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Abstract
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Document type: Article de périodique (Journal article)

Référence bibliographique
Ruelle, Jean ; Sanou, Mahamoudou ; Liu, Hsin-Fu ; Vandenbroucke, Anne-Thérèse ; Duquenne, Armelle ; et. al. Genetic polymorphisms and resistance mutations of HIV type 2 in antiretroviral-naive patients in Burkina Faso. In: AIDS research and human retroviruses, Vol. 23, no. 8, p. 955-64 (2007)
DOI: 10.1089/aid.2007.0034
Genetic Polymorphisms and Resistance Mutations of HIV Type 2 in Antiretroviral-Naive Patients in Burkina Faso

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ABSTRACT

Natural polymorphisms in the pol gene of HIV-2 may influence the susceptibility to antiretroviral drugs and the choice of treatment. We collected samples in centers for anonymous HIV testing in Ouagadougou, Burkina Faso, in patients supposedly naive for any antiretroviral treatment. Eighty-four samples were first tested as HIV-2 positive in Burkina Faso and then shipped to Brussels, Belgium, for confirmation of the serological status and plasma viral load. Fifty-two samples were confirmed as HIV-2 positive in Belgium. Twelve others were HIV-1 positive and 20 were dually reactive. Twenty-one of HIV-2 confirmed samples had an HIV-2 plasma viral load higher than 1000 copies/ml. These viruses were sequenced in the protease and reverse transcriptase genes and 17 sequences of the pol gene were obtained. Highly polymorphic positions were identified in protease and RT genes. Two samples harbored known resistance mutations: M184V RT mutation in one and Q151M with M184V in the other. Phylogenetic analysis showed that viruses in Burkina Faso did not cluster separately from published sequences from neighboring countries. The two resistant strains were unrelated. Our findings imply either that resistant viruses are circulating in Burkina Faso or that some individuals take unsupervised treatment. Both hypotheses present problems.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS TYPE 2 (HIV-2) infections are mainly found in West Africa, the origin of the epidemic.1–3 Although eight different subtypes have been described up to now, only subtypes A and B are regularly seen. Subtype A is globally the most abundant, but the geographic distribution can vary from almost only subtype A in countries surrounding Guinea-Bissau to a predominance of subtype B in the Ivory Coast.4–7

HIV-2 infections are characterized by a longer asymptomatic period compared to HIV-1, a lower plasma viral load, a lower transmission rate, and a reduced risk of mortality.8–10 Therefore, if we apply guidelines for the treatment of HIV-1 infection, most HIV-2-infected patients will not need antiretroviral (ARV) drugs.11,12 But it is not yet clear if the usual time to start ARV treatment is adequate for the control of HIV-2 infection and if the same combinations of drugs used for HIV-1 are optimal.

It has been shown that antiretroviral therapy has a modest impact on the CD4 count recovery.13,14 In HIV-2 infections, better virological and immunological outcomes are seen with protease inhibitor (PI)-containing regimens compared to nucleosidic reverse transcriptase inhibitor (NRTI)-only regimens,15,16 although some authors see no marked differences.14,17 Sensitivity to ARV drugs differs between HIV-1 and HIV-2. HIV-2 is naturally resistant to nonnucleosidic reverse transcriptase inhibitors (NNRTI) and fusion inhibitors (FI).18 Reduced sensitivity is observed for some PIs: nelfinavir, amprenavir, and atazanavir.19 Few recommendations exist for the treatment of HIV-2 infection20 and they are based on limited results.

Resistance mutations appear during ARV therapy. Their study is complicated by the presence of natural polymorphisms at positions known to be linked to resistance in HIV-1: 10V, 32I, 36I, 46I, and 71V in the protease gene21 and 118I and 215S in the RT gene in addition to the three positions linked to the resistance to NNRTI (181I, 188L, and 190A).22,23 Neverthe-
Serological tests in patients failing lopinavir seen to appear under a tenofovir-containing regimen clearly linked to failure of therapy: in the RT gene, K65R was less, some mutations appearing under treatment have been described in viruses from patients failing their ARV therapy.28,29

The rules and algorithms established for HIV-1 genotypic resistance do not apply to HIV-2. More studies are needed to compare mutations appearing under well-defined regimens and to define what sequence variations are natural polymorphisms. The present study is one step in this direction.

