# HIV-1 drug resistance genotypic profiles in children with undetectable plasma viremia during antiretroviral therapy

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## ABSTRACT

Treatment of HIV-1 infection with highly active antiretroviral therapy has led to sustained viral suppression in the plasma in a large number of children. However, studies have suggested that the integrated provirus in resting CD4+ T lymphocytes could be a source of reactivatable virus and maintain drug-resistant virus. We evaluated the resistance-related mutations in children receiving antiretroviral therapy with prolonged viral suppression. Thirty-two peripheral blood mononuclear cell samples from 16 children with viral loads that had been below detection limits for at least 12 months were obtained at two different time points and the DNAs sequenced. The median CD4 cell count was 1,016 cells/mm$^3$ (347-2,588) and 938 cells/mm$^3$ (440-3,038) at the first and second time points, respectively. The median follow-up time was 15 months (9-27). Six (37.5%) and seven (43.75%) of the 16 patients showed at least one NRTI-associated mutation in the first and second samples, respectively. Two out of 16 (12.5%) had an NNRTI-associated mutation at the first time point and three out of 16 (18.75%) at the second. In addition, 14 out of 16 (87.5%) had at least one PI-associated mutation at both time points. Despite plasma HIV-1 RNA suppression for at least 12 months, resistance-related mutations from previous antiretroviral failures could still be detected in archival virus. Furthermore, viral evolution occurred at the reverse transcriptase region in spite of viral suppression to levels below 400 copies/mL. Persistence of archival resistant virus may be relevant when considering future treatment options.

**Keywords**: children; HIV-1; prolonged viral suppression; antiretroviral therapy; antiretroviral resistance.

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## INTRODUCTION

Treatment of human immunodeficiency virus type 1 (HIV-1)-infected individuals with highly active antiretroviral therapy (HAART) has dramatically changed the course of the infection, reducing mortality and morbidity events associated with this disease.[1-3] After HAART, a great number of patients had plasma viremia reduced to below the detection limits of current sensitive assays.[4-6] Antiretroviral therapy (ART) in children has special features, and consequently, results obtained from clinical trials in adults may not be representative of the results obtained when using ART in children.[7,8] Studies have shown that there is poor maintenance of viral load suppression in children, with up to half of those studied showing viral rebound within a year of treatment.[9,10]

In patients on HAART, HIV-1 persistence is evidenced by free virus in the plasma; given the short half-life of free virus, this residual viremia indicates active virus production.[11] However, the virus is able to persist as a result of several potential mechanisms, such as its ability to establish a state of latent infection in resting memory CD4+ T cells.[12,13] Sensitive assays have demonstrated that resting memory CD4+ T cells retain replication-competent viral DNA that may be reactivated to produce virus even in patients with prolonged suppression of plasma viremia.[14]

Given the low fidelity of the HIV reverse transcriptase enzyme combined with the high replication rate of the virus, it is not surprising that even triple-class HAART therapy eventually fails in the vast majority of patients and is typically associated with the emergence of resistance to viral reverse-transcriptase (RT) and protease (PT) inhibitors, the currently used antiretroviral agents.[15-17]

To determine whether the integrated proviruses in resting CD4+ T cells are associated with the development of drug-resistance mutations, we studied a group of Brazilian children who had prolonged suppression of viral replication under ART.

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We declare no conflict of interest.

## METHODS

### Patients and study design

The study involved 32 samples of peripheral-blood mononuclear cells (PBMC) from 16 children followed up at the Pediatric Infectious Disease Clinic (CEADIPE) at the *Universidade Federal de São Paulo*, Brazil, (UNIFESP) at two different time points. The first sample was collected at inclusion, and the second after a minimum of nine months of follow-up. The inclusion criteria were (I) VL < 400 copies/mL with virological suppression (VL < 400 copies/mL) maintained for at least 12 months at baseline and (II) treatment with ART and only one viral rebound with > 1,000 copies/mL during the 12 months prior to baseline. The parents or guardians of the children signed informed consent documents, and the study was approved by the institutional review board. The samples were collected from October 2002 to March 2005.

The children were monitored every three months, when they were examined physically and blood samples were collected for serial measurements of laboratory markers of HIV-1 infection, such as T-cell subsets and viral loads, and for safety tests. There was not a uniform approach to the antiretroviral treatment in the background regimen given. Instead, each pediatrician administered the appropriate ART regimen and changed the drugs according to his/her interpretation of the data and international guidelines. Each clinician measured adherence by interviews with parents or guardians.

