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Antiretroviral Drug Resistance in Brazilian Children Infected by Human Immunodeficiency Virus Type 1

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Abstract

The purpose is to determine the prevalence of drug-resistance mutations in HIV-1 infected children under antiretroviral treatment from Brazil. Blood samples from sixty one human immunodeficiency virus type 1 (HIV-1) vertically-infected Brazilian children are studied. DNA was extracted from the samples, and a 1.0 kb fragment containing HIV-1 PR and RT-coding sequence were amplified by Nested Polymerase Chain Reaction sequencing. The HIV-1 sequencing, based on the polymerase gene sequences (protease and reverse transcriptase regions), was as follows; subtype B (83.6%), subtype F (9.8%) and B/F viral recombinant forms (6.6%). Two major protease inhibitor-resistance associated mutations, M36I and L90M, were most prevalent in our samples (32.8%), as well as the polymorphism L63P (42.6%). Many mutations associated with reduced susceptibility to nucleoside or non-nucleoside reverse-transcriptase inhibitors were detected: M184V (42.6%), M41L (37.7%), D67N (26.2 %), T215Y (24.6%), L210W (21%). This study showed that 85.2% of the studied population showed evidence of therapy failure, with the presence of viral genomic mutations associated with drug resistance.

Keywords: PCR; HIV-1; HAART; Resistance

Introduction

Combination therapy with protease (PR) and reverse transcriptase (RT) inhibitors can efficiently suppress human immunodeficiency virus (HIV) replication, however drug resistance emergence can occur and variants correlate strongly with therapeutic failure [1]. The human immunodeficiency virus type 1 (HIV-1) shows remarkable genetic diversity with many implications in pathogenesis, vaccine development, diagnosis, antiretroviral therapy (ARV) and drug susceptibility [2-4].

The development of HIV-1 resistance to antiretroviral drugs is considered to be a major contributing factor to the loss of plasma HIV-1 RNA suppression [5,6], limits options for alternative antiretroviral regimens and is the primary reason for a highly active antiretroviral therapy (HAART) failure over time [7,8].

The emergence of HIV-1 drug-resistance within treated patients is attributed to viral replication errors due to the fast replication rate. On an average, 10 billion HIV particles are produced everyday and, associated with the highly error prone nature of the viral reverse transcriptase (RT), at least one new mutation can be introduced in each new virus genome [9]. The selection of drug-resistant viruses during the therapy, associated with other factors such as, adherence, drug pharmacological factors, and host immune response pressure, also contribute to the evolution of HIV-1 drug-resistance in infected patients [10].

HIV-1 genetic forms may have variable biological properties and display different geographical prevalence, justifying the need for regional characterization and surveillance. The study of HIV-1 genetic variability in distinct regions of the world is also important for the design of HIV vaccines in the future, which may include the circulating subtypes [11]. Recent data indicate that viral subtypes may influence immune responses and the effectiveness of antiretroviral treatment [12,13].

Nevertheless, the selection of viral resistant strains is a major problem for the medical management of infected individuals and accounts for the transmission of these variants to non-infected people. This represents an important public health problem, particularly in areas where antiretroviral drugs have been widely used for many years [14,15].

The Brazilian Ministry of Health has been sponsoring free access to HIV treatment for AIDS patients since 1996 [16,17], supplying antiretroviral drugs to the majority of HIV-1-infected patients. To date, highly active antiretroviral therapy (HAART) options for the management of HIV-1 disease include six nucleoside and one nucleotide reverse transcriptase inhibitors (NRTIs), three non-nucleoside reverse transcriptase inhibitors (NNRTIs) and seven protease inhibitors (PI).

Drug resistance mutations to all of these inhibitors have been identified even in previously non-treated patients [15,18-20]. Experience based on clinical trials has led to the suggestion that HIV genotyping resistance determinations are of increasing diagnostic value in a number of clinical settings, including monitoring of drug resistance prior to therapy initiation or at the time of therapy failure [20]. Drug resistance mutations, identified at the time of treatment failure, have been associated with a poor response of the patient [5].

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To identify HIV-1 isolates resistant to multiple antiretroviral agents, we examined isolates from sixty one children with HIV-1 infection receiving HAART. Here, we report the development of a genotype resistance assay that can determine the susceptibility of HIV-1 to both Reverse Transcriptase and Protease inhibitors. The purpose of this study was the early detection of HIV-1 antiretroviral resistance mutants and to guide physicians in therapeutic management of HIV-1 in these infected patients failing HAART.

Materials and Methods

Subjects

Sixty one vertically HIV-1-infected children were included in this study; these patients were being followed at the Immunodeficiency Clinic at The State University of Campinas, São Paulo, Brazil. Entry criteria established an HIV-1 viral load of higher than 10,000 copies/ml of virus in plasma and at least 6 months in HAART. These patients were studied to evaluate resistance mutations that complicate treatment. Fifty seven had received HAART regimens previously, and just four had received their first HAART regimen. Informed consent was obtained from all patients or guardians and the protocol was approved by the Hospital's Ethics Committee. The periods of treatment during which the sample was obtained from each child are listed in table 1. The average HAART duration was 5 years and the antiretrovirals most used were zidovudine, lamivudine and nelfinavir. Thirteen children achieved an undetectable viral load, while the remainder never achieved this. Viral load, CD4 and CD8 count are listed in tables 2 and 3.

