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Full Length Research Paper

Antiretroviral drug resistance in HIV-1 infected patients receiving antiretroviral treatment in routine clinics in Cotonou, Benin

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A cross-sectional survey was conducted in 2008 to examine virological outcome and emergence of drug resistance in HIV-infected patients treated according to the national guidelines in an AIDS clinic in Cotonou, Benin. Median time on (antiretroviral therapy) ART of the 122 enrolled patients was 35 months. Viral load above 1,000 copies/ml was observed in 33 patients (27%) and 22.1% (27/122) harbored resistant strains: viruses from four, fifteen and eight patients were resistant to one, two and three drugs in their treatment regimen. The most frequent mutations were M184V and K103N in the reverse transcriptase gene. The proportion of patients with drug resistant mutations did not increase with time on ART. There seems to be a trend towards a lower prevalence of drug resistance mutations in patients who started with protease inhibitors-based regimens as compared to (Non-Nucleoside Reverse Transcriptase Inhibitor) NNRTI-based regimens. Further studies are necessary to evaluate if in the absence of laboratory monitoring, the use of PIs for the first-line ART should be reconsidered to minimize the risk of emergence of HIV drug resistance.

Key words: HIV, resistance, protease inhibitors, African countries, public health approach.

INTRODUCTION

The number of people receiving antiretroviral therapy (ART) in low- and middle- income countries has increased significantly reaching 5.2 million in 2009 (UNAIDS, 2010). Most of the countries use the public health approach recommended by World Health Organization (WHO), which was initiated in 2002 and updated in 2006 and 2010. This approach advises a standard first-line therapy - two Nucleoside Reverse Transcriptase Inhibitors, NRTIs, (3TC(/FTC)+AZT/TDF) and one Non-Nucleoside Reverse Transcriptase Inhibitor, NNRTI, (EFV/NVP) - together with treatment initiation and switch, guided by clinical disease progression and when possible CD4 cell counts monitoring (Gilks et al., 2006). If resources permit, it is now recommended to use viral load in a routine approach every 6 months or in a targeted approach to confirm clinical or immunological failure (WHO, 2010; Gilks et al., 2006). However, virological monitoring is still not feasible for the majority of patients on ART due to the absence of adequate laboratory facilities and the high cost of testing. HIV drug resistance genotyping is recommended at the population level for surveillance and monitoring, with the support of the WHO HIVResNet network of accredited laboratories

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Abbreviations: ART, Antiretroviral therapy; **ARV,** antiretroviral; **WHO,** World Health organization; **ANRS,** Agence Nationale de Recherche contre le SIDA; **NRTIs,** Nucleoside Reverse Transcriptase Inhibitors; **NNRTI,** Non-Nucleoside Reverse Transcriptase Inhibitor; **PI,** Protease Inhibitor; **TAMs,** Thymidine analogue mutations; **PR,** Protease; **RT,** Reverse transcriptase; **CRF,** Complex Recombinant Form; **URFs,** Unique Recombinant Forms.

(WHO, 2010). There is thus a need to assess virological outcomes in routine care settings in resource-limited countries in order to evaluate the effectiveness of antiretroviral programs, and to evaluate whether the second-line treatment recommended by WHO would still be effective.

Benin is a West African country with about eight million inhabitants. The seroprevalence is relatively low, estimated between 1 to 1.3% (UNAIDS, 2010). The number of patients on ART has increased rapidly since 2004, reaching now approximately 53% of the 29,000 people needing ART by the end of 2009, based on WHO 2010 guidelines (UNAIDS, 2010). ART in Benin is available since 2002 and is free of charge since 2004. The country adopted the 2006 WHO recommendation (WHO, 2006), But before 2006, for first line treatment, NNRTI as well as non-boosted protease inhibitor (indinavir) regimens were used.

Although PI use was not recommended in the 2002 WHO guidelines (Zannou et al., 2007), others countries like Senegal also used this approach (Vergne et al., 2003). Clinical follow-up was scheduled at two, four and eight weeks after ART initiation, and then every six months. Viral load quantification is recommended yearly. While viral load testing is technically available in Benin, routine utilization remained marginal due to an inappropriate financing mechanism under the national program and consequently proficiency decreased.

