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ANALYSIS OF HIV- TYPE 1 PROTEASE AND REVERSE TRANSCRIPTASE IN BRAZILIAN CHILDREN FAILING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)*

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SUMMARY

The aim of this study was to evaluate the genotypic resistance profiles of HIV-1 in children failing highly active antiretroviral therapy (HAART). Forty-one children (median age = 67 months) receiving HAART were submitted to genotypic testing when virological failure was detected. cDNA was extracted from PBMCs and amplified by nested PCR for the reverse transcriptase and protease regions of the *pol* gene. Drug resistance genotypes were determined from DNA sequencing. According to the genotypic analysis, 12/36 (33.3%) and 6/36 (16.6%) children showed resistance and possible resistance, respectively, to ZDV; 5/36 (14%) and 4/36 (11.1%), respectively, showed resistance and possible resistance to ddI; 4/36 (11.1%) showed resistance to 3TC and D4T; and 3/36 (8.3%) showed resistance to Abacavir. A high percentage (54%) of children exhibited mutations conferring resistance to NNRTI class drugs. Respective rates of resistance and possible resistance to PIs were: RTV (12.2%, 7.3%); APV (2.4%, 12.1%); SQV(0%, 12.1%); IDV (14.6%, 4.9%), NFV (22%, 4.9%), LPV/RTV (2.4%, 12.1%). Overall, 37/41 (90%) children exhibited virus with mutations related to drug resistance, while 9% exhibited resistance to all three antiretroviral drug classes.

KEYWORDS: HIV resistance; Antiretroviral therapy; Children; Treatment failure.

Abreviations used in the paper: HAART: Highly Active Antiretroviral Therapy: ZDV: Zidovudine; 3TC: Lamivudine; D4T: Stavudine; ABC: Abacavir; NVP: Nevirapine; EFV: Efavirenz; NFV: Nelfinavir; RTV: Ritonavir; SQV: Saquinavir; IDV: Indinavir; LPV: Lopinavir/RTV; APV: Amprenavir; RT: Reverse transcriptase; Pt: Protease; NRTI: Nucleoside analogue reverse transcriptase inhibitor; NNRTI: Non-Nucleoside analogue reverse transcriptase inhibitor; PI: Protease inhibitor; PBMCs: Peripheral blood mononuclear cells.

INTRODUCTION

The main goal of antiretroviral therapy is to suppress HIV replication to the greatest extent for as long as possible. Combination drug protocols for effective viral suppression require multiple daily doses of three or more medications over an indefinite period (HAART). Despite some progress in antiretroviral therapy, the lack of adequate pediatric formulations for certain drugs, together with maintaining the treatment regimen, have created a challenging situation for pediatricians providing treatment to infants and young children.

In Brazil, antiretroviral drugs are provided free of cost by the health system to HIV-1 infected patients. The use of the three- drugs combination therapy with children was begun in 1997 at our clinical practice, and although we noted prolonged survival of the pediatric population[6,10], we encountered other serious issues such as drug resistance. The emergence of HIV resistant strains during antiretroviral therapy is one of the main reasons for treatment failure in HIV-infected children[3]. Resistance of HIV-1 to antiretroviral agents results from mutations within the *pol* gene, which encodes for the viral reverse transcriptase (RT) and protease (Pt) regions, targets of currently used antiretroviral agents. In the present study, we analyze the genotypic resistance profiles of HIV in Brazilian children failing highly active antiretroviral therapy (HAART).

METHODS

The current study was conducted at Federal University of São Paulo, São Paulo, Brazil, between May 2000 and June 2001. The medical record review of 160 HIV-infected children attending the outpatient

