HIV-1 genotypic resistance profile of patients failing antiretroviral therapy in Paraná, Brazil

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ABSTRACT

Antiretroviral therapy (ART) has reduced morbidity and mortality related to human immunodeficiency virus (HIV) infection, but in spite of this advance, HIV mutations decrease antiretroviral susceptibility, thus contributing to treatment failure in patients. Genotyping HIV-1 allows the selection of new drugs after initial drug failure. This study evaluated the genotypic profile of HIV-1 isolates from treated (drug-experienced) patients in Paraná, Brazil. The prevalence of mutations in reverse transcriptase (RT) and protease (PR) genes were assessed. We analyzed 467 genotypes of patients with HIV-1 viral loads above 1,000 copies/mL. Mutations at HIV-1 RT and PR genes and previously used ART regimens were recorded. The most prevalent RT mutations were: 184V (68.31%), 215YF (51.6%), 103NS (46%), 41L (39.4%), 67N (38.54%), 210W (23.5%), 190ASE (23.2%), and 181C (17.4%). PR mutations were 90M (33.33%), 82ATFS (29%), 46I (26.8%) and 54V (22.2%). The prevalence of mutations was in line with previous national and international reports, except to non-nucleoside analogue reverse transcriptase inhibitors related mutations, which were more prevalent in this study. Previous exposure to antiretroviral drugs was associated with genotypic resistance to specific drugs, leading to treatment failure in HIV patients.

Keywords: HIV-1, genotype, antiretrovirals, drug-experienced patients.

[Braz J Infect Dis 2010;14(4):360-371]

INTRODUCTION

Since the last decade, antiretroviral therapy (ART) has been altering the course of human immunodeficiency virus (HIV) infection in Brazil, with dramatic decrease in mortality and morbidity. HIV emerged as a potentially treatable chronic infection.[1,2] In Brazil, in 2008, the estimated number of patients on combination of antiretroviral (ARV) classes, the so-called highly active antiretroviral therapy (HAART), was 185,000.[2]

Some factors, like poor compliance (related to toxicity or complexity of regimens), prescription of suboptimal treatments and the "genetic barrier" of some regimen promote a non-suppressive ART, which strongly contributes to the selection of resistant HIV-1 mutants.[3] High and continuous replication rate of HIV-1 *in vivo*, in addition to the error-prone nature of the viral enzymes, in the setting of inadequate levels of ARVs, lead to marked genetic variation with viral mutations that confer drug resistance.[4,5]

Development of drug resistance is one of the most serious obstacles to sustained suppression of HIV and, eventually, multidrug resistance can exhaust the patient's therapeutic options. This population with rising levels of plasma HIV RNA represents potential transmitters of HIV, including drug-resistant strains, to susceptible people.[3,6-8]

Since the first report of zidovudine resistance in clinical isolates from treated patients in 1989,[9,10] data on prevalence of resistance have been published in different geographic areas. Genotyping HIV-1 allows for mutations detection in HIV genome, particularly at the protease (PR) and reverse transcriptase (RT) genes. Based on a large knowledge accumulated and published by many authors about PR and RT mutations in the last 10 years, genotype report helps to target selection of new drugs after initial drug failure.[11-13]

Internationally, reports estimating the prevalence of antiretroviral resistance have shown epidemiological features of the global burden of resistance through the years.[14-19]

Submitted on: 02/25/2010
Approved on: 06/10/2010

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We declare no conflict of interest.

National epidemiological studies have also been reviewed. In 2002, Tanuri *et al*. published data from a Brazilian database.[20] From 2004 to 2007, many other authors showed data from the prevalence of antiretroviral resistance in adults from Bahia, São Paulo, Rio de Janeiro, Federal District and North Eastern states.[21-27]

The aim of this study is to analyze the genotypic profile of patients tested for resistance in the Brazilian state of Paraná, in order to determine the frequency of RT and PR mutations among patients failing ART.

MATERIAL AND METHODS

This study was approved by the Ethics Committee of Hospital de Clínicas of Federal University of Paraná (UFPR) and by the Laboratory Division of Brazilian AIDS Programme (PN-DST/AIDS) of Brazilian Ministry of Health (MH).

Study population and antiretroviral therapy

Adults on ARV treatment failure in Paraná, Brazil, with genotyping tests from 2002 to 2006 were selected. Genotypes were obtained as part of routine clinical care and indicated when a patient on ART presented virologic failure. The sources were genotypes from private patients and local genotype results from the National Genotyping Network (RENAGENO). An informed consent was signed by all patients before sample collection.

The observational analysis included patients' ART history, viral load (VL) in every ARV regimen failure, CD4 counts (nadir and last available count before genotyping), and PR and RT HIV-1 mutations. Therapeutic failure was defined as VL higher than 1,000 copies/mL after continuous use ART for more than three months. Patients whose tests were performed by RENAGENO had VL higher than 5,000 copies/mL.

Patients should have been exposed to at least two ARV, including: nucleoside analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside analogue reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). Patients were excluded when information about ART was missing or incomplete.

HAART was defined as a combination of at least three antiretroviral agents, including two NRTIs and a NNRTI or a PI. Some patients used boosted PIs. NRTI mono or double therapy were considered non-HAART regimen.

Genotypes description

Genotypings were done by polymerase chain reaction (PCR) amplification and DNA sequencing technique. The HIV-1 genomic region analyzed was POL, specifically the PR and RT codons. Genotypic sequencing was performed using commercially available assays, with over 70% of samples being tested with ViroSeq HIV-1 Genotype System®.

Reports from RENAGENO where performed by ViroSeq HIV-1 Genotype System® from Celera Diagnostics (Applied Biosystems). Reports of private patients were performed by one of the following assays: GenoSure® (by LabCorp), virco-TYPE HIV-1® (by VIRCO) and GeneSeq® (by Monogram, formerly ViroLogic Inc.).

The epidemiology of mutations found was described and no Genotype Algorithm was used to interpret results in this study.

