

Characterization of Drug-Resistance Mutations in HIV-1 Isolates From Non-HAART and HAART Treated Patients in Burkina Faso

W.M. Nadembega,^{1,2} S. Giannella,³ J. Simpoire,^{1,2,3} F. Ceccherini-Silberstein,³ V. Pietra,¹ A. Bertoli,³ S. Pignatelli,¹ M.C. Bellocchi,⁴ J.B. Nikiema,² G. Cappelli,³ A. Bere,² V. Colizzi,³ CP. Perno,^{3,4} and S. Musumeci^{5*}

¹Centre Médical Saint Camille de Ouagadougou (CMSC)—Centre d'Accueil et de Solidarité de Ouagadougou (CASO), Burkina Faso, Ouagadougou, Burkina Faso

²University of Ouagadougou, Ouagadougou, Burkina Faso

³University of Rome "Tor Vergata", Via Montpellier, Rome, Italy

⁴National Institute for Infectious Diseases "L. Spallanzani", Via Portuense, Rome, Italy

⁵Department of Pharmacology, Gynecology and Obstetrics, Pediatrics,

University of Sassari and Institute of Population Genetics, National Research Council (CNR), Alghero SS, Italy

Non-B HIV subtypes have been estimated to account for 88% of HIV infections in the world. These subtypes are particularly relevant in view of the availability of antiretroviral (ARV) drugs, since subtype-specific mutations are associated with drug-resistance in developing countries. Therefore, the *pol* gene sequences in HIV-1 isolates were examined from the three distinct groups of 39 infected patients from Ouagadougou in Burkina Faso: 17 patients who had not received any antiretroviral therapy (ART); 16 patients received ART, and 6 HIV-infected children, from infected mothers, received a single Nevirapine dose prophylaxis during birth. HIV-1 *pol*/sequencing was successful for 29 samples. As expected, all patients presented the common (non-B subtype) M36I polymorphism and 26/29 (90%) the K20I mutation. Phylogenetic studies showed high predominance of recombinant HIV-1 strains: CRF06_cpx 16/29 (55.17%), CRF02_AG 9/29 (31.03%), A1 2/29 (6.89%), G 1/29 (3.44%), and CRF09_cpx 1/29 (3.44%). Two twins showed, 6 months after birth, a NNRTI-mutation (Y181C/Y). During the same period, the twin mother presented a different NNRTI-mutation (V106I), thus suggesting that the different blood drug concentration may determine a different drug-resistance pathway. Among 17 non-highly active antiretroviral therapy (HAART) patients, 3/17 (17.64%) presented virus with reverse transcriptase (RT) mutations [V118I: 1/17 patients (5.88%), V179E: 2/17 patients (11.76%)]. 10/17 (58.82%) presented virus with minor protease (PR) mutations [L63P: 5/17 patients (29.41%), V77I: 3/17 patients (17.64%), L10I: 2/17 patients (11.76%)]. 4/17 patients did not show any PR and RT mutations (23.52%). Among six HAART-treated

patients, 6/6 and 3/6 had M36I and L63LP protease minor subtypes, respectively; and only two (33.33%) presented virus with K103N mutation. The low prevalence of drug-resistant associated mutations in Burkina Faso is encouraging. However, further studies with a larger cohort with a high non-B subtype prevalence are necessary to optimize ART in developing countries. **J. Med. Virol. 78:1385–1391, 2006.**

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INTRODUCTION

Worldwide, Sub-Saharan Africa is the region with the highest number of AIDS with 25 million HIV infected persons [Joint United Nations Programme on HIV/AIDS, 2004]. This epidemic is characterized by a high genetic diversity of HIV-1 strains with a specific geographical localization. As an example, the Southern Africa HIV epidemic is dominated by HIV-1 subtype C;

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*Correspondence to: Prof. S. Musumeci, Department of Pharmacology, Gynecology and Obstetrics, Pediatrics, University of Sassari, Viale San Pietro n. 43b—07100 Sassari, Italy. E-mail: smusumeci@tiscalinet.it