MATERIALS AND METHODS

Patient samples

Samples were collected between July and December 2005 in centers for anonymous HIV testing in Ouagadougou, Burkina Faso. Serological tests were first performed in the Lamizana Medical Centre and in the National Centre of Fight Against Tuberculosis (CNLAT). Samples were then shipped frozen to the AIDS reference laboratory of UCL, Brussels, Belgium where an HIV confirmation test, plasma viral load, and sequencing of protease and RT genes were performed.

Serological tests

In Burkina Faso HIV tests were performed following national consensus rules: one sensitive rapid test followed by one discriminant test. The first test was Determine (Abbott, Delkenheim, Germany) and the second was ImmunoCombi II (Organics, Strasbourg, France). Both were used following the manufacturers’ recommendations. In Belgium the Innolisa immunoblot (Innogenetics, Gent, Belgium) was used to assess the HIV-1, HIV-2, or dually reactive serological status on the Auto-Lia platform following the manufacturer’s short time protocol.

Viral load measurements

HIV-2 plasma viral load was assessed by a real-time polymerase chain reaction (PCR) in-house assay on a Lightcycler platform.28 The assay as described was modified to obtain a sensitivity of 50 RNA copies per ml. It used a quantified synthetic RNA as external standard. Viral RNA was extracted from 1 ml of plasma or serum by the Nuclisens Magnetic Isolation kit on a Mini-Mag apparatus (Biomérieux, Boxtel, The Netherlands). RNA was eluted in 40 μl buffer. Eight microliters was used for reverse transcription using the Transcripter first strand cDNA synthesis kit (Roche Diagnostics, Penzberg, Germany) with random hexamers (final concentration 60 μM) in a final volume of 20 μl. The real-time PCR was performed using the Lightcycler FastStart DNA MasterPLUS SYBR Green I, 100 μl kit (Roche Diagnostics, Penzberg, Germany) on a Lightcycler 2.0 platform. Each capillary contained 48 μl PCR grade water, 6 μl of each primer (in a final concentration of 0.6 μM—see ref. 30 for sequences), 20 μl of Master Mix provided in the kit, and 20 μl cDNA. Each run consisted of 40 cycles of amplification (95°C 15 sec, 60°C 40 sec, 72°C 30 sec) followed by a melting curve analysis.

HIV-1 plasma viral load was assessed by the Quantiplex HIV-1 RNA 3.0 assay (bDNA) (Bayer Health Care, Tarrytown, NY).

The overall median viral load was calculated by assessing the value of 50 copies (cp/ml) (1.7 log cp/ml) for samples with undetectable viremia. These values were not used for the calculation of the median detectable viral load. The value of 500,000 cp/ml (5.7 log cp/ml) was used for one HIV-1 sample with a viremia higher than 500,000.

HIV-2 genotype

RT-PCR and nested PCR were performed as described by Colson et al.28 and PCR products were analyzed in a 2% agarose gel with ethidium bromide. This protocol generated a 1507-bp fragment that covers the protease and the RT coding regions. Viruses of subtype B failed to be amplified. In this case, two separated nested PCR reactions were performed using 2 μl of RT-PCR product. One reaction amplified the RT coding region with the forward primer JR23 5’-TAATGACAGGCGACACCC-3’ and the reverse primer JR24 5’-TGTCCTGGCCAAATTTAG-3’, both in a final concentration of 0.2 μM, with PCR conditions as follows: 10 min at 94°C, 40 cycles of 30 sec at 94°C—45 sec at 57°C, 1 min 30 sec at 72°C, and finally 10 min at 72°C. The second reaction amplified the protease coding region, with the forward primer JR21 5’-AGACACCTACAAGGAGC-3’ and the reverse primer JR41 5’-TGTATGGATT-AGTAGGAGCG-3’ in the same experimental conditions.

