Genotype Testing and Antiretroviral Resistance Profiles from HIV-1 Patients Experiencing Therapeutic Failure in Northeast Brazil

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Genotype testing for HIV-1 drug resistance is useful for selecting antiretroviral drug regimens for patients experiencing therapeutic failure, but the optimal means for interpreting the test results is unknown because many HIV-1 protease and reverse transcriptase (RT) mutations contribute to drug resistance. This study identified common combinations of resistance mutations related to antiretroviral resistance profiles. From April 2002 to March 2004, 101 protease and RT sequences were determined for HIV-1 isolates from patients who were failing antiretroviral therapy. The resistance profile was evaluated using the Stanford Database program. Male patients predominated (76.2%), the median age was 38 years, the average CD4 count was 279.21 cells/mm^3 and the average viral load was 4.49 log. In relation to protease inhibitors (IP) 31 mutation patterns were detected, 49 mutation patterns were detected in Nucleoside RT Inhibitors (NRTI), and 17 patterns were found in the Non Nucleoside RT Inhibitors (NNRTI). K65R was detected in 5.9% of the isolates. The most frequent mutations were: L90M, M184V and K103N related to PI's, NRTI's and NNRTI's, respectively. The best antiretroviral susceptibility was found to be Lopinavir in the PI class and Tenofovir in the NRTI class. The top six mutation patterns accounted for 49% of the resistance to PI's, for 38.5% of NRTI resistance, and the top two mutation patterns accounted for 40.9% of resistance to NNRTI's.

Key-Words: Genotype test, mutation, HIV-1, protease, reverse transcriptase, antiretrovirals, resistance.

Antiretroviral therapy must be efficient to control HIV replication and to avoid selection pressure for the development of resistance mutations [1]. Continuing failing regimes is associated with an increase in resistance and the development of virus resistant to multiple drugs [2-8]. Genotype testing showed clinical utility in 4 of 5 prospective randomized studies [9], in contrast with Phenotypic tests which barely showed clinical utility in 1 of 4 prospective studies [10]. Several studies and clinical trials have compared the benefits of the two different resistance tests, and have demonstrated a significant benefit in the application of both tests when utilized by an HIV expert. The VIRADAPT [11] was the first prospective study, which evaluated Genotype tests in 108 French patients. The study was discontinued in the 6th month, due to a difference in benefit between the groups, which favored the Genotype test group. The study CPCRA-046/GART (Genotypic Antiretroviral Resistance Testing) was a multi-center study with 153 participants [12] that confirmed the benefit of the Genotype testing (p=0.0001) previously described. In addition, we mention the HAVANA [13] and NARVAL [9,14] studies that support these results. A goal-analysis study of 10 recently published studies (total of 2258 participants) showed increased virologic efficacy in patients whose therapy modification was guided by Genotype testing or virtual Phenotypic testing [15].

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Therefore, Genotype testing to identify HIV-1 antiretroviral resistance appears to select more efficient therapeutic regimens in patients developing therapeutic failure, and may be helpful in occupational accidents involving healthcare workers as well [16]. However how to optimally interpret these tests is unknown due to multiple mutations in the protease and RT which contribute to resistance [17]. Another important application for Genotype testing in our country is drug resistance surveillance in the Brazilian drug-naïve population [18,19] As a result, more information about the execution and interpretation of Genotype testing, and mainly individualization of the studies for each locality [20-22], is necessary for better application to patients' therapy [23].

This study evaluated the antiretroviral resistance profile through Genotype testing followed by utilization of the Stanford Database; identify resistance mutation patterns by analyzing protease and reverse transcriptase sequences of HIV-1 isolates obtained from a patient population experiencing therapeutic failure in Ceará, Northeast Brazil; and determine the main individual mutations for each antiretroviral class.

Materials and Methods

The study was conducted in the São José Infectious Disease Hospital in Ceará, Brazil from April 2002 to March 2004, in a patient population receiving antiretroviral therapy that presented with: failing double or triple therapy regimens which included a NNRTI, and first or second virologic failure of triple therapy regimen which included a PI. The study was retrospective in design. The samples were collected by LACEN (Central State Laboratory) in a total of 101 patients. The blood samples had Genotype tests performed using the ViroSeq system from Celera Diagnostics. A database was developed using the SPSS program, version 11 which was compatible

with the questionnaire used for data collection. For evaluation of mutation patterns, polymorphic positions were excluded for protease in codons: 10, 20, 33, 36, 63, 71, 77 and 93, and for reverse transcriptase: 98 and 179 [17]. During the analysis of individual mutations, all mutations encountered were included in the anaylsis. Mutations were analyzed with the Stanford Database to define the antiretroviral susceptibility profile [24].