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East Africa by HIV-1 subtypes A and D; West Africa by subtypes A and G; while Central Africa is the broadest reservoir of strains [Peeters, 2000; Peeters et al., 2003; Papathanasopoulos et al., 2003; Kandathil et al., 2005]. This large HIV-1 genetic diversity can be a constraint for antiretroviral (ARV) therapy, used by African persons infected with HIV. However, it has been demonstrated recently that highly active antiretroviral therapy (HAART) reduces dramatically mortality and morbidity in patients with HIV in both developing and developed countries [Frater et al., 2002; Vergne et al., 2003a]. Therefore, the majority of African Country Government, with the aid of WHO, UNAIDS, and non-governmental organizations (NGO), initiated the use of ARV for HIV patients and for Mother-to-Child-Transmission (MTCT) prevention program [Pignatelli et al., 2005; Simporé et al., 2006]. Burkina Faso, a developing country with a HIV-1 prevalence in 4.2% of adult population [UNAIDS/WHO, 2004] has adhered to these programs. A seroprevalence study in pregnant women in five sentinel towns in Burkina Faso (Bobo Dioulasso, Ouagadougou, Ouahigouya, Gaoua and Tenkodogo) showed a prevalence of HIV-1 infection of 6.94% (1997), 6.34% (1998), 5.84% (1999), 5.18% (2000), 4.74% (2001), and 4.40% (2002). From 1997 to 2000, the reduction in prevalence of HIV in these sentinel towns was not significant ($P = 0.067$). In contrast, from 2001, a progressive and uniform reduction of prevalence ($P = 0.014$) was observed [Rapport Ministère de la Santé du Burkina Faso, 2003]. In 2005, WHO and UNAIDS calculated 43,000 HIV-1 infected persons, in Burkina Faso needing ARV therapy. Nevertheless, the ARV therapy was used effectively only by 2000 HIV infected persons in the course of 2004. It is clear that in such situations it is extremely important to identify HIV mutations that constrain the antiretroviral therapy (ART).

The aim of this study was to characterize the prevalence of HIV-1 subtypes in Burkina Faso. The prevalence of key drug resistance mutations in drug-naïve and ARV-treated patients and the ARV response in HAART-treated patients, including the mother and the child, of the MTCT program were also determined. The HIV-1 *pol* gene, containing the reverse transcriptase (RT) and protease (PR), principal targets of antiretroviral drugs was also sequenced and examined.

MATERIALS AND METHODS

Patients

After informed consent, 39 HIV-1 patients from two Ouagadougou distinct sites were enrolled in this study. Samples were collected between December 2003 and June 2004. Twenty-two samples were coming from the "CMSC (Ouagadougou)," and seventeen from the "Centre d'Accueil et de Solidarité de Ouagadougou, CASO", a specialized center for the care of HIV infected persons. Patients were selected randomly among three distinct groups, regarding the ARV drug use. The three groups were composed by: (1) 17 patients not receiving any ARV drugs (male 6 and female 11, aged 27–55 years,

average 36.76 ± 8.58); (2) 16 patients receiving ARV drugs (male 5 and female 11, aged 25–49 years, average 35.87 ± 7.55); and (3) 6 HIV-1 infected children (male 1 and female 5, aged 6–18 months, average 8.33 ± 4.76), born to HIV-1 infected mothers who received a single dose Nevirapine (NVP) for prophylaxis during delivery. All ARV-treated patients received 6 months HAART therapy (2NRTI + 1NNRTI) except a mother of two HIV-1 infected twins who was treated with a single NPV dose during the delivery. The various drug combinations (or drug regimens) were: AZT/3TC + NVP (eight patients), DDI + D4T + EFV (three patients), 3TC + D4T + EFV (two patients), AZT/3TC + IDV (one patient), AZT/3TC + EFV (one patient), and single NVP dose (one patient). All children received a single NVP dose after birth.

T Lymphocytes Count

Blood was collected in EDTA containing tubes and, the absolute cell count of CD4+, CD8+, and CD3 T lymphocytes were enumerated by a FACS count (Becton Dickinson, San Jose, CA) in CMSC laboratory. Plasma samples were stored at -80°C before sequencing at the laboratory of Virology at the Department of Experimental Medicine of "Tor Vergata" University (Rome, Italy).

