Transmitted Drug Resistance Is Still Low in Newly Diagnosed Human Immunodeficiency Virus Type 1 CRF06_cpx-Infected Patients in Estonia in 2010

Radko Avi, Kristi Huik, Merit Pauskar, Valentina Ustina, Tõnis Karki, Eveli Kallas, Ene-Ly Jõgeda, Tõnu Krispin, and Irja Lutsar

Abstract

The presence of transmitted drug resistance (TDR) in treatment-naive HIV-1-positive subjects is of concern, especially in the countries of the former Soviet Union in which the number of subjects exposed to antiretrovirals (ARV) has exponentially increased during the past decade. We assessed the rate of TDR among newly diagnosed subjects in Estonia in 2010 and compared it to that in 2008. The study included 325 subjects (87% of all subjects tested HIV positive from January 1 to December 31, 2010). Of the 244 sequenced viral genomic RNA in the reverse transcriptase (RT) region 214 were CRF06_cpx, nine were subtype A1, three (one each) were subtype B and subtype C, CRF02_AG, and CRF03_AB; 15 viruses remained unclassified as putative recombinant forms between CRF06_cpx and subtype A1. HIV-1 TDR mutations in 2010 and 2008 (n = 145) occurred at similar frequency in 4.5% (95% CI 2.45; 7.98) and 5.5% (95% CI 1.8; 9.24) of the patients, respectively. In 2010, 2.5% (6/244) of the sequences harbored nonnucleoside reverse transcriptase inhibitor (NNRTI) (K103N and K101E), 1.6% (4/244) nucleoside reverse transcriptase inhibitor (NRTI) (M41L, M184I, and K219E), and 0.4% (1/244) protease inhibitor (PI) (V82A) mutations. Our findings indicate that in spite of the increased consumption of ARVs the rate of TDR in Estonia has remained unchanged over the past 3 years. Similar stabilizing or even decreasing trends have been described in Western Europe and North America albeit at higher levels and in different socioeconomic backgrounds.

Introduction


The Estonian HIV-1 epidemic is typical of the “new Eastern European HIV-1 epidemics” that broke out in 2000 mainly among young male IDUs and reached its highest prevalence in the European Union (1,053 per million inhabitants) by 2001. Surprisingly, the epidemic was mainly caused by a rare recombinant form CRF06_cpx and its next generation recombinants with subtype A1 viruses.1,2 During the past 10 years the proportion of HIV positives under highly active antiretroviral therapy (HAART) has rapidly increased from 1% in 2001 to 23% in 2010 (Ministry of Social Affairs, www.sm.ee).

In the “new Eastern European HIV-1 epidemics” the transmitted drug resistance (TDR) has been poorly monitored; data are mainly available for Latvia, Georgia, and some regions of Russia3-8 (http://hivdb.stanford.edu/). Furthermore, the studies have not followed the time trends, consist of very few patients with unbalanced risk categories, and use variable sampling strategies.

In Estonia the prevalence of drug resistance mutations (DRMs) among treatment-naive HIV-positive subjects has

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been monitored since 2005.\textsuperscript{9,10} The studies demonstrate a rapid rise of TDR between the years 2005–2006 and 2008 from 0\% to 5.5\%. However, these studies included different patient populations—chronically infected subjects in former and newly diagnosed patients in the latter study. In the current study we aimed to further follow-up the dynamics of TDR in Estonia and to compare it to the dynamics of TDR in the area of “new Eastern European HIV-1 epidemics” in the countries of the FSU.

**Materials and Methods**

According to the Estonian Health Board database (www.terviseamet.ee) 372 subjects with HIV infection were diagnosed for the first time from January 1 to December 31, 2010. HIV infection was screened by enzyme-linked immunosorbent assay (Vironostika HIV Uniform II Ag/Ab; bio-Mérieux, Marcy l’Etoile, France) and verified by the immunoblotting assay (INNO LIA HIV I/II Score Western Blot; Microgen Bioproducts Ltd., Surrey, UK) in the West-Tallinn Central Hospital HIV Reference Laboratory. Of all the 372 samples used for HIV-1 diagnosis about 150–200 µl of leftover serum in 325 (87\%) subjects was available for TDR testing. All patients were ARV naive and tested HIV positive for the first time. The HIV verification was carried out using personal ID codes by the Estonian Health Board, which excluded double reporting.

