Drug resistance prevalence and HIV-1 variant characterization in the naive and pretreated HIV-1-infected paediatric population in Madrid, Spain

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Received 9 May 2011; returned 6 June 2011; revised 17 June 2011; accepted 1 July 2011

Background: Drug resistance mutations affect antiretroviral therapy (ART) effectiveness in HIV-1-infected children, compromising long-term therapy. HIV-1 variants and drug resistance mutations were identified in HIV-infected children from Madrid, Spain.

Methods: Patients from the Madrid cohort of HIV-infected children (1993–2009) with available pol sequences or infected samples stored at the Spanish HIV-1 BioBank were selected. Specimens were used to perform new pol sequences when not available. HIV-1 variants were characterized by phylogenetic analysis. Resistance mutations were identified according to the International AIDS Society–USA list (2009).

Results: In 198 patients, pol sequences were recovered from routine resistance testing (n=98) or newly performed using stored plasma, lymphocytes or DNA (n=100). Patients were mostly Europeans (90%), with moderate to severe AIDS symptoms (65%), on ART (85%) when the specimen was sequenced and infected by subtype B (90%). Among the 19 HIV-1 non-B variants found, 58% were recombinants (8CRF02_AG, 1CRF08_BC, 1CRF12_BF and 1CRF13_cpx) and the rest were ‘pure’ non-B subtypes (1A2, 2C, 2D, 1F1, 1G and 1H). Transmitted drug resistance (TDR) mutations were detected in 13% of naive children; 4%, 7% and 10% for protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), respectively. Global resistance prevalence was higher (66%) among ART-exposed children; 37% for PIs, 54% for NRTIs and 35% for NNRTIs.

Conclusions: HIV-1 non-B variants infected 10% of the cohort during 1993–2009. Resistant viruses were present in 26.5% and 66% of naive and pretreated children, respectively. Our data suggest that TDR prevalence in children could be higher than that reported in adults in Spain. The provided data will help to improve clinical management of HIV-infected children in Spain.

Keywords: HIV/AIDS, antiretroviral therapy, drug resistance, paediatrics

Introduction

The virological, immunological and clinical outcomes in HIV-infected children have improved greatly with the introduction of highly active antiretroviral therapy (HAART).1 HAART significantly reduces the progression of HIV-1 disease and decreases AIDS-associated morbidity and mortality in the paediatric population.2 Antiretroviral therapy (ART) leads to HIV RNA viral load (VL) suppression, which prevents the evolution of viral drug resistance and allows normal immune function, leading to normal growth and development in most infected children.3 However, HIV-infected children must use long-term ART,4 increasing the development of drug resistance mutations and toxic effects, which limit the long-term effectiveness of HAART, leading to the selection of drug-resistant HIV-1 variants and virological failure events.5 Drug-resistant variants can also be selected...
during pregnancy in women treated with a sub-optimal regimen, which has serious implications for future ART success in infants.5

Spain has among the highest AIDS incidence rate and prevalence in women in Western Europe,7,8 which has a direct impact on the spread of the infection in infants. The Autonomous Community of Madrid (CAM) is the area most affected by HIV infection in Spain and this mainly occurred during the 'epidemic of heroin use' in the 1990s. The rates of mother-to-child transmission (MTCT) in the CAM have followed the same trend as in other neighbouring countries due to the implementation of health policies, pregnancy follow-up and ART administration in infected mothers. During 2009, 1037 new cases of AIDS were declared in Spain, and only 4 (0.4%) of these were caused by MTCT. However, between 1981 and June 2009 a total of 946 cases of AIDS caused by MTCT were reported in Spain. Of these, 237 cases were reported in the CAM.

