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Research paper

Stable level of HIV transmitted drug resistance in Estonia despite significant scale-up of antiretroviral therapy

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ABSTRACT

Background: Due to the widespread use of non-nucleoside reverse transcriptase inhibitors (NNRTI) as part of first-line therapies to curb the human immunodeficiency virus (HIV) epidemic in Eastern-European countries, transmitted drug resistance (TDR) is of serious concern in this region. Therefore, TDR and its associated risk factors were investigated among newly diagnosed HIV-1 subjects in Estonia.
Methods: This nationwide observational study included all newly diagnosed HIV-1 subjects from January 1 until December 31, 2013. Demographic and clinical data were collected using the national surveillance system and the Estonian HIV-positive patient database (*E*-HIV). Starting from RNA, the HIV-1 protease (PR) and reverse transcriptase (RT) region was sequenced and surveillance drug resistance mutations (SDRM) were determined. Sequences from previous studies in Estonia and from public databases were included to study epidemic trends and to determine TDR clusters by phylogenetic analysis.
Results: Out of 325 newly diagnosed HIV-1 infections, 224 were successfully sequenced (68%). As in previous studies from Estonia, the circulating recombinant form CRF06_cpx was the most prevalent HIV subtype (164/224, 74%). Fifteen strains displayed SDRM, giving a TDR rate of 6.7% (95% CI 3.9; 11.0). The most common SDRMs were associated with NNRTI (10/15, 4.5%), followed by PI (3/15, 1.3%) and NRTI (2/15, 0.9%). K103 N (8/15, 53%) was the most common SDRM. The level of TDR and mutational patterns were comparable to previous years. Twenty-six transmission clusters containing Estonian sequences were observed, of which 23/26 belonged to CRF06_cpx and 2/26 displayed evidence of TDR. The only risk factor associated with the presence of TDR was imprisonment (OR 5.187, CI 1.139–25.565, $p = 0.034$).
Conclusions: TDR remained stable at a moderate level in Estonia, K103N is the main SDRM with only one transmission-pair detected. We suggest screening for TDR at the time of diagnosis or prior to antiretroviral treatment initiation to tailor first-line regimens accordingly.
Summary: The third consecutive transmitted drug resistance (TDR) study demonstrated a stable TDR in Estonia. TDR reached 6.7% (moderate level) in 2013, with imprisonment being the only associated risk factor. Few drug resistance-associated transmission clusters were identified.

1. Introduction

Combination antiretroviral therapy (cART) has significantly reduced the morbidity and mortality of patients infected with human immunodeficiency virus type 1 (HIV-1) (Palella Jr. et al., 1998). However, acquired and transmitted drug resistance (TDR) has limited the success of treatment. In so-called old HIV-1 epidemics (Western Europe, USA), TDR rapidly increased up to 20% immediately after the roll-out of cART (Taiwo, 2009; Girardi, 2003). As suboptimal dual- or monotherapy and drug regimens with a low genetic barrier are rarely used anymore, TDR remains at 10–15% in these regions (Bannister et al., 2008; Frentz et al., 2012; Wheeler et al., 2010). The most prevalent surveillance drug resistance mutations (SDRM) in Western Europe are revertant mutations at reverse transcriptase (RT) position 215 and the non-nucleoside RT inhibitor (NNRTI) mutation K103 N (Frentz et al., 2014). The latter may be caused by the widespread use of

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NNRTI within first-line cART and the low genetic barrier to resistance for this specific drug class. Often, SDRM is spread via transmission clusters in Europe (mainly by subtype B viruses and the men having sex with men (MSM) population) (Pineda-Pena et al., 2014; Karlsson et al., 2012; Fearnhill et al., 2017).

Although the Eastern European HIV-1 epidemic has been ongoing since the late 1990s, comprehensive data on TDR from the Former Soviet Union (FSU) countries are scattered and mostly limited to small cohorts, in contrast to the situation in Western Europe and the USA. In Eastern Europe, cART was rarely used during the initial years of the epidemics and as a result, TDR was negligible in that time period (Avi et al., 2009). Importantly, the use of cART in Eastern Europe has increased considerably in the 2000s, mainly within the format of potent drug regimens rather than suboptimal mono- or dual therapy (Murray et al., 2014). The reported data of TDR from FSU countries vary but are generally below 10% [0% in Lithuania (Caplinskas et al., 2013), 2.1% in Ukraine (Fearnhill et al., 2017), 3–7% in Latvia (Kolupajeva et al., 2008; Balode et al., 2010), 0–13% in Russia (depending on the study) (Bobkova, 2013)].

With a population of 1.3 million (Statistics Estonia 2016), Estonia has experienced an HIV-1 epidemic typical to the FSU. The epidemic started in 2000 among young people who inject drugs (PWID) and remains to be predominantly driven by HIV-1 viruses classified as circulating recombinant form CRF06_cpx (Adojaan et al., 2005; Zetterberg et al., 2004; Ustina et al., 2001). According to international guidelines, cART is currently offered to all people living with HIV (PLWH) (Society EAC, 2015), free of charge and regardless of health insurance status. The percentage of patients being treated with cART has increased from 53% in 2010 to 88% in 2017 (Estonian HIV database (*E*-HIV), www.hiv.ut.ee). As TDR has been monitored since 2006 in Estonia (Avi et al., 2011; Avi et al., 2014a), we aimed to study the trend of TDR and its association with the roll-out of cART, as well as the risk factors associated with TDR in 2013.

2. Materials and methods

2.1. Study population

All serum samples of subjects who tested seropositive for HIV-1 for the first time at the primary testing site and had this diagnosis confirmed at the Reference Laboratory of West Tallinn Central Hospital between January 1 and December 31, 2013 were eligible for the study. HIV-1 reactivity was assessed by enzyme-linked immunosorbent assay (ELISA) or comparable test at the primary sites. Results were confirmed by ELISA (HIV Ag/Ab Combo Assay; Abbott Laboratories Diagnostics Division, IL, USA or HIV Combi PT; Roche Diagnostics GmbH, Mannheim, Germany) or by Western-blot (NEW LAV BLOT I and NEW LAV BLOT II, Bio-Rad Laboratories Inc., Hercules, California, USA) or if Western-blot negative then by HIV Ag and HIV Ag Confirmatory Test (both Roche Diagnostics GmbH, Mannheim, Germany). Samples were stored at -80 °C until HIV-1 genotyping.