PCR products were then purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Twenty nanograms of each PCR product was sequenced with the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Primers used for sequencing analysis were H2Mp328 and JR 40 5’-AGGATTAGTTGGAGGTGC-3’ for the protease coding region and JR 23, JR 24, JR25 5’-GACCTCCAACTAATCCT-3’ and JR 26 5’-GCGATATATGGTCTAAAGTG-3’ for the RT coding region. If the primers used for subtype A amplification did not succeed, the protease coding region was sequenced with primers JR21 and JR41, while the RT coding region was sequenced with JR23, JR34 5’-AGTTGACGTGGCCAAATTTA-3’ and JR35 5’-CGCCTCTTACTAATCCATA-3’. Products of the sequencing reaction were purified by ethanol-acetate precipitation and sequenced on an ABI Prism 310 genetic analyzer (Applied Biosystems).

Phylogeny

These sequences and sequences retrieved from GenBank for comparison were aligned with the Mega software MEGA version 3.131 followed by minimal manual editing. Phylogeny construction and evaluation were performed using the Phylip software package (version 3.66, University of Washington, Seattle, WA),32 with the neighbor-joining method (NJ) and the maximum likelihood method (ML). The F84 model was used to calculate the evolutionary distances. The robustness of the NJ tree was assessed with 1000 bootstrap replicates.
was statistically evaluated by bootstrap analysis with 1000 bootstrap samples. Since the ML method is already a statistical method (with a statistical evaluation of the branch length), no bootstrapping was done for it.

**GenBank accession numbers**

The protease and reverse transcriptase coding sequences studied here were submitted to GenBank and received accession numbers from EF090162 to EF090192. Published protease and RT sequences used for phylogenetic analysis were as indicated in Figs. 3 and 4.

**Ethical approval**

This study was approved by the Commission for biomedical ethics of the medical faculty of the Université Catholique de Louvain, Belgium and by the national ethical commission in Burkina Faso.

## RESULTS

### Serological status and plasma viral load

Eighty-four serum samples from anonymous HIV testing centers in Ouagadougou, Burkina Faso were diagnosed as HIV-2 positive or HIV-1 and -2 dually positive between July and December 2005. The age of the patients ranged from 19 to 53 years; 51 patients were women (60.7%) and 33 were men (39.3%). Table 1 shows a comparison between results obtained in Burkina Faso with the ImmunoCombII assay and results from the Inno-Lia confirmatory test. The positive predictive value of the ImmunoCombII assay is 94% for HIV-2 and 59% for dually reactive samples if Inno-lia is taken as a reference.

Among 52 confirmed HIV-2 infections, 20 had an undetectable viral load (VL). The detectable HIV-2 VLs ranged from 75 to 222,500 cp/ml (1.88–5.35 log cp/ml). It contrasts with the HIV-1 samples of this population where the VL was always detectable. Plasma viral load is lower in HIV-2 infection: median VL, were 3.78 and 2.66 log cp/ml, respectively, in HIV-1 and HIV-2 samples. The mean VL for samples with detectable viremia was 4.99 log cp/ml (77,104 cp/ml) and 4.35 log cp/ml (22,383 cp/ml), respectively, for HIV-1- and HIV-2-positive samples.

Among the 20 samples dually reactive with Inno-Lia 17 samples had an undetectable HIV-2 plasma VL (81%), a higher proportion than in the HIV-2 positive group where 20 out of 52 had an undetectable VL (38%). Similarly, only two samples (10%) had a plasma VL higher than 1000 copies/ml in the dually reactive group, compared to 21 out of 52 (40%) in the HIV-2 group. Insufficient volume was available to perform an HIV-1 VL in this group.