Our aim was to describe, depending on the treatment history, the virological outcome and the emergence of drug resistance in HIV-infected patients receiving ART in an AIDS clinic in Cotonou, the largest city in Benin.

METHODS

In a cross-sectional survey, plasma samples were collected from 122 adult HIV-1–infected patients (≥18 years) receiving ART in one AIDS clinic of Cotonou. All patients, receiving ART for at least 12 months, were consecutively enrolled between January and March 2008 during routine follow-up visits. After informed consent was obtained from patients, the demographic, epidemiologic, clinical and treatment history data were retrospectively retrieved from the medical records and anonymized for further analysis. Whole blood was collected in EDTA tubes. After centrifugation, plasma aliquots were stored at -80° C until viral load testing (Generic HIV VL assay, Biocentric) with a detection limit of 300 copies/ml (Rouet et al., 2007), and genotypic antiviral drug resistance testing (in-house nested PCR assay (Vergne et al., 2000) were performed. Techniques are detailed in Table 2.

RESULTS AND DISCUSSION

In this survey, the median age of the 122 adult HIV-1 infected patients was 38 years and ranged from 19 to 68 years (Table 1). The median time on ART was 35 months (IQR 22-47, range 12-91). At the beginning of treatment, median CD4 count was 86 cells/mm^3 (IQR 38-144). A large majority of patients (103/122, 84.4%) started their treatment with NNRTI+NRTI with, for 93/103 (76.2%), the combination AZT(43/93)/D4T(50/93)+3TC+NVP(17/93) /EFV(76/93); in 8.2% (10/103), ddI was used instead of AZT/D4T or 3TC, and the remaining 15.6% (19/122) started with a non-boosted protease inhibitor (PI) instead of an NNRTI (18 IDV and 1 NFV). Overall, 73/122 (59.8%) patients were still on their initial ART regimen, 30.3% changed regimen once, 7.4% twice and 2.5% three times. Median time for the first switch was 22.3 months (IQR 11.4-27.5). Main treatment changes were switch between AZT, D4T or ddI, and between NVP or EFV.

Major reasons of treatment switches were: toxicity, pregnancy, stock rupture and treatment standardization. At the time of the study, 84.3% of patients received AZT/D4T+3TC+NVP/EFV predominantly with AZT and EFV and 13.2% still received IDV in combination with NRTIs. The median CD4 count reached 338 cells/mm^3 (IQR 226-430).

For 36/122 (29.5%) patients, the plasma viral load was above 300 copies/ml (threshold of the technique), with a median of 5.02 $\log_{10}$ copies/ml (106,791 copies/ml) (Table 1). Genotypic antiviral drug resistance testing was performed on the 33 plasma samples with HIV-1 RNA levels equal or above 1,000 copies/ml (Table 2). For 3 samples, the 1,770 basepair *pol* fragment of the in-house PCR protocol could not be amplified and the HIV-1 PCR and Sequencing Procedures from the ANRS AC11 Resistance Study Group (www.hivfrenchresistance.org) were applied to amplify the protease (PR) and the reverse transcriptase (RT) regions separately. Finally, only for one sample, with VL=3.13 $\log_{10}$ copies/ml, PCR amplification was not successful.

The phylogenetic tree analysis (Vidal et al., 2006) of the 32 sequences (Table 2) showed that CRF02 was predominant (21/32, 66%) among the strains. Other HIV-1 variants found included subtype G (n=2), sub-subtype A3 (n=1), CRF06 (n=1) and 7 unique recombinant forms (URFs) represented by 1 CRF37/A, 2 CRF02/CRF06, 1 CRF02/U; 3 clustered with two complex recombinant strains (U/A/K) B76 and 99GR303 previously described in Benin (Baldrich-Rubio et al., 2001) and Greece (Paraskevis et al., 2001).