Spain has become one of the main entrance points of Africans into the European Union, mainly from sub-Saharan African countries with high HIV infection rates and a high prevalence of HIV-1 non-B variants.9,10 Owing to these population movements, all HIV-1 subtypes and multiple recombinants are circulating in Spain.11–13 Infection with certain non-B variants can have clinical implications, accelerating disease progression.14 Furthermore, non-B variants present clade-specific substitutions in positions related to drug resistance,15 which can accelerate the emergence of drug-resistant viruses, change or induce alternative pathways of resistance16 and influence the interpretation of genotypic resistance algorithms.17 Rapid subtyping tool reliability18 and VL quantification.19 Thus the proper detection and description of HIV-1 variants in representative cohorts is essential for further studies. The aims of this study were to identify the HIV-1 variants infecting patients from the Madrid cohort of HIV-infected children, summarize their clinical and epidemiological features and determine the prevalence of drug resistance mutations in viruses present in naive and ART patients.

Methods

Study population

The Madrid cohort of HIV-infected children has registered a total of 523 patients since the beginning of the epidemic in Spain. Today, 198 (38%) of these patients remain under clinical follow-up in paediatric units, 79 have transferred to adult units, 63 have been lost to follow-up and 183 have died. A total of 227 patients infected during their childhood were included in this study, selected according to sample (immortalized DNA, plasma or peripheral blood mononuclear cells (PBMCs)) or pol sequence availability. Available sequences from previous clinical routine drug resistance analysis performed since 1999 in hospitals where patients were under follow-up were recovered during 2010. HIV-1-infected specimens since 1993 from the remaining children without available pol sequences at December 2009 were recovered from the HIV-1 BioBank located in Hospital Gregorio Marañon, where they were stored and new sequences were performed. This study was approved by the Ethics Committees of all the institutions and hospitals involved.

HIV-1 diagnosis

Diagnosis of HIV-1 infection was previously confirmed in the study population by a positive RNA or DNA PCR for HIV in children aged less than 18 months and by two serial positive serological assays in children older than 18 months.

RNA and DNA extraction

For RNA extraction from HIV-1-infected plasma samples, an automated platform for the isolation of RNA based upon magnetic-silica technology was used employing NucliSensâ® easyMAG® instrumentation (bioMérieux, Durham, NC, USA), according to the manufacturer’s instructions. For viral DNA extraction from infected PBMCs, a commercial DNA isolation kit based upon column extraction was used (Qiagen Inc., Valencia, CA, USA).

HIV-1 pol sequencing and subtyping

HIV-1 pol sequencing and subtyping

HIV-1 RNA or proviral DNA amplification using an in-house reverse transcriptase (RT) nested PCR method was performed for the pol coding region (1121 bp), including the complete protease (PR) (297 bp) and partial RT gene as previously reported.19 CDNA synthesis was carried out using the Access Quickâ® RT–PCR system (Promega, Madison, WI, USA) and amplifications from generated cDNA or extracted proviral DNA using iProof™ High-Fidelity Master Mix (Bio-Rad, Hercules, CA, USA) or PCR Master Mix (Promega), following the manufacturers’ instructions. All pol sequences were subtyped by phylogenetic analysis as previously described.19 At least two representative sequences of each subtype and circulating recombinant form (CRF) within HIV-1 group M available at the moment of the analysis were taken as references. DNA sequences were aligned using the ClustalW program. The tree topology was obtained using the neighbour-joining method. The pairwise distance matrix was estimated using the Kimura two-parameter model within the DNADIST program, as implemented in the PHYLIP software package. Bootstrap re-sampling (1000 datasets) of the multiple alignments was performed to test the statistical robustness of the tree. The bootstrap cut-off was set at 700.

Genotypic drug resistance identification

Transmitted drug resistance (TDR) mutations among naive patients were classified using the WHO drug resistance mutation list20 and drug resistance mutations among pretreated patients, defined by the International AIDS Society (IAS)–USA,21 were manually located in each PR and RT sequence. Resistance mutations in the PR gene in pretreated patients were classified as primary (major) or secondary (minor) following the IAS–USA nomenclature. Genotypic drug resistance interpretation was performed using the genotypic resistance algorithms provided by Stanford (v.4.3.7) available from http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceFormalgorithms.

Statistical analysis

Confidence interval (CI) tests were performed with Epidat 3.1 (Pan American Health Organization). Significance was set at P<0.05.