Demographic data for the subjects were obtained from the Estonian Health Board (www.terviseamet.ee) as part of a national surveillance system. Data collected included gender, age, and indication for HIV testing. Clinical data [CD4+ T-cell count, HIV-1 viral load (VL), route of transmission, residence, date of clinical seroconversion if available, AIDS diagnosis, and hepatitis C serology] were obtained from *E*-HIV (Soodla et al., 2015) for subjects who had provided their informed consent for inclusion in the database. As not all patients were included in E-HIV, we also searched laboratory databases of 4 major hospitals for the first HIV-1 VL measurement after diagnosis.

Data on cART consumption were obtained from the Estonian State Agency of Medicines (https://www.ravimiamet.ee/en, n.d.; Ravimiamet, 2015).

2.2. Laboratory methods

All samples were tested for recent HIV infection using the limiting antigen (LAg) avidity enzyme immunoassay (EIA) (Sedia tm HIV-1 LAg Avidity EIA; Sedia Biosciences Corporation, Portland, OR, USA), as described elsewhere (Soodla et al., 2017) and regency period was defined at 140 days according to the protocol.

Viral RNA extraction, reverse transcription, and amplification were carried out as previously described (Avi et al., 2011). Briefly, HIV-1 viral RNA was extracted from 100 to 140 µL serum. Reverse transcription and nested polymerase chain reaction (PCR) were performed according to a modified protocol originally developed by Albert (Murillo et al., 2010) (Karolinska Institute, Stockholm, Sweden), using the JA272degen reverse transcription (RT)-PCR primer (5′-GGATAAATMTGACTTGCCCART-3′), and first-round PCR primers JA272degen and JA269degen (5′-AGGAAGGMCACARATGAARGA-3′), followed by second-round PCR primers JA270 (5′-GCTTCCCTCARATCACTCTT-3′) and JA271 (5′-CCACTAAYTTCTGTATRTCATTGAC-3′).

Second-round PCR products (HXB2 nt 2248-3333) were directly sequenced with ABI Prism BigDye Terminator 3.0 Cycle Sequencing Kits (Applied Biosystems; Life Technologies Corporation, Carlsbad, CA), using second-round PCR primers and additional sequencing primers JA274 (5′-AAAATCCATACAATACTCCA-3′), PRO-2B (5′-AATGCTYTTATTTTCTCTTCTGTCAATGGC-3′), and A(35)06EE (5′-TTGGTTGTACTTTAAATTTTCCAATAAGTCCTATT-3′).

2.3. Subtyping, compilation, quality control and phylogenetic analysis of sequence data

HIV-1 subtype classification of protease (PR)-RT sequences was performed using the online automated tools COMET v1.0 (Struck et al. 2014) and Rega v3 (Pineda-Peña et al. 2014), and verified by a manual phylogenetic approach using the 2010 curated subtype reference alignment extracted from the Los Alamos HIV Sequence Database (LANL) (http://www.hiv.lanl.gov).

To investigate the TDR trend in Estonia and to enable transmission cluster analysis, HIV-1 PR-RT sequences from a previous study in Estonia (Avi et al., 2014a) and HIV-1 PR-RT control sequences were extracted from LANL and GenBank (http://www.ncbi.nlm.nih.gov/genbank/). As the HIV-1 epidemic in Estonia is dominated by CRF06_cpx strains, all available HIV-1 CRF06_cpx PR-RT sequences at LANL were extracted as control sequences. For the other two subtypes that are mainly circulating in Estonia (subtype A1 and B), the 100 most similar sequences to each local HIV-1 sequence were retrieved using the standard nucleotide Basic Local Alignment Search Tool (BLAST, Camacho et al., 2009) and included as control sequences.

The quality of all sequences and the presence of surveillance drug resistance mutations (SDRM) were assessed using the CPR tool version 6.0 (http://cpr.stanford.edu/cpr.cgi) (Team RC, 2013). Duplicate sequences identified by the ElimDupes tool (LANL) and sequences that did not pass the quality control check were removed.

Separate datasets were constructed for CRF06_cpx, subtype A1 and B. As an out-group, the LANL subtype B reference sequences were included for the CRF06_cpx and A1 datasets (K03455, AY331295, AY173951, AY423387), and the LANL subtype C reference sequences for the subtype B dataset (U46016, U52953, AF067155, AY772699). Sequences were aligned with Muscle v3.7 (Edgar, 2004) and manually edited in AliView (Larsson 2014). Nucleotide positions encoding for SDRM (Team RC, 2013) were removed from the alignments to avoid the effect of drug-induced convergent evolution. For the CRF06_cpx, A1 and B datasets, the final alignments contained respectively 1178, 334 and 389 HIV-1 PR-RT sequences spanning 965, 956 and 947 nucleotide positions.

A maximum likelihood (ML) tree was inferred using the general time reversible (GTR) nucleotide substitution model with γ distribution and 1000 bootstrap replicates in RAxML v8.2.12 (Stamatakis 2014).

Transmission clusters were identified using a bootstrap support ≥98% (Hassan et al., 2017) and TDR transmission clusters were defined as transmission clusters with at least one sequence from the Estonian 2013 cohort that displayed SDRM.

2.4. Statistical analyses

Descriptive statistics and 95% confidence interval (CI) were calculated using R version 2.13.1 (Team RC, 2013). Continuous variables in clinical data were compared using the Wilcoxon Rank Sum test. Categorical variables were compared using Fisher's exact test. For SDRM risk analysis logistic regression or Firth's penalized-likelihood logistic regression was performed.