Blood samples and DNA extraction: Peripheral blood mononuclear cells (PBMC) proviral DNA was extracted virus. The proviral DNA were extracted with phenol/chloroform and then precipitated in ethanol. The erythrocyte lysis was followed by PBMC lysis. The sample was transferred for a tube containing 400 µl of extraction buffer (Tris-HCl [10 mM, pH 7.6], KCl [10 mM], $MgCl_2$ [10 mM], NaCl [0.4 M], EDTA [2 mM]) and 25 µl of sodium dodecyl sulfate (10%), and incubated at 55°C for 30 min. The supernatant was then purified by phenol-chloroform isoamilic alcohol (24:1) followed by purification with phenol-chloroform. DNA was precipitated with ethanol, resuspended in 25 µl of distilled water and stored at -20°C until use.

Polymerase Chain Reaction (PCR) conditions: Two different HIV-1 genomic regions were targeted for polymerase chain reaction (PCR) amplification: RT (reverse transcriptase) and PR (protease). PCR HIV-1-PCR was performed during two stages of the reaction: the first one with pairs of outer primers (5′ TAACTCCCTCTCAGAAGCAGGAGCCG 3′/5′ TAGGCTGTACTGTCCATTTAT 3′) the second one with inner primers (5′ CCTCAAATCACTCTTTGGCAAC 3′/5′ ATCAGGATGGAGTTCATAACCCATCCA 3'). Full length PR gene and a fragment corresponding to codons 1 to 219 of the RT gene were amplified at the end of the investigated amplicon sequence, which harbors most of the known resistance mutations to licensed antiretroviral drugs. The mixture for each reaction contained 0.5 µl of DNA, 50 mM of KCl; 10 mM of Tris-HCl (pH=8.4); 4.0 mM of $MgCl_2$; 2.0 pmol of each primer (DP10: 5′ TAACTCCCTCTCAGAAGCAGGAGCCG 3′/LR54: 5′ TAGGCTGTACTGTCCATTTAT 3′); 200 mM of the deoxyribonucleic mixture-dNTPs (dATP, dGTP, dCTP, dTTP) (GIBCO-BRL); 1.0 U of *Taq* DNA Polymerase (GIBCO-BRL). The target DNA was amplified using a Thermocycler PTC-100 Programmable Thermal Controller, MJ Research (Research Products). The reaction was initially performed using 3 cycles at 95°C for 3 minutes, 55°C and 72°C for 1 minute, followed by 35 cycles of amplification, each cycle having the following conditions: 95°C for 1 minute; 55°C for 45 seconds, 72°C; after the 35th cycle. A final cycle of 72°C was employed. N-PCR-The product from the first PCR (0.5 µl) was then transferred to a second test tube containing 50 µl of a product, same conditions of reaction described. The sample was amplified according to the first reaction, but utilizing a pair of inner primers to amplify 1.0kb. The product of PCR was cleaned with a Quiagen PCR Purification Kit.

Total of children	Ages (years)	Sex (%)		ACTG 076 (%)		Adherence (%)		Period of time under treatment (years)	Stage of disease (%)							
		Male	Female	No	Yes	No	Yes		A1	A2	A3	B2	B3	C1	C2	C3
61	7.5	61	39.3	93	6.8	72	28.5	5 years	3.3	6.6	3.3	26.2	9.8	1.6	29.5	49

ARV Drugs in current regimen									
ZDV	3TC	DDI	DDC	TDF	NFV	RTV	RTV+LOP	NVP	EFV
73.7	50.8	19	26.2	1.6	62.2	11.4	8.1	1.6	13.1

Table 1: Demographic and Clinical information about children.

Children	Exam´s moment value	Lowest value	Highest value	Value before ARV drugs
	CD4 CD8 REL	CD4 CD8 Rel	CD4 CD8 Rel	CD4 CD8
61	606.5 1275.5 0.52	381.9 1010.4 0.04	1352 1925 0.75	969 1914

Table 2: CD4, CD8 counts.

Medium (aproximately)			
Lowest value	Highest value	Exam´s moment value	Value before ARV drugs
Copy/ml Log	Copy/ml Log	Copy/ml Log	Copy/ml Log
5.008 2.44	1.108.411 6.25	84814 4.14	989.633 5.29

Table 3: Viral load counts.