The viral load was measured using the AMPLICOR HIV-1 MONITOR test (Roche Diagnostic Corporation, Indianapolis, IN).

RNA Extraction, RT-PCR, and Sequencing

RNA was extracted from 1 ml of each plasma sample using the QiaAmp Viral RNA (Qiagen GmbH, Hilden, Germany). RNA was collected in 50 μl of sterile nuclease-free water and stored at -80°C for further testing. cDNA was synthesized from 10 μl of extracted RNA by RT-PCR kit (Viroseq 2, Abbott). Twenty-nine samples were successfully amplified. The *pol* sequencing failed in six patients of the HAART group because of a low viral charge, not sufficient to obtain isolates. Genotyping analyses were carried out using Applied Biosystem ViroSeq HIV-1 Genotyping System. All procedures were performed according to the manufacturer protocol. Sequencing reactions were run in the capillary automated DNA sequencer (ABI model 3100 Applied). Sequences were examined by the software program for HIV analysis, and the obtained reports were submitted to the Stanford web site for Drug Resistance Algorithm (http://hivdb2.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb, Beta Test). The reference mutation list, reported in the Stanford HIV Drug Resistance Database [<http://hivdb.stanford.edu>], was used to evaluate resistance.

Phylogenetic Analysis

The sequences were compared with the reference sequences for HIV-1 subtypes (A, B, F, G, J, K) and circulating recombinant forms (CRFs), reported in the Los Alamos database (<http://hiv-web.lanl.gov>) and in

the Pubmed web sites (<http://www.ncbi.nlm.nih.gov>). The entire sequences were aligned by the CLUSTAL \times 1.80 program and subjected to manual adjustment by using the BioEdit software (BioEdit version 5.0.9). Phylogenetic analyses were performed with MEGA 2 program for the phylogenetic tree construction, according to neighbor-joining method. Using the TREEVIEW version 1.4 programs, additional phylogenetic trees were constructed by maximum parsimony methods.

RESULTS

Biological and clinical characteristics of the patients (12 men and 27 women) are described in Table I. Average ages for 33 non-HAART and HAART patients were 36.76 ± 8.58 (range 26–55) and 35.87 ± 7.55 (range 25–50) years, respectively. The CD4+ T cells were under 500 cells/ μ l in all patients. Seventeen non-HAART and five HAART patients had CD4+ T < 200 cells/ μ l, average 43.65 cells/ μ l (range 1–162) and 76.8 cells/ μ l (range 2–194), respectively. Three adults' non-HAART patients were at C stage (CDC) with clear signs of AIDS in the final stage. Seven non-HAART patients of the B stage (CDC) had at least one symptom of AIDS. Eleven HAART patients had a mean of 336.82 ± 100.14 cells/ μ l (Table I).

Phylogenetic Analysis

The amplification, sequencing and phylogenetic analyses of the 1.3 Kb region of HIV-1 *pol* gene (containing all known mutations associated with antiretroviral resistance) were completed successfully only for 29 plasma samples from 39 patients (see Table I).

Sequence analyses showed a high predominance of recombinant forms (28/29 isolates, 96.55%): CRF06_cpx was the most common circulating form (16/29 isolates, 55.17%), followed by CRF02_AG (11/29 isolates, 37.93%), and CRF09_cpx (1 isolate, 3.44%). Three (10.34%) patients only had clear subtype A1 (two isolates 6.89%), and G (one isolate 3.44%) (Fig. 1).

Among 17 non-HAART patients, 3 (17.65%) had viral isolates with minor RT mutations (V118I: 1 patient, V179E: 2 patients) and 10 (58.82%) had viral isolates with minor PR mutations (L63P: 5 patients, V77I: 3 patients, L10I: 2 patients) (Table II). The HIV viral load in these non-HAART patients was $80,200 \pm 72,600$ copies/ml. The number of CD4+/ mm^3 was 28 (1–162).

