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

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Abstract

The presence of transmitted drug resistance (TDR) in treatment-naïve 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

AFTER THE POLITICAL AND socioeconomic changes in the countries of the former Soviet Union (FSU) the rapidly growing number of intravenous drug users (IDUs) in the early 1990s generated a biological niche for blood-borne infections. This niche was first filled by the hepatitis B virus (HBV) and hepatitis C virus (HCV) and then followed by HIV-1 infection, which first started to spread in Odessa (Ukraine) in 1995. In the next 5 years HIV spread all over the FSU. An overwhelming proportion of HIV-positive subjects were young male IDUs infected by monophyletic and very homogeneous subtype A1 viruses. The other characteristics of the new FSU HIV-1 epidemic were eliminating almost entirely the period of mono- or dual antiretroviral (ARV) therapy and the use of “old type” first-line regimens containing thymidine analogue (TA) [zidovudine (ZDV), didanosine (ddI)] as backbones; the latter is still the case. In most FSU countries, including Estonia, the treatment is fully sponsored by the government.

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 Russia^{3–8} (<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-naïve HIV-positive subjects has

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been monitored since 2005.^{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 patients 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 immunoassay (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 μ l 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.⁹ Briefly, HIV-1 genomic RNA was extracted from 100–140 μ l 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'-GGATAAATMTGACTTGCCCAART-3'), first-round PCR primers JA272degen and JA269degen (5'-AGGAAGGMCA CARATGAARGA-3'), followed by second-round PCR primers JA270 (5'-GCTTCCCTCARATCACTCTT-3') and JA271 (5'-CCACTAAATTCTGTATRTCATTTGAC-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'-AAAATCCATACAA TACTCCA-3'), PRO-2B (5'-AATGCTYTTATTTCTCTTCTG TCAATGGC-3'), and A(35)06EE (5'-TTGGTTGTACTTTAAA TTTTCCAATAAGTCCTATT-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), AF286238 (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), AF414006 (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.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), T/t 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).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.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⁺ 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 1. CHARACTERISTICS OF HIV NEWLY DIAGNOSED SUBJECTS IN 2010

	Successfully sequenced	Not successfully sequenced
Population size, <i>n</i>	244	81
Males (%)	61.9	55.6
Age (years), median (IQR)	30.0 (25.0; 36.0)	29.0 (25.5; 36.0)
Reason for testing (%)		
Not reported	71.3	56.8
Imprisoned ^a	18.0	29.6
Blood and organ donors	2.5	1.2
Pregnancy screening	8.2	12.3

^aIndicates 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

ID	Age	Gender	Subtype	Reason for testing	Drug resistance mutations 2010			High	Interpretations			
					NRTI mutations	NNRTI mutations	PI mutations		Intermediate	Low	Possible low	
49	25	M	CRF06_cpx	Imprisoned	—	K103N	—	EFV, NVP	—	—	—	—
69	28	F	A1	Not reported	M41L	—	—	—	—	AZT, d4T	—	—
89	37	F	A1	Not reported	M41L	—	—	—	—	AZT, d4T	—	—
124	NA	F	CRF06_cpx	Imprisoned	—	K103N	—	EFV, NVP	—	—	—	—
169	29	F	CRF06_cpx	Not reported	M184I	—	—	3TC, FTC	—	ABC	ddI	—
236	27	M	CRF06_cpx	Imprisoned	K219E	—	—	—	—	—	AZT, d4T	—
246	21	F	CRF06_cpx	Pregnancy	—	K101E	—	—	NVP	EFV	ETR, RPV	—
253	26	M	CRF06_cpx	Imprisoned	—	K103N	—	EFV, NVP	—	—	—	—
273	30	F	CRF06_cpx	Not reported	—	—	V82A	—	IDV/r, NFV	ATV/r, LPV/r	SQV/r, FPV/r	—
298	31	M	CRF06_cpx	Not reported	—	K103N	—	EFV, NVP	—	—	—	—
311	37	M	CRF06_cpx	Imprisoned	—	K103N	—	EFV, NVP	—	—	—	—