RT and PR gene sequencing: The HIV-1 reverse transcriptase (RT) and Protease (PR) genes were determined automatically by the dideoxy chain termination method (5′ TGTGGTATTCCTAATTGAACTTCCC 3′/5′ CAATGGCCATTGACAGAAGA 3′), in a Megabace1000 Sequence Analyzer (Amersham Pharmacia Biotech), according to manufacturer's instructions, following Sanger's method. Sequence editing and alignments were performed using the ChromasPro® Program. Drug resistance mutation analyses of RT and PR genes were determined using the HIVdb program at the HIV RT and PR Sequence Database [21] (http://hivdb.stanford.edu/pages/algs/HIVdb.html). For subtyping analysis, samples were aligned based on software against a reference sequence set from the Los Alamos database (ClustalW, http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html). This is a computerized rule-based algorithm that provides the similarity of user-submitted sequences with the closest subtype reference sequence and classifies the virus to each anti-retroviral agent. In the PR gene, resistance mutations were classified as major or minor, according to recommendations of IASUSA [22]. Minor manual adjustments were made to improve the alignments.

Statistical analysis: The prevalence of HIV resistance mutations and HIV clades was estimated as the proportion of samples successfully tested with specific resistance mutations or different clades. The results were expressed in percentage and proportion (positive samples/total samples). The significance threshold followed was 5% ($p \leq 0.05$).

Results

We analyzed genotyping tests from the proviral DNA of 61 vertically-infected children from the Immunodeficiency Clinic at the State University of Campinas, São Paulo, Brazil. All samples from 61 patients (60.6% males) were successfully amplified in the PR and RT regions of the HIV-1 DNA. In our study, 85.2% of the HIV-1 infected studied children, who are under anti-retroviral treatment and with viral load counts of closer to 10.000 copies/ml, presented viral genomic mutations, associated at least with one antiretroviral resistance.

The region of HIV-1 genes selected for amplification through the Nested-PCR, presented one fragment of 1.0 kb. The HIV-1 gene *pol*, whose region is more susceptible to mutations, was detected by Nested-PCR in patients being followed at the Immunodeficiency Clinic at the State University of Campinas, São Paulo, Brazil.

Based on the genotyping database, 51 (83.6%) samples were classified as subtype B, followed by six (9.8%) viral recombinant forms between subtypes B and F and four (6.6%) were subtype F. The antiretrovirals current used in regimen are described in table 1.

Only one sample (1.6%) did not show any mutation associated with ARV resistance. The frequency of ARV mutations in the RT gene of HIV-1 positive children was: M184V (42.6%), M41L (37.7%), D67N (26.2%), T215Y (24.6%), L210W (21%), K70R (16.4%) and E44D (11.5%), which are associated with NRTIs resistance and K103N (6.6%), which is associated with NNRTIs resistance. The most prevalent mutations associated with IP were: L63P (42.6%), M36I (32.8%), L90M (32.8%), V77I (29.5%), I93L (23%), V82A (14.8%), I54V (14.8%) and K20R (13.1%). The frequencies of all mutations associated with ARV drugs are listed in figures 1 and 2, divided by ARV categories (NRTIs/NNRTIs and PI).

For PR sequences, at least two major mutations, M36I (32.8%) and L90M (32.8%), were detected in 20 sequences. In addition to the resistance-associated mutations found within the PR and RT regions,

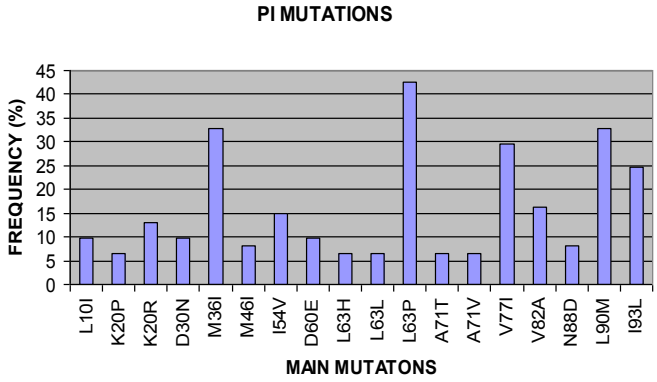

Figure 1: Prevalence of resistance-associated main mutations in the Protease gene.

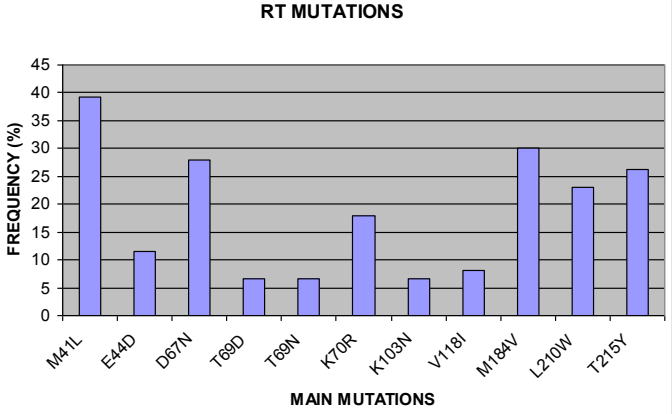

Figure 2: Prevalence of resistance-associated main mutations in Reverse Transcriptase gene.

many other polymorphisms were detected. In the PR gene, the most frequent polymorphism occurred at codon L63P (42.6%), which is associated with resistance to PIs when present with other mutations [23].