No correlation was found among the lymphocyte CD4+ number and HIV viral load. Regarding codon mutations associated with drug resistance, as expected, all patients had the common (non-B subtype) M36I and the K20I mutations.

Among the six HIV-1 infected children who received a single dose of nevirapine, 5/6 (83.3%) had the CRF06 recombinant form and 1/6 (16.66%) the A1 subtype. The isolated minor protease (PR) was: 6/6 (100%) M36I, 4/6 (66.66%) K20I, 2/6 (33.33%) L63P, and 1/6 (16.66%) L10V or A71AV. Only in two, 6 months old twins (33.3%) with NNRTI, the virus Y181YC mutation was isolated (Table III).

Interestingly, 6 months after birth, the twin's mother had different V106I NNRTI-associated mutation and an additional V82I mutation, characteristic of C and G HIV subtypes (Table IV).

Among six HAART patients 3/6 (50%) had the CRF02, 2/6 (33.3%) CRF06 and 1/6 (16.6%) CRF09 subtypes. The major protease V82I mutation was present in 1/6 (16.6%) patients. The minor protease mutations were 5/6 (83.33%) K20I, 6/6 (100%) M36I, 3/6 (50%) L63LP. The minor NNRTI mutations were 2/6 (33.33%) K103N and 1/6 (16.66%) V106I (Table IV). The HIV viral load was $133,200 \pm 121,400$ copies/ml. The number of CD4+ was $238/\text{mm}^3$ (range 2–493), significantly higher than that found in non-HAART patients.

DISCUSSION

This study is the first report of HIV antiretroviral resistance analysis in Burkina Faso and confirms a large variability of the circulating strains in this country. HIV-1 strains circulating in Burkina Faso, by phylogenetic and genotypic drug-susceptibility analysis of HIV-*pol* frame, show an evident predominance (16/29, 55.1%) of the HIV-1 CRFs recombinant form, in particular the CRF06.

Oelrichs et al. [1998] described the frequent CRF06_cpx recombinant form in Burkina Faso. The reference strain, AUBFP90 was a mosaic of A, G, J, and K subtypes. This CRF is becoming the predominant circulating form in Ouagadougou, Burkina Faso [Ouedraogo-Traoré et al., 2003] and was reported frequently in West Africa in association to CRF02_AG [Palella et al., 1998; Bellocchi et al., 2005].

In the definition of antiretroviral resistance, major and minor protease mutations are distinguished.

TABLE I. Characteristics of non-HAART and HAART Patients Including Six Children Who Received Nevirapine Prophylaxis

Characteristics	Non-HAART patients	Children HAART patients		Total
N	17	6	16	39
CDC stage and CD4+ cell count/ μ l				
C: CD4+ < 200 cell/ μ l	17	NA	5	22
B: 200 < CD4+ < 500 cell/ μ l	0	NA	11	11
A: CD4+ > 500 cell/ μ l	0	NA	0	0
RT and PR sequencing	17	6	6	29

NA: not available.