Mutations definition

Mutations associated with reduced antiretroviral drug susceptibility are described below and were based on the compilation of data reported by the Stanford University HIV Drug Resistance Database, updated in 2005 and 2008,[11,28] and the International AIDS Society – USA Panel Guidelines reports from 2000 to 2008.[12,29-41]

Mutations associated with reduced susceptibility to NRTIs are at RT gene positions 41, 43, 44, 62, 65, 67, 69, 70, 74, 75, 77, 115, 116, 118, 151, 184, 208, 210, 215, 218, 219, 221, and 228. Thymidine associated mutations (TAMs) are M41L, D67N, K70R, L210W, T215YF, K219QE. Accessory mutations include K43EQ, E44AD, V118I, H208Y, D218E, H221Y, and L228HR. The non-thymidine associated mutations are K65R, K70EG, L74V, Y115F, M184VI. Additional mutations are related to treatment with NRTI with no clear phenotypic significance, and include D67GE, T69DSAN, V75AS, K219NR. Multi-NRTI resistance mutations that confer resistance to all NRTIs are 69 insertion, A62V, V75I, F77L, F116Y, Q151M.

NNRTIs-associated mutations included amino acid substitutions at RT gene positions A98G, L100I, K101E/P, K103N/S, V106A/M/I, V108I, V179D/E/F/T, Y181C/I/V, Y188C/H/L, G190A/S, P225H, F227L/C, M230L, and K238T.

Major PI mutations included amino acid substitutions at PR gene positions D30N, V32I, L33F, M46I/L, I47V/A, G48V/M, I50L/V, I54M/L, Q58E, T74P, V82A/F/L/T/S, I84V/A/C, N88D/S, and L90M. Minor PI mutations included the substitutions in positions L10F/I/R/V, V11I, I13V, G16E, K20M/R/I/T, L23I, L24I, E34Q, E35G, M36I/V, K43T, F53L, I54V/T/A/S, D60E, I62V, L63P, I64L/M/V, A71V/T/I, G73S/T, L76V, V77I, N83D, I85V, L89V, and I93L/M of PR.

Database construction

Collection Data was stored at Access 2007 for Windows Vista and exported to Excel 2007 for Windows Vista for analyses. There were 393 variables, including every TR and PR mutations, data about previous ART, and demographic data.

Statistical analysis

Statistical analyses were performed with STATA 8.2 for Windows (Statacorp, Texas).

The distribution of categorical variables was compared using the Chi square-test. Odds ratio was used to compare differences in frequencies. We studied the association between mutations and sex, as well as changes of mutations prevalence throughout the years.

The association between the presence of mutation and previous exposure to certain ARV was assessed by univariate and multivariate analyses. Dependent variables were the mutations, and the ARV exposure was the independent variable.

The significance level of 0.05 and a Confidence Interval (CI) of 95% were used.

RESULTS

From more than 600 genotypes performed from 2002 to 2006, 467 genotypes of 467 patients from Paraná State were evaluated. The genotypes of paediatric patients and the exams of patients whose ART history could not be elucidated were excluded. For patients with more than one genotype, the most recent one was included. RENAGENO performed 73.7% of the genotypes (Table 1). PR and RT subtype was defined in 389 (83.3%) patients, most of them (61.4%) were subtype B, 20.6% subtype C and 4.9% were subtype F. About 13.1% of the patients showed mixtures in PR and RT subtypes.

Mean age of patients in study was 41.12 ± 9.1 years, and most of them (n = 308; 66%) were males. Mean viral load in patients with failing regimens – including NRTI, NNRTI, and PI – was of 1,000 to 30,000 copies/mL. Most patients (72%) had AIDS and 56.7% had mean nadir CD4 count lower than 150 cells/mL. After ART, 59.6% had CD4 count above 200 and 31% above 350 cells/mL, in spite of virologic failure.

Patients were exposed to 6.69 ± 2.94 ARVs and 4.03 ± 2.45 ART combinations. The three ARV classes (NRTI, NNRTI, and PI) were used by 296 (63.4%) patients. Seventy three (15.6%), 89 (19.1%) and 9 (1.9%) patients used PI plus NRTI, NNRTI plus NRTI and NRTI double therapy, respectively.

NRTI double therapy was the most frequent initial regimen (Table 2). From patients starting on two NRTI, 59.16% received a PI containing HAART and 36.12% received a NNRTI containing HAART as subsequent therapy.

HAART used when genotype was requested included lamivudine in 80.94%, a NNRTI in 50.54% and a boosted PI in 35.55% (Figure 1).

Mean duration of drug exposure was longer for NRTIs and shorter for some PIs, like saquinavir (Table 3).

Table 1. Characterization of the study genotypes by date of collection and methodology of performance

	Number (n)	Frequency (%)
Date of genotype		
2002	22	4.71
2003	30	6.42
2004	117	25.05
2005	186	39.83
2006	112	23.98
Genotype assay		
ViroSeq System® - Applied biosystems (Renageno)	344	73.66
VircoTYPE HIV-1® (VIRCO)	83	17.77
GenoSure® (LabCorp)	22	4.71
GeneSeq® (Monogram, ViroLogic)	18	3.85

PR = protease, RT = reverse transcriptase.

Table 2. Patterns of initial and last antiretroviral regimen used

	Number (n)	Frequency (%)
Initial regimen		
NRTI + PI	179	38.33
NRTI + boosted PI	6	1.28
NRTI + NNRTI	65	13.92
Monotherapy (AZT)	27	5.78
NRTI double therapy	191	40.89
Last regimen		
NRTI + PI	95	20.34
NRTI + boosted PI	166	35.55
NRTI + NNRTI	236	50.54
Monotherapy (AZT)	0	0
NRTI double therapy	9	1.93

NRTI = nucleoside analogue reverse transcriptase inhibitors, NNRTI = non-nucleoside analogue reverse transcriptase inhibitors, PI = protease inhibitors, AZT = zidovudine.