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Informed Consent for Human Experimentation Guidelines: Progress of this study was annually reviewed and approved by the Institutional Review Board of the Federal University of São Paulo (UNIFESP), CEP # 362/00, ensuring compliance with all guidelines for human experimentation set down by the DHHS and UNIFESP. Informed consent was obtained from the caregivers of all included children.
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clinic (CEADIPe) identified 63 eligible children for this study. Eligibility requirements included: age < 18 years; current antiretroviral treatment composed by at least 3 drugs (2 NRTIs plus 1 PI, or 2 NRTIs plus 1 NNRTI); displaying one of the following criteria for virologic failure[16]: a less than 10-fold decrease from baseline HIV RNA levels in those receiving two NRTIs and one PI, or a less than 5-fold decrease from baseline HIV RNA levels in those receiving two NRTIs plus one NNRTI after 12 weeks of therapy; lack of a sustained decrease in HIV RNA copy number of 1.5 to 2.0 log10 from baseline; repeated detection of HIV RNA in patients who had undetectable levels prior to beginning therapy; or a persistent increase in HIV RNA levels after beginning of treatment greater than 3-fold in children aged two years or more, or greater than 5-fold in children under two years of age.

Of the 63 eligible children 41 composed the study population (Table 1). Reasons for not participating included: blood sample not available during the study period; and caregiver refusal. All children (median age = 67 months) included had been receiving HAART (2 NRTIs plus 1 PI, or 2 NRTIs plus 1 NNRTI) for a median period of 12 months. The caregivers of the study participants signed an informed–consent form previously approved by the local institutional review board.

Table 1
General characteristics of the study population at the time of genotypic testing. VL, stands for the $\log_{10}$ of plasma viral load

Total of Patients (n)	41	
Median age (months) (range)	66 (12-232)	
Transmission route (n)		
Vertical	38	
Transfusional	3	
Clinical category according to CDC*	n	%
A	12	29.3
B	12	29.3
C	15	36.6
N	2	4.9
Laboratory Parameters at genotyping	Median	Range
Log_{10} viral load (copies/ml)	4.15	2.1- 6.4
CD4 (X 10^6 cell/L)	960	52-2748
CD8 (X 10^6 cell/L)	1469	411-7017

*CDC indicates Centers for Disease Control and Prevention

Quantification of HIV-1 RNA and T-cell subsets in peripheral blood: HIV-1 RNA copy numbers (viral load) were measured using a quantitative assay (NASBA, Biomérieux, France) with a lower quantitation limit of 80 copies/ml. T-lymphocyte subsets in peripheral blood were quantified by flow cytometry.

HIV-1 genotyping: DNA was extracted from PBMCs and amplified by nested PCR for the reverse transcriptase (RT) and protease (Pt) regions of the *pol* gene. Direct, bidirectional, dideoxynucleotide terminator cycle sequencing of the PCR product was performed using the OpenGene™DNA Sequencing System as described elsewhere[7]. Sequences were analyzed and manually proofread and edited.

Analysis of drug-resistance mutations: Consensus guidelines for resistance testing[8] were used to define well-characterized, drug-resistance mutations. These mutations and the drugs to which they are related are (primary mutations are denoted by an asterisk): Ritonavir (RTV), K20M/R, V32I, L33F, M36I, M46I/L, I54L/V, A71T/V, V77I, V82A/F/S/T*, I84V*, and L90M*; Nelfinavir (NFV) L10I, K30N*, M36I, M46I, G48V, A71T/V, V77I, I84V*, N88D, L90M*; Amprenavir (APV) V32I, M46I/L, I47V, I50*, I84V* Zidovudine (ZDV), M41L, D67N, K70R*, L210W, T215F/Y* and K219Q; Didanosine (ddI), K65R, L74V*, and M184I/V; Stavudine (d4T), V75T; 3TC, E44D*, V118I*, and M184*; Nevirapine (NVP), L100I, K103N*, V106A*, V108I*, Y181C/I*, Y188C/H/L*, and G190A*; Efavirenz L100I, K103n*, Y188C/H/L*, P236L*; and multinucleoside, Q151M, 69 insertion. Strains with genetic mixtures of mutant and wild-type sequences at amino acid sites that code for major drug resistance were considered to be drug-resistant.