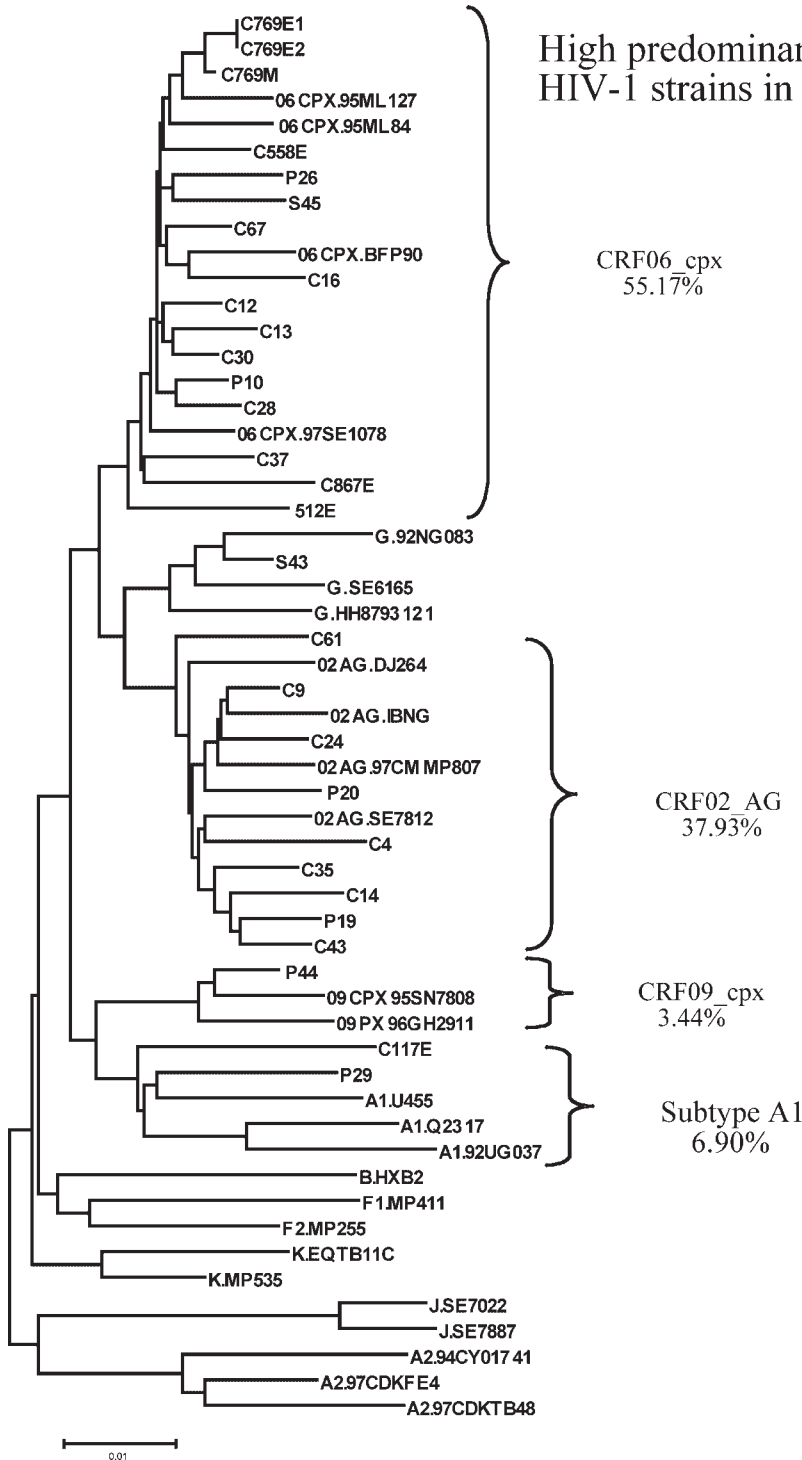


Fig. 1. Pol phylogenetic tree of 29 HIV-1 isolates from patients of Ouagadougou.

The first mutation is responsible of an alteration of the drug-viral target enzyme link. The second, which appears always after the major mutation, has no effect on the viral resistance but, often, increases the viral fitness that was influenced by the presence of a major mutation.

V118I, a mutation associated with NRTI resistance, was found only in one non-HAART isolate. This mutation was classified as NAMS (multi-nRTI resistance) and contributes to NRTI resistance. It was present frequently in patients who received Zidovudine and Lamivudine [Stoekli et al., 2002]. The study ACTG

TABLE II. Recombinant Form Subtypes, Minor Protease (PR) and Reverse Transcriptase (RT) Mutations in 17 Non-HAART Patients