The wild-type virus was present in 23 (4.9%) patients. Mutations conferring resistance to only one ARV class were seen in 66 (14.1%) patients. Resistance-mutations to two ARV classes were detected in 241 (51.6%) patients, and most of them were resistant to NRTI and NNRTI (59.34%), followed by resistance to NRTI and PI (38.6%), and to PI and NNRTI (2.1%). Resistance mutations to the three classes was detected in 137 (29.3%) patients.

Any NRTI mutation was present in 405 (86.7%) patients. M184V alone was detected in 64 (13.7%) patients. The prevalence of NRTI-related mutations is showed in Figures 2 and 3. Most patients (88%) were on thymidine analogues at the time of genotype and 99.8% of patients were previously exposed to a thymidine NRTI, 45% had more than three TAMs, and 21.8% had both M41L and L210W mutations, but 32.8% had no TAMs.

NNRTI-related mutations were present in 316 (67.7%) patients and the most frequent were K103NS, G190AES, and Y181C. The prevalence of NNRTI-related mutations present in the group of NNRTI-exposed patients is listed in Figure 4. Three out of 73 (4.10%) patients exposed only to PI and NRTI presented NNRTI-related mutations: one had K103N mutation, the other patient had Y181C and G190A mutations, and the third one presented Y181C, K101E and A98G mutations.

Major PI mutations were present in 239 (51.2%) patients and minor PI mutations were present in 455 (97.4%). The most frequent major PI mutation was L90M. The prevalence of PI-related mutations in the group of patients exposed to PI is listed in Figures 5 and 6. Two out of 89 patients (2.3%) exposed only to NNRTI e NRTI had major PI-related mutations: one had 30N mutation and the other patient had D30N, N88D and L90M mutations.

Exposure to each ARV class was significantly related to the presence of resistance mutations conferring resistance to these classes. NNRTI-related mutations occurred more often after NNRTI exposure (OR 10.57, 95% CI = 4.03-22.73, p = 0.000). Likewise, major PI mutations were more frequent after PI exposure (OR 4.55, 95% CI = 2.45-8.46, p = 0.000). Initial therapy consisting of two NRTIs (double therapy) was significantly related to a higher frequency of TAMs (OR 1.69, 95% CI = 1.12-2.54, p = 0.01) and the higher number of TAMs was also more prevalent in the group of patients who was exposed to double therapy (OR 1.16, 95% CI = 1.05-1.28, p = 0.004). NNRTI based HAART as first therapy was associated to lower prevalence of TAMs (OR 0.41, 95% CI = 0.24-0.71, p = 0.0009).

M184IV was related to lamivudine exposure (OR 26.21, 95% CI = 5.66-121.42, p = 0.0000). After multivariate analysis, TAMs were associated to longer exposure to d4T, and patients exposed to ddI and TDF showed significantly more

Figure 1: Overall antiretroviral regimen use (%) – The dark grey columns represent the frequency of patients on each ARV at the time of genotype. The light grey columns represent the frequency of patients ever exposed to each ARV since the beginning of treatment.

Nucleoside analogue reverse transcriptase inhibitors: AZT – Zidovudine, d4T – Stavudine, 3TC – Lamivudine, ddI – Didanosine, ddC – Zalcitabine, ABC – Abacavir, TDF – Tenofovir.
Non-nucleoside analogue reverse transcriptase inhibitors: EFV – Efavirenz, NVP – Nevirapine, DLV – Delavirdine.
Protease Inhibitors: RTV – Ritonavir, SQV – Saquinavir, IDV – Indinavir, NFV – Nefinavir, ATV – Atazanavir, APV – Amprenavir, SQV/r – Saquinavir/ritonavir, Indinavir/ritonavir (IDV/r), ATV/r – Atazanavir/ritonavir, APV/r – Amprenavir/ritonavir, LPV/r – Lopinavir/ritonavir, DRV/r – Darunavir/ritonavir.

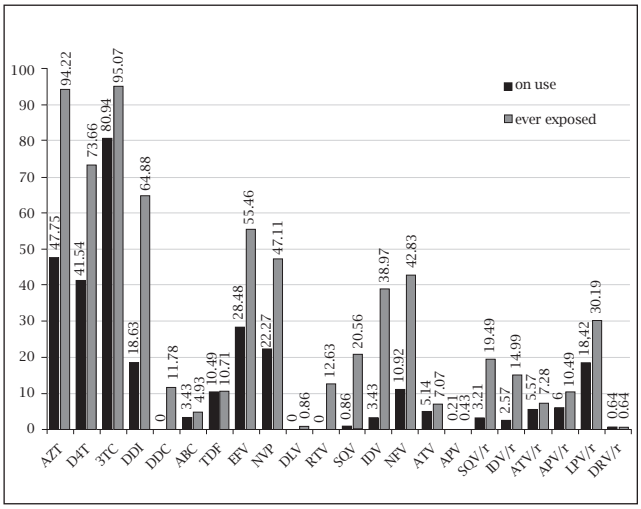


Table 3. Mean duration of each antiretroviral exposure

Antiretroviral	Mean duration of exposure (months)
Zidovudine	26
Stavudine	23
Lamivudine	40
Didanosine	8.5
Zalcitabine	11
Abacavir	6
Tenofovir	9
Efavirenz	10.5
Nevirapine	10
Delavirdine	3
Ritonavir	8
Saquinavir	2
Indinavir	11
Nelfinavir	11
Atazanavir	10
Amprenavir	6
Saquinavir/ritonavir	8.5
Indinavir/ritonavir	8
Atazanavir/ritonavir	7
Amprenavir/ritonavir	9
Lopinavir/ritonavir	3
Darunavir/ritonavir	10.5

Figure 2: Prevalence (%) of NRTI-related mutations I – The horizontal columns represent the frequency of NRTI-related mutations (Thymidine analogue mutations, Accessory and Additional mutations) in the 467 patients evaluated. Each column is described at the legend on the right. Additional mutations are 67G, 69DAN and 75A. Accessory mutations are 43EQ, 44AD, 181I, 208Y, 218E, 221Y and 228H. TAMs are 41L, 67N, 210W, 215FY and 219EQ. There were no 67E, 69S, 75S and 228H mutations in these patients.