RESULTS

Thirty three of 41 children exhibited complete sequences from both the RT and Pt regions. Thirty-six sequences from the RT, and 38 sequences from the Pt regions were available for analysis. According to the genotypic analysis (GuideLines ™ Rules 5.0, Visible Genetics)[7], 12 of 36 (33.3%) children showed resistance to ZDV, while 6 showed possible resistance to this drug (16.6%); 5/36 (14%) and 4/36 (11.1%) showed resistance or possible resistance, respectively, to ddI; 4/36 (11.1%) showed resistance to 3TC and D4T; and 3/36 (8.3%) showed resistance to Abacavir. A high percentage of children exhibited mutations conferring resistance to Nevirapine (44.4%) and Efavirenz (38.8%) (Fig. 1). Tables 2 and 3 show mutations related to decreased susceptibility to antiretroviral drugs and the antiretroviral therapy history, respectively. The major mutations related to PI resistance were D30N (n = 2), M46I (n = 3), V82A (n = 7) and L90M (n = 2), corresponding to the following respective rates of resistance and possible resistance to the different drugs: RTV (12.2%; 7.3%); APV (2.4%, 12.1%); SQV (0%, 12.1%); IDV (14.6%, 4.9%), NFV (22%, 4.9%), LPV/RTV (2.4%, 12.1%) (Fig. 2).

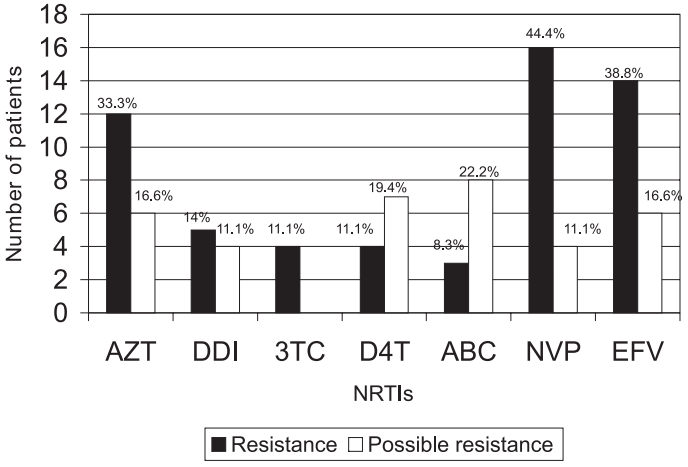

Fig. 1 - Incidence of resistance or possible resistance to NRTI and NNRTI in HIV- infected children failing HAART. *ZDV= Zidovudine; 3TC=Lamivudine; D4T=Stavudine; ABC= Abacavir; NVP= Nevirapine; EFV= Efavirenz*

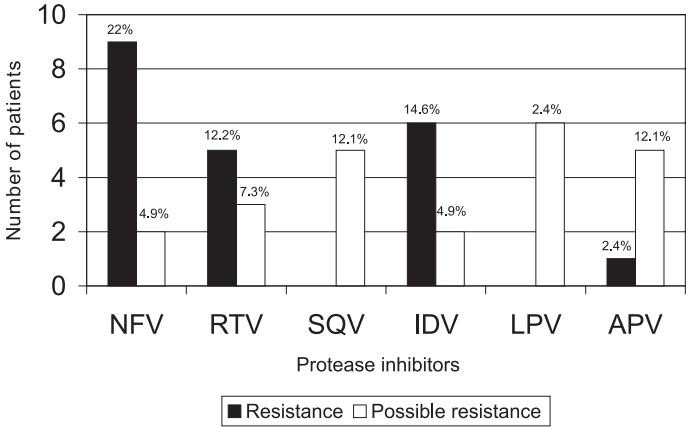

Fig. 2 - Incidence of resistance or possible resistance to PIs in HIV infected children failing HAART. *NFV=Nelfinavir; RTV= Ritonavir; SQV= Saquinavir; IDV= Indinavir; LPV= Lopinavir/RTV; APV= Amprenavir*

The percentages of patients with mutations related to drug resistance varied according to the drug classes employed: 57% for NRTI, 54% for NNRTIs, and 67% for PIs.

Three of 33 (9%) children with complete sequences from both the protease and reverse transcriptase regions of the *pol* gene showed resistance to all three drugs classes (MDR), while 4/33 (12%) children showed no genotypic resistance at all.

By the time genotypic testing was performed, 19 children showed a PI-based HAART, while eight (42%) showed resistance to this drug class. Three of eleven children (27%) previously exposed to PIs and receiving NNRTI-based HAART were resistant to at least one PI.

