

# Prevalence of antiretroviral drug resistance in untreated persons newly diagnosed with HIV-1 infection

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## SUMMARY

Current knowledge of HIV-primary resistance indicates that the prevalence of transmitted resistant strains has increased to substantial levels over the past few years, with a wide variation depending upon a number of factors. New infections with a virus strain already resistant to antiretroviral drugs, namely non-nucleoside reverse transcriptase inhibitor (NNRTI), have a negative impact on initial treatment response and also shorten the time to first virologic failure. The aim of this study was to determine the prevalence of antiretroviral drug resistance by a genotypic test in a population with newly diagnosed HIV-1 infection at a clinical centre in Bologna between June 2006 and September 2007.

**KEY WORDS:** HIV-primary resistance, HAART, Genotypic test

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## INTRODUCTION

The widespread use of highly active antiretroviral therapy (HAART) in developed countries has led to a dramatic improvement in rates of morbidity and mortality for individuals with HIV-1 infection. Unfortunately, the frequent drug resistance developing during HAART has proved a major obstacle to the successful long-term management of the disease. Since 1993 primary resistance to HAART has been reported in untreated patients (Erice *et al.*, 1993; Angarano *et al.*, 1994) and transmission of drug-resistant viruses has been shown to occur following infection by different routes, including heterosexual and homosexual intercourse, intravenous drug use and

vertically from mother to child (Bode *et al.*, 1999; Veenstra *et al.*, 1995).

Current knowledge of HIV-primary resistance indicates that the prevalence of transmitted resistant strains has increased to substantial levels over the past few years, with a wide variation depending upon a number of factors. New infections with a virus strain already resistant to antiretroviral drugs, namely non-nucleoside reverse transcriptase inhibitor (NNRTI), have a negative impact on initial treatment response and also shorten the time to first virologic failure (Violin *et al.*, 2004; Garcia-Lerma *et al.*, 2004; Little *et al.*, 2002; Borroto-Esoda *et al.*, 2007; Pozniak *et al.*, 2006; Kuritzkes *et al.*, 2008). In addition, the emergence of resistance to non-nucleoside reverse transcriptase inhibitors has recently been associated with a greater risk of negative outcome (Hogg *et al.*, 2006). Although it is not well known how long mutations persist in naïve patients' plasma, several reports indicate that transmitted drug resistant strains could persist for years as domi-

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nant quasiespecies (Fox *et al.*, 2006; Pao *et al.*, 2004; Delaugerre *et al.*, 2004; Barbour *et al.*, 2004). On the basis of these literature reports the current guidelines (Department of Health and Human Service guidelines for antiretroviral therapy) recommend resistance testing in chronically infected persons before starting therapy.

The aim of this study was to determine the prevalence of antiretroviral drug resistance by a genotypic test in a population with newly diagnosed HIV-1 infection at a clinical centre in Bologna between June 2006 and September 2007.

## MATERIAL AND METHODS

### *Population*

Eighty-one drug-naïve HIV-1-infected persons with recent diagnosis of seropositivity attending the Infectious Diseases Unit of S. Orsola Hospital, Bologna were recruited from June 2006 to September 2007.

Eligibility criteria included age >18 years and antiretroviral-drug-naïve status according to personal interview.

Seroconverters were defined according to the presence of either of the following criteria:

1. a negative or indeterminate HIV antibody enzyme-linked immunosorbent assay result associated with a positive plasma HIV RNA result, or
2. an initially negative test for HIV antibody followed by a positive serology result within 12 months. Primary HIV infection was confirmed by avidity test. Each specimen was analysed in duplicate, diluted 1:10 respectively in phosphate-buffered saline (PBS, reference dilution) and 1 M Guanidine (G, test dilution). The avidity index (AI) was calculated with the equation of the S/CO ratio of the test dilution (mean of replicate) over the S/CO ratio with the reference dilution (mean of replicate).