Results

Study population selection

A total of 227 patients infected during childhood with HIV-1 in the period 1993–2009 were enrolled in this study. HIV-1 pol sequences were obtained from genotypic resistance analysis in 98 patients from routine testing in the corresponding hospitals. In the remaining 129 children with available infected samples, 100 additional sequences were newly performed from successful
**Clinical features of the study population**

All clinical and epidemiological features from the 198 children included in the study by 31 December 2009 were recorded (Table 1). Patients were mainly female (59%), perinatally infected (96%), born in Spain (88.5%), and on ART (85%) by sample collection time. Most infections (67%) occurred during 1990–1999, although sequenced samples for the study were recovered in 76% of children after 1999.

**Phylogenetic characterization of HIV-1 variants**

HIV-1 subtype B was the most prevalent (90%) variant found by phylogenetic analysis of pol sequences. It was present in 179 of the 198 children under study infected during the 1993–2009 period (Table 1). The 19 non-B variants identified in the study cohort were confirmed with high bootstrap values (>700) in phylogenetic trees. Among the 19 (10%) HIV-1 non-B variants found by phylogenetic characterization, 42% corresponded to ‘pure’ non-B subtypes or sub-subtypes (1A2, 2C, 2D, 1F1, 1G and 1H) and 58% were inter-subtype recombinant strains ascribed to CRFs (8CRF02_AG, 1CRF08_BC, 1CRF12 BF and 1CRF13_cpx). No unique recombinant forms (URF) were found in the study population.

Figure 1 shows the HIV-1 non-B subtypes and recombinants defined using PR and partial RT sequences (1302 bp) available for 17 non-B variants. The 19 non-B subtypes and recombinants found during the study period corresponded to 4 (21%) native children born to native Spanish parents and 15 (79%) children born to a foreign infected biological mother or father coming from India (1 case) or sub-Saharan Africa.

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**Table 1. Baseline features of the 198 patients**

<table>
<thead>
<tr>
<th>Features</th>
<th>Patients, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
</tr>
<tr>
<td>male gender</td>
<td>81 (41)</td>
</tr>
<tr>
<td>adopted</td>
<td>44 (22)</td>
</tr>
<tr>
<td><strong>HIV-1 transmission</strong></td>
<td></td>
</tr>
<tr>
<td>perinatal</td>
<td>190 (96)</td>
</tr>
<tr>
<td>blood transfusion</td>
<td>5 (2.5)</td>
</tr>
<tr>
<td>sexual</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>unknown</td>
<td>2 (1)</td>
</tr>
<tr>
<td><strong>Origin</strong></td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>175 (88.5)</td>
</tr>
<tr>
<td>other European countries</td>
<td>3 (1.5)</td>
</tr>
<tr>
<td>Africa</td>
<td>12 (6)</td>
</tr>
<tr>
<td>America</td>
<td>7 (3.5)</td>
</tr>
<tr>
<td>Asia</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td><strong>HIV-1 infection year</strong></td>
<td></td>
</tr>
<tr>
<td>1980–89</td>
<td>31 (16)</td>
</tr>
<tr>
<td>1990–99</td>
<td>133 (67)</td>
</tr>
<tr>
<td>2000–09</td>
<td>34 (17)</td>
</tr>
<tr>
<td><strong>Year of specimens</strong></td>
<td></td>
</tr>
<tr>
<td>1993–99</td>
<td>36 (18)</td>
</tr>
<tr>
<td>2000–04</td>
<td>83 (42)</td>
</tr>
<tr>
<td>2005–09</td>
<td>67 (34)</td>
</tr>
<tr>
<td>unknown</td>
<td>12 (6)</td>
</tr>
<tr>
<td><strong>ART status of specimens collected</strong></td>
<td></td>
</tr>
<tr>
<td>drug naive</td>
<td>30 (15)</td>
</tr>
<tr>
<td>under ART</td>
<td>168 (85)</td>
</tr>
<tr>
<td>PI experienced</td>
<td>166 (84)</td>
</tr>
<tr>
<td>NNRTI experienced</td>
<td>126 (64)</td>
</tr>
<tr>
<td>NRTI experienced</td>
<td>92 (46)</td>
</tr>
<tr>
<td><strong>Infecting HIV-1 variants</strong></td>
<td></td>
</tr>
<tr>
<td>B subtype</td>
<td>179 (90)</td>
</tr>
<tr>
<td>non-B variants</td>
<td>19 (10)</td>
</tr>
</tbody>
</table>