DISCUSSION

Our analyses of HIV-1 from samples obtained at the time of virological failure revealed a high incidence of virus with mutations

Table 2
Genotypic profiles of viral isolates and antiretroviral exposure for each patient

ID	Clinical stage of patient (CDC)	NRTI Mutations	NNRTI Mutations	PI Mutations	Drugs used in therapy[a]
1	B2	none	K103N	L10V;M36I	***D4T/ddI/EFV,***AZT
2	C3	D67N;K70R;F116Y	K103N	L63P;V77I	***D4T/ddI/EFV***AZT;3TC;RTV
3	A1	L210W;T215Y	Y188L	M36I	***D4T/ddI/EFV***;AZT
4	B3	M41L	K103N	M46I;L63P;V77I;L90M	***D4T/NVP/NFV;***AZT;3TC;RTV
5	C3	M41L;D67N;K70R;T215Y	none	L10V;K70R;M36I;I54V;L63P;V82A	***AZT/ddI/NFV***;D4T;3TC;RTV
6	B3		V179I;Y181I;G190A	L10I;K20R;M36I;I54V;L63P;A71V;V82A	***D4T/NVP/NFV***;AZT;3TC;RTV
7	B2	T215F	A98G;L100I;K103N	L10I;K20R;M36I;I54V;L63P;V82A	***D4T/ddI/EFV***;AZT;3TC;RTV
8	C3	M41L;D67N;K70R	L100I;K103N;V108I	L24I;M36I;	***D4T/ddI/EFV***;AZT;3TC;RTV
9	C2	none	none	none	***AZT/ddI/NFV***;RTV
10	B1	M184V;T215Y	A98E	L63P;V77I;I93L	***AZT/ddI/NFV***;RTV
11	C3	none	none	D30N;M36I;L63P;N88D	***AZT/ddI/EFV***;D4T;3TC;NFV
12	C2	none	none	D30N;M36I;L63P;A71V;V77I;N88D;I93L	***D4T/ddI/EFV***;AZT;3TC;NFV
13	C3	none	none	K20R;M36I;F53L;L63Q	***D4T/ddI/EFV***
14	B3	T215Y	K103N;Y188L;G190A	M36I;L63P;V77I	***D4T/ddI/EFV***;AZT
15	A1	D67N;T69N;K70R;M184V;T215F;K219Q	A98G	ND	***D4T/ddI/NFV***;AZT;3TC;RTV
16	N3	M41L	none	L63P	***D4T/ddI/NVP;***AZT;3TC
17	C3	none	none	M36I;L63S	***D4T/3TC/EFV***;ddI;NFV
18	C3	none	K103N	none	***D4T/NFV/NVP***;AZT;3TC
19	C3	M41L;L74V	K101E	M36I	***D4T/ddI/RTV;***AZT;3TC;NFV
20	A2	M41L;D67N;T69D;K70R;T215Y	K103N;V106A	A71V;V82A	***D4T/ddI/NVP;***AZT;3TC;NFV
21	A2	ND	ND	L63Q	***D4T/3TC/NVP***;AZT;ddI
22	B2	K70R;T215Y	K103N	L10V	***AZT/ddI/EFV***
23	A2	M41L;M184I;T215Y	A98G	M36I;I47V	***D4T/ddI/EFV;***AZT;3TC;NFV
24	B3	ND	ND	M36I;L63P	***D4T/ddI/NVP***;AZT;3TC
25	A1	M41I;D67N;T69R;V75A;T215Y	K103N;V106A	L63S;A71V;V77I;V82A;I93L	***D4T/ddI/EFV***;AZT
26	A1	none	none	ND	***D4T/ddI/NFV***;AZT;3TC;RTV
27	B3	M41L;D67N;T215Y	none	M36I;L63P;V77I;L90M	***D4t/ddI/NFV***;AZT;3TC
28	A2	ND	ND	I93L	***D4T/ddI/NVP;***AZT;3TC;NFV
29	A2	P236L	K103N	ND	***AZT/ddI/RTV***; EFV;D4T;3TC
30	A2	none	none	L63P	***AZT/ddI/RTV***
31	C3	M184V	none	M36I;I54V	***D4T/ddI/RTV***;AZT;3TC;NFV
32	C2	ND	ND	M36I	***D4T/3TC/NFV***;AZT;ddI;NVP
33	N2	K70R	K103N	K20R;M36I;I54V;L63P;A71V;V82A;I93L	***D4T/ddI/NFV***;AZT;RTV
34	A1	T69N;K70R;T215Y	none	M36I;L63T;I93L	***D4T/ddI/NFV;***AZT
35	B1	none	none	M46I;L63H	***AZT/ddI/RTV***
36	B2	none	none	none	***D4T/ddI/NFV***;AZT;3TC
37	C2	M184V	K103N	V77I	***D4T/ddI/EFV***;AZT;3TC;RTV
38	C3	none	none	L10I;V32I;M46I;I54V;A71V;V82A	***D4T/ddI/EFV; AZT;NFV***
39	C2	none	none	L63P;V77I;I93L	***D4T/ddI/NFV/EFV;***AZT;3TC;RTV
40	A1	V118I	A98G;Y181C	V32I	***D4T/ddI/NVP***;AZT;3TC
41	B2	ND	ND	L63P	***D4T/ddI/NVP;***AZT;RTV