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.

recombinant forms between CRF06_cpx and subtype A1 by initial analysis using the ML phylogenetic tree, SimPlot software, and the Rega Subtyping tool. Phylogenetic analysis indicated that 88% (214/244) of sequences were clustered with CRF06_cpx reference sequences, which indicates high similarity to the Estonian CRF06_cpx viruses. Nine sequences (4%) formed a monophyletic cluster with the Eastern European subtype A1 viruses. Few cases of subtype B ($n=3$), subtype C, CRF02_AG, and CRF03_AB ($n=1$ for each) viruses were found. The reanalysis using the Rega subtyping tool also indicated a high prevalence of CRF06_cpx sequences (80%; 194/244).

Altogether, 11/244 strains (4.5%; 95% CI 2.45; 7.98) possessed DRMs; no dual or triple class resistance was observed. DRMs were found in nine CRF06_cpx and three subtype A1 viruses (Table 2). The most common DRMs were those against nonnucleoside reverse transcriptase inhibitors (NNRTIs) ($n=6$; 2.5%). Five viruses possessed K103N and one K101E mutation. The nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) mutations were found in four cases (1.6%), composed of mutations M41L, M184I, and K219E in two (0.8%), one (0.4%), and one (0.4%) of the cases, respectively. Protease inhibitor (PI) resistance mutation V82A was found in one virus (0.4%). Of the 11 described NRTI DRM positions two were polymorphic (M184IM and K219EK). The available demographic and clinical data of transmitted DRM possessing subjects are presented in Table 2. In addition to DRMs, RT region substitutions V108I and V179E associated with DR were described in one (0.4%) and six (2.5%) viral strains, respectively. PR polymorphisms I13V (233/244, 95%), K14R (199/244, 82%), G17E (189/244, 77%), K20I (215/244, 88%), E35D (193/244, 79%), M36I (241/244, 99%), R41K (216/244, 89%), L63H (179/244, 73%), H69K (236/244, 97%), and L89M (223/244, 91%) were also found to be highly prevalent. The DRMs interpretation of the 2010 sequences revealed that most of the high and intermediate level resistance was against EFV (efavirenz) and/or NVP (nevirapine) (Table 2).

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.⁹ 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.⁹ 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.^{3-5,8} The

TABLE 3. CLINICAL CHARACTERISTICS, TRANSMITTED DRUG RESISTANCE MUTATIONS, AND THEIR INTERPRETATION IN NEWLY DIAGNOSED PATIENTS POSSESSING TRANSMITTED DRUG RESISTANCE MUTATIONS IN THE 2008 NEWLY DIAGNOSED POPULATION

ID	Age	Gender	Subtype	Reason for testing	Drug resistance mutations 2008				Interpretations			
					NRTI mutations	NNRTI mutations	PI mutations		High	Intermediate	Low	Possible low
10	NA	M	CRF06_cpx	Not reported	—	L90LM	—		NFV	SQV/r	ATV/r, FPV/r, IDV/r	LPV/r
21	NA	F	CRF06_cpx	Not reported	M184V	—	—		3TC, FTC	—	ABC	ddl
54	NA	M	CRF06_cpx	Not reported	—	K103N	—		EFV, NVP	—	—	—
83	NA	M	B	Not reported	M41L, A62V, T215C	—	M46L, L90M		NFV	AZT, d4T, ATV/r, FPV/r, IDV/r, SQV/r	ABC, ddl, TDF, LPV/r	3TC, FTC
142	NA	M	CRF06_cpx	Not reported	—	K103KN	—		EFV, NVP	—	—	—
153	NA	F	CRF06_cpx	Not reported	—	—	M46I		—	—	NFV/r	ATV/r, FPV/r, IDV/r, LPV/r
163	NA	F	B	Not reported	M41L, A62V, T215DN	—	M46L, L90M		NFV	AZT, d4T, ATV/r, FPV/r, IDV/r, SQV/r	ABC, ddl, TDF, LPV/r	3TC, FTC
176	NA	F	CRF06_cpx	Not reported	M184I	K103N, V108L, V179E, P225H	—		3TC, FTC, EFV, NVP	—	ABC	ddl

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; SQV/r, saquinavir; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

relatively low levels of TDR are also characteristic of the new HIV epidemic in other FSU countries.^{7,13,14} It is important to emphasize that in most Eastern European countries no systematic large-scale studies have been conducted yet.