Sex	Age years	CD4/mm ³	Recombinant form subtypes	Minor protease mutation			Reverse transcriptase mutation	
							NRRT ^a	NNRTI ^a
F	33	90	CRF02_AG	M36I	K20I			
F	30	162	CRF06_cpx	M36I	K20I	L63P		
F	29	72	CRF02_AG	M36I	K20I			
F	36	93	CRF06_cpx	M36I	K20I	L63P		
M	38	28	CRF02_AG	M36I	K20I			
M	54	58	CRF06_cpx	M36I	K20I			V179E
F	27	18	CRF02_AG	M36I	K20I			
F	29	53	CRF06_cpx	M36I	K20I	L63P		
F	30	37	CRF02_AG	M36I	K20I			
F	55	19	CRF06_cpx	M36I	K20I	L63P	V77I	L10I
M	42	1	CRF06_cpx	M36I	K20I		V77I	V118I
M	40	55	CRF06_cpx	M36I	K20I			V179E
F	43	28	CRF02_AG	M36I	K20I			
M	26	2	CRF02_AG	M36I	K20I			
F	35	10	CRF02_AG	M36I	K20I			
M	42	9	CRF06_cpx	M36I	K20I	L63P	V77IV	
F	36	7	CRF06_cpx	M36I	K20I		L10I	

^aSee abbreviations.

136 demonstrated that the V118I mutation was selected by the zidovudine/didanosine [Shafer et al., 1995]. Unfortunately, the significance of this mutation is unknown [Romano et al., 2002]. The V179E RT subtype was present in two non-HAART patients but, these subtypes were not associated with antiretroviral drug resistance [Montes et al., 2004; Nkengasong et al., 2004; Kantor, 2005]. Three non-HAART patients with CRF06_cpx showed at least one RT mutation while, the protease gene was relatively conserved. The V118I mutation in the non-B subtype was associated with a high resistance level to protease inhibitors. It is considered a common polymorphism of G subtype and represents a mutation due to treatment [Shafer et al., 2001; Holguin et al., 2002; Vergne et al., 2003a,b; Kantor, 2005].

An accessory V118I/V mutation, rarely present in non-HAART isolates, occurred in a patient with AZT resistance mutation [Delaugerre et al., 2001].

The observation that ten patients of the HAART group did not have a minor protease mutation, while all non-HAART patients had K20I and M36I mutations is interesting for the future of ART treatment in Burkina Faso. It could be a signal of the HAART efficacy on viral replication, as shown by the significant increase

of CD4 + 238 (4–493)/mm³ cells. Nevertheless, the possibility that the drug resistant mutations remains underestimated is always present. In fact, HAART patients had higher CD4+ levels. Three patients, with CD4+ less than 25/mm³, had minor protease K20I and M36I mutations whilst, only in one of these the reverse transcriptase mutation (NNRT) K103N was documented. The minor protease L63LP mutation is frequent in non-HAART patients [Kozal et al., 1996]. Its prevalence was increased in patients who failed to protease inhibitor drugs even if this mutation was not associated with significant increase of IC50 for protease inhibitors.

The major PR mutation (V82IV) was present only in one HAART patient with the reverse V106I transcriptase mutation. This last mutation was not associated with high resistance levels of nevirapine, delavirdine, and efavirenz. RT K103N and K103KN mutations, isolated in two of the HAART patients, reduce substantially the clinical efficacy of all NNRTIs in use. Two patients with the same treatment (AZT/3TC + NVP) and a mother who was treated with NVP the MTCT prevention showed K103N/KN and V106I mutations, respectively. The twins showed a RT Y181CY mutation. V106A or V106M mutations, in contrast to V106I, are known to be associated with resistance to NVP, DLV,

TABLE III. Recombinant Form Subtype, Minor Protease (PR) and Reverse Transcriptase (RT) Mutations in 6 HIV-1 Children Treated With NVP During Birth

Sex	Age months	Recombinant form subtype	Minor protease mutation ^a	Reverse transcriptase mutation NNRTI ^b
F	6 ms	CRF06_cpx	K20I, M36I	
F	7 ms	A1	L10V, M36I	
F twins	6 ms	CRF06_cpx	K20I, M36I, L63P	Y181CY
F twins	6 ms	CRF06_cpx	K20I, M36I, L63P, A71AV	Y181CY
M	18 ms	CRF06_cpx	M36I	
F	7 ms	CRF06_cpx	K20I, M36I	

^aThe M36I mutation was documented in all children.

^bSee abbreviations.