TAMs = thymidine analogue mutations, NRTI = nucleoside analogue reverse transcriptase inhibitors.

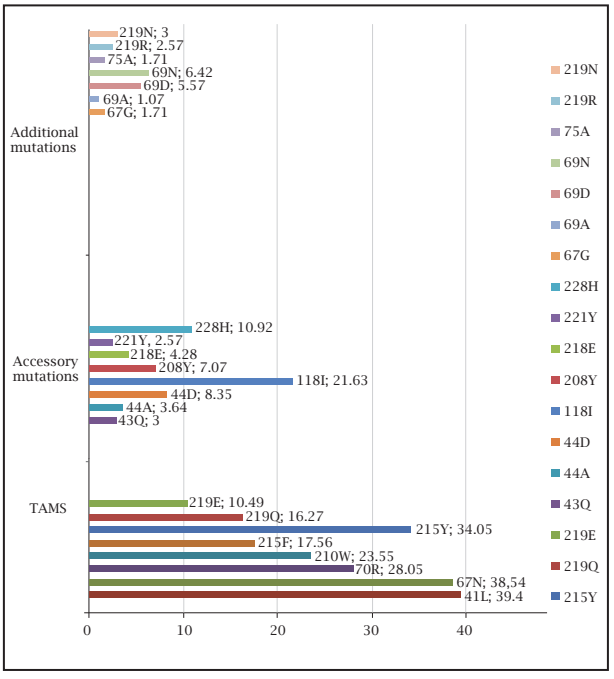


Figure 3: Prevalence (%) of NRTI-related mutations II – The horizontal columns represent the frequency of NRTI-related mutations (non-thymidine analogue mutations, and multi-nrti resistance mutations) in the 467 patients evaluated. Each column is described at the legend on the right. Non-TAMs are 115F, 75M, 75T, 74I, 74V, 65R e 184V. Multi-NRTI Resistance Mutations are 62V, 75I, 77L, 116Y and 151M and the insertion 69.

TAMs = thymidine analogue mutations, NRTI = nucleoside analogue reverse transcriptase inhibitors.

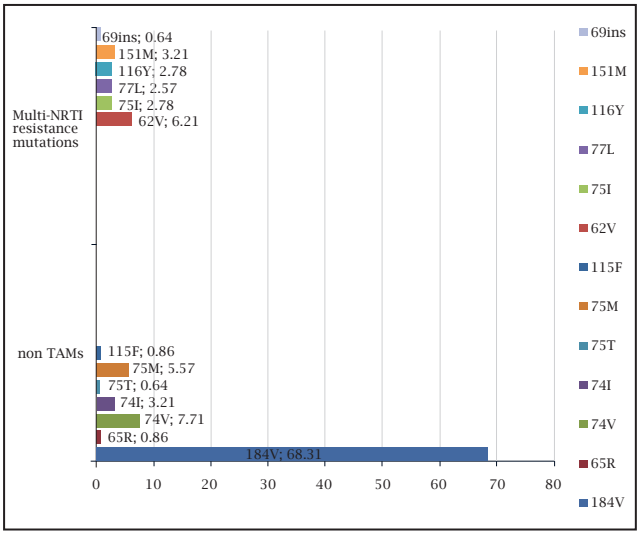


Figure 4: Prevalence (%) of NNRTI-related mutations – The vertical columns represent the frequency of NNRTI-related mutations in the 382 patients exposed to NNRTIs. Each column is described at the legend on the right. The mutations 179T, 181IV and 188H were not present in these patients.

NNRTI = non-nucleoside analogue reverse transcriptase inhibitors.

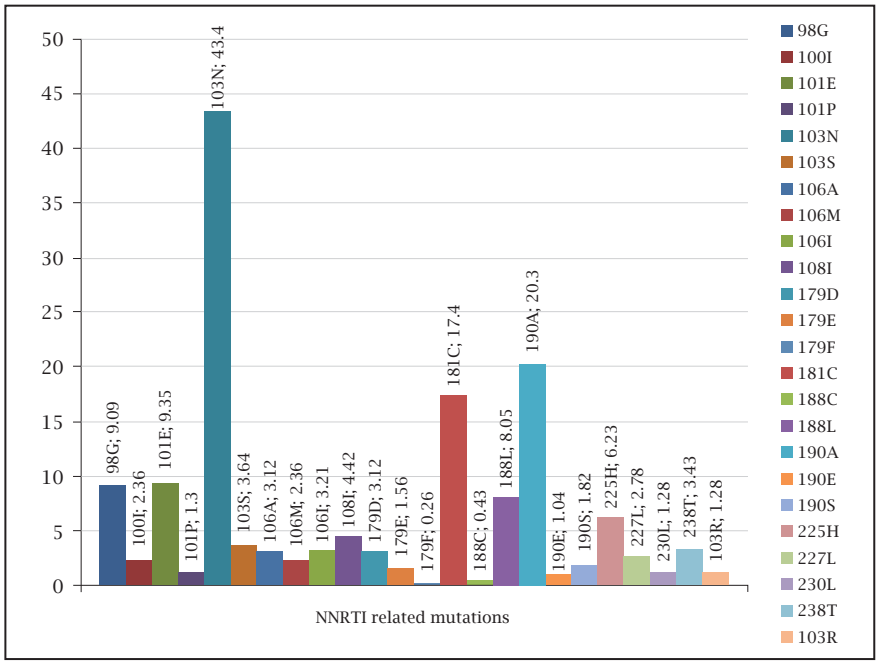


Figure 5: Prevalence (%) of Major PI-related mutations – The vertical columns represent the frequency of PI-related mutations in the 366 patients exposed to PIs. Each column is described at the legend on the right (30N, 32I, 33F, 46IL, 47AV, 48VM, 50LV, 54ML, 58E, 74P, 76V, 82ATFS, 84V, 88DS, 90M). The mutations 84AC were not present in these patients.

PI = protease inhibitor.