Major genotypic mutations associated with ART drug resistance were detected in 27 patients, that is 22.1% of the total patients and 84.4% of patients with viral load above 1,000 copies/ml. Detailed results are shown in Table 2. Among the 27 patients with resistant strains, 4, 15 and 8 were resistant to respectively one, two and three drugs of the actual regimen. 4/27 strains were resistant to drugs from the NNRTI-class only, 21 to NRTI+NNRTI, one to NRTI+PI and one to drugs from all three classes. Because the predominant first-line regimen included drugs, EFV/NVP and 3TC, with low genetic barrier to resistance, 85.2% of viral strains from patients with antiretroviral resistance mutations were resistant to at least two of the three drugs from their regimen, and more than 90% were resistant to NVP/EFV and 3TC/FTC. The proportion of patients who failed their treatment and

Table 1. General information on the study population, virological failure and drug resistance.

Study population	n	122	
Sex	Female	69	56.5%
	Male	53	43.5%
Age (years)	Median (IQR)	38	(31-45)
	Range	19-68	
First-line antiretroviral regime	3TC/DDI+AZT/D4T/DDI+NVP/EFV	103	84.4%
	3TC+AZT/D4T+NVP/EFV	93	76.2%
	with DDI	10	8.2%
	2NRTI (AZT/D4T/DDI/3TC) + 1PI (IDV or NFV (n=1))	19	15.6%
CD4 counts at treatment initiation	Median (IQR) cells/mm^3	86	(38-144)
CD4 counts at enrollment	Median (IQR) cells/mm^3	338	(226-430)
First line ARV regime and time on ART			
12-23 months on ART	3TC/DDI+AZT/D4T/DDI+NVP/EFV	32/32	100%
24-35 months on ART	3TC/DDI+AZT/D4T/DDI+NVP/EFV	41/41	100%
≥ 36 months on ART	3TC/DDI+AZT/D4T/DDI+NVP/EFV	30/49	61%
	2NRTI (AZT/D4T/DDI/3TC) + 1PI (IDV or NFV (n=1)	19/49	39%
Treatment switch	Total	49/122	40.2%
	1 swith	37/49	
	2 switches	9/49	
	3 switches	3/49	
Viral load > 300 c/ml	n	36/122	29.5%
	Median VL (IQR) (Log c/ml)	5.02	3.9-6.0
	Range	2.67-7.51	
Viral load > 300 c/ml and time on ART	12-23 months on ART	10/32	31.3%
	24-35 months on ART	11/41	27.5%
	≥ 36 months on ART	15/49	30%
Viral load ≥ 1,000 c/ml	n	33/122	27.0%
PCR amplification of samples with VL ≥ 1,000 c/ml	n	32/33	
Presence of at least one major drug resistance mutation in amplified samples	n	27/32	84.4%
Genotypic drug resistance to different drug classes	NNRTI only	4/27	14.8%
	NRTI + NNRTI	21/27	77.8%
	NRTI + PI	1/27	3.7%
	NRTI+NNRTI+PI	1/27	3.7%
Presence of at least one major drug resistance mutation in amplified samples and time on ART	12-23 months on ART	7/32	21.9%
	24- 35 months on ART	9/41	22%
	≥ 36 months on ART	11/49	22.5%

Table 1. Contd.

Presence of at least one major drug resistance mutation in patients on 36 months ART and initial ARV regime	2NRTI + 1 NNRTI	9/30	30%
	2NRTI + 1 PI	2/19	10.5%

with drug resistance strains are comparable to those reported in some African countries (Barth et al., 2010) including Togo, a neighboring country (30.8%) (Dagnra et al., 2011). Results were slightly higher than the rate of 16.9% ART resistance reported after 24 months on treatment in Cameroon (Kouanfack et al., 2009) but lower than the rate of 58.7% of Beninese patients with detectable viral load after 18 months of ART (Ogouyemi-Hounto et al., 2010).

All 23 viruses with NRTI resistance harbored the M184V/I mutation; 10 harbored also TAMs with T215Y/F in six patients, M41L in five patients, K70R in four patients, L210W and D67N in three patients and K219Q/E in two patients. L74V mutation was found in two cases. Viruses from eight patients accumulated sufficient TAMs resulting in resistance to AZT/D4T and two patients (7.4%) harbored viruses with mutations that confer also resistance to ABC, a drug they never received, recommended for second-line treatment in the 2006 but not in 2010 WHO guidelines. Possible resistances to TDF, a drug recommended for second-line ART by WHO in 2010, were found for four patients including two with intermediate resistance also to ABC related to presence of M41L, D67DN, M184V, T215Y and L210W mutations.