### Amplification of HIV-1 pol from proviral DNA

PCR amplification of purified proviral DNA was performed as previously described.[18] The cycling conditions were as follows: 5 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C, followed by 25 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The product was purified, and RT and protease regions were sequenced.

### Genotypic analysis of HIV-1 isolates

Genotypic HIV-1 drug resistance was determined using the Applied Biosystems ViroSeq HIV-1 Genotyping Kit. The HIV-1 protease and reverse transcriptase regions were analyzed using an ABI 377 sequencer and associated Sequence Navigator software. Drug-resistance-associated mutations were defined according to the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu) and antiretroviral resistance profile defined according to the Brazilian National AIDS guidelines for antiretroviral resistance interpretation (RENAGENO: http://www.aids.gov.br).

## RESULTS

### Population characteristics

The main characteristics of the study population at baseline are summarized in Table 1. The median follow-up time was 15 months (9-27). Fourteen patients (87.5%) were on HAART at the time of the first sample, and two (12.5%) were receiving two NRTIs. The median CD4+ T-cell count was 1,016 cells/mm$^3$ (347-2,588) and 938 cells/mm$^3$ (440-3,038) at the first and second time points, respectively. All children had HIV-1 RNA plasma levels below the detection limits on entry to the study (400 copies/mL). There were no differences in immune and clinical classifications for the two time points.

### Resistance-associated mutation profiles

Thirty-two PBMC DNA samples were extracted from the 16 patients who had had HIV RNA levels < 400 copies/mL

*Table 1. Baseline characteristics of the study population*

| | Parameter | Patients (n = 16) |
|---|---|---|
| | Age (years)[a] | 11 (6 - 15) |
| | Male sex (%) | 8 (50%) |
| **Therapy** | First time point | Second time point |
| 2 NRTI + 1PI | 10 | 9 |
| 2 NRTI + 1 NNRTI | 2 | 4 |
| 2 NRTI + 2 PI | 1 | 1 |
| 2 NRTI + 2 PI + 1 NNRTI | 1 | 1 |
| 2 NRTI | 2 | 1 |
| **Baseline CD4 cell count (cells/mm$^3$)** | 1,016 (347-2,588) | 938 (440-3,038) |
| **CDC[b] clinical classification** | | |
| N | 1 | 1 |
| A | 3 | 3 |
| B | 6 | 6 |
| C | 6 | 6 |
| **Immune classification** | | |
| 1 | 4 | 4 |
| 2 | 6 | 6 |
| 3 | 6 | 6 |

[a]Median (range); [b]Centers for Disease Control and Prevention Classification.

for at least 12 months after they started ART. Thirteen patients maintained suppression of viral replication throughout the study. Genotypic drug resistance of all samples was determined by amplification and HIV-1 sequencing.

Fifteen out of the 16 individuals (93.75%) had at least one mutation related to decreased susceptibility to antiretroviral therapy. The percentage of patients with mutations related to drug resistance varied according to the drug classes used, with 31.25%, 12.5% and 31.25% having NRTI, NNRTI and PI-related mutations, respectively, at the first time point, and 37.5%, 18.75% and 31.25% having NRTI, NNRTI and PI-related mutations, respectively, at the second time point. Figures 1 and 2 show all the mutations found for both time points for each reverse-transcriptase and protease region.

Two out of the 16 children (12.5%) were resistant to all three drug classes whereas 9/16 (56.25%) did not have any resistance-associated genotypic mutations at the first time point. At the second time point, 3/16 (18.75%) were resistant to all three drug classes.

According to the genotype analysis, three out of the 16 children (18.75%) were resistant to ZDV, and 2/16 (12.5%) had intermediate resistance to this drug; 1/16 (6.3%) and 2/16 (12.5%) were resistant or had intermediate resistance, respectively, to ddI; 2/16 (12.5%) were resistant to 3TC and ABC; and 3/16 (18.75%) were resistant to d4T. A small percentage of children were found to have mutations conferring resistance to nevirapine (12.5%) and efavirenz (6.3%) at the first time point.