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*aPatient distribution among the CAM hospitals; Hospital La Paz (n=51), Hospital Gregorio Marañón (n=47), Hospital 12 de Octubre (n=34), Hospital Carlos III (n=30), Hospital de Getafe (n=24), Hospital Niño Jesús (n=7), Hospital Príncipe de Asturias (n=4) and Hospital de Móstoles (n=1).**

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*Countries of origin: Spain (175), Portugal (1), Romania (1), Poland (1), Equatorial Guinea (6), Cameroon (2); Morocco (2), Mozambique (1), Nigeria (1), Ecuador (2), Guatemala (2), Honduras (1), Peru (1), Venezuela (1) and India (1). Six of the 175 children from Spain had a foreign mother or father coming from Equatorial Guinea (2), Morocco (1), Democratic Republic of Congo (1), Senegal (1) or Cameroon (1).*
Table 2. Characteristics of the 198 patients with available pol sequence by December 2009

<table>
<thead>
<tr>
<th>Features</th>
<th>Total (n=198)</th>
<th>Infected by resistant viruses (n=119)</th>
<th>Infected by wild-type virus (n=79)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alive [n (%)]</td>
<td>194 (98.0)</td>
<td>116 (97.5)</td>
<td>78 (98.7)</td>
</tr>
<tr>
<td>Mean age, years (SD)</td>
<td>15.2 (5)</td>
<td>15.9 (4.8)</td>
<td>14.5 (5.1)</td>
</tr>
<tr>
<td>Carrying HIV-1 non-B variants [n (%)]</td>
<td>19 (9.6)</td>
<td>5 (4.2)</td>
<td>14 (17.7)</td>
</tr>
<tr>
<td>Clinical follow-up [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>paediatric unit</td>
<td>151 (76.3)</td>
<td>89 (74.8)</td>
<td>62 (78.5)</td>
</tr>
<tr>
<td>adults unit</td>
<td>27 (13.6)</td>
<td>18 (15.1)</td>
<td>9 (11.4)</td>
</tr>
<tr>
<td>lost to follow-up</td>
<td>16 (8.1)</td>
<td>9 (7.6)</td>
<td>7 (9.0)</td>
</tr>
<tr>
<td>deceased</td>
<td>4 (2.0)</td>
<td>3 (2.5)</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Clinical status (CDC) [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>asymptomatic, N1</td>
<td>1 (0.5)</td>
<td>0 (0)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>low symptoms, A</td>
<td>65 (32.8)</td>
<td>38 (32.0)</td>
<td>27 (34.2)</td>
</tr>
<tr>
<td>moderate symptoms, B</td>
<td>47 (23.7)</td>
<td>26 (21.8)</td>
<td>21 (26.5)</td>
</tr>
<tr>
<td>severe symptoms, C</td>
<td>82 (41.4)</td>
<td>53 (44.5)</td>
<td>29 (36.7)</td>
</tr>
<tr>
<td>unknown</td>
<td>3 (1.6)</td>
<td>2 (1.7)</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Viral load (HIV-1 RNA copies/mL) [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>116 (58.6)</td>
<td>70 (58.8)</td>
<td>46 (58.2)</td>
</tr>
<tr>
<td>51–500</td>
<td>22 (11.1)</td>
<td>17 (14.3)</td>
<td>5 (6.3)</td>
</tr>
<tr>
<td>&gt;500</td>