[a] Drug abbreviations: AZT, zidovudine; 3TC, lamivudine; DDI, didanosine; D4T, stavudine; NVP, nevirapine; EFV, efavirenz; RTV, ritonavir; NFV, nelfinavir. The present regimen included the drugs listed in boldface, italic type.

Table 3
Antiretroviral therapy history of 41 children failing HAART

Therapeutic history	n	Patients ID
Less than 3 prior ARV schemes	21	1, 3, 8, 9, 13, 14, 17, 20, 21, 22, 23, 24, 25, 26, 30, 31, 33, 34, 35, 40, 41
≥ 3 prior ARV schemes	20	2, 4, 5, 6, 7, 10, 11, 12, 15, 16, 18, 19, 27, 28, 29, 32, 36, 37, 38, 39
First ARV therapy		
Monotherapy	8	5, 6, 10, 15, 18, 19, 21, 37
Double therapy	25	1, 2, 3, 4, 7, 11, 12, 13, 14, 16, 17, 20, 23, 24, 25, 27, 28, 29, 30, 32, 34, 36, 38, 39, 40
HAART	9	8, 9, 22, 26, 30, 31, 33, 35, 41
Time since first ARV scheme		
< 2 years	13	1, 3, 9, 10, 13, 14, 17, 31, 34, 35, 38, 41
2-3 years	16	2, 8, 11, 12, 16, 20, 22, 23, 24, 25, 28, 30, 31, 33, 36, 40
> 3 years	14	4, 5, 6, 7, 15, 18, 19, 21, 26, 27, 29, 32 37, 39
Previous treatment without PI	14	2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 17, 19, 20, 23, 26, 28, 31, 33, 37, 38, 39, 41
Previous treatment with PI	23	1, 3, 14, 16, 18, 21, 24, 25, 27, 29, 32, 34, 36, 40
Patients receiving the first ARV scheme	4	13, 22, 30, 35

related to drug resistance (37/41; overall 90%), with percentages varying according to specific drug classes (57% for NRTI, 54% for NNRTIs, and 66% for PIs).

The major mutations associated with resistance to drugs not being used when the genotyping tests were performed included those related to Lamivudine (M184V; patient 10), or either Ritonavir or Indinavir (V82A; patients 5, 6, 20, 25, 33) and NNRTIs (K103N; patient 33). In some cases, these mutations may have been selected during a previous regimen and maintained by subsequent treatments[15]. However, this is unlikely to be the case for patients 10, 25 and 33, who exhibited principal mutations related to drugs to which they had not been previously exposed. In these three cases, a plausible explanation may be an uncommon cross-resistance phenomenon, such as M184V selected by DDI in patient 10, or V82A by Nelfinavir in patient 25, or less likely, primary resistance from mother-to-child transmitted resistant strains, which is probably the case for K103N in patient 33.

Only four children were NRTI naive when HAART was begun and were experiencing their first therapeutic failures when submitted to genotypic testing (patients 13, 22, 30 and 35). Most children (90%) had been treated with antiretroviral nucleoside RT inhibitors before beginning of HAART. Consequently, many triple drug regimens included one or two NRTIs already used by the children, and this may be one of the reasons for difficulty in adequate suppression of viral replication and the subsequent failure of ARV treatment in this population.