Analysis revealed association between some mutations and previous exposure to ARV drugs. M184V mutation were associated with previous exposure to Lamivudine and Didanosine, T215Y/F/S/N mutations were related to a prior use of estavudine and didanosine [18].

The NRTI antiretrovirals most used were zidovudine, lamivudine and didanosine. Zidovudine was associated with the M41L mutation (37.7%) and T215Y (24.6%). M184L, that was commonly found (42.6%), correlated with lamivudine resistance. Another mutation found was D67N, which was related to resistance to all NRTI. The PI antiretroviral most used in children was nelfinavir, which was related to mutations M36I and L90M. Found in 32.8% of individuals, L90M causes resistance to saquinavir and nelfinavir [8]. The M36I mutation is associated with the resistance to each of the PIs when presented with other mutations and was present in 32.8% of the sequences.

In total, 85.2% of HIV-1 infected patients presented viral genomic mutations associated with drug resistance. Mutations associated with PI resistance were found in 78.7% of the viral sequences and those associated with RTI resistance represented 72.1%. Among these sequences containing RTI resistance-associated mutations, 72.1% were associated with NRTI resistance and 21.3% with NNRTI resistance.

The inferred levels of resistance were deduced from the amino acid sequences. For each sample, the PR and RT regions were classified in to one of the following levels of drug resistance: susceptible, intermediate resistance and resistant. Table 4 show NRTI/NNRTI and IP mutations and the type of resistance occurred in patients. Of the 61 samples, 85.2% showed resistance to at least one of the antiretrovirals analyzed. The drugs used in treatment are zidovudine (73.7%), lamivudine (50.8%), didanosine (19%), zalcitabine (26.2%), tenofovir (1.6%), nelfinavir (62.2%), ritonavir (11.4%). Ritonavir associated with lopinavir (8.1%), nevirapine (1.6%), efavirenz (13.1%).

Discussion

Human immunodeficiency virus type 1 (HIV-1) drug resistance arises from mutations in the viral genome, specifically in the regions that encode the molecular targets of therapy [24], in such a way that the protease (PR) and reverse transcriptase (RT) enzymes are no longer inhibited by drugs, allowing the virus to replicate freely [20].

Studies performed in developing countries and, more recently, in Brazil have demonstrated substantial benefits from ARV treatment in terms of survival and quality of life for patients with AIDS [25,26].