### *Peripheral blood CD4 lymphocytes*

Peripheral blood CD4 lymphocytes were counted by flow cytometry (FACScan, Becton & Dickinson, Mountain View, CA) using commercially available monoclonal antibody (Becton-Dickinson) and the count of T CD4+ cells was more than 200 cells/mm<sup>3</sup> in all patients.

### *HIV-RNA viremia*

Blood collected in EDTA was separated from the cell fraction by centrifugation at 2500 rpm for 20 min and then stored at 80°C until use.

All the plasma samples were analyzed for HIV-1 RNA level using the Quantiplex HIV-RNA-3.0 assay (Siemens, Tarrytown, NY, USA) according to the manufacturer's instructions. HIV RNA levels were expressed as copy number per ml of plasma and the lowest detection limit of the assay was 50 copies/ml.

### *Genotypic analysis of plasma*

Plasma viral RNA was extracted using activated silica in a column format (QIAamp viral RNA kit; QIAGEN, Hilden, Germany), with processing performed according to the instructions provided by the manufacturer, and resuspended in 60 µl of RNA diluent. An aliquot of 17 µl of extracted RNA eluate was mixed with RT-PCR reagent using the Trugene HIV-1 Genotyping kit (Siemens). This step amplifies the entire protease region and the first 250 codons of reverse transcriptase as a single amplicon.

The cDNA product was used for the sequencing reactions (CLIP) employing three pairs of labeled primers that sequence the protease reading frame (one pairs) and the beginning and the middle of the reverse transcriptase reading frame (one pair each). Three CLIP sequencing of the codons 4-99 of protease and codons 35-247 of RT was performed in both 5' - and 3' - directions using the Cy5 - labeled sense primers and Cy5.5-labeled antisense primers.

The forward and reverse sequences were aligned and compared to the consensus sequence of a lymphadenopathy-associated virus type 1 (HIV-1<sub>LAV-1</sub>). The amino acid changes associated with resistance to NRTIs, NNRTIs and PIs were identified using the FDA approved OpenGene DNA Sequencing System resistance interpretation algorithm, (GuideLines Rules 12.0, Siemens, Tarrytown, NY, USA).

### *HIV-1 subtyping*

HIV-1 subtypes were determined from HIV-1 *pol* gene sequences obtained with the Trugene HIV-1 genotyping system. Subtype assignment was done by phylogenetic analysis using reference strains of known subtype derived from the Los Alamos database ([www.hiv.lanl.gov](http://www.hiv.lanl.gov)). The neigh-

bor-joining method was used to compare the sequences to pairwise distance matrices generated using the Kimura 2-parameter distance estimation method with a transition/transversion ratio of 2.0.

The consistency of the phylogenetic clustering was tested using bootstrap analysis with 100 replicates. Bootstrap values above 70 were considered sufficient for subtype assignment. CRFs and their breakpoint locations were accurately identified with the bootscan analysis using SimPlot 3.5.1 software.

## RESULTS

### Patients

Between June 2006 and September 2007, 81 patients were newly diagnosed with HIV-1 infection at the S. Orsola-Malpighi Hospital of Bologna, but only 56 (69%) met the inclusion criteria. Their baseline demographic and clinical characteristics are summarized in Table 1: 94.6% of the patients were Caucasian, 91% Italian, and included 47 men and 9 women, aged 20-70 years.

Intensive medical evaluation excluded a history of drug abuse and transmission was established to be by sexual contact in all subjects.

In particular, 41 patients (73.2%) reported unprotected homosexual relations as a risk factor

and 16 patients (28.6%) unprotected heterosexual relations.

### CD4 and viral load

CD4 cell count showed variable levels ranging from 215 to 1796 (mean baseline value of 505 cells/mm<sup>3</sup>). All patients enrolled in the study showed a moderate to high level of viral replication, ranging from 1.1x10<sup>2</sup> to >5x10<sup>5</sup> HIV-RNA copies/ml (mean viral load 1.05x10<sup>5</sup> HIV-RNA copies/ml).