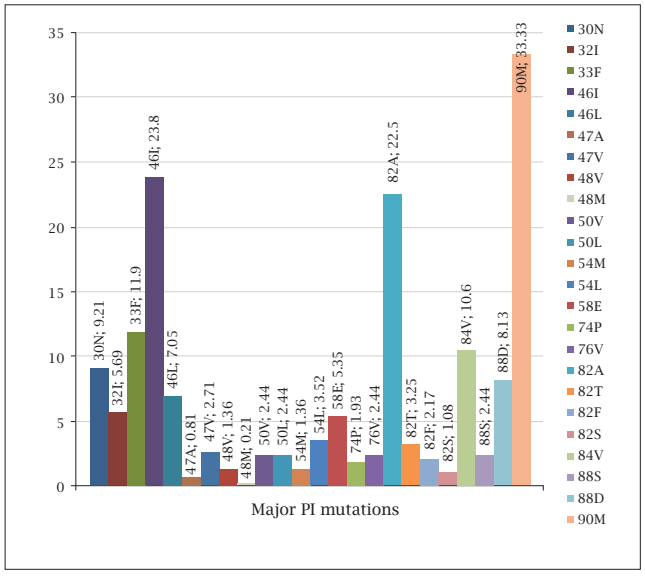
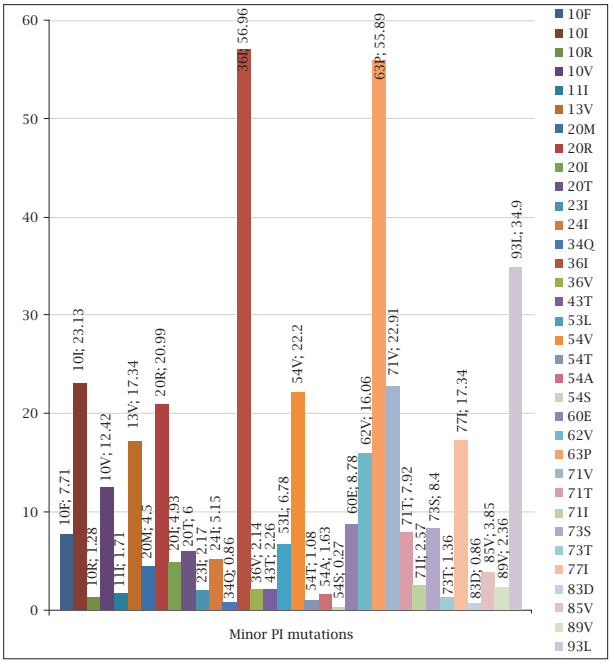


Figure 6: Prevalence (%) of Minor PI-related mutations – The vertical columns represent the frequency of PI-related mutations in the 366 patients exposed to PIs. Each column is described at the legend on the right (10FIRV, 11I, 13V, 20MIR, 23I, 24I, 34Q, 36IV, 43T, 53L, 54VTAS, 60E, 62V, 63P, 71VIT, 73ST, 77I, 83D, 85V, 89V, 93L). The mutations 16E, 20T, 35G e 64LMV were not present in these patients.

PI = protease inhibitor.



TAMs. Mutations on codons 74, 75 and 115 of RT were associated to longer exposure to TDF and ddI. On multivariate analysis tenofovir exposure was significantly associated to the presence of 74IV (OR 2.67, 95% CI = 1.27-5.63, p = 0.010) and 115F (OR 1.97, 95% CI = 1.01-53.60, p = 0.048) (Table 4). Likewise, the presence of mutations on codons 103, 101 and 106 was associated to exposure to EFV and NVP. Mutations on codons 181, 190 and 227 were significantly associated to NVP exposure, and at codons 100, 108, 179 and 225, to EFV exposure (Table 5).

Exposure to full dose ritonavir was related to the presence of 33F, 46IL, 47AV, 48V, 50V, 58E, 76V, 82ATFS, 84V, 90M. Exposure to indinavir was related to 32I, 33F, 46IL, 58E, 74P, 76V, 82ATFS, 84V, 90M. Exposure to boosted indinavir was related to 33F, 46IL, 58E, 54ML, 82ATFS, 90M. Exposure to saquinavir was related to 32I, 33F, 48V, 76V, 82ATFS, 84V, 90M. Exposure to boosted saquinavir was related to 33F, 46IL, 48V, 58E, 82ATFS, 84V, 90M. Exposure to nelfinavir was related to 30N, 33F, 46IL, 50L, 58E, 88DS, 90M. Exposure to atazanavir was related to 50L, 74P, and, to boosted atanavir, to 32I, 33F, 46IL, 47AV, 54ML, 82ATFS, 90M. Exposure to boosted lopinavir was related to 46IL, 47AV, 48VM, 50V, 54ML, 58E, 76V, 82ATFS, 84V, 90M. Exposure to amprenavir was related to 32I, 54ML, 58E, and, to boosted amprenavir, to 33F, 46IL, 47AV, 50V, 54ML, 58E, 82ATFS, 84V, 90M. Exposure to boosted darunavir was related to 32I, 33F, 47AV, 50V, 54ML, 76V.

After logistic regression analysis, lopinavir/ritonavir exposure was not a risk factor to the presence of G48VM after controlling for SQV and SQV/r exposure (OR 8.67, 95% CI = 0.85-87.98, p = 0.068). SQV exposure was not associated with the presence of L76V after controlling for DRV/r and LPV/r exposure (OR 3.02, 95% CI = 0.68-13.67, p = 0.146). The presence of 33I was associated only with the exposure to LPV/r, IDV and IDV/R, but not with NFV (OR 1.55, 95% CI = 0.75-3.18, p = 0.234) or DRV/r (OR 4.74, 95% CI = 0.40-56.94, p = 0.320).

The presence of major PI mutations, TAMs and other NRTI-related mutations was significantly associated with the exposure to a higher number of HAART regimens and a higher number of ARVs (Table 6).

When the frequency of ARV classes' exposure was analyzed, the use of NNRTIs, PIs and exposure to the three ARV classes was similar for patients who were genotyped from 2002 to 2006. There was a significant increase of tenofovir use and 3TC at the moment of test, and an overall decrease of exposure to thymidine analogues from 2002 to 2006 (Table 6).