Several findings in this study illustrate the consequences of sequential, non-potent regimens, with subsequent, sub-optimal responses to more potent schemes. Serial monotherapy with NRTIs may have selected for thymidine-associated mutations (TAM), along with resistance to ddI, ABC and 3TC. Conceivably by the time HAART was begun, truly potent, antiretroviral regimen for these children could not be developed due to cross-resistance to the "new" NRTIs. Not surprisingly, the responses to PIs or NNRTIs were transient, with selection of resistance mutations related to these drug classes. Our patients were extensively treated with Didanosine and Stavudine, although we found a low incidence of mutations at codons 74 and 75, consistent with other published reports[5,9]. Although the children in this study had never received ABC, a high percentage of resistance to this drug (8.3%) was found, according to one of the following rules from Guidelines rules 5.0 [3]: NRTI 2, NRTI 3, NRTI 22. These findings again suggest cross-resistance.

Four children carried no resistance mutations in the context of a rising plasma HIV-1 RNA levels. In these cases, poor adherence to the treatment regimen should be thoroughly investigated, although cellular resistance, and/or the low, or theoretically, occasionally low, sensitivity of the test in detecting resistant strains (low negative predictive value) may be the cause[12].

Our results confirm the close relationship between therapeutic failure and genotypic resistance in children, as also shown for adults[1,14,16]. The present study was not designed to assess whether the resistance found is the cause or the consequence of therapeutic failures. However, it is important to emphasize the possible interacting roles of adherence to the treatment regimen, regimen potency, and pharmacokinetics that may negatively influence the effectiveness of antiretroviral therapy in children.

Results like those shown here emphasize the importance of considering the appropriate moment at which to initiate ARV therapy, as well as the choice of adequate, suppressive, antiretroviral drugs. This may be especially important in children who exhibited a naturally high viral load compared to adults[2,11]. Thus, viral suppression with less potent regimens may not be effective considering the subsequent loss of efficacy in the succeeding regimens. The longer a child remains on a suboptimal suppressive regimen, the greater the likelihood of secondary mutations developing, and the greater the risk of subsequent, cross-resistance and drug failure[13].

RESUMO

Análise da protease e transcriptase reversa do HIV-1 em crianças com falha terapêutica em uso de terapia anti-retroviral altamente eficaz (HAART)

O objetivo deste estudo foi avaliar o perfil de resistência genotípica do HIV-1 em crianças com falha terapêutica ao tratamento anti-retroviral (HAART). Quarenta e uma crianças (idade mediana = 67 meses) em uso de HAART foram submetidas ao teste de genotipagem no momento da detecção de falha ao tratamento. Foi realizada extração de cDNA de células periféricas mononucleares e amplificação do mesmo (regiões da transcriptase reversa e protease do gene *pol)* através de *PCR-nested*. O perfil genotípico foi determinado através do seqüenciamnto de nucleotídeos. De acordo com a análise genotípica, 12/36 (33,3%) e 6/36 (16,6%) crianças apresentaram, respectivamente, resistência e possível resistência ao AZT; 5/36 (14%) e 4/36 (11,1%), respectivamente, eram resistentes e possivelmente resistentes ao ddI; 4/36 %11,1%) apresentaram resistência ao 3TC e D4T, e 3/36 (8,3%) eram resistentes ao ABC. Uma alta porcentagem de crianças (54%) apresentou mutações relacionadas à resistência aos inibidores da trancriptase reversa não-análogos de nucleosídeos. As taxas de resistência e possível resistência aos inibidores da protease foram, respectivamente: RTV (12,2%; 7,3%); APV (2,4%; 12,1%); SQV (0%; 12,1%); IDV (14,6%; 4,9%); NFV (22%; 4,9%); LPV/RTV (2,4%; 12,1%). No total, 37/41 (90%) crianças apresentaram vírus com mutações relacionadas à resistência a alguma droga, sendo que 9% delas tinham vírus resistentes às três classes de drogas anti-retrovirais disponíveis.

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