### Phylogenetic analysis and prevalence of mutations correlated to drug resistance

HIV-1 RT and PR sequences derived from plasma of patients analyzed showed that most (97%) viral strains belonged to subtype B.

Among non-B, subtype A1, CRF01\_AE and CRF02\_AG were detected in three patients (5.4%). The overall weighted prevalence of patients with primary mutations was 10.7%, (6/56) (Table 2). The highest prevalence of resistance mutations was observed for nucleoside reverse transcriptase inhibitors (8.9%, 5/56) associated predominantly with thymidine analogue mutations (TAMs) including T215 revertants; in particular the T215D mutation was found in four patients (7.1%, 4/56). The weighted prevalence of patients with mutations associated with resistance to non-nucleoside reverse transcriptase inhibitors (NNRTI) was

TABLE 1 - Characteristics of persons newly diagnosed with HIV-1 infection in Bologna, June 2006-September 2007.

Patient characteristic		Total number	Subtype B	Subtype non B
Age	Median(range)	34 (20-70)	34 (20-70)	31 (23-40)
Sex	Male	47 (83.9%)	45 (95.7%)	2 (4.3%)
	Female	9 (16.1%)	8 (88.9%)	1 (11.1%)
Risk group	Homosexual	41 (73.2%)	40 (97.6%)	1 (2.4%)
	Heterosexual	15 (26.8%)	13 (86.7%)	2 (13.3%)
	IVDU	0	0	0
Ethnicity	Caucasian	53 (94.6%)	51 (96.2%)	2 (3.8%)
	Black-African	2 (3.6%)	1 (50%)	1 (50%)
	Hispanic	1 (1.8%)	1 (100%)	0
CD4 count (cells/mm <sup>3</sup> )	Median (range)	505 (215-1796)	507 (215-1796)	409 (229-693)
Country of birth	Italy	51 (91%)	50 (98%)	1 (2%)
	other	5 (9%)	3 (60%)	2 (40%)

TABLE 2 - Characteristics of newly diagnosed with HIV-1 infection persons presenting antiretroviral drug resistance mutations.

Pt	Age	Risk group	Ethnicity	Avidity Index	CD4	VL	Subtype	RT Res.	PI Res.	Low Level of Res.	Inter/high Level of Res.
M1	30	MSM	C	0.40	1122	1800	B	D67N, K70R, K103N, M184V K219Q		ABC D4T	AZT 3TC FTC EFV NVP DLV
M2	34	MSM	C	0.50	730	120000	B	T215D		AZT D4T	
M3	56	MSM	C	0.55	690	86000	B	A62V L210W T215D		ABC DDI TDF	AZT D4T
M4	31	MSM	C	0.45	394	200000	B	T215D		AZT D4T	
F5	41	HTS	C	0.75	345	28000	B	T215D		AZT D4T	
M6	43	MSM	C	0.30	606	110000	B		M46L	ATZ NFV	

1.78% (1/56). The global prevalence of patients with protease inhibitor associated mutations was 19.6% (11/56). Major mutations associated with PI resistance were found in 1.8% (1/56) and minor mutations in 17.9% (10/56) of patients, respectively.

Based on the Stanford interpretation algorithm, among the patients with resistance mutations, 6/6 (100%) had low level NRTI resistance mutations and 1/6 (16.7%) had low-level PI resistance. Intermediate/high level resistance occurred in 2/6 (33.3%) patients for the NRTI and 1/6 (16.7%) for the NNRTI. Lastly, 1/6 (16.7%) patients showed dual class NRTI and NNRTI resistance.

#### *Risk factors associated with drug resistance mutations*

All the patients with resistance mutations were Caucasian, Italian and subtype B. There was a trend for higher prevalence of resistance in males (5/6, 83.3%) than females (1/6, 16.7%) and in homosexuals (5/6, 83.3%) compared with heterosexuals (1/6, 16.7%). There was no association between detection of resistance and age, CD4 and HIV-1 viral load. There were no independent predictors of detection of resistance mutations in the multivariable analysis.