Viral RNA extraction, reverse transcription, and amplification were carried out as previously described.\textsuperscript{5} Briefly, HIV-1 genomic RNA was extracted from 100–140 µl of serum. The reverse transcription and nested polymerase chain reaction (PCR) were performed according to a modified protocol originally developed by Dr. Jan Albert (Karolinska Institutet, Stockholm, Sweden) using the RT-PCR primer JA272degen (`5′-GATAATTGTTGCTGACCCART-3′), first-round PCR primers JA272degen and JA269degen (`5′-AGGAAGGMCACARATGAARGA-3′), followed by second-round PCR primers JA270 (`5′-GCCTCCAATAGTCCTATT-3′) and JA271 (`5′-CCACTTAAYTTGCTGATRTCATTGAC-3′). The second-round PCR products were directly sequenced using the ABI Prism Big Dye 3.0 fluorescent terminator sequencing chemistry (Applied Biosystems; Life Technologies Corporation, Carlsbad, CA) with the second-round PCR primers and additional sequencing primers JA274 (`5′-AAAATCCATAACATCTCCTCCGTAATGCC-3′), PRO-2B (`5′-AATGCTYTTATTTTCCTCTTGTCATTAGGC-3′), and A(35)06EE (`5′-TTGTTGTAATCTTAAAATTTCCTCTCTATT-3′). Sequences were assembled using Vector NTI software (Invitrogen, Carlsbad, CA) and aligned by adding subtype reference sequences from the Los Alamos HIV Sequence Database (accession numbers AF004885 [subtype A1], DQ676872 [A1], AF286528 [A2], K03455 [B], U52953 [C], K03454 [D], AF077336 [F1], AY371158 [F2], AF084936 [G], AF190127 [H], EF614151 [J], AJ249235 [K], U54771 [CRF01_AE], AY271690 [CRF02_AG], AF140006 [CRF03_AB], AB286851 [CRF06_cpx], AJ245481 [CRF06_cpx], and AF064699 [CRF06_cpx]) and recent sequences from Estonia and Russia [AY500393 (A1), AY535659 (CRF06_cpx), and DQ400856 (CRF06_cpx)] (www.hiv.lanl.gov) using MEGA 5 software.\textsuperscript{11} The tree was rooted by the HIV-1 group O sequence (L20587). The maximum likelihood (ML) phylogenetic tree was calculated with MEGA 5 with default values (substitution model: Tamura-Nei), bootstrap replications: 500. The bootstrap cut-off value of 80 was used to define the sequences belonging to a certain subtype or transmission cluster. The identification of putative recombinant forms between CRF06_cpx and A1 was conducted with SimPlot software using the bootscanning method with the following parameters: Window 200 bp, Step 20 bp, GapStrip on, Reps 100, Kimura (two-parameter), T1 2.0, and the NJ. HIV sequence subtyping was also performed by Rega Subtyping tool version 2.0 (http://dbpartners.stanford.edu/RegaSubtyping/; last accessed May 2013).

The distribution of DRMs was analyzed by the Stanford HIV Drug Resistance Database Calibrated Population Resistance (CRP) Tool version 6.0 using the WHO 2009 list of surveillance DRMs (SDRM 2009).\textsuperscript{12} For the analysis of natural polymorphisms, the pol region nucleotide sequences were converted into amino acids using MEGA 4.0 software and polymorphic positions were defined as described in the International AIDS Society USA Database (www.iasusa.org/, accessed December 2011).

A control group consisted of 145 sequences sampled between April 1, 2008 and November 30, 2008 from all 545 newly diagnosed subjects in 2008 as described in detail previously.\textsuperscript{9} All diagnostic and resistance testing methods in the previous and in the present study were similar. The failure rate of the resistance testing was 28\% in 2008. For the consistency in the determination and interpretation of DRMs the 2008 sequences were reanalyzed with the algorithms used in the 2010 study.

Descriptive statistics together with 95\% confidence interval (CI) were calculated using program R (version 2.13.1, www.r-project.org; last accessed January 2012). The levels of TDR were compared with the Fisher exact test.

**Results**

The RT-PCR and pol region sequencing (PR codons 6–99 and RT codons 1–241) were successfully performing in 244 subjects (75\%) of whom 61.9\% were male and the median age was 30 years (IQR = 25.0; 36.0). The available demographic data are presented in Table 1. The plasma HIV-1 RNA levels and CD4\textsuperscript{+} T cell counts were not available in any of the analyzed samples. As shown in Table 1, a larger number of samples had failed the genotypic resistance testing among imprisoned subjects compared to those not in prison (Table 1).