Ethics statement

This study was approved by the Research Ethics Committee of the University of Tartu, in full accordance with the Declaration of Helsinki and Good Clinical Practice. All subjects in *E*-HIV signed their informed consent to participate in the cohort.

3. Results

3.1. Characteristics of the study population in 2013

In 2013, a total of 325 subjects were newly diagnosed with HIV-1 and included in the study (Table 1). The majority (61%) were men, displayed a median age of 32 years and were living in Tallinn, Ida-Virumaa and Narva. Heterosexual transmission (55%) and intravenous drug use (21%) were the two most commonly self-reported risk factors.

Table 1
Characteristics of newly HIV-1 diagnosed populations in 2013.

	Total population (%)	Sequenced (%)	Not sequenced (%)
Number of subjects	325	221	104
Males, N (%)	199 (61)	131 (60)	68 (65)
Age in years, median (IQR)	32 (27–41)	33 (28–42)	32 (27–39)
Reason for testing			
Clinical suspicion of HIV infection	88 (27)	61 (28)	27 (26)
Screening (pregnancy, blood donors)	36 (11)	24 (11)	12 (12)
Indicator diagnosis (STD, TB)	9 (3)	7 (3)	2 (2)
Indicator risk (PWID, imprisonment, known contact)	79 (24)	53 (24)	26 (25)
Unknown or other	113 (35)	76 (34)	37 (36)
Self-reported risk factor			
Heterosexual	181 (56)	121 (55)	60 (58)
PWID	69 (21)	48 (22)	21 (20)
MSM	6 (2)	3 (1)	3 (3)
Unknown or other	69 (21)	49 (22)	20 (19)
County of residence			
Ida-Virumaa and Narva	132 (41)	93 (42)	39 (38)
Tallinn	96 (30)	68 (31)	28 (27)
Other	20 (6)	13 (6)	7 (7)
Unknown	77 (24)	47 (21)	30 (29)
Known clinical seroconversion	6 (2)	5 (2)	1 (1)
Known negative HIV-1 test within last 2 years	61 (19)	41 (19)	20 (19)
Stage of infections by limiting antigen (LAg) avidity testing			
Recent	127 (39)	92 (42)	35 (34)
Long-term	160 (49)	129 (58)	31 (30)
Unknown	38 (12)	0 (0)	38 (37)

Note: IQR – interquartile range, PWID – people who inject drugs, MSM – men having sex with men, STD - sexually transmitted disease, TB – tuberculosis.

All patients were treatment-naïve at sampling and 39% (127/325) were recently infected according to LAg avidity assay. Data on HIV-1 viral load (VL) and CD4+ cell count were available for 176/325 (54%) and 173/325 (53%) subjects, respectively. At the time of diagnosis, median HIV-1 viral load was 4.9 $\log_{10}$ (IQR 4.2–5.5) copies/mL, median CD4+ T-cell count was 366 cells/μL (IQR 206–540), and 84/173 (49%) subjects presented with CD4+ T-cell counts of < 350 cells/μL. Chronic hepatitis C infection was diagnosed in 22/113 patients (19.5%). For 36 subjects (11%) no leftover sample was available for sequencing and for 65 samples (20%) no sequence could be obtained, leaving 224 (69%) strains in the final subset (Fig. 1, Table 1). The patient characteristics of this final subset was comparable to the characteristics of the entire study population.

3.2. HIV-1 subtypes and transmitted drug resistance in 2013

CRF06_cpx was identified as the most prevalent subtype (185/224, 83%), followed by subtype A1 (23/224, 10%), B (7/224, 3%) and unique recombinant forms (URF) (9/224, 4%). Of the patients infected with a CRF06_cpx strain, 46% were recently infected whereas the proportion of recent infections was non-significantly lower in HIV-1 subtype A1 and B infected patients (respectively 13% and 0%).

In total 15/224 strains displayed any SDRM giving an overall TDR prevalence rate of 6.7% (95% CI 3.9; 11.0) (Table 2 and Table 3). No dual- or triple-class resistance was detected. The most common SDRM was associated to NNRTI (10/224, 4.5%), more specifically 8 strains displayed K103N, one strain Y181C and another strain G190E (Table 2). SDRM associated to nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) were found in only 2 cases (2/224, 0.9%), both of which were M41 L. Protease mutation M46I was detected twice (2/224, 0.9%) and L90M once (1/224, 0.4%), resulting into a TDR rate of 1.3% against PI. TDR was found in 5.4% CRF06_cpx (10/185), in 13% A1 viruses (3/23), and 14% subtype B virus (1/7).

3.3. Transmission clusters within the Estonian HIV-1 epidemic

All HIV-1 CRF06_cpx strains that were collected in Estonia belonged to a single clade, although supported with a bootstrap of 87%, within the global CRF06_cpx diversity (Fig. 2a). This Estonian CRF06_cpx clade contained only 11 sequences that were sampled in other countries (Finland (Girardi, 2003), Belarus (Taiwo, 2009), United Kingdom (Taiwo, 2009), Russia (Taiwo, 2009) and Norway (Taiwo, 2009)). Within the Estonian CRF06_cpx epidemic, 23 transmission clusters were observed of which 13 clusters contained at least one Estonian HIV-1 sequence collected in 2013. Out of these 13 transmission clusters, 4 also contained Estonian strains that were collected in 2008 or 2010. Eleven clusters were pairs, one transmission cluster contained 3 strains and one contained 4 strains. Two newly diagnosed HIV-1 patients from 2013 displayed TDR mutation K103 N and clustered into a pair, while another strain also with the K103 N mutation formed a pair with a wild-type strain sampled in 2010 in Estonia.