## DISCUSSION

This study determined that the prevalence of antiretroviral drug resistance in an unselected population of HIV-1-infected individuals diagnosed between June 2006 and September 2007 was 10.7%. This prevalence is very close to the 10.4% rate reported in a multicentre European study of 2208 drug-naïve patients tested in 1996-2002, which included data from 19 countries (Wensing AMJ *et al.* 2005). The new data on the same cohort (SPREAD programme) from 1245 HIV-1-infected individuals in 17 countries diagnosed in 2002-2003 reported a prevalence of 9.1% (SPREAD Programme 2008). The prevalence of antiretroviral drug resistance was higher (16.1%) in a previous study of selected populations of drug-naïve HIV-1 infected patients in Italy (the ICoNA Cohort over the period 1996-2001) than in our population (Violin M *et al.* 2004).

As shown in table 3, the prevalence of resistance to NRTI (8.9%) in our cohort was lower than that observed in the ICoNA study (15.2%) and SPREAD programme which had already detected a decrease of this prevalence from 13% (1996-2001) to 5.4% (2002-2003). The predominance of single thymidine-associated mutations (TAMs)

TABLE 3 - Prevalence of resistance in our cohort compared to the Italian and European data.

City/Country	Bologna	ICONA (Italy)	CATCH (Europe) 1996-2001	SPREAD (Europe) 2002-2003
Total resistance	10.7%	16.1%	10.4%	9.1%
Resistance to NRTI	8.9%	15.2	13%	5.4%
Resistance to NNRTI	1.78%	0.9%	9.8%	3%
Resistance to PI	1,8%	2.7%	3-4%	2.6%

and 215 revertants can be explained by a combination of factors. TAMs are selected by the thymidine analogues (zidovudine and stavudine). For this reason, transmission of viruses with solitary TAMs most likely reflects a predominant circulation of these viruses at points in time (e.g. late 1980s and early 1990s) when there was extensive use of non-suppressive mono and dual therapies with thymidine analogues. Introduction of highly active antiretroviral therapy in the mid-1990s caused a more equal distribution of resistance among the three classes in patients with treatment failure. As a result, more recent transmission patterns show a relative decrease in the proportion of NRTI resistance.

We found a higher prevalence of resistance to NNRTI with respect to the ICoNA study, but lower than the European data. It is difficult to account for this finding because only one patient in our cohort had an HIV strain with the K103N mutation. However, as a solitary mutation may dramatically affect the susceptibility of NNRTIs, we support the recommendations for baseline resistance testing for newly diagnosed individuals and, if necessary, subsequent customizing of initial therapy.

The prevalence of resistance to PI was lower than that reported in the ICoNA cohort and SPREAD programme and this is a good result also because the effect of single PI mutations on first-line therapy is expected to be limited in the era of boosted PIs because multiple mutations have to be generated before therapy fails.

With respect to the risk factors for antiretroviral drug resistance, the multivariable analysis did not include an independent predictor of detection of resistance mutations.

The prevalence of newly diagnosed patients infected with non-B subtypes and circulating re-

combinant forms is very high in some European countries (43.9% in U.K.) and is traditionally associated with immigration (Booth CL *et al.* 2007). The prevalence of patients infected with non-B subtype in our cohort was only 5.4% (one case subtype A1, one case CRF01\_AE and one CRF02\_AG) and may be due to the poorer access to health facilities and antiretroviral therapy by immigrants. The prevalence of homosexual males in our cohort was 73.2%, a higher prevalence than that noted in the SPREAD programme (44%). Several literature reports have demonstrated prevalences of transmitted antiretroviral drug resistance higher than 20% in major US cities with large populations of homosexual men and a long period of access to antiretroviral treatment (Grant *et al.*, 2003; Wensing *et al.*, 2003; Truong *et al.*, 2006). For this reason, as Bologna is an Italian city with a large entrenched homosexual population, we expected a higher prevalence of transmission of resistant HIV strains.

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