### Sequencing of reverse transcriptase and protease genes from viral RNA

Fourteen protease sequences were obtained (11 from subtype A and 3 from subtype B). Similarities of nucleotide sequences when compared to the reference sequences ROD and EHO varied from 91 to 96% for subtype A, and from 93 to 95% for subtype B. Highly polymorphic positions were found at positions 14, 40, and 65 (see Fig. 1 for details). Positions 17, 36, 43, 46, and 70 were also variable within the subtype A group. In this small number, positions 17 and 46 were always linked: G17 with I46 or D17 with V46. Conserved domains were observed from residues 1 to 10, 21 to 33, 47 to 56, 78 to 88, and from residues 94 to 99. Within these domains, we found only variations at residues 7, 96, and 99. We saw no mutation associated with PI resistance in HIV-1, if we exclude natural polymorphisms found in the majority of HIV-2 strains at positions 10, 32, 36, 46, and 71.

Seventeen reverse transcriptase sequences were analyzed. Eleven cluster within subtype A (65%) and six within subtype B (35%). Similarities of nucleotide sequences compared to ref-

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**Table 1. Serological Status and Viral Load of 84 HIV-2 Seropositive Samples Collected from Anonymous HIV Testing Centers in Ouagadougou, Burkina Faso**

<table>
<thead>
<tr>
<th>Serological status following determine/immunocomb</th>
<th>HIV-2</th>
<th>HIV-1/2 dually reactive</th>
<th>Total</th>
<th>HIV-2 VL (cp/ml)</th>
<th>HIV-1 VL (cp/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1</td>
<td>3 (6%)</td>
<td>9 (26%)</td>
<td>12 (14%)</td>
<td>Not done</td>
<td>VL &lt; 1000:7 50 &lt; VL &lt; 1000</td>
</tr>
<tr>
<td>HIV-2</td>
<td>47 (94%)</td>
<td>5 (15%)</td>
<td>52 (62%)</td>
<td>VL &gt; 1000:21 50 &lt; VL &lt; 1000:10 VL &lt; 50:20 Not done</td>
<td>Not done</td>
</tr>
<tr>
<td>HIV-1/2</td>
<td>0</td>
<td>20 (59%)</td>
<td>20 (24%)</td>
<td>VL &gt; 1000:2 50 &lt; VL &lt; 1000:17 VL &lt; 50:17</td>
<td>Not done*</td>
</tr>
<tr>
<td></td>
<td>50 (100%)</td>
<td>34 (100%)</td>
<td>Total: 84 (100%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Insufficient sample volume.

VL, viral load; cp/ml, genome copies per ml.
erence sequences ROD and EHO varied from 91 to 95% for subtype A, and from 88 to 92% for subtype B. Highly polymorphic positions with four or more different amino acids on all samples were found at positions 8, 11, 102, 117, 126, 135, 166, 167, 176, 179, 180, 195, 200, 203, 227, and 251. The most conserved domains were residues from position 67 to 81, from 91 to 100, and from 148 to 161. Except for variations directly related to subtype A or B, we saw no patterns of mutations, but our patient group is probably not large enough to observe relations between polymorphisms. Surprisingly two samples harbored primary resistance mutations to nucleosidic reverse transcriptase inhibitors: one with M184V (V05-06106) and one with Q151M and M184V (V05-06116). On the nucleotidic level, codon 151 corresponded to CAG in seven samples, CAA in seven others, CAR (mixed G/A populations) in two samples, and AUG in the one where methionine replaced glutamine.