Overall distribution of resistance mutations related to the three classes had no significant difference throughout the years, except for the significantly higher prevalence of accessory mutations (Figures 7 and 8). Some isolated mutations became significantly more prevalent over time (Table 7).

Some TAMs, V118I and PI mutations, like L90M, were more frequent in the HIV-1 isolates of male patients (Table 8).

Table 4. Multivariate analyses to NRTI exposure and presence of TAMs

Mutation	ARV exposure	OR*	95% IC	p-value
TAMs	Exposure to AZT	0.99	0.44-2.28	0.999
	Longer AZT exposure	1.16	1.00-1.34	0.051
	Exposure to d4T	1.02	0.65-1.61	0.926
	Longer d4T exposure	1.25	1.09-1.43	0.001
	Exposure to TDF	3.36	1.39-8.15	0.003
	Exposure to ddI	1.85	1.23-2.78	0.007
74IV	Longer ddI exposure	1.42	1.12-1.78	0.0031
	Longer TDF exposure	2.71	1.54-4.77	0.0005
75TM	Longer ddI exposure	1.42	1.05-1.90	0.0214
	Longer TDF exposure	2.73	1.33-5.62	0.0064
115F	Longer ddI exposure	2.64	1.22-5.74	0.0141
	Longer TDF exposure	9.04	1.36-59.89	0.0225

TAMs = thymidine analogue mutations, NRTI = nucleoside analogue reverse transcriptase inhibitors, AZT = zidovudine, d4T = stavudine, TDF = tenofovir, ddI = didanosine.
OR = odds ratio, CI = confidence interval.
*Odds ratio for the occurrence or not of mutations when patients were exposed to these ARVs..

Table 5. Multivariate analyses to NNRTI exposure and presence of NNRTI related mutations

Mutation	ARV exposure	OR*	95% IC	p-value
100I	Exposure to EFV	12.05	2.50-58.17	0.0001
108I		2.11	1.00-4.53	0.0484
179DEF		9.18	1.16-72.92	0.0107
225H		10.97	3.86-31.19	0.0000
181C	Exposure to NVP	9.92	4.58-21.49	0.0000
190ASE		4.26	2.50-7.29	0.0000
227L		3.87	1.04-14.37	0.0291
101EPH	Exposure to EFV and NVP	2.04	1.02-4.06	0.0383
		2.30	1.13-4.67	0.0178
103NS		3.92	2.52-6.09	0.0000
		4.28	1.17-15.70	0.0168
106AM		8.31	1.04-66.47	0.0168
		3.46	1.47-8.17	0.0025

NNRTI = non-nucleoside analogue reverse transcriptase inhibitors, EFV = efavirenz, NVP = nevirapine.
OR = odds ratio, CI = confidence interval.
*Odds ratio for the occurrence or not of mutations when patients were exposed to these ARVs.

Table 6. Amount of ARV exposure and presence of mutations

Mutation	ARVs and regimens	OR*	95% CI	p-value
TAMs	Increasing number of HAART regimen	1.11	1.03-1.20	0.0082
TAMs-Accessory mutations		1.11	1.02-1.20	0.0094
Multi-NRTI resistance mutations		1.15	1.02-1.30	0.0247
NNRTI mutations		1.09	1.01-1.18	0.0301
Major PI mutations		1.28	1.19-1.37	0.0000
TAMs	Increasing number of ARV exposure	1.08	1.01-1.15	0.0214
TAMs-Accessory mutations		1.10	1.02-1.17	0.0056
NRTI-Additional mutations		1.14	1.05-1.24	0.0017
Multi-NRTI resistance mutations		1.13	1.02-1.25	0.0208
Major PI mutations		1.26	1.18-1.34	0.0000

ARV = antiretroviral, TAMs = thymidine analogue mutations, HAART = highly active antiretroviral therapy, NRTI = nucleoside analogue reverse transcriptase inhibitors, NNRTI = non-nucleoside analogue reverse transcriptase inhibitors, PI = protease inhibitors OR = odds ratio, CI = confidence interval.
*Odds ratio for the occurrence or not of mutations when patients were exposed to an increasing number of ARVs and therapeutic regimens.

Table 7. Evidence of changes on ARV exposure, and PR and TR mutations from 2002 to 2006

	OR*	95% CI	p-value
ARV exposure			
On lamivudine	1.43	1.14-1.78	0.0016
On tenofovir	1.94	1.46-2.57	0.0000
Exposure to tymidine analogues	0.72	0.55-0.94	0.0159
NRTI-related mutations			
M184V	1.25	1.04-1.50	0.0200
L210W	1.28	1.04-1.57	0.0172
K43EQ	1.45	1.08-1.95	0.0127
L228H	1.44	1.09-1.91	0.0094
NNRTI-related mutations			
V108I	1.44	1.03-1.99	0.0313
M230L	2.40	1.11-5.19	0.0256
PI-related mutations			
M46IL	1.33	1.09-1.63	0.0055
I47AV	2.01	1.19-3.40	0.0095
Q58E	1.59	1.08-2.34	0.0183
I54ML	1.61	1.03-2.53	0.0376
T74P	2.18	1.16-4.09	0.0155

ARV = antiretroviral, NRTI = nucleoside analogue reverse transcriptase inhibitors, NNRTI = non-nucleoside analogue reverse transcriptase inhibitors, PI = protease inhibitors.
OR = odds ratio, CI= confidence interval.
*Odds ratio for the occurrence or not of mutations when patients were evaluated from 2002 to 2006. This represents an increase of mutations and ARV exposure.

Figure 7: Evolution of prevalence of mutations over the years – The graphic represents the frequency of patients with wild-type virus, and with each class mutation pattern. These frequencies were stratified in the genotype samples from 2002, 2003, 2004, 2005, and 2006.

NRTI = nucleoside analogue reverse transcriptase inhibitors, NNRTI = non nucleoside analogue reverse transcriptase inhibitors, PI = protease inhibitor.

