

RESISTENCE PROFILE AND SUBTYPING IN HIV-1 INFECTED PATIENTS IN BOTUCATU, BRAZIL

Rejane M. T. Grotto¹, Vanessa B. Garcia¹, Lenice R. Souza², Domingos A. Meira², Paulo E. A. Machado¹, Maria Inês M. C. Pardini¹.

¹ Hemocentro de Botucatu, Faculdade de Medicina, UNESP, Botucatu, Brazil.

² Departamento de Doenças Tropicais e Diagnóstico por Imagem, Faculdade de Medicina de Botucatu, Botucatu, Brazil.

Corresponding author:

Rejane M. T. Grotto, Laboratório de Biologia Molecular, Divisão Hemocentro, Faculdade de Medicina de Botucatu, UNESP, Distrito de Rubião J/R s/n, 18618-000 Botucatu, Brazil.

Tel.: +55 14 3811-6041

Fax: +55 14 3811-6041

E-mail: regrotto@uol.com.br

Key words: genotyping, HIV-1, resistance

ABSTRACT

The efficiency of antiretroviral treatment for HIV is related to the TCD₄ cell number and plasma viral load. The detection of mutations in the protease and reverse transcriptase gene that confer resistance to drugs has revealed a strong association between treatment failure and the selection of resistant mutants. HIV viral RNA isolated from plasma was used as source for RT-PCR amplification and automatic sequencing (genotyping method) of the protease and reverse transcriptase gene in 132 patients from the Tropical Diseases Department, UNESP, Botucatu, SP, Brazil to determine the resistance profile and virus subtype. Twenty-one percent of the samples presented genotypes associated with resistance to the two classes of antiretroviral (reverse transcriptase inhibitors and protease inhibitors), suggesting the occurrence of multi drug-resistance. Most mutations of the reverse transcriptase gene were: M184V (36.8%), M41L (27.6%), D67N (26.3%) and K103N (23.6%) and most mutations of the protease gene were: L63P (46%), M36I (38.1%) and V77I (21%). It was also observed that 82.9% of the samples belonged to the B subtype. From 132 patients, 23 were followed-up at times and genotyping was performed at time D0 (start point of study) and at the time of possible failure. These 12 reached high viral load at an early time, suggesting the occurrence of therapeutic failure, and 7 (58.3%) presented mutations associated with resistance to antiretroviral drugs, suggesting an association between treatment failure and the selection of resistant mutants.

INTRODUCTION

AIDS (Acquired Immune Deficiency Syndrome) is the result of an infection caused by the Human Immunodeficiency Virus (HIV) (Abbas et al. 1998).

The HIV is highly variable and nowadays two types of HIVs have been present in the world: HIV-1 and HIV-2. While HIV-2 has been restricted to the African continent, HIV-1 has spread to all the world and can be clustered in three groups according to phylogenetic analyses of HIV-1 sequences: M (*major*), O (*outlier*) and N (*new*). In these groups, amino acid sequences present 30% of similarity in *gag* region and 47% in *env*. The M group is subdivided into nine subtypes: A, B, C, D, F, G, H, J, K, which present amino acid sequences with differences of 15% in *gag* and 22% in *env* (Taveira et al. 2002).

The subtype B is predominant in Brazil, although a refined analysis by Diaz in 2002 showed regional tendencies. In São Paulo and Rio de Janeiro states and the northeast region there is a

predominance of subtype B (80%); however, subtypes F, C and recombinant forms have also been found. In the center-west and north regions, the predominance was of subtype B (70%) followed by F (30%). In Rio Grande do Sul the subtype C was found in 67% of the cases, followed by B (30%), recombinant forms (22%) and F (3%). In Parana the subtype C was found in 32% of the cases. Nowadays, HIV-1 mutants and recombinant forms are dispersed in all the national territory, constituting an obstacle for prevention, diagnosis and treatment (Diaz 2002).

Moreover, the emergence of drug resistance, a consequence of the viral genetic variability and the selection of resistant variants, constitutes a great obstacle for antiretroviral treatment (Roberts et al. 1988, Miller 1998, Deeks et al. 1999, D' Aquila 2000, Richman 2000).

Antiretroviral treatment has been accomplished by two classes of drugs, the reverse transcriptase inhibitors and the protease inhibitors (Blanchet et al., 1998; Ministerio da Saúde, 2000). Most of the time, these classes have been used in combined therapy, improving the antiretroviral effect, elevating the T CD4 lymphocyte number and reducing the plasma viral load. This increase of the activity potentiates the therapeutic effect or presents action synergism in different sites of the HIV replication cycle (Ministerio da Saúde 2004).

