance mutations described in the latest version of the International AIDS Society-United States of America (IAS-USA) list of mutations and in the Stanford list of mutations.\(^18,19\)

Results

Study population

Among the 198 patients included in the study, 100 were from Bamako and 98 from Segou. Out of the 198 patients, 132 were women (67%) and 66 were men (33%). The median age was 34 years (range: 5–69), and the median CD4 cell count was 375 cells/mm\(^3\) (range: 9–1672). The detuned assay revealed that
only seven patients were supposedly infected for <6 months, all from Segou. There was no evident difference in characteristics between the two patient groups (Table 1).

**Phylogenetic analysis of the PR and RT sequences**

Amplified products were not obtained for either PR or RT in 5 of the 198 samples (in one sample from Bamako and four from Segou); the RT gene alone was not amplified in a further seven samples (two from Bamako and five from Segou). Phylogenetic trees showed that the CRF02_AG form was the most prevalent, identified in 73 of the 99 samples analysed in Bamako (74%) and 66 of the 94 samples in Segou (70%) (Figure 1a). The distribution of the other subtypes in Mali was as follows: 22 CRF06_cpx (11%), 6 A (3%), 5 CRF09_cpx (3%), 2 G (1%), 2 F2 (1%), 1 CRF01_AE (0.5%) and 1 CRF18_cpx (0.5%), with a greater diversity in Bamako than in Segou (Figure 1b). The remaining 15 isolates were not clustered with any of the known subtypes in the phylogenetic trees. These viruses displayed pol gene mosaicism, involving different subtypes; CRF02_AG was present in all these isolates (Figure 1c). The strains from the seven recently infected patients did not form a cluster, according to the phylogenetic analyses. All the sequences are available on GenBank with accession numbers EU330647–EU330839.

**Resistance mutation analysis of PR and RT sequences**

According to the 2007 IAS-USA list of mutations, two major resistance mutations, L33F and M46L, were identified in PR, among 193 isolates analysed. M46L, known to be associated with indinavir resistance, was identified in a subtype A isolate. L33F, a major resistance mutation for tipranavir but secondary for lopinavir as well as indinavir, was found in a CRF02_AG isolate. Moreover, many minor mutations, associated with PI resistance in subtype B, were found. Table 2 summarizes the frequencies of these different mutations for each subtype. One CRF02_AG isolate harboured an asparagine insertion between amino acids 37 and 38 of PR.

The RT sequence analysis for 186 isolates revealed 21 RT mutations, each characterized by a single isolate from a single patient, except for one patient who harboured two RT mutations, V90I and A98G, on a CRF02_AG form (Table 3). Thus, according to the 2007 IAS-USA list of mutations, prevalence rates of mutations associated with nucleoside RT inhibitor (NRTI), non-NRTI (NNRTI) and PI resistance were 1.6% (3/186), 10% (18/186) and 1% (2/193), respectively, in 2006 (Figure 2). When recalculated according to the 2007 Stanford list of mutations, the prevalence rates of primary resistance in 2006 for NRTI, NNRTI and PI were as follows: 1% (2/186), 1.6% (3/186) and 0%, respectively; the main difference between IAS-USA and Stanford lists of mutations is related to NNRTIs. There was no statistical difference between overall primary resistance between 2005 and 2006, according to either the 2007 IAS-USA list or the Stanford list of mutations: 11.5% (95% CI: 7–16) versus 11% (95% CI: 6.6–15.4), and 3% (95% CI: 0.6–5.4) versus 2.5% (95% CI: 0.3–4.7), respectively. There was also no statistical difference for NRTI, NNRTI and PI resistance between 2005 and 2006 (Figure 2).

**Discussion**

We studied the genetic variability of HIV-1 PR and RT genes in 198 HIV-1-infected patients who had never received ARV treatment. Patients included in this study came from two cities in Mali, Bamako and Segou, and were consecutively diagnosed with HIV-1 in 2006.

Although samples were taken from patients at the time of HIV diagnosis, the detuned assay revealed that only seven of them were very recently infected (<6 months). Thus, HIV diagnosis in Mali appears to occur later in the natural development of infection, as in many developing countries: indeed, the median CD4 cell count observed in this population was 375 cell/mm³.

<table>
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<th>Bamako</th>
<th>Segou</th>
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<td>men</td>
<td>35</td>
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<tr>
<td>Age (years)a</td>
<td>34 (5–69)</td>
<td>34 (11–61)</td>
<td>34 (5–69)</td>
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<td>CD4 count (cells/mm³)a</td>
<td>398 (150–902)</td>
<td>371 (9–1672)</td>
<td>375 (9–1672)</td>
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<tr>
<td>Recently infectedb</td>
<td>0</td>
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*aValues shown are median and range.

*bAccording to the results of the detuned ELISA.*
Bamako than in Segou, which may be explained by the fact that Bamako is the main city of Mali with a high rate of commercial and migratory exchanges.

The analysis of HIVDR transmission is important in countries where ARV treatment is being rapidly expanded. Transmission of resistant viruses could jeopardize the...
Table 2. Frequency of mutations in the PR gene according to the subtype

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<th>G16</th>
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<th>M36</th>
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*aSubscript indicates the number of isolates in which the amino acid was found. Mutations in the 2007 IAS-USA list are shown in bold.