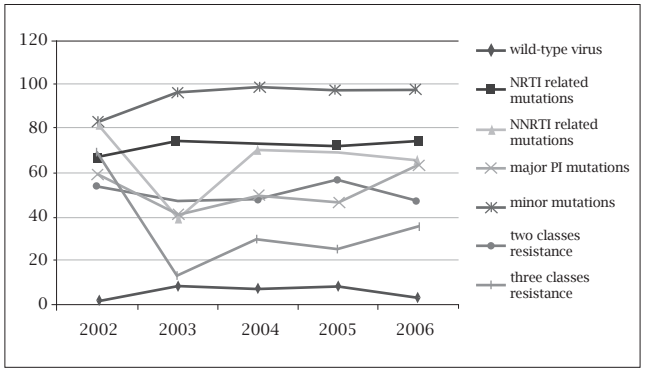


Figure 8: Evolution of NRTI-related mutations over the years – The graphic represents the frequency of patients with NRTI-related mutations. These frequencies were stratified in the genotype samples from 2002, 2003, 2004, 2005, and 2006. All mutations had the same prevalence pattern over the years except the Accessory mutations and the M184V, which have increased from 2002 to 2006. * (OR 1.25, 95% CI = 1.04-1.50, p = 0.02), ** (OR 1.23, 95% CI = 1.02-1.47, p = 0.0277).

TAMs = thymidine analogue mutations, NRTI = nucleoside analogue reverse transcriptase inhibitors , OR = odds ratio, CI = confidence interval.

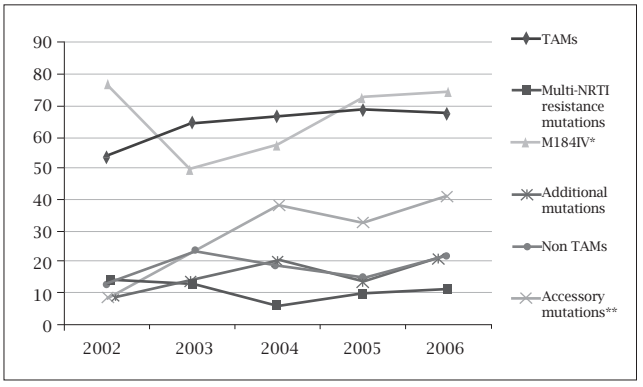


Table 8. Prevalence of mutations according to gender

Mutation	Male sex (n = 308)		Female sex (n=159)		OR	95% IC	p-value
	Number (n)	Frequency (%)	Number (n)	Frequency (%)			
M41L	138	44.8	46	28.9	1.99	1.32-3.02	0.0009
D67N	135	43.8	45	28.3	1.98	1.30-3.00	0.0011
L210W	88	28.6	22	13.8	2.49	1.41-4.20	0.0004
T215Y	118	38.3	41	25.8	1.79	1.17-2.74	0.0069
> 3 TAMs	160	51.9	50	31.4	1.26	1.13-1.40	0.0000
V118I	76	24.7	25	15.7	1.76	1.06-2.90	0.0261
M46I	72	23.4	16	18.2	2.73	1.51-4.91	0.0005
V82A	64	20.8	19	11.9	1.93	1.11-3.37	0.0181
L90M	93	30.2	31	19.5	1.79	1.12-2.84	0.0132
I54V	66	21.4	16	10.1	2.4	1.35-4.40	0.002
L24I	17	5.5	2	1.3	4.59	1.04-20.29	0.0273

TAMs = thymidine analogue mutations, OR = odds ratio, CI = confidence interval

DISCUSSION

This study aimed to estimate HIV drug resistance in patients receiving ART in Paraná, Brazil, from 2002 to 2006. This first estimate of the prevalence of mutations in our region shows a high frequency of resistance, similar to other Brazilian sites but higher for some patterns of mutations.

Almost all patients were exposed to thymidine NRTIs and lamivudine. Double therapy was the initial regimen in more than one third of patients. These features explain the high number of NRTI related mutations.

About half of patients were exposed to NNRTIs and most of them presented NNRTI related mutations. The PI used by most patients was nelfinavir followed by unboosted indinavir, representing the pattern of drug usage in the 90's, when most of these patients started therapy.

The increasing exposure to tenofovir was significant and reflects the availability of this drug after 2002 in clinical trials and in 2004 delivered by the PN-DST/AIDS.

About 15% of patients were exposed to boosted and unboosted atazanavir and 30% to boosted lopinavir, the most used PI nowadays, also reflecting the time of inclusion of

these drugs in the PN-DST/AIDS during the study period. Less than 1% of patients were exposed to darunavir, PI available since 2004 in clinical trials.

The most frequent mutations in RT were associated to lamivudine and NNRTIs exposure, which confirms the low genetic barrier of these drugs.[42]

Due to high prevalence of exposure to thymidine analogues and the presence of TAMs, K65R is expected to occur in a very low prevalence, as in the presence of multiple TAMs K65R is rarely selected on the same human immunodeficiency virus type 1 genome *in vivo*.[43]

In this study, the prevalence of mutations was significantly higher in the group of patients exposed to more drugs and more regimens. This was the case for TAMs, NRTI accessory mutations, NNRTI related mutations and major PI mutations. Initial double therapy was also significantly associated to more TAMs.

Patients starting HAART with a regimen including NNRTI presented less TAMs, probably because, besides avoiding double therapy, the tolerability of this regimens promoted better adherence. The same was not seen in the group starting HAART with a regimen including unboosted PIs, frequently a less tolerable regimen. Additionally, patients starting with unboosted PIs were exposed to higher number of drugs (6.99 versus 4.75) and regimens (3.75 versus 2.29) than patients starting with NNRTIs.

The low prevalence (less than 5%) of mutations associated with classes not previously used may represent primary resistance, acquired resistance or a false positive result. For patients exposed only to NNRTI and NRTI presenting major PI mutations, primary resistance is a possibility, since PI where first available to treatment for this group of patients tested in the early 2000's.