The ML phylogenetic analysis was conducted on 229 sequences out of 244, as 15 sequences were considered obvious

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Successfully sequenced</th>
<th>Not successfully sequenced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population size, n</td>
<td>244</td>
<td>81</td>
</tr>
<tr>
<td>Males (%)</td>
<td>61.9</td>
<td>55.6</td>
</tr>
<tr>
<td>Age (years), median (IQR)</td>
<td>30.0 (25.0; 36.0)</td>
<td>29.0 (25.5; 36.0)</td>
</tr>
<tr>
<td>Reason for testing (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not reported</td>
<td>71.3</td>
<td>56.8</td>
</tr>
<tr>
<td>Imprisoned\textsuperscript{a}</td>
<td>18.0</td>
<td>29.6</td>
</tr>
<tr>
<td>Blood and organ donors</td>
<td>2.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Pregnancy screening</td>
<td>8.2</td>
<td>12.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Indicates statistically different distribution between populations (p<0.05).

IQR, interquartile range.
Table 2. Clinical Characteristics, Transmitted Drug Resistance Mutations, and Their Interpretation in Newly Diagnosed Patients Possessing Transmitted Drug Resistance Mutations in the 2010 Newly Diagnosed Population

<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Gender</th>
<th>Subtype</th>
<th>Reason for testing</th>
<th>NRTI mutations</th>
<th>NNRTI mutations</th>
<th>PI mutations</th>
<th>High</th>
<th>Intermediate</th>
<th>Low</th>
<th>Possible low</th>
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<tbody>
<tr>
<td>49</td>
<td>25</td>
<td>M</td>
<td>CRF06_cpx</td>
<td>Imprisoned</td>
<td></td>
<td>K103N</td>
<td>EFV, NVP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>28</td>
<td>F</td>
<td>A1</td>
<td>Not reported</td>
<td>M41L</td>
<td></td>
<td></td>
<td></td>
<td>AZT, d4T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>37</td>
<td>F</td>
<td>A1</td>
<td>Not reported</td>
<td>M41L</td>
<td></td>
<td></td>
<td></td>
<td>AZT, d4T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>NA</td>
<td>F</td>
<td>CRF06_cpx</td>
<td>Imprisoned</td>
<td></td>
<td>K103N</td>
<td>EFV, NVP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>169</td>
<td>29</td>
<td>F</td>
<td>CRF06_cpx</td>
<td>Pregnancy</td>
<td>M184I</td>
<td></td>
<td>3TC, FTC</td>
<td></td>
<td>ABC</td>
<td></td>
<td>ddl</td>
</tr>
<tr>
<td>236</td>
<td>27</td>
<td>M</td>
<td>CRF06_cpx</td>
<td>Imprisoned</td>
<td>K219E</td>
<td></td>
<td></td>
<td></td>
<td>AZT, d4T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>246</td>
<td>21</td>
<td>F</td>
<td>CRF06_cpx</td>
<td>Not reported</td>
<td>K101E</td>
<td></td>
<td>NVP</td>
<td></td>
<td>ETR, RPV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>253</td>
<td>26</td>
<td>M</td>
<td>CRF06_cpx</td>
<td>Imprisoned</td>
<td>K103N</td>
<td>EFV, NVP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>273</td>
<td>30</td>
<td>F</td>
<td>CRF06_cpx</td>
<td>Not reported</td>
<td></td>
<td>V82A</td>
<td></td>
<td></td>
<td>IDV/r</td>
<td></td>
<td>SQV/r</td>
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<tr>
<td>298</td>
<td>31</td>
<td>M</td>
<td>CRF06_cpx</td>
<td>Not reported</td>
<td>K103N</td>
<td>EFV, NVP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>311</td>
<td>37</td>
<td>F</td>
<td>CRF06_cpx</td>
<td>Imprisoned</td>
<td></td>
<td>K103N</td>
<td>EFV, NVP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3TC, lamivudine; ABC, abacavir; ATV/r, atazanavir; AZT, zidovudine; d4T, stavudine; ddI, didanosine; EFV, efavirenz; ETR, etravirine; F, female; FPV/r, fosamprenavir; FTC, emtricitabine; IDV/r, indinavir; LPV/r, lopinavir; M, male; NA, not available; NFV, nelfinavir; NVP, nevirapine; RPV, rilpivirine; SQV/r, saquinavir; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

The phylogenetic analysis revealed seven potential direct or indirect transmission subclusters (defined by bootstrap values above 80%) inside the Estonian CRF06_cpx cluster, each containing two viruses, except for one that contained three viruses. One such cluster involved two K103N DRM-possessing viruses suggesting the presence of potential DRM transmission. In another cluster, one out of two viruses possessed the M184I mutation.

As described in our recent study, in the control group of 2008 samples of HIV, genotyping was successfully performed in 72% of the subjects. The reanalysis of sequences revealed that the TDR level was 5.5% (95% CI 1.8; 9.24). The available demographic, clinical, TDR mutation, and interpretation of data of TDR mutation-possessing subjects are presented in Table 3. Fisher’s exact test did not indicate any statistical difference between the levels of TDR in 2008 (4.5%) and in 2010 (5.5%) (p=0.80).