Although therapeutic failure is related to several factors, such as adherence, pharmacological factors, host immunological condition, the main concern has been the drug resistance due to mutations in the viral genetic material (D' Aquila 2000). The lack of proofreading of HIV-1 reverse transcriptase leads to the high rate of mutation unrepaired and transmitted (Holland et al. 1982, Biochemistry 2001). Drug resistance appears as consequence of the mutations in the viral genotype that lead to alterations of amino acids in the reverse transcriptase and/or protease sequence, leading to inferior susceptibility to that usually observed in drugs. Therefore, once the mutant is selected by the selective pressure of the therapy, resistance appears (Condra 1998, Richman et al. 2000).

The frequent development of resistance to drugs makes it difficult to maintain the infected patient, because mutations that cause resistance to one antiretroviral can lead to cross-resistance to other drugs of the same class, so the choice of a rescue therapy for patients in therapeutic failure has been difficult in medical practice. The assays of resistance analysis are useful at this moment for the selection of the most appropriate rescue therapy, but its usefulness is still being established to anticipate the answer to the therapy (Miller 1998, Deeks et al. 1999).

In this context, laboratory tests for identification of resistance to antiretroviral, have been evaluated in clinical assays in the attempt at an early determination of the most appropriate rescue therapy to permit continuity of the treatment (Ministerio da Saúde 2004).

At the moment, drug resistance determination has been evaluated by genotypic and phenotypic assays. The genotyping consists in the detection of specific point mutations that lead to

a change of amino acids in specific sites which link to the drug resistance (Richman et al. 2000, Chaix-Couturier et al. 2000). Phenotyping is an assay involving viral culture that measures drug-susceptibility *in vitro* (Ballard et al. 1998). In that way, the resistance tests constitute one further progress in the treatment of patients infected by HIV-1.

In this paper we performed genotyping of the HIV-1 *pol* region of the patients attended by the Viral Load National Program in Botucatu region in order to infer the circulating subtypes and antiretroviral resistance.

MATERIAL AND METHODS

In this study we included 132 HIV-1 seropositive patients, 61 were women (46,2%) and 71 men (53,8%), with age above eighteen years, attended by the Tropical Disease Area and Image Diagnosis of the Medical College (FMB), UNESP, with medical orders for a plasma viral load test, between January 2000 and June 2001. The approval of the Committee of Ethics in Research of Botucatu Medical College was made by official document n.º 1292 in 10/19/2001.

The samples were divided into four groups according to the plasma viral load (PVL).

Group 1: Patients with PVL between 80 - 10.000 copies/ mL (N = 29).

Group 2: Patients with PVL between 11.000 - 30.000 copies/mL (N = 33).

Group 3: Patients with PVL between 31.000 - 100.000 copies/mL (N = 33).

Group 4: Patients with PVL above 100.000 copies/mL (N = 37).

From 132 evaluated patients, 23 were naive to therapy or were submitted to a change in antiretroviral therapy, making it possible to evaluate these along time, thus constituting a new group (Group 5). Of these, 16 were male and seven female, with ages varying between 20 and 67 years with a medium of 37 years.

METHODOLOGY

Viral RNA was isolated from plasma by the method of Boom (Boom et al. 1990) and it was used as a source for genotyping. The technique consisted in a reverse transcription followed by PCR reactions, whose products were analyzed by a system of automatic sequencing.

The extracted RNA (5µL) was reverse transcribed in a mix containing 15ng/µL of Random Primer pd(N)6 (Invitrogen), 0.7U of RNA guard (Pharmacia), 0.5mM of dNTP (Pharmacia), 1X AMV-RT Buffer (Invitrogen), 1.8U of SuperScript II (Invitrogen) in a reaction total volume of 20µL. This reaction was incubated at 42°C for 90 minutes.

The PCR for amplifying the HIV-1 polymerase gene was performed in a mix containing 1X Buffer (Invitrogen), 3.5 mM of MgCl₂ (Invitrogen), 0.4mM dNTP (Pharmacia), 0.2pmol of Kozal 1 primer (5'-CAGAGCCAACAGCCCCACCA-3'), 0.2pmol of Kozal 2 primer (5'-TTTCCCCACTAACTTCTGTATGTCATTGACA-3') (Kozal et al. 1996), 2.5U of Taq Recombinant DNA Polymerase (Invitrogen – 5U/μL) and 10 μL of cDNA in a reaction total volume of 50μL. Amplification conditions were 94°C for 10 minutes, followed 35 cycles [94°C-30 seconds, 55°C-30 seconds, 72°C-2 minutes] and 10 minutes at 72°C.