At the second time point, four out of the 16 children (25%) were resistant to ZDV whereas 2/16 had intermediate resistance to this drug (12.5%); 1/16 (6.3%) and 3/16 (18.75%) were resistant or had intermediate resistance, respectively, to ddI; 3/16 (18.75%) were resistant to 3TC and ABC; and 4/16 (25%) were resistant to d4T. Three out of 16 (18.75%) were found to have mutations conferring resistance to nevirapine.

**Figure 1:** Mutations found in the reverse transcriptase region at both time points.

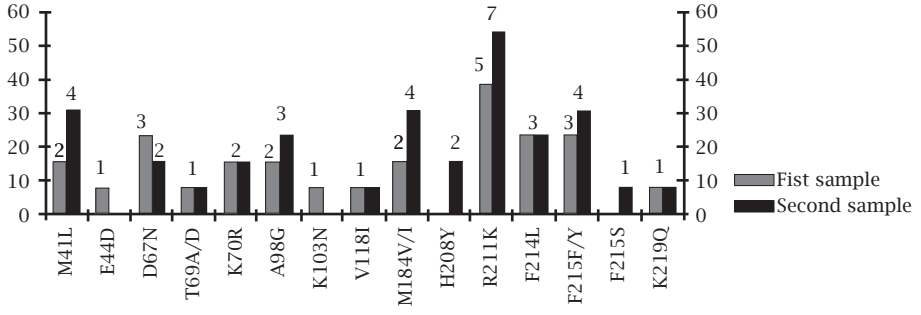


**Figure 2:** Mutations found in the protease region at both time points.

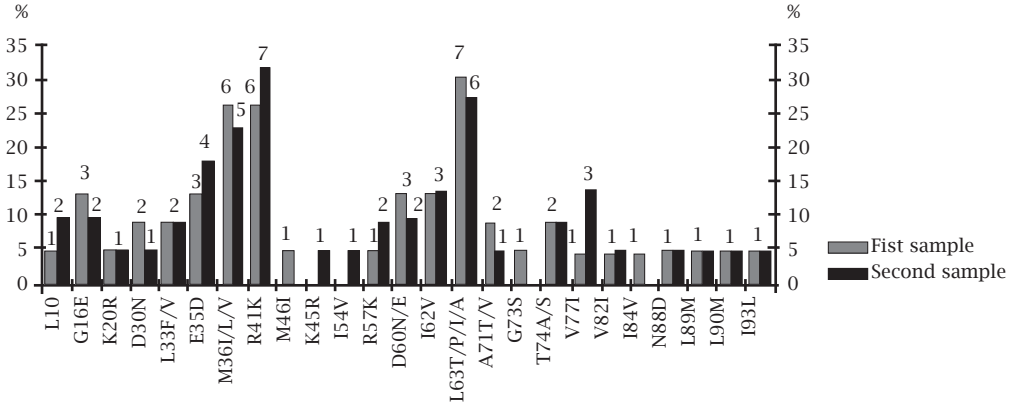

Table 2 shows the mutations associated with decreased susceptibility to each antiretroviral class and the antiretroviral-therapy history for each patient. The major mutations related to PI resistance in the first sample were D30N (n = 2), M46I (n = 1), V82I (n = 1), I84V (n = 1) and L90M (n = 1), corresponding to the following rates of total resistance and intermediate resistance to the different drugs, respectively: RTV (12.5%; 6.3%); APV (6.3%; 0%); SQV (12.5%; 12.5%), IDV (6.3%; 12.5%), NFV (18.8%; 6.3%), LPV/RTV (0%; 0%). The corresponding figures for the second sample were: D30N (n = 1), M46I (n = 0), V82I (n = 1), I84V (n = 0) and L90M (n = 1), corresponding to the following rates of total resistance and intermediate resistance to the different drugs, respectively: RTV (6.3%; 0%); APV (6.3%; 0%), SQV (6.3%; 12.5%); IDV (6.3%; 6.3%), NFV (12.5%; 6.3%), LPV/RTV (0%; 0%).