The descriptive statistical analysis was carried out using the SPSS Program, version 11. For comparative analysis the Epi Info Program version 6 was used, considering two standard deviations with confidence interval values of 95%. The tests were defined statistically significant when the p value <.05.

Results

Characteristics of the study population are found in Table 1. Patients initiated antiretroviral therapy between July/81 and September/02. With regards to the prior utilization of antiretrovirals, we found the percentage of patients which had used each drug to be the following: Zidovudine 82.2%, Lamivudine 79.2%, Stavudine 64.4%, Didanosine 77.2%, Zalcitabinae 10.9%, Efavirenz 27.7%, Nevirapine 23.8%, Nelfinavir 43.6%, Indinavir 27.7%, Saquinavir 9.9%, Ritonavir 26.7% (used as an active drug), Lopinavir 4% and Amprenavir 1% Amprenavir 1 (1%). Atazanavir was not utilized. Double therapy was utilized in 65 regimens before triple therapy was initiated, but we were not able to evaluate the impact of double therapy on resistance because these regimens were changed for HAART before Genotype testing. These patients were failing three or more regimens at the time Genotype testing was performed [25]. From 101 isolates, 7.9% did not present with resistance mutations, 9.9% had mutations associated with one class of drugs (7.9% NRTI, 1% NNRTI and 1% PI), 73.3% to two classes (38.6% NRTI and PI, and 34.7% NNRTI and NRTI), and 8.9% to all classes.

Table 1. Epidemiologic, clinical and immuno-virologic characterization of patients experiencing therapeutic failure, Northeast, Brazil, from 2002 to 2004 (N=101)

Characteristic	Value (%)
Sex	Male 77 (76.2%)
	Female 24 (23.8%)
Age*	Median = 38 years (variation 6-66)
HIV Diagnoses date	July/81 to September/02
AIDS #	67 (66.3%)
Symptomatic patients	24 (23.8 %)
CD4	Median = 251 cells/mm^3 (2-1300)
Viral load	Median=33,000 copies/mL (1,600-1,900,000)
Log	Median = 4.5

*N=95. #AIDS: CMV retinitis, Kaposi sarcoma, pneumocystis pneumoniae, neurotoxoplasmoses, esophageal candidiasis, tuberculosis and atypical mycobacteriosis, invasive cervical cancer and recurrent bacterial pneumonia (> 2 episodes in 1 year).

In the protease gene, 97 (96%) had sequences with a resistant mutation in at least one of the 21 positions associated with resistance. L63P was the mutation most frequently encountered (73.3%) when considering all resistance positions in the protease gene. Excluding polymorphic positions, the main mutation was L90M (24.8%). Among the 101 sequences, 91 (90.1%) had one or more mutations in the 18 positions conferring resistance to NRTIs. M184V was the most common mutation found (60.4%) [23]. T215Y was the second most common, occurring in 42.6% sequences (presentation T215F/Y in 3% of cases), followed by M41L in 40.6% [26,27].

For NNRTIs, 44 (43.5%) of the sequences contained one or more mutations in the 12 positions associated with resistance. K103N was the most frequent mutation found (26.7%).

The antiretroviral susceptibility profile using the Stanford Database can be seen in Table 2. Of the samples evaluated, 49 (48.5%) demonstrated resistance to PIs, with 31 different patterns found. The six main mutation patterns, excluding polymorphic positions, corresponded to 49% of the sequences found. Analysis of these patterns by the Stanford Database was also performed (Table 3). In the NRTI class, we found 49 mutation patterns and the six standard corresponded to 38.5% of mutation sequences (Table 4). These patterns contained an average of 4 resistance mutations to NRTIs (minimum 1 and maximum 5).

K65R was found in 5.9% (6) patients, occurring alone in 3 patients, and in combination with M184V, or with M184V and K219E, or with M41L in one patient.