<td>53 (26.8)</td>
<td>29 (24.4)</td>
<td>24 (30.4)</td>
</tr>
<tr>
<td>unknown</td>
<td>7 (3.5)</td>
<td>3 (2.5)</td>
<td>4 (5.1)</td>
</tr>
<tr>
<td>Range of CD4+ T cell count (cells/mm(^3)) [n (%)]</td>
<td>13 (6.6)</td>
<td>6 (5.0)</td>
<td>7 (8.9)</td>
</tr>
<tr>
<td>&gt;1500</td>
<td>66 (33.3)</td>
<td>41 (34.5)</td>
<td>25 (31.6)</td>
</tr>
<tr>
<td>751–1500</td>
<td>51 (25.8)</td>
<td>37 (31.1)</td>
<td>14 (17.7)</td>
</tr>
<tr>
<td>500–750</td>
<td>27 (13.6)</td>
<td>16 (13.5)</td>
<td>11 (13.9)</td>
</tr>
<tr>
<td>&lt;200</td>
<td>38 (19.2)</td>
<td>16 (13.4)</td>
<td>22 (27.9)</td>
</tr>
<tr>
<td>unknown</td>
<td>3 (1.6)</td>
<td>2 (1.7)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>ART by December 2009 [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>drug naive</td>
<td>7 (3.5)</td>
<td>1 (0.8)</td>
<td>6 (7.6)</td>
</tr>
<tr>
<td>stopped treatment(^b)</td>
<td>11 (5.6)</td>
<td>5 (4.2)</td>
<td>6 (7.6)</td>
</tr>
<tr>
<td>under ART</td>
<td>178 (89.9)</td>
<td>112 (94.2)</td>
<td>66 (83.5)</td>
</tr>
<tr>
<td>unknown</td>
<td>2 (1.0)</td>
<td>1 (0.8)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>Sequenced specimen [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA-derived sequence(^c)</td>
<td>42 (21.2)</td>
<td>19 (16.0)</td>
<td>23 (29.1)</td>
</tr>
<tr>
<td>RNA-derived sequence(^d)</td>
<td>156 (78.8)</td>
<td>100 (84.0)</td>
<td>56 (70.9)</td>
</tr>
<tr>
<td>Numbers of ART regimens by December 2009 [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7 (3.5)</td>
<td>1 (0.8)</td>
<td>6 (7.6)</td>
</tr>
<tr>
<td>1</td>
<td>12 (6.1)</td>
<td>7 (5.9)</td>
<td>5 (6.4)</td>
</tr>
<tr>
<td>2</td>
<td>24 (12.1)</td>
<td>12 (10.1)</td>
<td>12 (15.2)</td>
</tr>
<tr>
<td>3</td>
<td>30 (15.2)</td>
<td>16 (13.5)</td>
<td>14 (17.7)</td>
</tr>
<tr>
<td>4–6</td>
<td>72 (36.4)</td>
<td>46 (38.7)</td>
<td>26 (32.9)</td>
</tr>
<tr>
<td>7–12</td>
<td>49 (24.7)</td>
<td>35 (29.4)</td>
<td>14 (17.7)</td>
</tr>
<tr>
<td>&gt;12</td>
<td>1 (0.5)</td>
<td>1 (0.8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>unknown</td>
<td>3 (1.5)</td>
<td>1 (0.8)</td>
<td>2 (2.5)</td>
</tr>
</tbody>
</table>

\(^a\)Classification according to the CDC. 1994 revised classification system for human immunodeficiency virus infection in children less than 13 years of age; Official authorized addenda: human immunodeficiency virus infection codes and official guidelines for coding and reporting ICD-9-CM. MMWR 1994; 43 (No. RR-12): 1–10.

\(^b\)Patients with previous but not current exposure to ART.

\(^c\)DNA-derived sequences were obtained from immortalized DNA (n=5) and PBMCs (n=37) available from HIV-1 BioBank.