The Nested PCR for amplifying the HIV-1 reverse transcriptase gene was performed in a mix containing 1X Buffer (Invitrogen), 2.5 mM of MgCl₂ (Invitrogen), 0.4mM dNTP (Pharmacia), 0.2pmol of Frenkel 1 primer (5'-GTTGACTCAGAT TGGTTGCAC-3'), 0.2pmol of Frenkel 2 primer (5'-GTATGTCATTGACAGTCCAGC-3') (Frenkel, et al. 1995), 2.5U of Taq Recombinant DNA Polymerase (Invitrogen – 5U/μL) and 5 μL of PCR product in a reaction total volume of 50μL. Amplification conditions were 94°C for 10 minutes, followed 35 cycles [94°C-1 minute, 55°C-1 minute, 72°C-1:30 minutes] and 10 minutes at 72°C.

The Nested PCR for amplifying the HIV-1 protease gene was performed in a mix containing 1X Buffer (Invitrogen), 2.5 mM of MgCl₂ (Invitrogen), 0.4mM dNTP (Pharmacia), 0.2pmol of DP10 primer (5'-TAACTCCCTCTCAGAAGCAGGAGCCG-3'), 0.2pmol of DP11 primer (5'-CCATTCCTGGCTTTAATTTTACTGGTA-3') (Cerqueira et al. 2004), 2.5U of Taq Recombinant DNA Polymerase (Invitrogen – 5U/μL) and 5 μL of PCR product in a reaction total volume of 50μL. Amplification conditions were 94°C for 10 minutes, followed 35 cycles [94°C-30 seconds, 55°C-30 seconds, 72°C-1 minute] and 10 minutes at 72°C.

The amplification was confirmed by visualization of the fragment of expected size (400bp for protease and 800bp for reverse transcriptase) in 2% agarose gel.

Nested PCR products used for sequencing were purified using a Concert™ Rapid PCR Purification System Kit (Gibco), according to the manufacturer's specifications. Sequencing of the Nested PCR products was performed with The BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.0 (Applied Biosystems) and analyzed on an ABI Prims™ 377 DNA Sequencer (Applied Biosystems), according to the manufacturer's instructions.

The sequences created for the HIV-1 Reverse Transcriptase and Protease region of each patient included in this study were analyzed using the program of sequence alignment BioEdit and after obtaining a consensus sequence; the same was analyzed by the algorithm seq-HIV of the Stanford University Site (<http://hivdbstanford.edu/>).

RESULTS AND DISCUSSION

From 132 samples included in this study, 76 had genotyping concluded by this method. The limitations of this technique, in most cases, concentrated on the amplification phase. Lack of amplification for both regions (RT and PR) was observed in 33 of the 132 samples tested, six for the protease region and seven for the reverse transcriptase region. Amplification difficulties in these cases were already expected because these samples had plasma viral load lower than 1000 copies RNA/mL. Concordant data with this paper have been obtained in other studies (Perelson et al. 1996, Ballard et al. 1998, Shafer et al. 2001). Another critical phase was sequencing, for which ten samples were not finished because the generated sequences were of poor quality. This method limitation is concordant with other studies (Clevenbergh et al. 2000, Dunne et al. 2001). For obtaining high quality sequence data in these cases technical alterations should be made.

Drug resistance is an important factor in antiretroviral therapy failure. However, resistance is generated as a result of the presence of mutants and drug selective pressure. Wild type virus is predominant in the antiretroviral therapy lack, although many variants can be present in lower replicative rate. The introduction of antiretroviral drugs leads to the inhibition of wild type virus replication, drugs susceptible, creating conditions for the increase of the mutants' number. This virus, drug resistant, has the capacity to replicate in the antiretroviral's presence. At this moment therapy failure is identified by the elevation of the plasma viral load (Ballard et al. 1998, Condra 1998, Perrin & Amalio 1998, Geretti 2001).

The mutations found in the protease (PR) gene were L63P (46%); M36I (38.1%); V77I (21%); L10I e V82A (17.7%); I93L (11.4%); L10V (10.5%); K20R (7.9%); A71V, M46I and L90M (6.6%); L63H e I54V (5.3%) e L63S (3.9%). The mutations found in the reverse transcriptase (TR) gene were M184V (36.8%); M41L (27.6%); D67N (26.3%); K103N (23.6%); K70R (17.1%) and T215Y (11.8%).