Patient	Mutation IP	Mutation NRTI/ NNRTI	ARV	Susceptible	Low-level resistance	Intermediate resistance	High-level resistance
1	D30N, N88D, K20I, L63LP, V77I	M41L, E44D, D67N, V118IV, T215Y, K103N, Y181C	AZT,DLV, EFV, NVP, TDF, DDI, D4T, NFV	APV, IDV,LPV,RTV	ATV,SQV,3TC, FTC,	ABC,D4T, DDI, TDF	NFV, AZT,DLV,EFV,NVP
2	N88D	E44D, D67N, K70R, M184V,	AZT,3TC, DDI, NFV, RTV, LPV,	APV, IDV,LPV,RTV, TDF, DLV, EFV, NVP	ATV, NFV, SQV, ABC, AZT, D4T, DDI,		3TC, FTC
3	L10I, L33V	NONE	NFV, AZT, DDI, D4T, NFV	APV, ATV, IDV, LPV, NFV, RTV, 3TC, ABC, AZT, D4T, DDI, FTC, TDF, DLV, EFV, NVP			
4	V32I, L10V, K20T, L63H, A71T	M41L, L210W, T215Y, K101E, K103R, G190A	EFV, DDI, D4T, NFV, EFV, KALETRA,TDF, 3TC, RTV, LPV		APV, ATV, IDV, LPV, NFV, RTV, SQV, 3TC,FTC, DLV,	ABC,D4T, DDI, TDF, EFV	NVP, AZT,
5	NONE	L210W	DDI,D4T,RTV,AZT, RTV, LPV, RTV	APV, ATV, IDV, LPV, NFV, RTV, SQV, 3TC, FTC,DLV, EFV, NVP	ABC, AZT, D4T, DDI, TDF,		
6	V77I	NONE	AZT, DDI, 3TC, NFV	APV,ATV, IDV, LPV, NFV, RTV, SQV, 3TC, ABC, AZT, D4T, DDI, FTC, TDF, DLV, EFV, NVP			
7	M46I, G73C, L90M, L63P, V77I	M41L, E44D, D67N, L74I,V118I, T215Y, V106I, V108I	AZT, DDI, NFV, D4T, RTV, 3TC, LPV, RTV, EFV, TDF, ABC, APV, TDF		LPV, 3TC, FTC, DLV, EFV, NVP	APV, ATV, IDV, RTV, SQV, D4T, TDF	NFV, AZT, ABC, DDI
8	M46L, I54V, V82A, A71I	A62V, M184V, K103S, V106M	AZT, 3TC, RTV, NFV, D4T, EFV, DDI,	AZT, D4T, TDF	ABC, DDI,	APV, ATV, LPV, SQV,	IDV, NFV, RTV, DLV, EFV, NVP, 3TC,FTC
9	L63H	M41L, D67E,	AZT, DDI, 3TC, EFV, NFV	APV,ATV, IDV, LPV, NFV, RTV, SQV, 3TC, FTC, DLV, EFV, NVP	ABC, D4T, DDI, TDF	AZT	
10	L90M, L63P, A71T,	NONE	AZT, DDI, NFV, SQV	3TC, ABC, AZT, D4T, DDI, FTC, TDF, DLV, EFV, NVP	APV, ATV, IDV, LPV, RTV	NFV, SQV	
11	L63P, V77I, I93I/L,	NONE	AZT, 3TC, RTV, NFV	APV, ATV, IDV, LPV, NFV, RTV, SQV, 3TC, ABC, AZT, D4T, DDI, FTC, TDF, DLV, EFV, NVP			
12	D30N, N88D, L90M, M36I, L63P	M41L, M184V, L210W, T215Y	AZT, DDI, 3TC, RTV, NFV, LPV, EFV,	DLV, EFV, NVP	APV, IDV, LPV, RTV,	ATV, SQV, ABC, AZT, D4T, DDI, TDF	NFV, 3TC, FTC
13	L63P, I93L	NONE	AZT, DDI, D4T, 3TC, NFV, EFV	APV, ATV, IDV, LPV, NFV, RTV, SQV, 3TC, ABC, AZT, D4T, DDI, FTC, TDF, DLV, EFV, NVP			
14	M36I, L63L/P	NONE	AZT, DDI, 3TC, RTV, D4T, NFV, LPV	APV, ATV, IDV, LPV, NFV, RTV, SQV, 3TC, ABC, AZT, D4T, DDI, FTC, TDF, DLV, EFV, NVP			
15	L63P, V77I, I93L	M41L, D67N, K70R, T215F, K219Q	AZT, DDI	APV, ATV, IDV, LPV, NFV, RTV, SQV, 3TC, FTC, DLV, EFV, NVP		ABC, DDI,TDF,	D4T, AZT,

16	NONE	T69I/N/S/T, K70R	AZT, DDI, RTV	3TC, ABC,FTC, TDF, DLV, EFV, NVP	AZT, D4T, DDI		
17	NONE	M41L, T215N/S/T/Y	AZT, DDI, NFV	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, FTC, DLV, EFV, NVP		ABC, AZT, D4T, DDI, TDF	
18	D30N,M36I,V77I	M41L,M184V	AZT, DDI, 3TC, RTV, NFV, EFV, LPV	APV, ATV, IDV, LPV, RTV, SQV, AZT, TDF, DLV, EFV, NVP	ABC, D4T, DDI, TDF	NFV,	3TC, FTC,
19	M36I, D60E, L63P	NONE	AZT, DDI, NFV, 3TC, EFV, RTV,	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
20	L63P, I93L	NONE	AZT,DDI, D4T, NFV	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
21	D30N, N88D, K20R,M36I, D60E	M41L	AZT, DDI, NFV, D4T, 3TC, LPV	APV, IDV, LPV, RTV, 3TC, FTC, DLV, EFV, NVP	ATV, SQV, ABC, AZT, D4T, DDI, TDF		NFV
22	N88S, K20T, M36I	M41L, V75M, M184V, L210W, A98G, Y181C	AZT, DDI, 3TC, NFV, D4T, NVP	APV, LPV, RTV, SQV,	IDV, AZT, TDF, EFV	ATV, NFV, ABC, D4T, DDI,	3TC, FTC, DLV, NVP
23	L63T, I93L	NONE	AZT, DDI, 3TC, NFV, LPV, RTV	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
24	K20R	T215Y, K103N	AZT, DDI, NFV, 3TC, LPV, RTV, D4T, EFV	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC,FTC	ABC, D4T, DDI, TDF	AZT	DLV, EFV, NVP
25	M36I, D60E, V77I	M41L, E44D/E, D67N, L210W, V106I	AZT, DDI, 3TC, NFV, D4T	APV, ATV, IDV, LPV, NFV, RTV, SQV,DLV, NVP, EFV	3TC, FTC	ABC,AZT, D4T, DDI, TDF,	
26	L10I, L63P, I93L	NONE	AZT, DDI, NFV	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
27	NONE	NONE	AZT, DDI, 3TC	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
28	V32I, M46I, I54V, V82A, L90M, L33M, L63P	D67N, T69N, K70R, M184V, K101E, K103N	AZT, DDI, EFV, 3TC, ABC, D4T, LPV, RTV, NFV, TDF		ABC, D4T, DDI,TDF	AZT	APV, ATV, IDV, LPV, NFV, RTV, SQV, 3TC, FTC, DLV, EFV, NVP
29	M46I, L90M, L63P, A71I, V77I	M184V, T215Y	AZT, DDI, 3TC, EFV, NFV	DLV, EFV, NVP,	LPV, AZT, D4T, TDF	APV, ATV, IDV, RTV, SQV, ABC, DDI	NFV, 3TC, FTC,
30	M36I, L63P, V77I,	NONE	AZT, DDI, 3TC, D4T, NFV	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
31	M46L, I54V, V82A, L90M, L10I, K20R, M36I, L63T, A71V, I93L		AZT, RTV, DDI, D4T, 3TC, LPV	DLV, EFV, NVP	3TC, ABC, FTC, TDF	AZT, D4T, DDI,	ATV, IDV, LPV, NFV, RTV, SQV,
32	G73A, L90M, L63P, V77I, I93L	D67N, K70R, V118I, M184V	AZT, DDI, NFV	DLV,EFV,NVP	APV, ATV, IDV, LPV, RTV, ABC, AZT, D4T, DDI, TDF	SQV,	3TC, FTC
33	M36I, L63S	NONE	AZT, DDI, 3TC, NFV, EFV, NVP, D4T	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
34	V771,	M184V, L210W	AZT, DDI, 3TC, RTV, NFV,EFV	APV, ATV, IDV, LPV, NFV, RTV, SQV,AZT, TDF, DLV, EFV, NVP	ABC, D4T, DDI,		3TC, FTC