Among the 26 NNRTI resistant viruses, 16 (61.5%) harbored the K103N mutation. Three viruses were predicted to be resistant to ETV, a new NNRTI, and two to be possible resistant. Two resistant viruses to ETV had mutations Y181C and H221Y and the third had V90I, K101R, V179I and Y181C. A study in Malawi showed that, when diagnosis of treatment failure is based on clinical observations and CD4 cell counts only, extensive NRTI and NNRTI resistance emerges, with 23% of patients showing resistance profiles com-promising second-line ART (Hosseinipour et al., 2009). In the Malawi study, the predominantly acquired mutations were K70E and K65R, associated with TDF resistance in the absence of this drug in ART regimens. Interestingly, these mutations were not found in our study and are possibly related to the absence of subtype C in Benin, in contrast to Malawi where subtype C predominates.

The two PI resistant viruses were derived from patients who received Indinavir, one virus was resistant to NRTIs (3TC) and PI (IDV and NFV and possible resistance to SQV/r) and the other virus, who had a treatment history with all three classes, was resistant to NRTI (3TC/FTC, D4T, AZT), NNRTI (EFV, NVP) and PI (IDV, FPV/r and NFV and possible resistance to LPV/r and SQV/r). Two others viruses showed possible resistance to SQV/r, a drug never received by the patients, due to the accumulation of L10M/I, I15V and K20I.

In contrast with other studies (Johannessen et al., 2009; Kouanfack et al., 2009), virological failure and the proportion of patients with drug resistant mutations reported here does not increase with ART duration: 21.9, 22 and 22.5% of patients harbored resistant HIV strains after 12-23, 24-35 and ≥36 months of ART (Table 1). A total of 49 patients were for more than 36 months on ART: 30 patients starting on NRTI+NNRTI and, interestingly, all the 19 patients who started their treatment with a combination of two NRTIs and one PI. In this group of patients, 15 out of the 49 had a detectable viral load (≥300 copies/ml) and this proportion was higher for patients who started on NRTI+NNRTI regimens, 12/30(40%) versus 3/19 (15.8%) for patients on NRTI+PI, but the difference is not significant (Pearson's chi-square test: p=0.07).

The same trend is observed for drug resistance, with 9/30 (30%) versus 2/19 (10.5%) for those starting with NNRTI or PIs respectively (p=0.11). Although our data were obtained from a limited number of patients, they suggest that initial therapy with PI-based regimens resulted in lower rates of resistance in absence of biological monitoring, which is similar to observations by other teams (Gupta et al., 2008; Lima et al., 2008).

We used a cross-sectional approach and, only patients still on ART were tested. We have no information about the number of patients who are lost to follow-up and on their virological and HIV resistance status. We have also no information on how many patients died or decided to stop their treatment, therefore the drug resistance level we found is most likely a minimal estimate. Another limitation of our study is that we have no information on presence or not of resistance mutations at baseline. However, in general, surveillance of transmitted HIV drug resistance according to WHO guidelines in ART-naive populations from Burkina-Faso, Côte d'Ivoire and Senegal showed low prevalences (<5%) (Ayouba et al., 2009; Bertagnolio et al., 2011). However some exceptions can exist: in the study of Dagnra et al. (2009) in Togo, 8 of 53 (15%) patients who are in need for treatment and assumed to be ARV naive, already harbored viruses with drug-resistance mutations (Dagnra et al., 2009) or 5-15% rates were also seen in few countries (Bertagnolio et al., 2011).