\(^d\)RNA-derived sequences were obtained from plasma samples newly performed in 58 patients and recovered from routine drug resistance analysis in 98 cases.
Figure 1. Phylogenetic analysis of pol sequences ascribed to HIV-1 non-B variants. The bootstrap cut-off was set at 700. Bootstrap values are shown as percentages. The tree was performed with the 17 non-B sequences including both PR and partial RT coding regions (1302 bp). The 19 non-B variants identified in the study cohort were confirmed with high bootstrap values (>700) in additional phylogenetic trees, including the two specimens with only PR or RT sequences available (data not shown).
Among the 198 HIV-1-infected children under study, pol sequences were analysed in 30 (15%) before using any ART. Seven of these (23%) were infected by non-B subtypes (1A2, 2D, 1G and 3CRF02_AG) and the rest by subtype B strains. Three naive patients infected with non-B variants were born in Spain, while the rest came from Equatorial Guinea (3) and Nigeria (1). All of them acquired HIV-1 infection through MTCT, except two patients with unknown infection routes (Table 1). Sequences from 30 naive subjects were amplified from 16 plasma specimens (14 PR/RT and 2 RT), 8 PBMC specimens (6 PR/RT, 1 RT and 1 PR), 2 DNA specimens (1 PR/RT and 1 RT) and the remaining 4 (all PR/RT) were recovered sequences. Four of the 30 naïve children (13%) were infected with HIV-1 resistant variants carrying at least one resistance mutation to one or more drug family. For protease inhibitors (PIs), only major mutations were considered. One of the four children carrying resistant viruses was infected by a non-B variant (CRF02_AG), and they were infected in 1994 (1), 1997 (1), 2004 (1) and 2005 (1). Thus the global TDR mutations to any analysed drug family appeared in 13% of naive children; 4%, 7% and 10% for PIs, nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), respectively (Table 3). Regarding TDR mutations for PIs, NRTIs and NNRTIs found among the 30 naïve patients, M46L was the only major PI resistance mutation found, and was present in one virus from 1 of the 26 patients with available PR sequences. The frequencies of NRTI resistance mutations in naive children were 6.9% (for M41L) and 3.4% (for both L210W and T215S). The frequency of NNRTI resistance mutations was 3.4% (for both K103N and P225H). All TDR mutations from naïve children were recovered from RNA-derived sequences. More than a quarter of 119 children carrying resistant virus presented <25% CD4+ T cells (Table 2).

### High level of drug resistance among children on ART

One hundred and sixty-eight of the 198 children (85%) with analysed sequences were on ART by the time specimens were collected. Among these, 156 (93%) were infected by subtype B viruses and 12 patients harboured non-B variants (2C, 1F1, 1H, 5CRF02_AG, 1CRF08_BC, 1CRF12_BF and 1CRF13_cpx). Five of these non-B-infected children were native Spanish and the rest came from Equatorial Guinea (n=3), Mozambique (n=1), Cameroon (n=1), Congo (n=1) and India (n=1). PR/RT sequences were analysed in 125 pretreated children, only PR in 32 and only RT in 11. Sequences from pretreated children were amplified from 42 plasma specimens (33 PR/RT, 5 PR and 4 RT), 29 from PBMCs (22 PR/RT, 1 PR and 6 RT), 3 from DNA (2 PR and 1 RT) and 94 recovered sequences (70 PR/RT and 24 PR).

Two-thirds of the 168 children on ART at specimen collection time were infected with mutants resistant to any drug family (Table 3). Three of the 111 children carrying resistant viruses were infected by non-B variants (1C, 1H and 1CRF02_AG). Thus global resistance prevalence was higher among ART-exposed children than in naïve children (66% versus 13%). Considering drug families, 32% of children were infected by viruses resistant to PIs, 46% of children were infected by viruses resistant to NRTIs and 29% of children were infected by viruses resistant to NNRTIs (Table 3). As expected, more children were infected by viruses resistant to two or more drug classes among pretreated versus naive individuals; 30% (50/168 patients) versus 0% (0/30 patients) (Table 3). Among pretreated patients in the cohort, a similar high rate of global resistance provided by RNA (136 patients) and DNA (32 patients) analysis was found (68% versus 56%; P=0.273), although significantly higher for PIs (41% versus 18%; P=0.036) and for NNRTIs (42% versus 10%; P=0.002). According to the infecting HIV-1 variant, we observed a significantly higher global prevalence of drug-resistant mutations among children harbouring subtype B compared with non-B variants (Table 4), mainly due to a significantly higher NNRTI resistance.