35	V77I	NONE	AZT, DDI, NFV, 3TC	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
36	I54V, V82M, L90M, K20R, M36I, D60E	M41L,E44A, D67N, V118I, L210W, T215Y, K219N, K103N	AZT, DDI, 3TC, RTV, NFV, EFV, LPV, RTV,		FTC	APV, ATV, IDV, LPV, RTV, SQV, TDF, DLV, EFV,NVP	NFV,ABC, AZT, D4T, DDI, DLV, EFV, NVP
37	L90M, M36I, D60E, V77I	M41L,E44D, D67N, T69D, L210W, T215Y	AZT, DDI, 3TC, D4T, NFV, RTV,	DLV, NVP, EFV,	APV, ATV, IDV, LPV, RTV, SQV,3TC, FTC	NFV, ABC,TDF	AZT, DDI, D4T
38	L63L/P, V77I	D67N, K70R	AZT, DDI, 3TC, NFV, D4T	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, DDI, FTC, DLV, EFV,NVP	D4T, TDF	AZT,	
39	D30N, N88D, K20K/M, L63P, I93L	A62V, T69N/T, V75I, F77L, F116Y, Q151M	DDI, NFV, D4T, AZT, ABC	APV, IDV, LPV, RTV, DLV, EFV, NVP	ATV, SQV, ABC, AZT, D4T, DDI, TDF	3TC, FTC, TDF,	ABC, AZT, D4T, DDI, NFV,
40	L10I, I93L	NONE	AZT, DDI, NFV, EFV	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
41	L90L/M, M36I	M41L/M, M184V	AZT, DDI, 3TC, RTV, NFV, EFV, D4T, LPV	AZT, TDF, DLV, EFV, NVP	APV, ATV, IDV, LPV, RTV, ABC, D4T, DDI,	NFV, SQV,	3TC, FTC
42	L90M, K20I, M36V, L63H	M184V, L210W	AZT, DDI, 3TC, NFV	AZT, TDF, DLV, EFV, NVP	APV, ATV, IDV, LPV, RTV, ABC, D4T, DDI,	NFV, SQV,	3TC, FTC
43	D30N, M46I, L63A, A71A/T, V77I	D67N, L210W, T215Y, Y188N/Y	AZT, DDI,NFV, ABC, 3TC, LPV, RTV, NVP	SQV, 3TC, FTC,	APV, ATV, IDV, LPV,RTV, DLV, EFV,NVP	ABC, D4T, DDI, TDF	NFV, AZT, ABC, DDI
44	V32I, L90M, K20T, M36I, D60E, A71V, V77I, I93F/I/L/V	M41L, K70R	AZT, DDI, NFV	3TC, FTC, DLV, EFV, NVP	LPV, ABC, D4T, DDI, TDF	APV, ATV, IDV, RTV,SQV, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP	NFV
45	L63P	T69N, F116Y, Q151L/M	DDI, D4T, NFV	APV, ATV, IDV, LPV, NFV, RTV, SQV, DLV, EFV,NVP	3TC, FTC, TDF,	ABC	AZT, DDI, D4T
46	V82A, K20R, M36I	M41L, T215Y, K101K/T	AZT, DDI, 3TC, RTV, NFV, EFV	DLV.EFV.NVP, 3TC, FTC	APV. ATV, LPV, SQV,	IDV, NFV, RTV, ABC, AZT, D4T, DDI,TDF, DLV, EFV,NVP	
47	V82M, L90M, M36I, L63P	E44A/E, D67N, T69D	RTV, AZT, 3TC, DDI	DLV, EFV, NVP	APV, LPV, ABC, AZT, D4T, FTC, TDF,3TC	ATV, IDV, RTV, SQV,DDI	NFV
48	I54V, V82A, L63P	M41L, D67E, T69S/G	AZT, 3TC, NFV, RTV, DDI, LPV	DLV, EFV, NVP	APV, SQV, 3TC, FTC,	ATV, IDV, LPV, NFV, RTV,ABC,D4T,TDF, DLV, EFV,NVP	AZT, DDI, D4T
49	L90M, L10I/L, K20K/R, M36I/M	M184V	AZT, DDI, 3TC, RTV, EFV, LPV	AZT, D4T, TDF, DLV, EFV, NVP	APV, ATV, IDV, LPV, RTV,	NFV, SQV,	3TC, FTC
50	L90M, K20T, M36I, L63L/P, A71V	M41L, T69A, M184V, T215Y	AZT, 3TC, RTV, NFV	DLV, NVP, EFV	APV, ATV, IDV, LPV,RTV, SQV,TDF	NFV, ABC, AZT, D4T, DDI	FTC, 3TC
51	M361	NONE	AZT, DDI 3TC, EFV, NVP	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
52	V77I	M41L, D67N, K70R, M184V, T215Y, K219Q	AZT, DDI, 3TC, NFV, LPV, RTV, EFV	APV, ATV, IDV, LPV, NFV, RTV, SQV, DLV, EFV,NVP		ABC, DDI, TDF,	3TC, AZT, D4T, FTC
53	V82A, L90M, L10V, K20M, L33I, M36I, L63P, I93L	M184V	AZT, DDI, 3TC, RTV, NFV	AZT, D4T, TDF, DLV, NVP, EFV	ABC, DDI,	APV, ATV,LPV, SQV,	IDV, NFV, RTV, 3TC, FTC
54	L10I, L63P, V77I, I93L	M184V	AZT, DDI, 3TC	APV, ATV, IDV, LPV, NFV, RTV, SQV,AZT, D4T,TDF, DLV, EFV,NVP	ABC, DDI,		3TC, FTC
55	I54V, V82A, L90M,D60E, L63P, A71T	A62A/V, T69S/G, K70R, M184V	AZT, DDI, NFV, RTV, D4T, TDF, LPV, SQV	DLV, EFV, NVP		APV, ATV, LPV, ABC, AZT, D4T, DDI, TDF	IDV, NFV, RTV, SQV, 3TC, FTC