The Estonian HIV-1 subtype A1 epidemic is embedded within a larger A1 epidemic of Former Soviet Union (FSU) countries (Fig. 2b). Only two transmission pairs were observed of which one pair consisted of two wild-type strains collected in Estonia and the other pair consisted of wild-type strains collected in Estonia and Ukraine. A larger cluster of 13 subtype A1 strains was observed in Estonia, however not robustly supported by bootstrap analysis (< 50%). Within this cluster, two strains with RT mutation M41 L and one strain with PR mutation M46I were observed.

Only 7 subtype B strains were detected in the samples of the HIV-1 patients diagnosed in 2013 and they were dispersed over the entire subtype B tree that was constructed (Fig. 2c). Nevertheless, one transmission pair was observed containing 2 wild-type strains collected in 2013.

Altogether 25 strains from 2013 belonged to a transmission pair or

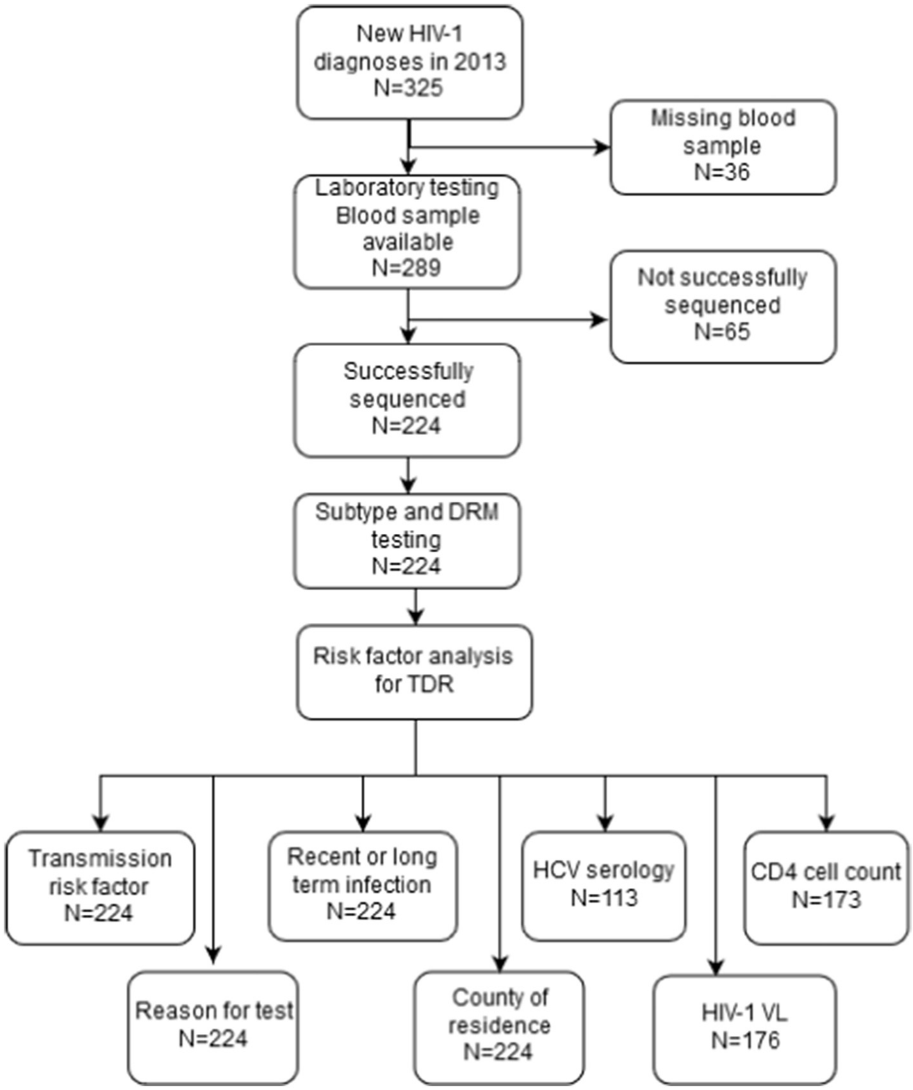

Fig. 1. Flowchart for TDR study in Estonia in 2013.

Table 2
Clinical characteristics of newly diagnosed HIV-positive subjects with transmitted drug resistance in 2013.

Gender	Age (years)	Subtype	Reason for testing	Route of transmission	Stage of infection	Baseline HIV-1 viral load (log_{10} copies/mL)	Baseline CD4+ T -cell count (cells/μL)	SDRM
F	43	A1	NA	heterosexual	Long term	2.3	562	M46I
F	42	CRF06_cpx	clinical suspicion	heterosexual	Recent	3.4	218	M46I
M	37	B	clinical suspicion	heterosexual	Long term	4.7	479	L90 M
M	30	A1	imprisoned	PWID	Long term	4.0	372	M41 L
M	23	A1	imprisoned	PWID	Long term	4.4	671	M41 L
M	44	CRF06_cpx	screening	heterosexual	Long term	5.3	61	K103 N
F	51	URF	clinical suspicion	heterosexual	Long term	NA	NA	K103 N
M	30	CRF06_cpx	imprisoned	PWID	Long term	5.1	511	K103 N
F	52	CRF06_cpx	screening	unknown	Long term	NA	NA	K103 N
F	34	CRF06_cpx	NA	heterosexual	Recent	NA	NA	K103 N
M	29	CRF06_cpx	screening	heterosexual	Recent	NA	NA	K103 N
M	29	CRF06_cpx	clinical suspicion	PWID	Long term	NA	NA	K103 N
M	20	CRF06_cpx	imprisoned	PWID	Recent	6.5	528	K103 N
M	26	CRF06_cpx	NA	PWID	Long term	5.3	55	Y181C
M	27	CRF06_cpx	clinical suspicion	heterosexual	Long term	NA	NA	G190E

Note: F – female, M- male; NA – not available; URF – unique recombinant form; PWID – people who inject drugs; stage of infection determined by Lag avidity assay and SDRM defined according to Bennett et al. (Team RC, 2013).

Table 3
Risk factors of SDRMs in HIV-1 sequences obtained from newly diagnosed patients in Estonia in 2013.