Table 2. Susceptibility profile to antiretroviral drugs found after Genotype testing and analysis using the Stanford Database algorithm (N=101)

Antiretroviral drug	S (%)	R (%)	I (%)
Tenofovir	40 (39.6)	1 (1)	60 (59.4)
Zidovudine	35 (34.7)	36 (35.6)	30 (29.7)
Lamivudine	34 (33.6)	62 (61.4)	5 (5)
Estavudine	29 (28.7)	26 (25.7)	46 (45.5)
Didanosine	26 (25.7)	20 (19.8)	55 (54.5)
Abacavir	26 (25.7)	19 (18.8)	56 (55.4)
Entricitabine	34 (33.6)	62 (61.4)	5 (5)
Dilaverdine	58 (57.4)	40 (39.6)	3 (3)
Efavirenz	56 (55.4)	40 (39.6)	5 (5)
Nevirapine	56 (55.4)	45 (44.6)	-
Amprenavir	62 (61.4)	4 (4)	35 (34.7)
Atazanavir	56 (55.4)	7 (6.9)	38 (37.6)
Indinavir	61 (60.4)	11 (10.9)	29 (28.7)
Lopinavir	66 (65.3)	2 (2)	33 (32.7)
Nelfinavir	52 (51.5)	30 (29.7)	19 (18.8)
Ritonavir	62 (61.4)	10 (9.9)	29 (28.7)
Saquinavir	64 (63.4)	8 (7.9)	29 (28.7)

R: resistance, I: intermediary resistance and S: susceptible.

In the NNRTI class, 17 mutational patterns were detected, and the two main patterns corresponded to 40.9% of all sequences (Table 5).

Nelfinavir was utilized as the only protease inhibitor in 26 patients (25.7%), whose Genotype test analysis showed the presence of 9 patterns with D30N (30N+77I+88D = 3,

Table 3. Most common resistance patterns in 49 sequences from patients with resistance mutations to Protease Inhibitors (PI)

Pattern	Nº	% patients	% Cumulative	Drug susceptibility						
				APV	ATV	IDV	LPV	NFV	RTV	SQV
90M	9	18.4	18.4	I	I	I	S	I	I	I
30N88D	5	10.2	28.6	S	S	S	S	R	S	S
46I90M	3	6.1	34.7	I	I	I	I	R	I	I
30N	3	6.1	40.8	S	S	S	S	I	S	S
54V82A	2	4.1	44.9	I	I	I	I	I	I	I
46L90M	2	4.1	49	I	I	I	I	R	I	I

AP: Amprenavir, ATV: Atazanavir, IDV: Indinavir, LPV: Lopinavir, NFV: Nelfinavir, RTV: Ritonavir and SQV: Saquinavir. R: resistance, I: intermediary resistance and S: susceptible.

Table 4. Top resistance patterns in 91 sequences from patients with resistance mutations to Nucleoside Reverse Transcriptase Inhibitors (NRTI).

Pattern	Nº	%	% Cumulative	Drug susceptibility						
				ABV	AZT	3TC	D4T	DDI	FTC	TDF
184V 41L184V	14	15.4	15.4	S	S	R	S	S	R	S
215Y 41L118I	5	5.5	20.9	I	I	R	I	I	R	I
184V210W215Y 41L67N 118I184V	4	4.4	25.3	I	I	R	I	I	R	I
210W215Y 67N70R	4	4.4	29.7	R	R	R	R	R	R	I
184V219Q 41L184V	4	4.4	34.1	I	I	R	I	I	R	S
210W215Y	4	4.4	38.5	I	I	R	I	I	R	I

ABV: Abacavir, AZT: Zidovudine, 3TC: Lamivudine, D4T: Estavudine, DDI: Didanosine, FTC: Emtricitabine and TDF: Tenofovir. R: resistance, I: intermediary resistance and S: susceptible.

Table 5. Top resistance patterns in 44 sequences from patients with resistance mutations to Non Nucleoside Reverse Transcriptase Inhibitors (NNRTI).

Pattern	Nº	%	% Cumulative	Drug susceptibility		
				DLV	EFV	NVP
103N	12	27.3	27.3	R	R	R
181C190A	6	13.6	40.9	R	R	R
103N190A	3	6.8	47.7	R	R	R
100I103N	3	6.8	54.5	R	R	R
190A	3	6.8	61.3	S	I	R
103N181C	3	6.8	68.1	R	R	R
181C	3	6.8	74.9	R	I	R

DLV: Delavirdine, EFV: Efavirenz and NVP: Nevirapine. R: resistance, I: intermediary resistance and S: susceptible.

30N+36I+88D = 2, and 30N+36I+46L, 30N+77I, 30N+36I, 30N+36I+46I+77I+88D = 1), corresponding to 34.6%, and 6 patterns with L90M (36I+90M = 4, 90M = 1, 77I+90M = 1) that corresponded to 23%. In addition to these, four were isolated with 77I, three with 36I, one with 36I+88D and three patterns isolated did not contain PI mutations.