56	L90M/L	M41L, E44D, D67N, T69D, V118I, L210W, T215X	AZT, DDI, RTV, NFV, 3TC	DLV, EFV, NVP	APV, ATV, IDV, LPV, RTV, 3TC,FTC,	NFV, SQV, ABC, TDF	AZT, DDI, D4T
57	NONE	T69A	AZT, DDI, NFV	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, ABC, AZT, D4T, DDI, FTC,TDF, DLV, EFV,NVP			
58	L33F, M46I, I50V, I54V, K20R, M36I, L63P, A71V	M41L, D67N, K70R, V75M, M184V, L210W, T215Y, V179D, G190Q	AZT, DDI, 3TC, D4T, NFV, RTV, EFV, LPV		DLV	ATV, SQV,TDF	APV, IDV, LPV, NFV, RTV,3TC, ABC, AZT, D4T, DDI, FTC, EFV,NVP
59	L33F,I54V, V82A, L90M, L10V, M36L, L63H	M41L, L210W, A98G, K101E, G190A	AZT, ETC, DDI, NFV, RTV, D4T, EFV	3TC, FTC,	ABC, DDI, TDF, DLV,	APV, ATV, LPV, AZT, D4T,EFV	IDV, NFV, RTV, SQV,NVP
60	I54V, V82A, K20R, M36I, L63A	T215Y	AZT, DDI, 3TC, RTV, NVP, LPV	DLV, EFV, NVP, 3TC, FTC,	APV, SQV, ABC, D4T, DDI, TDF	ATV, IDV, LPV, NFV, RTV, AZT	
61	L63P, V77I, I93L	NONE	AZT, DDI, RTV, NFV, 3TC, ABC, EFV	APV, ATV, IDV, LPV, NFV, RTV, SQV,3TC, AZT,ABC, D4T, DDI, FTC, TDF, DLV, EFV, NVP			

Table 4: Mutations (NRTI/ NNRTI and IP) and the type of resistance occurred in patients.

However, the development of drug resistance remains the most serious obstacle to maintain the suppression of HIV replication by in HAART therapy infected patients [27].

Several clinical trials have shown a significant correlation between drug resistance and virologic response to a new treatment regimen when prior therapy has failed [5,28,29]. The analysis of an association between previous exposure to antiretroviral drugs and resistance associated mutations shows the impact of the use of these drugs in HIV-1 drug resistance [30].