	OR	Lower CI	Upper CI	*P* value
Risk factor				
Heterosexual	1.0			
PWID	1.350	0.373	4.308	0.627
MSM	1.908	0.014	22.254	0.699
Unknown or other	1.005	0.239	3.427	0.994
Reason for testing				
Clinical	1.0			
Imprisonment	**5.187**	**1.139**	**25.656**	**0.034**
Coinfection	1.114	0.008	13.306	0.945
Other	3.343	0.511	19.166	0.191
Indicator risk	0.727	0.068	4.636	0.745
Screening	1.857	0.293	10.216	0.483
Median CD4 cell count (cells/µL)				
1 unit	1.0	0.998	1.003	0.874
Median HIV-1 viral load in Log_{10} (copies/mL)				
1 $\log_{10}$ unit	0.587	0.304	1.133	0.112
County of residence				
Ida-Virumaa	1.0			
Tallinn	0.583	0.172	1.980	0.387
Other	0.778	0.090	6.695	0.819
Viral hepatitis				
HBV	1.0			
HCV	0.306	0.002	2.684	0.345
Infection stage				
Recent infection	1.0			
Long term infection	0.488	0.15	1.582	0.232
HIV-1 subtype:				
CRF06_cpx/A1	1.0			
CRF06_cpx	0.304	0.058	3.075	0.262
A1	0.854	0.116	9.866	0.883
B	1.154	0.078	17.054	0.911
URF	1.667	0.010	57.023	0.789

Note: Univariate logistic regression or Firth's penalized-likelihood logistic regression analysis was used.
SDRM – surveillance drug resistance mutation as determined by CPR tool (WHO list 2009) (Team RC, 2013).
CI – confidence interval; PWID – people who inject drugs; HCV – hepatitis C virus; HBV – hepatitis B virus; indicator risk – PWID, imprisonment, known contact, tuberculosis diagnosis, sexually transmitted disease diagnosis; URF – unique recombinant form.

cluster (25/224, 11%).

3.4. Risk factors associated with TDR

Most subjects with SDRM lived in Northeast Estonia (10/15), were male (10/15), had been infected by heterosexual contact (8/15), and had median CD4+ T-cell counts of 377 (IQR 212–571) cells/µL. Comparison of these patients with the population without SDRMs revealed no significant difference. The SDRM ratio was higher among imprisoned subjects compared with all others (OR 5.187, CI 1.139–25.565, $p = 0.034$). Analyzed risk factors are presented in Table 3.

3.5. Trends of TDR and its association with ART consumption

From 2003 until 2013, overall cART consumption increased remarkably from 0.106 defined daily doses (DDD) per 1000 persons/day in 2003 to 3.346 DDD/1000 persons/day in 2013. The most commonly used cARTs had a NRTI backbone (1.66), with either PI (0.94) or NNRTI (0.75) (Fig. 3). In 2013, the most commonly used NRTI backbones were abacavir/lamivudine (0.82), tenofovir/emtricitabine (0.41), and zidovudine/lamivudine (0.41). Lopinavir/ritonavir was the most commonly used PI (0.52), and efavirenz was the most commonly used NNRTI (0.67). Over the years, TDR remained stable at approximately 5.6%, and only slightly varied between 4.5% (95%CI 1.8; 9.2) in 2010 and 6.7% (95%CI 3.9; 11.0) in 2013. No major changes in subtype distribution have been observed, but an upward trend of subtype A1 has been noted.

4. Discussion

This study was conducted in Estonia to examine TDR from 2008 to 2013. The results show that the point-estimate of TDR has remained at a moderate level of 6.7% (95%CI 3.9; 11.0) in 2013, despite a significant scale-up of ART. Approximately 50% of all SDRM involved K103 N, likely because of widespread use of the low genetic barrier drug efavirenz. The CRF06_cpx viruses that initiated the HIV epidemic in early 2000 are still the most common clade but increase in A1 subtype was observed in 2013. A less reported finding was screening due to imprisonment as a risk factor for TDR. The Estonian CRF06_cpx epidemic contained one large infection cluster, including almost exclusively of sequences sampled in Estonia, however supported with a bootstrap value lower than 90 or 98% (Hassan et al., 2017). Most probably, the limited phylogenetic signal that is associated to conserved stretches such as PR-RT in combination to a dense sampling of highly similar sequences impedes the characterization of this cluster as a transmission cluster. Fortunately, we were able to identify 23 smaller transmission clusters in the CRF06_cpx epidemic (bootstrap support > 98%), of which 13 clusters contained at least one Estonian HIV-1 sequence collected in 2013. One transmission pair with K103 N was detected.

The main strength of this study was analysis of the entire country, as well as use of the same SDRM testing methodology since year 2000 (Avi et al., 2009; Avi et al., 2011; Avi et al., 2014b). As data were directly reported from the HIV Reference Laboratory to the Health Board, we are confident that we captured all new HIV-1 diagnoses. In 2013, we also made every effort to identify recently infected subjects by interrogating laboratory and clinical databases, along with use of LAg-assay testing (described elsewhere (Soodla et al., 2017)), rather than relying on self-reporting.

Status as a MSM or an immigrant has previously been associated with TDR (Hofstra et al., 2016; Descamps et al., 2013; Beyrer et al., 2012), but both these risk groups are rare among PLWHs in Estonia (Ruutel et al., 2015). In contrast, we found imprisonment to be the only risk factor for the presence of SDRM. Similar results were demonstrated in a study performed in Italy, in which 22% of inmates had TDR (Sanarico et al., 2016). Although the reasons for this trend were not investigated, one could speculate that the intermittent imprisonment and thus interruptions of cART while subjects are not incarcerated could contribute to the emergence of SDRM. Intermittent incarceration is also characteristic of PWID (common route of HIV transmission among inmates), which could explain nonadherence to cART and the emergence of SDRM. Our results show that in order to prevent TDR targeted interventions are urgently needed among the prison population in Estonia. There was one transmission pair among imprisoned PWIDs similar to heterosexual transmission pair with K103 N mutation. The reason could be relatively low TDR among CRF06_cpx viruses or very small number of subtype B viruses.