Discussion

The current study, one of the most comprehensive studies conducted concerning a new Eastern European HIV-1 epidemic, demonstrates that in spite of a significant increase in the consumption of ARV agents in recent years, the prevalence of TDR among newly diagnosed subjects infected almost entirely with non-subtype B HIV-1 viruses CRF06_cpx is still around 5%. Furthermore, the levels compared to 2008 have remained stable. Until now there have been very few studies in which the rate of TDR has been monitored and reported among the entire yearly cohort of newly diagnosed HIV-1-positive subjects in a single, albeit a small, Eastern European country.

Our previous observation concerning the increase of the TDR from 0% in 2005 to 5.5% and its stabilization at 4.5% in 2010 is in agreement with the data from the relatively well-investigated countries of Latvia and Georgia.
<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Gender</th>
<th>Subtype</th>
<th>Reason for testing</th>
<th>NRTI mutations</th>
<th>NNRTI mutations</th>
<th>PI mutations</th>
<th>High</th>
<th>Interpretations</th>
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<tbody>
<tr>
<td>10</td>
<td>NA</td>
<td>M</td>
<td>CRF06_cpx</td>
<td>Not reported</td>
<td>—</td>
<td>L90LM</td>
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<td>21</td>
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<td>F</td>
<td>CRF06_cpx</td>
<td>Not reported</td>
<td>M184V</td>
<td>—</td>
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<td>3TC, FTC</td>
<td>—, ABC, ddI</td>
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<tr>
<td>83</td>
<td>NA</td>
<td>M</td>
<td>B</td>
<td>Not reported</td>
<td>M41L, A62V, T215C</td>
<td>K103N</td>
<td>M46I, L90M</td>
<td>NFV</td>
<td>—, ABC, d4T, ATV/r, LPV/r</td>
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<td>142</td>
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<td>—, ABC, d4T, ATV/r, LPV/r</td>
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<td>F</td>
<td>CRF06_cpx</td>
<td>Not reported</td>
<td>—</td>
<td>—</td>
<td>M46I</td>
<td>—</td>
<td>—, NFV/r, ATV/r, FPV/r, LPV/r</td>
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<tr>
<td>163</td>
<td>NA</td>
<td>F</td>
<td>B</td>
<td>Not reported</td>
<td>M41L, A62V, T215DN</td>
<td>—</td>
<td>M46I, L90M</td>
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<td>176</td>
<td>NA</td>
<td>F</td>
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<td>Not reported</td>
<td>M184I</td>
<td>K103N, V108I, V179E, P225H</td>
<td>—</td>
<td>3TC, FTC, EFV, NVP</td>
<td>—, ABC, ddI</td>
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</table>

3TC, lamivudine; ABC, abacavir; ATV/r, atazanavir; AZT, zidovudine; d4T, stavudine; ddI, didanosine; EFV, efavirenz; ETR, etravirine; F, female; FPV/r, fosamprenavir; FTC, emtricitabine; IDV/r, indinavir; LPV/r, lopinavir; M, male; NA, not available; NFV, nelfinavir; NVP, nevirapine; RPV, rilpivirine; SQV/r, saquinavir; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.
A few limitations of the study should be noted. First, we conducted the analysis when the HIV infection was first diagnosed not when the patient was infected. Since actively replicating viruses with DRM may revert back to a wild-type state, the actual number of transmitted DRMs may be underestimated. Moreover, this bias can be affected by the time between infection and diagnosis, which may have changed with time. In the case of the Estonian epidemic it is likely that many patients diagnosed today were infected in the early or mid-2000s when prophylactic measures (e.g., syringe exchange programs) were still not prevalent. Second, the current study used population-based sequencing in HIV genotyping, which makes it possible to describe only prevailing HIV mutation quasispecies. Third, a highly homogeneous viral population could interfere with the identification of transmission clusters or sample cross-contamination. The latter is not unique to our study. Very similar or identical sequences have also been reported in other monophyletic HIV-1 epidemics among IDUs in the FSU or Southeast Asia.

In summary, TDR among newly diagnosed HIV-positive subjects in Estonia is still low, remaining at a level of around 5%. Thus, DRM tests are not recommended prior to introducing ARV therapy at present. A further monitoring of TDR mutations in Estonia and other regions of the FSU should improve our understanding of similarities and differences between Western and new Eastern European HIV-1 epidemics.

Sequence Data

The nucleotide sequences of IN regions have been submitted to GenBank and the accession numbers are JX431619–JX431862.

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Author Disclosure Statement

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