Conclusion

The virological failure (VL≥1000 cp/ml) in patients routinely followed in HIV/AIDS clinic in Cotonou, Benin,

Table 2. Treatment history and drug resistance mutations in 33 patients on ART with virological failure (VL≥1,000 c/ml)

Months on ART	Initial ART regimen	Regimen if ART switches	Viral load[1] (log c/ml)	Subtype[2]	Drug resistance[3]	NRTI genotypic mutations	NNRTI genotypic mutations	IP genotypic mutations
13	D4T, 3TC, EFV	D4T, 3TC, NVP	4.90	URF (U/A/K)	3TC/FTC, ABC, NVP, EFV, (ETV)	L74LV, M184V	K101EK, Y181C, G190A	
14	AZT, 3TC, EFV		3.62	CRF06	EFV, NVP		K101E	
14	AZT, 3TC, NVP	AZT, 3TC, EFV	5.35	CRF02	3TC/FTC, D4T, AZT, (ABC, TDF), EFV, NVP	M41L, D67DN, K70KR, M184V, T215Y	K103N	
16	AZT, 3TC, EFV		3.13	Negative PCR				
18	D4T, 3TC, EFV		3.04	URF (CRF02/06)	3TC/FTC, EFV, NVP, (SQV/r)	M184V	K103N	L10M, I15V, K20I
19	D4T, 3TC, NVP	AZT, 3TC, NVP	5.42	CRF02	3TC/FTC, EFV, NVP	M184V	K103NS, V106A	
19	AZT, 3TC, NVP		6.27	CRF02	3TC/FTC, D4T, AZT, EFV, NVP, ETV	M184V, T215FIST	Y181CY, H221HY, M230LM	
22	AZT, 3TC, EFV		6.46	CRF02	EFV, NVP		K103N, P225H	
27	D4T, DDI, EFV		5.69	A3	3TC/FTC, EFV, NVP, (ETV)	M184MV	K101EK, V179I, Y181C	
28	D4T, 3TC, NVP	D4T, 3TC, EFV	4.96	CRF02	3TC/FTC, EFV, NVP, ETV	M184V	K103N, Y181C, H221Y, P225HP	
29	AZT, 3TC, EFV		4.75	CRF02	3TC/FTC, EFV, NVP	M184V	K103N, M230L	
29	D4T, 3TC, EFV	D4T, 3TC, NVP	5.11	CRF02	3TC/FTC, D4T, AZT, EFV, NVP	M184V, T215F	K103N, M230L	
29	AZT, 3TC, EFV	AZT, 3TC, NVP	3.02	CRF02	-			
32	D4T, 3TC, EFV	D4T, 3TC, NVP	6.24	CRF02	3TC/FTC, EFV, NVP	M184V	Y188L	
32	AZT, 3TC, EFV		5.69	CRF02	3TC/FTC, D4T, AZT, EFV, NVP	M184V, T215Y	K103N	
35	AZT, 3TC, EFV		5.88	CRF02	- (SQV/r)			L10IL, I15IV, K20I
35	AZT, 3TC, EFV		4.72	G	3TC/FTC, EFV, NVP	M184V	K101EK, K103N	
35	AZT, 3TC, NVP	D4T, 3TC, NVP	4.26	URF (U/A/K)	3TC/FTC, NVP, EFV, ETV	M184V, L210LW	V90I, K101R, V179I, Y181C	
35	D4T, 3TC, EFV	D4T, 3TC, NVP	3.96	URF (CRF02/U)	3TC/FTC, NVP, EFV	M184V	K101E, G190A	
40	AZT, 3TC, EFV		6.42	URF (U/A/K)	3TC/FTC, NVP, EFV	D67DN, K70R, M184V	K103N	
41	AZT, 3TC, EFV		6.00	CRF02	3TC/FTC, EFV, NVP	M184V	K103N, P225H	
42	AZT, 3TC, EFV		6.03	CRF02	EFV, NVP		K103N	
42	AZT, 3TC, EFV		6.49	CRF02	-			

Table 2. Contd.