For patients treated with Indinavir (N=6), the main mutation found was 63P (66.6%), followed by 36I (50%), 82A (50%) and L90M (16.6%). In the NNRTI class, when the initial regimen utilized EFV (N=23), the following were found as the main mutations: 103N (60.8%), 190A (21.7%), 108I (13%) and 181C (8.7%). When NVP was utilized (N=21), we found 181C (52.4%), 103N (38.1%), 190A (38.1%) and 108I (4.7%) were the main mutations. There was not a significant difference in the predominance of the 103N mutation with prior use of Efavirenz or Nevirapine (p=0.13), nor for 190A (p=0.23) or 108I (p=0.33). However, there was a significant difference for the mutation 181C (p=0.0015) with prior use of NVP.

Table 6. Resistance mutation profiles found in therapeutic failure subgroups based on antiretroviral class (N=101)

Therapeutic Class	Resistance mutation	1st failure N=49 (%)	2nd failure N=37 (%)	Multifailure N=15 (%)
Protease	L33W/F	0	0	1 (6.6)
Inhibitors (PI)	V82A/F/L/T	3 (6.1)	4 (10.8)	6 (49)
	I84V	1 (2)	0	3 (20)
	L90M	6 (12.2)	8 (21.6)	12 (80)
Nucleoside Reverse	M41L	16 (32.6)	13 (35)	12 (80)
Transcriptase Inhibitors	D67N	15 (30.6)	11 (29.7)	6 (40)
(NRTI)	K70R	13 (26.5)	8 (21.6)	0
	L210W	12 (24.5)	10 (27)	13 (86.6)
	T215Y/F	21 (42.8)	17 (45.9)	13 (86.6)
	K219Q/E	11 (22.4)	8 (21.6)	0
	E44D	3 (6.1)	1 (2.7)	3 (20)
	V118I	7 (14.3)	7 (18.9)	6 (40)
	M184V	27 (55.1)	26 (70.6)	8 (53.3)
	K65R	4 (8.1)	2 (5.4)	0
	69	3 (6.1)	1 (2.7)	3 (20)
Non Nucleoside Reverse	K103N	14 (28.6)	10 (27)	3 (20)
Transcriptase Inhibitors	Y181C/I	8 (16.3)	4 (10.8)	3 (20)
(NNRTI)	Y188C/L/H	2 (4)	1 (2.7)	1 (6.6)
	G190A/S	6 (12.2)	7 (18.9)	4 (26.6)

When the main individual mutation profiles were analyzed in first failure, second failure, and multi-failure (three or more regimens failure) subgroups, the results shown in Table 6 were obtained.

Discussion

After evaluating the antiretroviral susceptibility utilizing the Stanford Database, we found the best profile for the PIs to be Lopinavir (65.3%), verifying the importance of the fact that it contains the largest genetic barrier to resistace, meaning a greater accumulation of mutations is necessary for the development of resistance [28]. Although it was not utilized in any of the patients, Atazanavir demonstrated a high resistance profile (55.4%). Therefore it presents a lower genetic barrier, as fewer resistance mutations are necessary to cause therapeutic failure. The most prevalent mutation in this class was L90M, which confers cross-resistance to all members of the class [6,29-31]. This mutation contributed to resistance of drugs not previously utilized, such as Atazanavir or even Lopinavir and Amprenavir, which were utilized in a limited number of patients. With regards to the NNRTI class, the susceptibility profile of Efavirenz and Nevirapine was 56.4%. As there was no difference in the resistance profile for the two drugs it suggests a strong relation of cross-resistance in this class. K103N was the most frequent resistance mutation, occurring in 27 samples, and is responsible for cross-resistance to all members in the NNRTI class [29].

K65R was found in 5.9% of the sequences isolated, therefore was described as rare. Other studies have described the K65R mutation as rare, occurring only in 2 to 3% of patients experiencing multi-drug failure which had previously received Tenofovir. This could be due to a possible antagonism between the K65R mutation and the NAMs [32,33]. However in our study this was not verified because the mutation was found to be more frequent. The mutation was associated with NAMs 50% of the time (M184V in 1 patient, M184V and K219E in 1 patient, and M41L in 1 patient). Therefore we need further follow up of patients using NRTI's in order to evaluate cross-resistance to Tenofovir.

Conclusion

Evaluation of Genotype tests in patients with therapeutic failure is important in order to understand resistance patterns that will help guide future selection of drug regimens. This study demonstrated the superior susceptibility profile of drugs with a higher genetic barrier, such as Tenofovir and Lopinavir. The main mutations for each class of drugs represented a significantly high percentage among all of the standards encountered. Mutations L90M, M184V and K103N were more frequent for the PI, NRTI and NNRTI, classes, respectively. These results correspond to findings in the world literature. These mutations are extremely important as they confer cross-resistance among drugs within the same antiretroviral class.

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