### Phylogenetic analysis

To investigate the diversity of strains circulating in Burkina Faso, we constructed phylogenetic trees with two different methods: neighborhood joining and maximum likelihood. Through GenBank we collected nucleotidic sequences of protease and reverse transcriptase from viruses isolated in West African countries surrounding Burkina Faso. For the protease coding sequence, the 14 sequences of this study were compared to 32 published sequences (Fig. 3), and our 17 sequences of the RT coding sequence were compared to 22 others (Fig. 4). Countries of origin of published sequences are detailed in the legends of these figures. We show here only the phylogenetic trees obtained with the neighborhood joining methods, but results obtained with maximum likelihood were very similar. First, we did not observe a specific cluster of strains from Burkina Faso compared to the strains from other countries, although two strains (V05-06082 and V05-06087) are closely related. Second, patients from this study all cluster with strains from sub-

**FIG. 1.** Protease amino acid sequence alignment. Eleven subtype A samples and three subtype B samples were aligned with two reference strains: ROD and EHO (respectively, subtype A and B). Within each subtype, samples were classified from the highest to the lowest plasma viral load. When a mixture with two different populations was present, the amino acid differing from the consensus was retained in the alignment.
FIG. 2. Reverse transcriptase sequence alignment (from amino acid 1 to 255). Eleven subtype A samples and six subtype B samples were aligned with two reference strains: ROD and EHO (respectively, subtype A and B). Within each subtype, samples were classified from the highest to the lowest plasma viral load. When a mixture with two different populations was present, the amino acid differing from the consensus was retained in the alignment. X at positions 85 in sample V05–06116 and 176 in sample V05–06116 stand for complex mixtures.
type A or subtype B. Third, we saw no association between samples from Burkina Faso with a particular neighboring country. Finally, the two strains harboring resistance mutations (V05-06106 and V05-06116) are both from subtype A but are not phylogenetically related, suggesting that the two patients had no relationship to each other.

DISCUSSION

Resistance mutations are selected in HIV-2 under ARV treatment. Published data show that despite some similarities, resistance profiles differ from HIV-1 and frequencies of known mutations also vary. This underscores the need for guide-
lines for the treatment of HIV-2 infection on the one hand, and for specific genotype interpretation rules on the other hand. Descriptions of natural polymorphisms present in naïve populations before any ARV treatment help in the design of these algorithms. We analyzed samples collected from patients in Burkina Faso, as part of an anonymous HIV screening program, and sequenced viral RNA. The first issue raised was the serological diagnosis of HIV-2 infection. Table 1 shows discordances between ImmunoComb hIV-1 and 2 (Organics, Strasbourg, France) and Inno-Lia (Innogenetics, Gent, Belgium). Predictive positive value was particularly low for the diagnosis of mixed infection: 59% if Inno-Lia is taken as reference. These double-reactive samples may reflect antibody cross-reactions during the test, or may be effectively related to the concomitant presence of both types of HIV. As we analyze the plasma viral load values obtained in HIV-2 reactive and dually reactive samples, we see that the proportion of samples with undetectable viremia is higher in the dually reactive population, 81% vs. 38% in the HIV-2-positive population. This suggests that within the dually reactive group, either there is a proportion of falsely positive samples for HIV-2, or that a higher HIV-1 replication inhibits the replication of HIV-2, but no data support this putative interaction. The samples that eventually appeared to be HIV-1 infected on serological confirmation all had a detectable viral load for HIV-1. This confirms the serological diagnosis with Inno-Lia. As the discrimination between HIV types 1 and 2 is of importance for medical care, the diagnosis of coinfection should ideally be confirmed by detection of provirus from both types in circulating lymphocytes. Unfortunately, PCR assays are not yet available in many regions of the world. The quality of the serological discrimination between HIV types 1 and 2 should therefore be improved.

Genotypic analysis revealed several polymorphic positions. In the protease codon 14, 40 and 65 were the most variable ones, in accordance with published data. Other positions also showed variability, but we observed conserved regions that match with functionally active domains described in the HIV-1 protease: the N-terminal from residues 1 to 10, the sequence around the active site from residues 21 to 33, the top of the flap from residues 47 to 56, the second loop of the β-sheet from residues 78 to 88, and the C-terminal domain from residues 94 to 99. Codon 30, located near the catalytic triad within the active site, was always an aspartate; therefore the reduced activity of nefllavir and amprenavir in HIV-2 is not due to a D30N mutation. We observed no mutation linked to PI resistance in this group of naïve patients, except natural HIV-2 polymorphisms that are linked to resistance in HIV-1.21 Residue at position 82 was always an isoleucine rather than a valine, a change possibly linked to resistance in some HIV-1 drug resistance algorithms. In the reverse transcriptase, positions 8, 11, 102, 122, 176, 211, and 228 were the most variable. Other polymorphic positions distributed along the whole sequence were observed (see results), including positions 5 and 35 described elsewhere as rare mutations under treatment.23 The most conserved domains were related to functional domains of the polymerase.37