Diaz in 2002 demonstrated that mutations in the 71, 63, 90, 36 and 46 codons of the protease gene and in the 184, 215, 219, 41 and 70 codons of the reverse transcriptase gene are frequently associated with resistance, in agreement with the results obtained here. In addition, Tanuri et al. (2002) showed that the presence of the M184V (48%); T69D (47%); T215Y/F (46%) and M41L (39%) in the reverse transcriptase gene and the L90M (26%) and double substitution in L90M and V82A (6%) in the protease gene have been associated with resistance emergence. In this context, agreement was observed with the data here obtained regarding the frequency of M184V (45%) and

M41L (27.6%). Other mutations found were T215Y (11.8%) and L90M (6.6%), however in a lower frequency than found by Tanuri et al. (2002).

This study observed that 58 samples (76%) presented susceptibility to all protease inhibitors, 18 (23%) showed resistance to all protease inhibitors, and 1% resistant to Nelfinavir.

As for the nucleoside reverse transcriptase inhibitors (NRTI), it was observed that 34 samples (45%) presented virus susceptible to all the drugs of this class, while 16 (21%) were resistant to all NRTI. The other fractions showed different resistance combinations to NRTI.

As for the non-nucleoside reverse transcriptase inhibitors (NNRTI), we observed that 39 samples (51%) presented susceptible virus to all NNRTI, 35 (46%) resistant to all drugs of this class and two (3%) resistant to Efavirenz (EFV) and Nevirapine (NVP).

Other studies have found similar results. Diaz et al. (2000) found a frequency from 80% resistance to the zidovudine (AZT) and didanosine (ddI) and less than 50% resistance for Saquinavir and Ritonavir (Diaz et al. 2000).

It is evident that there was a larger resistance rate regarding the reverse transcriptase inhibitors. This result may be due to the fact of this drug class was the first to be used in monotherapy (Sabino 2002, in publication). It is also observed in the studied patients, the resistance to all the protease and reverse transcriptase inhibitors, suggesting a cross-resistance: Abacavir is responsible for several mutations in the reverse transcriptase gene in vitro, including M184V, L65R, L74V and Y115F. Other inhibitors of the nucleosides reverse transcriptase inhibitors can be responsible for specific mutations that leads crossed resistance to Abacavir. The resistance to Efavirenz is more commonly associated with the mutation K103N and this leads to crossed resistance with Nevirapine and Delavirdine (Deeks et al. 1999).

In the case of AZT, several mutation combinations have been found in the 41, 67, 70, 210, 215 and 219 codons, which lead to resistance to AZT, estavudine (d4T), didanosine (ddI) and abacavir (Stanford 2005).

In the analysis of the frequency of the subtypes of the HIV-1 we observed that most analyzed samples (82.9%) presented subtype B virus; 5.3% subtype F and 11.8% recombinant forms (Table 1).

Table 1: HIV-1 Subtypes according reverse transcriptase and protease regions

Subtype according to Protease region	Subtype according to Reverse Transcriptase region		
	B	F	K
B	82.9%	0	1.3%
D	2.6%	1.3%	0
F	6.6%	5.3%	0

The results of this study show a larger incidence of the subtype B (82.9%), being concordant with other studies that evaluated the HIV-1 genetic diversity in Brazil. Diaz found a frequency of 83.87% of the subtype B in the São Paulo state (Diaz 2002), while Tanuri et al. (2002) verified a frequency of 76.7% of the same subtype in Rio de Janeiro.

Considering therapy failure due to resistance emergence, 23 patients were followed up until plasma viral load showed an increase equal or superior to 0.5 log (Group 5).

Due to the methodological limitations already discussed, enough data could only be obtained for the analysis of 16 patients.

From the 16 analyzed patients, four (25%) presented therapeutic effectiveness with the instituted antiretroviral therapy and they did not present any mutation associated with resistance to the drugs, which probably justifies the efficiency of the treatment.

However 12 patients (75%) were in therapeutic failure. Of these, seven (58.3%) presented mutations that led to resistance, most of which were in the area of the reverse transcriptase, which can be justified by the fact that reverse transcriptase inhibitors have been introduced first in the market in Brazil in 2000 and that the longest time of use of that class of drugs made possible the appearance of great amount of virus resistant to them. Added to that the fact that the reverse transcriptase gene is more extensive than that of the protease (Los Alamos 2005), increasing the probability of occurrence of mutations. Besides, a lot of mutations found were already present before the introduction of the therapy. Those discoveries can justify the lack of response of those patients to the instituted therapy. In the other patients the failure cannot be associated with resistance to drugs that shows the influence of other factors in the failure of antiretroviral therapy.

Although the results of this study show the relevance of the genotyping test to patient treatment, these tests should not be used as a main criterion for modification of antiretroviral therapy. Such a decision should be based on laboratory and clinical parameters, the change of therapy being supported by several parameters (Hirsh et al. 2000).

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