***Table 2. Genotypic profiles of viral isolates and antiretroviral exposure for each patient at both time points***

| ID | ITRN | ITRNN | IP | TARV* |
|---|---|---|---|---|
| **01-1** | V118DV | None | **G16E;** L24*; V32G; I47R | ZDV; NVP; ***ddI; d4T; NFV*** |
| **01-2** | None | None | **G16E** | ZDV; NVP; ***ddI; d4T; NFV*** |
| **02-1** | R211G | None | **K20R; R41K; A71V; I93L** | ZDV; ***3TC; ddI; RTV*** |
| **02-2** | **M184V;** R211G | None | **K20R; R41K; A71V; I93L** | ZDV; ***3TC; ddI; RTV*** |
| **03-1** | E44K; D67N; R211K | G190K | G16E; D30N; E34K; E35N; M36I; R41K; M46I; G48E; D60N; I62V | ZDV; ***3TC; d4T; RTV*** |
| **03-2** | M41L; M184V; R211K; T215Y | None | G16E; E35D; M36I; R41K; K45R; I62V | ZDV; ***3TC; d4T; RTV*** |
| **04-1** | R211K | None | E35D; M36I; R41K; L63S | ddI; NFV; ***d4T; 3TC; EFV*** |
| **04-2** | R211K | None | E35D; M36I; R41K; L63S | ddI; NFV; ***d4T; 3TC; EFV*** |
| **05-1** | M41L; V118IV; M184V; F214L; T215F | A98G; L100LV; K103N; V106I | D30N; L33F; M36L; R57K; D60E; L63P; A71V; N88D | 3TC; d4T; NFV; ***ddI; ZDV; EFV*** |
| **05-2** | M41L; K70KQ; V118I; M184V; H208Y; F214L; T215F | A98G; V106I | D30N; L33F; M36L; R41KR; R57K; D60E; L63P | 3TC; d4T; NFV; ***ddI; ZDV; EFV*** |
| **06-1** | None | None | None | ZDV; RTV; EFV; ***3TC; d4T; NFV*** |
| **06-2** | R211KR | G190GR | None | ***ZDV;*** RTV; ***EFV; 3TC***; d4T; NFV |
| **07-1** | R211X | None | V77I; V82I | ZDV; EFV; NVP; ***ddI; d4T; RTV*** |
| **07-2** | R211K | None | R57K; V77I; V82I | ZDV; EFV; NVP; ***ddI; d4T; RTV*** |
| **08-1** | E44DE; F214L | None | L63I | ZDV; ddI; EFV; ***d4T; 3TC; NFV*** |
| **08-2** | F214L | M230IM | L63S; V77I | ZDV; ddI; EFV; ***d4T; 3TC; NFV*** |
| **09-1** | V118D | None | L63A; T74AT | ZDV; ddI; ***d4T; 3TC; NFV*** |
| **09-2** | M41L; T215S | A98G | L10I; L63A; T74AT; V77I | ZDV; ddI; ***d4T; 3TC; NFV*** |
| **10-1** | None | None | E35D; I47M; G48R; L63P | ZDV; ddI; EFV; d4T; 3TC |
| **10-2** | V118D | None | E35D; L63P | ZDV; ddI; d4T; 3TC; EFV; NVP |
| **11-1** | None | None | M36I | ZDV; ddI |
| **11-2** | None | None | L63H | ZDV; ddI |
| **12-1** | R211K | None | G16E; L33V; E34K; G73S; L89M | ZDV; ddI; 3TC; d4T; RTV |
| **12-2** | R211K | None | L33V; L89M | ZDV; ddI; 3TC; d4T; RTV |
| **13-1** | M41L; M184V; T215Y | None | R41K; D60E; I62V; L63P | ZDV; EFV; 3TC; d4T; RTV |
| **13-2** | M41L; M184V; T215Y | None | R41K; D60E; I62V; L63P | ZDV; EFV; 3TC; d4T; RTV |
| **14-1** | D67N; T69D; K70R; R211K; | A98G | L10I; E35D; M36V; R41K; I62V; L63P; T74S; I84V; L90M | ZDV; 3TC; NFV; RTV; ddI; d4T; EFV; LPV/r |
| **14-2** | D67N; T69D; K70R; H208Y; R211K; F214L; T215F; K219Q | A98G | L10I; E35D; M36V; R41K; I54V; I62V; L63P; T74S; L90M | ZDV; 3TC; NFV; RTV; ddI; d4T; EFV; LPV/r |
| **15-1** | D67N; K70R; R211K | None | M36I; L63S | ZDV; 3TC; RTV |
| **15-2** | D67N; K70R; R211K | None | M36I; L63S; | ZDV; 3TC; RTV |
| **16-1** | None | None | R41K; L63P; L90V 3TC; ABC; LPV/r | ZDV; RTV; d4T; ddI; NFV; EFV; |
| **16-2** | None | None | R41K; L63P; N88D 3TC; ABC; LPV/r | ZDV; RTV; d4T; ddI; NFV; EFV; |

* Drug abbreviations: ZDV, zidovudine; 3TC, lamivudine; ddI, didanosine; d4T, stavudine; NVP, nevirapine; EFV, efavirenz; RTV, ritonavir; NFV, nelfinavir.; LPV, lopinavir; ABC, abacavir. The patients' regimens at the time the study was carried out including the drugs listed in boldface, italic type.