The drug resistance mutations for PIs, NRTIs and NNRTIs observed in pretreated children are shown in Figure 2. The major resistance mutations found in over 8% of children receiving ART were: for PIs, L90M (14.6%), D30N (27.9%), M41L (25.7%), T215Y (22.8%), K70R (16.9%), L210W (16.9%), M184V (15.4%) and K219Q (11%); and for NNRTIs, K103N (16.2%).
All 198 PR and/or RT sequences from the HIV-1 pol gene from all infected subjects reported in this study have been submitted to GenBank. Accession numbers for subtype B sequences are HQ426714–HQ426892 and for non-B variants are HQ426893–HQ426911.

Discussion

This study describes for the first time the HIV-1 variants infecting patients enrolled in the Madrid cohort of children infected with HIV during the period 1993–2009, their clinical and epidemiological features and the prevalence of drug resistance mutations for different drug families in viruses present in naive and pretreated patients at sample collection.

**HIV-1 non-B variants are present in the Madrid paediatric cohort**

The major finding is that HIV-1 non-B variants infect almost 1 in 10 patients in this large Spanish paediatric cohort, most of whom are under current follow-up. The data reveal that non-B variants were mainly introduced through perinatal infection in the first half of the 1990s through foreign infected mothers, mainly coming from countries with a high prevalence of non-B variants.22 Only 21% of the children infected by non-B variants...
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had native Spanish parents. The observed prevalence of HIV-1 infections with non-B variants (10%) agrees with previous reports in infected adults from Madrid and Spain,11,13 where an increasing prevalence has been reported in recent years.11

HIV-1 subtype diversity represents a challenge for clinical management of HIV-1-infected individuals.23 The HIV-1 clade can impact on disease progression and response to ART.24 Although no evidence of subtype-determined virological response to ART has been found in previous Paediatric European Networks,25 differences in disease progression have been confirmed for adult and children infected with clade D variants,14,26 as well as subtype-associated differences in the rate of CD4+ cell decline.23

Since infection in newborns under suboptimal antiretroviral prophylaxis of their mothers increases the risk of drug resistance acquisition, early resistance genotyping of HIV-1-infected newborns is essential,27 mainly in resource-limited settings where ART is being scaled up.28 However, limited data on antiretroviral drug resistance rates are available in well-established paediatric cohorts. Our study reports for the first time the rate of HIV-1 drug resistance in naive and pretreated children in a large paediatric cohort, the Madrid cohort of HIV-infected children.

High rate of TDR among naive patients in the cohort of HIV-infected children in Madrid

The estimated TDR rate (13%) found among the naive paediatric cohort studied was similar to the reported TDR data for adults in Spain (13.6%), although significantly higher in non-B versus B subtypes (18.7% versus 10.6%).17 The TDR rate in naive children in Spain was also similar to that reported in other adult cohorts across Europe and the USA, ranging from 5% to 15%.29–31 TDR rates observed in our cohort were higher than in other surrounding countries, such as France, where 10% of perinatally infected children born during 1997–2004 carried resistant HIV-1 variants.52 Lower TDR rates (6.8%) were also found in the multicentre Collaborative HIV Paediatric Study (CHIPS) cohort in the UK.33 Regarding drug families, TDR for NRTIs and NNRTIs in the 30 naive subjects studied from the Spanish cohort was higher (7% and 10%, respectively) than that reported for 44 naive patients from the CHIPS cohort (6.8%) from 1998 to 2004.31 The TDR rate observed in our cohort could be explained by the use of suboptimal antiretroviral prophylaxis in their mothers, mainly infected when only monotherapy was available. It could also be due to suboptimal ART regimes in foreign mothers coming from low-income countries, where prophylaxis is not always well implemented. Unfortunately, the maternal treatment history of children with TDR was not available.