A substantial proportion of SDRM in Europe belong to drug resistance transmission clusters: 43% in Belgium, 27–55% in Sweden, 33% in Ukraine (Pineda-Pena et al., 2014; Karlsson et al., 2012; Fearnhill et al., 2017) and > 40% according to latest study of European and Canada cohorts (Paraskevis et al., 2019) similarly to our finding of 33% (5/15). Most of the clusters reported in Europe are formed by subtype B viruses and carry common SDRM (e.g., K103N) (Pineda-Pena et al., 2014). Although the K103N mutation is dominant in Estonia, our study found only one transmission pair with SDRM among CRF06_cpx viruses, suggesting that infections with drug resistant HIV-1 strains in Estonia, in contrast to those in Western Europe, are independently selected. These results also highlight the positive

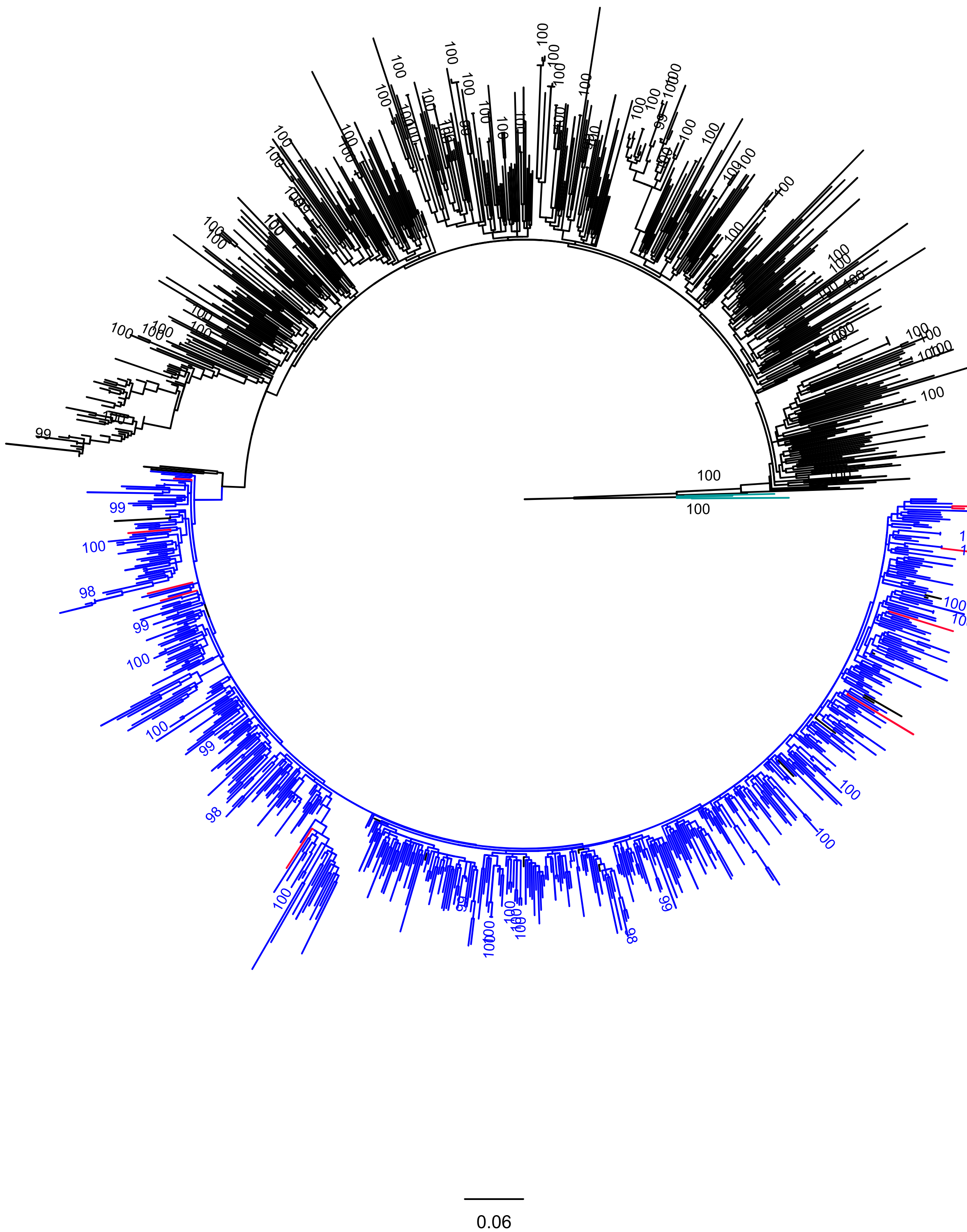

Fig. 2. a. Maximum likelihood (ML) tree of newly diagnosed HIV-1 CRF06_cpx subjects in Estonia in 2013. Wild-type HIV-1 strains sampled in Estonia are colored in blue and strains sampled in Estonia with evidence of TDR in red. Only bootstrap values ≥ 98% are shown. b. Maximum likelihood (ML) tree of newly diagnosed HIV-1 subtype A1 subjects in Estonia in 2013. Wild-type HIV-1 strains sampled in Estonia are colored in blue and strains sampled in Estonia with evidence of TDR in red. Subtype B or C reference sequences that were used as outgroup are colored in green. Only bootstrap values ≥ 98% are shown. c. Maximum likelihood (ML) tree of newly diagnosed HIV-1 subtype B subjects in Estonia in 2013. Wild-type HIV-1 strains sampled in Estonia are colored in blue and strains sampled in Estonia with evidence of TDR in red. Subtype B or C reference sequences that were used as outgroup are colored in green. Only bootstrap values ≥ 98% are shown.
Maximum likelihood (ML) tree of newly diagnosed HIV-1 patients in Estonia in 2013, per subtype. Wild-type HIV-1 strains sampled in Estonia are colored in blue and strains sampled in Estonia with evidence of TDR in red. Subtype B or C reference sequences that were used as outgroup are colored in green. Only bootstrap values ≥98% are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