## DISCUSSION

Although HIV-RNA viral loads reach undetectable levels less frequently in HIV-infected children than they do in adults,[19,20] we describe a selected group of children on ART who experienced long-term viral suppression. Several findings in the present study confirm that resistance can develop when the viremia level is below 400 copies/mL.

When Cohen Stuart *et al.*[21] evaluated plasma samples with an average viral load of 76 copies/mL, they found that 72.7% of the viruses had resistance mutations to one or more drugs. However, when De la Rosa *et al.*[1] studied adults' PBMC with viremia levels < 50 copies/mL, they found that only 40% of viruses had one or more mutations related to resistance.

Our analysis showed a high frequency of viruses (94%) with resistance-related mutations at both time points, with percentages varying according to the drug class. At the time the first samples were collected, there was a significantly higher percentage of viruses with mutations selected by NRTIs and PIs (31.3%) than of viruses with mutations selected by NNRTIs (12.5%). At the time the second samples were collected, 37.5% and 31.3% of the viruses were observed to have mutations selected by NRTIs and PIs, respectively, while the corresponding figure for NNRTIs was 18.8%.

Naeger LK and Struble KA[22] showed that lopinavir/ritonavir response rates were less than 30% when protease substitutions at M46, I54 or I84 were present at baseline. Two patients (ID 03 and 14) in the present study continued to have undetectable viral loads even in the presence of these mutations. However, the majority of sequences obtained from the latent reservoir showed a virus with mutations conferring resistance to drugs that were part of failed prior regimens. A striking result was the fact that this "archival" drug-resistant virus persisted despite continued treatment with the relevant drug with no viral replication.

In one patient (ID 14) who had previously been exposed to several therapeutic regimens containing ZDV, 3TC, NFV and RTV, the viral load first reached undetectable levels in October 2001 while the patient was undergoing the eighth therapeutic regimen, which contained d4T, ddI, EFV and LPV/r. Although the genotypic profile showed mutations associated with resistance to the three classes of drugs and sensitivity only to LPV/r, this patient continued to have complete viral suppression (< 400 copies/mL) at the time the study was completed. The probable explanation for this is related to a reduction in viral fitness due to mutations.[23] A similar finding was described by Ghosn *et al.*[24] in two patients with a virus that was resistant to at least two of the three drug classes in use, one of whom maintained complete viral suppression for 24 months and the other for 48 months.

Another critical issue relates to two children who used the first antiretroviral therapy (ID 11 and 15). Although maintaining undetectable viral loads, one of them had mutations selected by a nucleoside analogue and had the same profile in the two samples, while the other showed no relevant changes.

It is important to emphasize that three out of the six children who showed non-nucleoside related mutations (IDs 05, 06 and 14) were receiving HAART containing this class of drug. With regard to the other three, one of them (ID 08) had been exposed to EFV in a prior regimen, and the other two (IDs 03 and 09) had never been exposed to an NNRTI before.

To summarize, we observed that 62.5% of the patients (10/16) had no relevant changes in the genotypic profile at the RT region. In 83.3% (5/6), new substitutions could be found at the second time point. These were all related to resistance (codons 41, 98, 184, 208 and 215), suggesting viral evolution. Some mutations found at the first time point were not present at the second time point (codons 44, 67 and 103). In the PR region, we observed that 37.5% of the patients (6/16) had no relevant changes in the genotypic profile. In 30% (3/10), new substitutions could be found in the second sample, all related to polymorphisms. The D30N mutation found in the first sample (ID 03) was not present in the second sample.

Despite our success in controlling viral replication, several children had mutant strains present in the lymphocytes, although this did not reflect any clinical or immunological deterioration. However, the persistence of the archival resistant virus may be relevant when considering future treatment options.

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