The association between exposure to thymidine analogues and higher number of TAMs was not neither significant in univariates or multivariate analyses as almost all patients were exposed to these drugs, but the longer time of exposure (specially to d4T) was significantly associated to more TAMs. Tenofovir and ddI exposure were significantly associated to higher number of TAMs not as a causal relationship, but because patients with exposure to more regimens had more TAMs and consequently were exposed to tenofovir and ddI since 2004.

Almost 5% of patients had no mutations in RT and PR, similarly to other Brazilian reports, where the prevalence of wild-type virus was less than 10% of samples.[21,23,25,26] Only one national study showed even higher prevalence of wild-type virus (15.3%).[20]

The most prevalent mutation occurred at codon 184 (68.31%), as reported in São Paulo (64%),[24] Rio de Janeiro (67%),[23] and Northeast Brazil (66%).[25] In Santos, from 2006, this mutation was even more prevalent (88%).[27] In Paraná, when samples from 2006 were analysed, the prevalence of M184V was of 75%, consistent with the significant increase of lamivudine exposure.

The frequency of mutations was high for all classes of antiretrovirals, which was expected for patients who had already been on different antiretroviral regimens. Predominantly, primary mutations were associated with NRTIs and NNRTIs. The high prevalence of TAMs (67.24%) was similar to that reported in São Paulo (73%).[24]

The K65R mutation was not reported in most national studies and has occurred in less than 1% of the patients in this study. At Brazilian Northeast region this mutation was seen in 3.4% of patients and, specifically in the state of Ceará, in 5.9% of cases.[23,26]

The overall prevalence of NNRTI mutations (67.7%) was higher than in other Brazilian series, probably because recently described NNRTI mutations (codons 101, 106, 179) were included in the analysis. Most of other Brazilian studies reported NNRTI prevalence of mutations of 30 to 55.4%.[21,23,25,26] In Santos, the overall NNRTI prevalence was not explicit, but K103N occurred in 52% of patients, whereas in these data from Paraná it was 43.4%.[27] At Brazilian Northeast region, the specific mutations were even higher, with 62% substitutions in codon 103, 38.7% in codon 190 and 181 in codon 29.2%.[25] At Rio de Janeiro, K103N was seen in 27.7%, but the the frequency of other mutations was similar to what was observed in Paraná: G190S/A in 22.12%, and Y181C/I in 17.4%.[23]

The PI related mutations were comparable to many other series. Mutation at codon 90 was the most frequent, followed by codons 46 and 82, prevalences similar to 25.5, 23.7 and 21.1%, respectively reported by Cavalcanti *et al.* (2005).[23] In the other Brazilian studies, L90M was also the most frequent, present in 26 to 37% of the cases.[20,21,23,24,44]

Compared with international data, these patients present lower prevalence of wild-type virus, probably because in other sites – where genotype was available earlier in the setting of virologic failure – more wild-type virus can be demonstrated. Other reason may be the lower exposure to less effective regimens, including less than two active agents. The prevalence of wild-type virus ranged from 14 to 22% in many trials.[14,16,17,45,46]

The pattern of PI and NRTI resistance mutations in Paraná was similar to some international series. PI resistance mutations occurred in 41 to 53% and NRTI related mutations occurred in 71 to 80% of samples from Spain, France, Italy and USA.[14,17,45-48]

Data from other countries showed lower prevalence (25 to 52%) of NNRTI mutations than this study.[14,15,17,45-48]

The most prevalent mutations worldwide were at codons 184 and 103 of TR, and 90 of PR, but they were less frequently than in this sample. M184V/I, K103N and L90M occurred respectively in 49.27 and 31% of patients in Spain and in 58.31 e 31% of patients in France.[44,48] Napravnik

et al. (2007) reported 79, 58 and 59% of M184V/I, K103N and L90M mutations respectively, but the analysis included only patients with three-class resistance, which explains this high prevalence.[19] Rhee *et al.* (2004) reported K65R in 1.9% of patients and Costagliola *et al.* (2007) reported it in 5% of cases.[16,49]

The prevalence of mutations conferring resistance to the three ARV classes was similar and even higher than other national and international studies in which it has been shown to be about 17 to 21%.[25,46-48]

No decrease in the prevalence of mutations has been observed in the sample from Paraná over the years, but some European studies did show an overall decrease in prevalence of TR and PR mutations, reflecting a better response to HAART.[40-52]

Differences in gender related prevalence are not widely described, but Di Giambenedetto *et al.* (2007), according to data from some mutations in Paraná, considered male sex an independent predictor of drug resistance.[50]

In terms of NNRTIs as future treatment options for this group of patients, it has been found that mutations on RT codons 181 and 190 occurred in more than 20% of patients, which could compromise etravirine efficacy if used in a suboptimal regimen. The new PI darunavir is an important future option to this group, as mutations limiting DRV/r response occurred in less than 10%, and PR mutations 50V, 54ML and 76V occurred in less than 5% of patients.

CONCLUSION

In conclusion, we showed a high frequency of resistance mutations to antiretroviral drugs in patients with virologic failure on ART in Paraná, from 2002 to 2006. This was more important to the NNRTI class compared to other studies. No reduction in the prevalence of mutations over the years was seen, as described in other countries. Prevalence of mutations and multidrug-resistant viruses needs to be monitored in the future in relation to the evolution of HAART.

Hopefully, the diminishing practice of sequential monotherapy and the availability of more effective and better tolerated combination regimens, since 2006, will improve suppression of viremia, resulting in prevention of acquired resistance.

ACKNOWLEDGEMENTS

The authors would like to thank Dra. Silvia Maria Gomes de Rossi, for giving us access to the genotypes at Centro Regional de Especialidades Barão, Curitiba. Our gratitude also is extended to Dra. Lilian Amaral Inocêncio, from the Programa Nacional de DST/AIDS for helping with the approval process of the tests studied. Assistance of Patrícia Monteiro, Roberta Terfiglio, Gaston Picchio and Eric Lefebvre, helping with the availability of tests from VIRCO is appreciated.

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