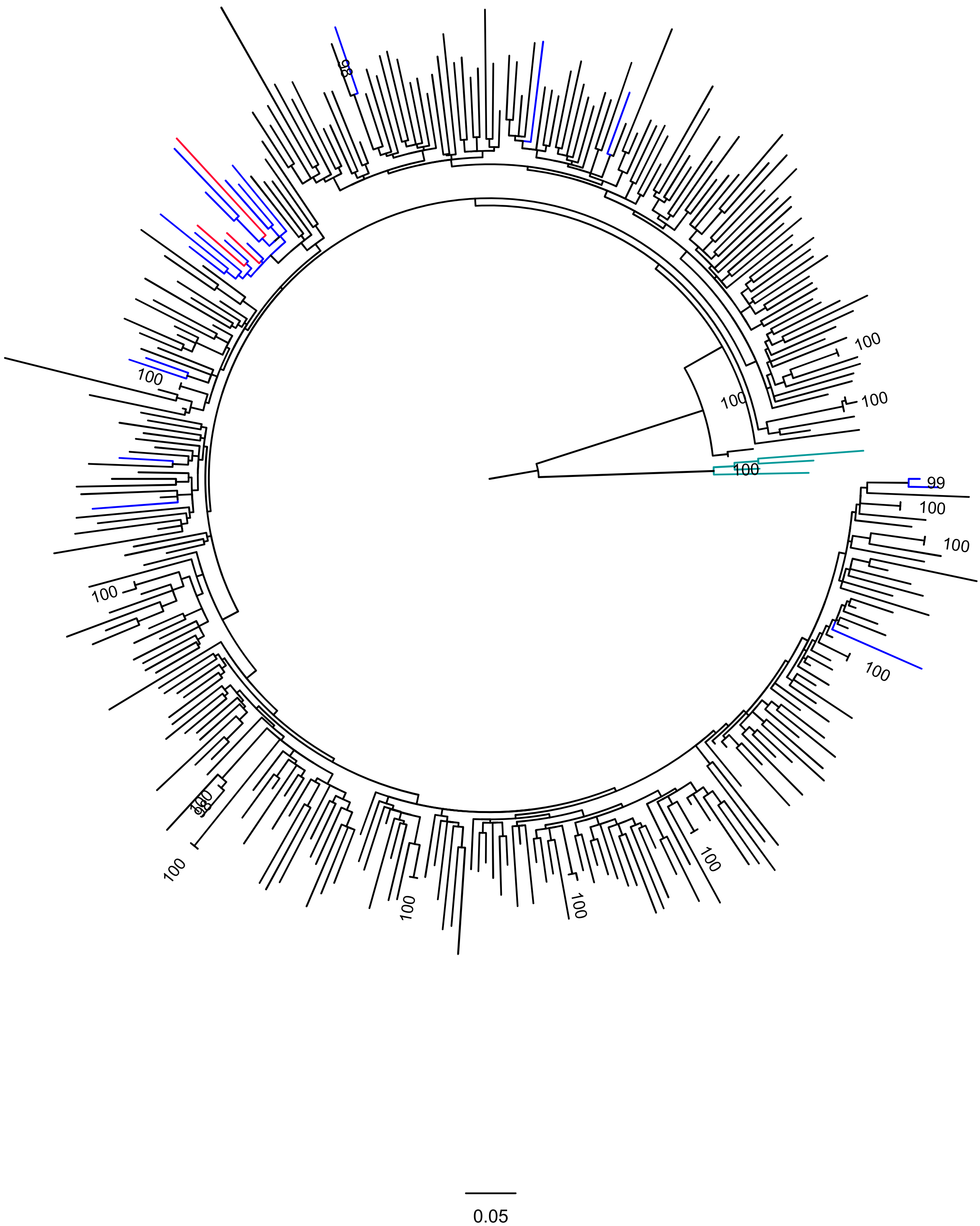

Fig. 2. (*continued*)

value of needle-exchange programs. Despite every effort, we cannot exclude the fact that transmission clusters are underestimated because of the monophyletic and highly similar sequences of Estonian CRF06_cpx viruses, which render identification of transmission clusters difficult.

In recent years, the increased number of new HIV-1 diagnoses and changes in treatment guidelines have resulted in a scale-up of cART in Estonia. The moderate level of TDR in Estonia reported here is in line with findings of Eastern European, as well as other industrialized countries (Karlsson et al., 2012; Tostevin et al., 2017; Hauser et al., 2017). In this context, the only difference between Eastern and Western Europe is the steady increase of TDR in FSU, in contrast to the steady decline in Western European countries (Frentz et al., 2012; Hofstra et al., 2016; Rhee et al., 2015; Snedecor et al., 2014; D'Costa et al., 2017).

Although the Estonian epidemic continues to be dominated by CRF06_cpx viruses, there has been an increase in the prevalence of subtype A1 (from 1.4% in 2008 to 10% in 2013). It is likely imported to