46	D4T, 3TC, EFV	AZT, 3TC, NVP			3.94	URF (CRF02/06)	3TC/FTC, NVP, EFV	M184V	G190A	
47	D4T, 3TC, IDV				3.70	CRF02	3TC/FTC, IDV, NFV, (SQV/r)	M184V		K20I, M46I, G73S, I84V
47	D4T, 3TC, EFV				6.13	CRF02	3TC/FTC, ABC, D4T, AZT, (TDF), EFV, NVP	M41L, D67N, M184I, L210W, T215Y	V106M	
47	AZT, DDI, IDV				6.95	CRF02	-			
51	DDI, 3TC, EFV	AZT, 3TC, EFV	DDI, 3TC, NFV	D4T, 3TC, IDV	5.15	G	3TC/FTC, D4T, AZT, (TDF), EFV, NVP, IDV, NFV, FPV/r, (LPV/r, SQV/r)	M41L, T69N, K70R, L74V, M184V, K219Q	L100I, K103N	L10I, K20I, M36I, I54V, A71V, L90M
53	AZT, 3TC, NVP				4.69	CRF02	3TC/FTC, EFV, NVP	M184V	K103N, Y181C	
53	D4T, 3TC, EFV	AZT, 3TC, NVP			6.36	CRF02	EFV, NVP		K103N	
61	D4T, DDI, IDV	DDI, 3TC, IDV	AZT, 3TC, NVP		4.91	URF (CRF37/A)	3TC/FTC, D4T, AZT, (ABC, TDF), NVP, EFV	M41L, M184V, L210W, T215Y	G190A	
62	DDI, 3TC, EFV	AZT, 3TC, EFV			5.09	CRF02	3TC/FTC, D4T, AZT, EFV, NVP	M41L, K70R, M184V, K219E	K103N, P225H	
66	D4T, 3TC, EFV	AZT, 3TC	DDI, ABC, EFV		3.73	CRF02	-			

AZT, Zidovudine; **3TC**, lamivudine; **FTC**, emtricitabine; **ddI** , didanosine; **d4T**, stavudine; **ABC**, abacavir; **TDF**, tenofovir; **EFV,** efavirenz; **NVP,** nevirapine; **ETV**, etravirine; **IDV**, Indinavir; **SQV**, Saquinavir; /**r** , boosted with ritonavir.

Drugs and resistance mutations between brackets indicate possible resistance.

1: HIV-1 RNA levels in plasma were measured using the RT-PCR test «Generic HIV VL assay» (Biocentric, Bandol, France) with a detection limit of 300 copies/ml (Rouet et al., 2007) using ABI Prism 7000 Sequence Detection System (Applied Biosystems, France).

2: Genotypic antiviral drug resistance test: amplification using in-house nested PCR assay previously described (Vergne et al., 2000) given 1,770 pol fragment. After purification, the amplified fragments were directly sequenced using a BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems,France) on an Applied Biosystems 3130 XL genetic analyzer. Genetic subtypes/CRFs were determined by phylogenetic tree analysis as previously (Vidal et al., 2006).

3: Amino acid sequences were analyzed for the presence of mutations in PR and RT genes with the drug resistance interpretation algorithm from ANRS (version July 2010).

was 27% after a median ART period of 35 months, and 22% patients harbored drug resistant strains. Globally, 19% were resistant to two or three drugs of their actual regimen. More than 90% of patients, with HIV resistant strains, were resistant to NVP/EFV and 3TC/FTC. Our results show that the strategy to monitor patients in these clinics needs to be improved.

In Benin at Cotonou, equipment and infrastructure for viral load testing is available but is not used routinely. In this context, the recommendation will be to analyse the reasons associated with the absence of viral load testing, because the 2010 WHO guidelines recommends now viral load testing when possible, to control adherence and

for early detection of virological failure in order to avoid the accumulation of resistance mutations, which could made the second line treatment less active. In five cases, no drug resistance was observed, therefore a second viral load testing after the reinforcement of adherence, could allow to identify whether this was related to adherence and avoid early switch to second line. An important finding of our study is that in most cases of drug resistance the WHO recommended second line of treatment would be active.

The trend that initial treatment with PI-based regimens seems to yield less resistance over time requires further analysis, within the context of universal access to ARV in resource-limited countries (Adlington et al., 2009; Harrigan et al., 2009). Moreover, the recent availability of cheap and simple generic protease inhibitors via the Clinton Foundation (http://www.clintonfoundation.org/) permits to reconsider the use of PIs for the first-line ART in resource-limited countries and need to be further evaluated.

These results are useful for clinicians managing patients and provide some indications on ARV program's effectiveness in patients still on treatment. Surveillance of transmitted drug resistance and monitoring of ART resistance at sentinel sites should be implemented, in order to inform health authorities and policy makers on the efficiency of first- and second-line ART and convey recommendations on future ART strategies (Bennett et al., 2008).

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