High resistance in children on ART

Among the 198 children studied, 85% were on ART and 61.6% had been treated with 4–21 different ART regimens by December 2009, presenting good immunological and clinical status. The number of children with ≥ 200 CD4+ T cells was high, similar to other European paediatric cohorts with long-term outcomes,32 and higher than in low-income areas such as Africa.36

This situation is different in low-income countries, such as those in sub-Saharan Africa, where 2.3 million children younger than 15 years have HIV infection3 and < 30% have received treatment.35 In paediatric HIV treatment cohorts in sub-Saharan Africa, children started ART with a poorer immunological status and presented severe immunodeficiency in nearly 70%, compared with 41% in our Spanish cohort. Moreover, the lower number of ART regimens, shorter treatment experience, lack of guarantees of ART per life and lack of extended resistance and viroemia testing predict that infections with resistance variants could be even higher among children infected in low-income countries.

Resistance mutations to PIs, NRTIs, and NNRTIs were nine (37% versus 4%), seven (54% versus 7%) and three (35% versus 10%) times higher, respectively, when comparing pretreated and naive children in the study cohort. As a limitation, this study may not be extrapolated to other settings due to the special characteristics of our study cohort and the small sample size of naive patients. In our cohort, resistant viruses were found in two-thirds (66%) of pretreated children, as in multicentre adult cohorts receiving ART.36 Almost one in three (30%) was infected with viruses resistant to two or more drug classes, reinforcing the importance of the use of new therapies not always approved for paediatric use.37 Another limitation is that although the majority of resistance analyses were performed in plasma-associated RNA, in 42 (10 naive and 32 pretreated) of the 198 children with no available plasma specimens, resistance was analysed in proviral DNA, resulting in lower resistance to PIs and NNRTIs. Resistance mutations found in proviral DNA can reveal hidden archived drug resistance from previous regimens, providing a more expansive historic record of drug resistance relative to plasma. Trends of drug resistance mutations over time were not analysed due to the difficulty in establishing time periods with an equal number of specimens.

In children failing first-line therapy, selection of specific mutations is differentially affected by distinct HIV-1 subtypes.38 HIV-1 non-B variants present natural polymorphisms in drug-associated positions in the absence of ART selection,15,39 which can affect the susceptibility to certain drugs and can accelerate the selection and persistence of strains carrying certain primary drug resistance mutations.40–42 However, the impact of each natural polymorphism on the efficacy of ART remains unknown.

The literature also reports the longer presence of variants carrying resistance mutations in patients infected by certain HIV-1 non-B variants, including the K103N mutation, in children on NNRTIs.34 In our study K103N was the most prevalent NNRTI resistance mutation observed among pretreated children, in agreement with a previous study performed in different cities in Spain.43 Due to the increasing frequency of patients infected with non-B variants in our country,1 and their unknown long-term ART evolution, all infected children should be tested for HIV-1 subtypes, mainly those with mothers from endemic countries for non-B variants.

There is an urgent need for resistance testing as well as the development of new therapies based on different retroviral targets in drug-experienced children with therapeutic failure events. It is essential to use optimized ART for each individual, based on resistance information, to avoid suboptimal regimens and early resistance acquisition and to reduce long-term consequences of incomplete virological control. The reported data will help improve clinical management of the HIV-infected paediatric population in Spain.
Acknowledgements

We thank Almudena García Torre for her excellent technical assistance in the search for specimens from HIV-1 BioBank. We also thank all paediatricians from the cohort, as well as the responsible professionals from the routine resistance testing in the participant hospitals for providing pol sequences of enrolled patients when available.

Members of the Madrid cohort of HIV-infected children


Funding

This work was supported in part by grants from Fondo de Investigaciones Sanitarias (FIS) from Ministerio de Ciencia e Innovacion (grants PI09/00284 and PI07/0236) and from Fundacion para la Investigacion y Prevencion del SIDA en España (FIPSE; grant 360829/09). M. de M. is supported by Instituto Ramon y Cajal de Investigacion Sanitaria and by FIS PI07/0236. G. Y. is supported by Consejeria de Educacion de la Comunidad de Madrid and Fondo Social Europeo (FSE). A. H. is supported by Agencia Lain Enralga.

Transparency declarations

None to declare.

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