Brazil is a huge country and differences in the patterns of HIV-1 subtype distributions have been identified among the geographic regions. HIV-1 subtype B is the most prevalent in our country, followed by subtype F [17,31-34]. One Brazilian research group demonstrated that almost 92% of the virus circulating in the state belongs to subtype B, suggesting the maintenance of a higher prevalence of HIV-1 subtype B, even with the introduction of other non-B subtypes [35]. In our study, we found 83.6% of viruses belonged to subtype B. Our results reflect the subtype distributions previously observed in other Brazilian regions, since subtype B was the most prevalent. In 51 samples (83.6%), six samples (9.8%) appear to represent recombinant forms of subtypes B and F, a finding also previously described in Brazil [36,37]. The remaining four samples (6.6%) were of subtype F.

In the group receiving antiretroviral therapy, some RT gene mutations were reported to occur at codons M41L, D67N, T215Y, L210W and K70R. Mutations at positions 41, 67, 70, 210, 215 and 219, named nucleotide excisions mutations (NEMs) are associated with clinical resistance to all NRTIs, with the probable exception of lamivudine [38]. Analyzing the resistance-associated mutations to RT inhibitors in our sequence samples, we observed that the M41L mutation was the most prevalent among B and non-B subtypes. The association detected between mutations and exposures to specific drugs were statistically significant: prior use of zidovudine was associated with M41L mutation. This substitution, associated with another mutation, T215Y, confers intermediate-to-high level resistance to zidovudine and stavudine [18]. Patients that have received zidovudine as monotherapy or as a combination therapy with nucleoside analogues for more than one year, are at risk of selecting variants with the zidovudine resistance mutations at RT codon 215 or at RT codons 215 and 41 [7,39-43]. In our study, twelve patients presented the RT codon 41 mutation, associated with the RT codon 215 mutation. The presence of zidovudine resistance mutations at both RT codons 215 and 41 also conferred an increased risk of progression of the disease and death [44,45]. In our study, one patient with this mutation died.

The M184V mutation was found in a high proportion in our patients and is associated with early virological failure during ARV therapy, concomitant with lamivudine [46], in patients receiving monotherapy with this drug [47].The frequency of the mutation is in agreement with those described for HIV patients failing HAART [48]. This drug is largely used in Brazil due to its practibility and low toxicity [49].

Another common mutation found was D67N (26.2%), which contributes to some degree of resistance to each of the NRTIs, probable resistance to zidovudine, and not to resistance to lamivudine [18].

Analyzing the genotyping resistance profile of the protease-associated mutation, L63P (42.6%) was the most prevalent in our study and is associated with resistance to PIs [18]. Position 63 is the most polymorphic codon of the PR, which may explain this result [21].

Other PR mutations were observed at high frequencies, these included M36I, L90M, V77I and I93L. The L90M mutation causes resistance to saquinavir and nelfinavir, and when presented with other mutations also compromise the activity of another drug [8]. The M36I mutation is associated with resistance to each of the PIs when presented with other mutations; this profile highlights the concern regarding the management of PIs. The mutation described, V77I, is presented in our study and is a common polymorphism, associated with resistance to nelfinavir.

The higher prevalence of resistance mutations in the PR region, when compared with RT, could be associated with the extensive use of PI for the treatment of infected patients in Campinas [50].

One study demonstrated that the mutations, I54V, V82A, D30N, and N88D, were presented in children receiving combination

antiretroviral therapy who have undetectable loads [51,52]. Another study with PI-naive-infected children of 2 to 17 years in age, showed that 84% had NRTI mutations-codons 215 (66%), 41 (42%), 67 (37%), 210 (33%) and 70 (32%) [53].

In summary, all mutations detected by resistance genotyping analysis were related to the ARV therapy prescribed and 85.2% of the HIV-1 infected children failing therapy presented several mutations in the viral genome, associated with drug resistance.

The knowledge of resistance profiles of virus strains from each locality may be helpful to guide the treatment of HIV-infected children, reducing effective public health costs. This is particularly important for São Paulo, where we treated 145 HIV-1 positive children and the antiretroviral drugs were first made available and the patients of this site have probably been exposed to anti-HIV therapy for longer than individuals from other sites. The selection and circulation of drug-resistant viruses may represent a serious public health problem and may compromise the entire Brazilian AIDS program for the control of HIV transmission and AIDS morbidity/mortality by ARV therapy. Moreover, these drug resistant strains may be transmitted to non-infected individuals, contributing to spread of resistance among the Brazilian population [5,14,15,17]. The maintenance of resistance genotyping programs, supplied for HIV-1 failing patients, is important for the management of HAART therapies and changes over time.

Conflict of Interest

No conflict of interest to declare. This study was approved by the Research Ethics Committee of the Faculty of Medical Science of the State University of Campinas-UNICAMP.

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