TABLE IV. Recombinant Form Subtype, Protease Minor Subtype and *Pol* Mutations in HAART Patients

Sex	Age years	CD4/mm ³	1° Therapy	2° Therapy	Recombinant form subtype	Protease major subtype	Protease minor subtype	Reverse transcriptase NNRTI ^a
F	33	296	AZT/3TC + NVP					
M	38	220	AZT/3TC + NVP		CRF02_AG		K20I, M36I, L63LP	K103N
F	28	427	AZT/3TC + NVP					
F	27	393	D4T + DDI + EFV	AZT/3TC + NVP				
F	34	194	DDI + D4T + EFV	AZT/3TC + IDV				
F	35	21	AZT/3TC + NVP		CRF02_AG		K20I, M36I	K103N
F	40	384	AZT/3TC + NVP					
M	49	2	AZT/3TC + EFV		CRF02_AG		K20I, M36I	
F	45	246	3TC + D4T + EFV					
M	50	18	D4T + DDI + EFV		CRF06_cpx		K20I, M36I, L63LP	
F	36	230	AZT/3TC + NVP					
F	28	493	AZT/3TC + IDV					
M	39	342	3TC + D4T + EFV					
M	37	220	AZT/3TC + NVP					
F	25	454	AZT/3TC + NVP		CRF09_cpx		L10I, M36I	
F	30	149	NVP		CRF06_cpx	V82IV	K20I, M36I, L63LP	V106I

^aSee abbreviations.

EFV, and NNRTI [Conway et al., 2001; Shafer et al., 2001; Brenner et al., 2003; Milinovic and Martinez, 2004]. These results demonstrate that the phenomenon of antiretroviral-drug resistance appeared also in Burkina Faso, in spite of the limited use of HAART due to the scarce availability and the high cost.

The antiretroviral drugs used for HIV infection determined a significant reduction in morbidity and mortality for HIV in Europe and in United States where the HIV-1 B subtype predominates [Palella et al., 1998]. The same effect was also observed in non-B HIV-1 infected patients.

ARV was available in Burkina Faso since 1996 through private pharmacies in Ouagadougou. In a first stage, it was used without an effective control. Now, the ARVs are coordinated by Ministère de la Santé of Burkina Faso, which monitors the use of ARVs by HIV infected persons [Nguyen et al., 2003].

The situation of Burkina Faso is easy to control. In fact, antiretroviral drug resistance tested in this study showed only one major protease mutation (V821V) in one HAART patient. The influence of this mutation in CRF06_cpx is not established and the consequence on the resistance to protease inhibitors cannot be presumed. Clearly, these results provide an overview of anti-HIV therapy and of the effect of treatment in Ouagadougou, Burkina Faso. The data confirm the prevalence of CRF06_cpx in Ouagadougou, Burkina Faso. Antiretroviral drug tests showed the presence of key mutations in non-HAART patient isolates. More investigations are necessary to determine the most frequent mutations present before ARV therapy. Since treated patients develop mutations during the ARV therapy, this phenomenon could lead to treatment failure.

The Mother-to-Child Transmission program allowed the CMSC, Ouagadougou, to reduce the HIV vertical transmission to the 10.3% [Simpore et al., 2006], which is well below the percentage (25–50%) reported in the

literature before NVP prophylaxis [Ahmadou et al., 2001]. However, the efficacy of NVP could be blanketed by the appearance of resistance to the drug that starts when the drug is used alone for the prevention of vertical transmission [Eshleman et al., 2001; Wainberg, 2004a,b]. From the above observations an advantage appears to favor the use of NVP.

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
ARV	antiretroviral
AZT	zidovudine
CDC	Centers for Disease Control and Prevention
CRF	circulating recombinant form
D4T	stavudine
DDI	didanosine
DLV	delavirdine
EFV	efavirenz
FACS	fluorescence activated cell sorter
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
IDV	indinavir
MTCT	mother-to-child-transmission
NAMs	multi-nRTI resistance
NNRTI	non-nucleoside reverse transcriptase inhibitors
NGO	non-governmental organization
NRTI	nucleoside reverse transcriptase inhibitors
NVP	nevirapine
PR	protease
RT	reverse transcriptase
3TC	lamivudine

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