The low level of TDR in the current study could be explained by several factors. First, the overwhelming majority of patients in the region of the FSU including Estonia have never been exposed to pre-HAART suboptimal ARV regimens containing one or two ARV agents. Second, because this is a relatively new epidemic the proportion of patients on ARV treatment is still low (approximately 23% in 2010) (Ministry of Social Affairs). Furthermore, even those on treatment are still receiving their first regimen and have not developed resistance yet.¹⁵ Third, the majority of infections are caused by drug-sensitive and monophyletic HIV-1 strains (CRF06_cpx or A1) originating most probably from the areas in which ARV treatment was not available before the Estonian outbreak.¹ Fourth, approximately 70% of patients visiting the treating physicians for the first time possess CD4⁺ T cell counts <350 cell/ml (Estonian HIV database, www.esid.ee). It suggests that newly diagnosed subjects were most probably infected a relatively long time ago during a period when ARV treatment was rare in Estonia.

In the current study and among the samples collected in 2008 the most prevalent DRM was K103N. The high prevalence of K103N has been described in all larger European TDR studies. K103N confers high-level resistance to all first-generation NNRTIs including EFV. EFV belongs to the most commonly used NNRTI-based first-line regimen in Estonia (EFV+ZDV+3TC) as well as to the preferred first-line regimen [EFV+tenofovir (TNF)+FTC] in Europe (www.europeanaidsclinicalociety.org). Somewhat unexpected was the low level of M184IV in the study samples as both K103N and M184IV have been described as by far the most common DRMs among treatment-failed patients in Estonia.¹⁶ A similar absence of M184IV has been demonstrated in some Western European transmitted DRM studies.^{17,18}

The level of TDR may be influenced by several demographic and social characteristics of the populations. First, an important source of TDR at the population level involves the proportion of patients under nonsuppressive ARV therapy carrying viruses with DRMs.¹⁹ According to the Estonian HIV database half of the patients (56%) receiving HAART have detectable viral loads (>50 cp/ml) that are higher than the corresponding rates from Western Europe (15% in some cohorts).²⁰ Second, the proportion of patients needing but not receiving ARV treatment could potentially influence the rate of TDR. According to modeling, the proportion of such subjects is about 60% in Estonia.²¹ We could speculate that these patients facilitate the expansion of the HIV-1 epidemic. However, as the overall TDR rate in Estonia is low it is likely that they are infected with wild-type viruses and therefore will contribute very little to TDR. The dynamics of TDR may also be influenced by the risk groups involved in HIV-1 epidemics.^{22–24} IDUs in general are the most difficult population to manage as their compliance with medical care including adherence to ARV therapy is in general low. In recent years, the rate of IDUs among newly infected patients in Estonia has diminished as expected; in 2010 they accounted for 30% (Estonian HIV database). The TDR could also be spread via vertical transmission. In Estonia this plays a minor role as the mother-to-child transmission rate is low (around 1%).¹⁵

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 quasiespecies. 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.^{2,9,10,25–27}

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.

Acknowledgments

The authors are grateful to Maarit Maimets and Päivi Pütsep for critical manuscript review. Financial support came from the Center of Excellence of Translational Research of Neuroimmunology (project SFOS WP1-NeuroAIDS), the Basic Financing and the Target Financing of Estonian Ministry of Education and Research (grant SF0180004s12), the Estonian Science Foundation (grants ETF8856, ETF8004, and ETF8415), the European Union through the European Regional Development Fund, and the Archimedes Foundation.

Author Disclosure Statement

No competing financial interests exist.

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