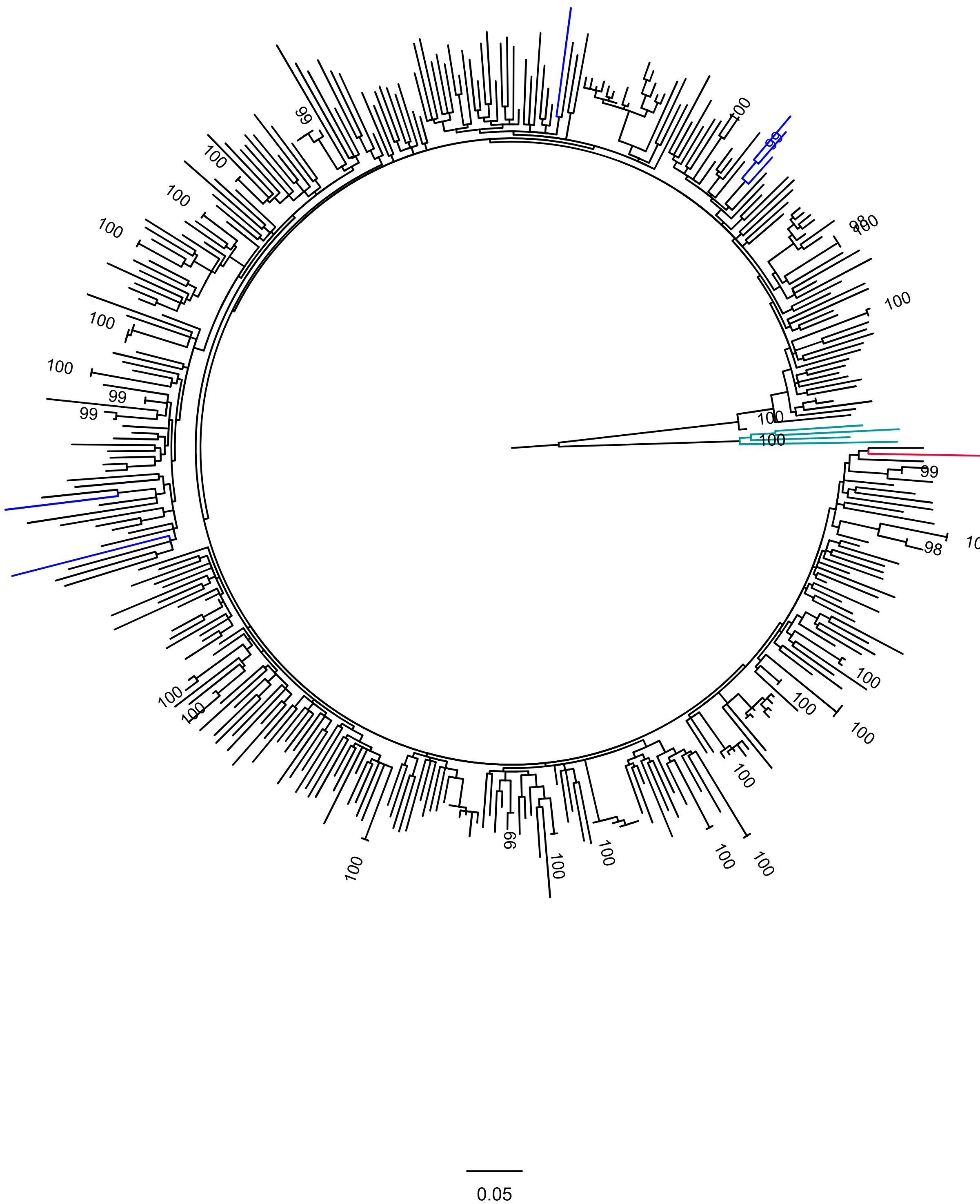

Fig. 2. (*continued*)

Estonia from Russia where this is a dominant subtype. According to the phylogenetic analysis HIV-1 subtype A1 has formed a local cluster in Estonia, although not robustly supported, and continues to spread domestically. Although not statistically significant the proportion of TDR among subtype A1 viruses is greater than among CRF06_cpx (13% vs 5.4%, respectively). The dynamic of this cluster and further changes should be carefully monitored in future.

In conclusion, the cART scale-up over the last 10 years has resulted in a stable rate of TDR in Estonia, characterized by notable prevalence of K103N mutation and one drug resistance transmission pair. We suggest screening for TDR in Estonia at the time of diagnosis or prior to cART initiation to predict the efficacy of first-line therapy and to monitor further dynamics of TRD. The high rate of TDR among imprisoned subjects calls for targeted prevention in this risk group.

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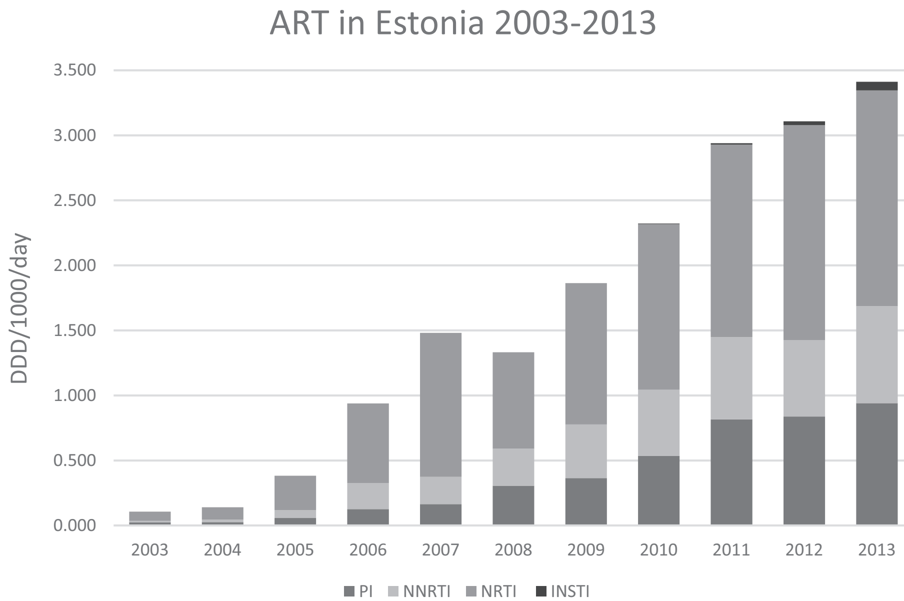

Fig. 3. Use of antiretrovirals in DDD/1000/days in Estonia 2003–2013 as reported by the Estonian State Agency of Medicines. Dark grey bars indicate PI, light grey bars NNRTI, grey bars NRTI and black bars INSTI usage in DDD/1000/day over the years.

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