Table 3. Frequency of mutations in the RT gene according to the subtype

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*aSubscript indicates the number of isolates in which the amino acid was found. Mutations in the 2007 IAS-USA list are shown in bold.
effectiveness of treatment, particularly where regimen choices are limited. WHO recommends assessing HIVDR in specific geographical areas with restricted resources, such as Mali, where transmitted HIVDR is likely to be observed. ARVs have been made available in Mali since 1997, and free since 2004. The recommended first-line regimen is a fixed-dose combination stavudine/lamivudine/nevirapine (Triomune), currently prescribed free of charge in the majority of newly treated patients. The alternative first-line regimens are as follows: zidovudine/lamivudine/efavirenz and zidovudine/lamivudine/nevirapine. Moreover, the recommended second-line regimen is abacavir/didanosine/indinavir, and the alternative drugs are tenofovir and lopinavir. In the present study, the overall prevalence of primary resistance is 11.5% in Mali in 2006, according to the 2007 IAS-USA list of mutations (NRTI: 1.5%, NNRTI: 9% and PI: 1%). In 2005, the prevalence of primary resistance was estimated to be 2% in newly diagnosed and untreated patients, according to the IAS-USA list of 2005. When recalculated with the 2007 IAS-USA list, the overall prevalence is then estimated to be 11% in 2005; indeed, the main difference between 2005 and 2007 IAS-USA lists of mutations is the absence of etravirine mutations in 2005, because this NNRTI was not available at that time. So, there is no significant difference between 2005 and 2006 in the overall prevalence or in the prevalence of mutations for the different ARV classes. When the overall resistance prevalence is calculated with the Stanford list, there is a significant difference in the IAS-USA list (11% versus 3% in 2005 and 11.5% versus 2.5% in 2006). This difference is mainly due to some etravirine mutations (V90I, A98G and V106I) that are present in the IAS-USA list, but not in the Stanford list explaining the results for NNRTIs: 10% versus 1% in 2005 and 9% versus 1.5% in 2006. These results suggest that etravirine mutations occur as a natural polymorphism in the non-B subtypes and are not related to the exposure to this ARV, because it is not yet available in Mali. An alternative explanation could be that these mutations are related to long-term exposure to NNRTIs such as nevirapine and which were then transmitted to a drug-naive individual. Moreover, the results show that the IAS-USA list, the favourite list used in epidemiological studies, is not relevant for this purpose, and the Stanford list needs to be validated.

L33F and M46L mutations are the first PI mutations to be detected in studies of primary resistance in Mali. L33F mutation is associated with resistance to tipranavir and lopinavir, and M46L mutation with resistance to tipranavir, lopinavir and indinavir, the latter being available for second-line treatment in Mali. In the Stanford list, L33F and M46L are considered as polymorphic, because they were found in 1% of the subtype A and 1.5% of subtype G sequences, respectively. Thus, we cannot exclude that these mutations may only be polymorphisms, but it is also possible that they arose from a transmitted virus resistant to second-line treatment, specifically for M46L.

Twenty-one mutations associated with resistance were found in the RT gene. Three of these are associated with NRTI resistance, consistent with the use of NRTIs in first-line HAART regimens available in Mali: K219Q, which is a thymidine analogue mutation, was found in two isolates and V75I, which belongs to the MDR complex, is known to increase resistance when the Q151M mutation is also present (but it does not induce resistance alone). A T215P mutation, never described in previous studies, was found in one isolate; this genetic location is
associated with resistance to thymidine analogues and may be a revertant form of T215Y. Among the 18 NNRTI resistance mutations identified in this study, only 3 of them can be considered as transmitted drug resistance mutations (Y181C, V108I and K101E) and are consistent with the large use of Triomune in Mali. The 15 other NNRTI resistance mutations can be considered as polymorphisms related to non-B subtype, such as etravirine-related mutations.

WHO recommends monitoring transmitted drug resistance; they have described a threshold survey method categorizing prevalence rates of transmitted HIVDR to relevant ARV drugs and drug classes as <5%, 5% to 15% or >15%. This method is based on a maximum of 47 specimens from consecutively diagnosed individuals who meet criteria for recent HIV infection, such as infected young women under the age of 25 years who are in their first pregnancy. Although this is effective and requires a small number of samples, this method is difficult to implement because of stringent inclusion criteria. The methods used in the present study required a larger number of samples but no demographic criteria were applied for inclusion of study subjects. The only potential limitation of this type of analysis is the underestimation of the prevalence of resistance mutations. Indeed, resistance mutations disappear over time without selection pressure in naive patients. However, some of them can persist for several months. Indeed, K219Q and Y181C mutations can persist for up to 9 and 25 months, respectively, without selection pressure. In the present work, these mutations were found in infected patients with CD4 cell counts of 354 and 372 cells/mm³ for two isolates harbouring K219Q mutation and 376 cells/mm³ for Y181C. Findings from the detuned assay suggested that these patients had probably been infected for more than 6 months. These mutations may have persisted for more than 6 months in these patients, or the patients may have been superinfected with a resistant virus.

In conclusion, our findings describe the circulation of HIV strains with mutations conferring resistance to three classes of inhibitors available in Mali, in patients who have never received ARV treatment. The prevalence of resistance mutation transmission was 2.5% in 2006, using the 2007 Stanford list. This is similar to the prevalence in 2005 (3% according to the 2007 Stanford list). Although the 2006 prevalence calculated with the 2007 Stanford list does not include PI mutations, we identified two PI mutations that have not been described in previous primary resistance studies in Mali. Although the overall resistance prevalence remains low—consistent with the findings from other West Africa countries, where prevalence rates are lower than 5%—it is necessary to regularly monitor the development of primary resistance in Mali, especially with the appearance of transmitted viruses conferring resistance to second-line ARV treatment.

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Transparency declarations

None to declare.

References

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