Even though global sequence similarity ranged from 88 to 95% in the reverse transcriptase compared to reference strains ROD and EHO, the number of polymorphic positions observed at the protein level is higher than what is observed for HIV-1 in untreated patients. A possible explanation is that the mean time between primoinfection and genotype is longer in HIV-2 cohorts with a higher number of generations and more mutations, but one should consider that HIV-2 may have a lower replication rate than HIV-1. We have no way to measure it in this study since patients were anonymously screened for HIV infection. Another theoretical explanation could be a difference in the error rate of reverse transcriptases between the two types of HIV, but it has been documented that fidelities of both enzymes are similar.39

Two different samples had known resistance mutations in the reverse transcriptase: Q151M and M184V in the first one and M184V in the second. These are two frequent mutations appearing under ARV treatment in HIV-2: the Q151M mutation results in phenotypic resistance to zalcitabine and didanosine, while the M184V mutation confers resistance to lamivudine. The Q151M mutation also causes resistance to zidovudine, but the pathway used is different from HIV-1: an increase in exclusion of triphosphate zidovudine (AZTP) from the active site has been described.40 At the nucleotide level, CAG and CAA glutamine codons were equally present at position 151 in the reverse transcriptase. Two or three changes are needed to obtain the methionine codon ATG observed in the sample with the 151M mutation. This mutation is therefore highly unlikely without antiretroviral selective pressure. At position 184 one single nucleotide change is sufficient to transform the methionine codon to valine.

The presence of resistance mutations is surprising for different reasons. First, patients were supposed to be naïve of any antiretroviral treatment since they were not aware of their HIV seropositivity at the time of sampling. Second, the M184V mutation causes a loss of replicative capacity41 and is usually not persistently detected after HIV-1 treatment interruption.42 These patients could have been infected by someone on antiretroviral therapy, but even in this case a back mutation from 184 V to M offers a selective advantage to the virus.

Because phylogenetic analysis showed that these two cases were not related, the presence of resistance mutations could thus not be linked to a single strain. Statistically valid conclusions should be drawn only after testing more patients. Circulation of resistant strains has been documented for HIV-1 in rural Burkina Faso.43 If the transmission of HIV-2 resistant strains is effective in West Africa, this may jeopardize the use of certain antiretrovirals. We could not exclude the possibility that these two patients have been on unsupervised antiviral therapy and would have lied about their unknown HIV status for social reasons. In any case our observation indicates that caution is needed in the implementation of therapy, taking into account the possibility of uncontrolled distribution of antiretrovirals.

In the phylogenetic analysis, different clusters including the viruses from Burkina Faso were apparent. The same results were obtained for protease and reverse transcriptase alignments using two different methods. This confirms that HIV-2 has been established in West African countries for a long time. This contrasts with other parts of the world: in South Korea, a study showed that all HIV-2 viruses were phylogenetically related to a single cluster, probably imported by a single recipient.44 Our study described polymorphisms naturally present in the protease and reverse transcriptase of HIV-2 isolated from antiretroviral naïve patients. The impact of these polymorphisms on ARV susceptibility is unknown; in addition, we should con-
sider the potential transmission of drug-related mutations. Combined to phenotypic data this should help to set up specific recommendations for HIV-2 resistance testing.

ACKNOWLEDGMENTS

This work was partially supported by the Prix Pierre and Colette Bauchau 2004.

REFERENCES


HIV-2 POLYMORPHISM IN BURKINA FASO


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