DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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188

RADKO AVI

Natural polymorphisms and transmitted drug resistance in Estonian HIV-1 CRF06_cpx and its recombinant viruses

TARTU UNIVERSITY PRESS

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Dissertation has been accepted for the commencement of the degree of Doctor of Philosophiae on August 24th, 2011 by the Council of the Faculty of Medicine, University of Tartu, Estonia

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Commencement: November 2nd, 2011

Publication of this dissertation is granted by University of Tartu

This research was supported by the European Regional Development Fund and European Social Fund.

ISSN 1024–395x
ISBN 978–9949–19–865–8 (trükis)
ISBN 978–9949–19–866–5 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimuse nr. 627

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LIST OF ORIGINAL PUBLICATIONS

1 Avi, R., Huik, K., Sadam, M., Karki, T., Krispin, T., Ainsalu, K., Paap, P., Schmidt, J., Nikitina, N., Lutsar, I., (2009) "Absence of genotypic drug resistance and presence of several naturally occurring polymorphisms of human immunodeficiency virus-1 CRF06_cpx in treatment-naive patients in Estonia." J Med Virol **81**(6): 953–8

2 Avi, R., Huik, K., Sadam, M., Karki, T., Krispin, T., Ainsalu, K., Paap, P., Schmidt, J., Nikitina, N., Lutsar, I., (2010) "Characterization of integrase region polymorphisms in HIV type 1 CRF06_cpx viruses in treatment-naive patients in Estonia." AIDS Res Hum Retroviruses **26**(10):1109–13

3 Avi, R., Huik, K., Pauskar, M., Ustina, V., Karki, T., Krispin, T., Ainsalu, K., Paap, P., Schmidt, J., Nikitina, N., Lutsar, I., (2011) "Emerging transmitted drug resistance in treatment-naïve human immunodeficiency virus-1 CRF06_cpx-infected patients in Estonia." Scand J Infect Dis **43**(2):122–8

The dissertant was in charge of study designs, conduction, data analysis and writing of all three articles. The samples used for analyses described in articles 1 and 2 were collected by the physicians and nurses caring for the HIV positive subjects or by the personel of HIV anonymous cabinets. The samples used for analyses described in articles 3 were obtained from the HIV Reference Laboratory of the West Tallinn Central Hospital.

ABBREVIATIONS

3TC	lamivudine
aa	amino acid
ABC	abacavir
AIDS	acquired immunodeficiency syndrome
APV	amprenavir
ARV	antiretroviral
ATV	atazanavir
ZDV	zidovudine
BD	beckton dikinson
CRF	circulating recombinant form
d4T	stavudine
ddC	zalcitabine
ddI	didanosine
DLV	delavirdine
DR	drug resistance
DRM	drug resistance mutation
DRV	darunavir
EFV	efavirenz
EMA	European Medicine Agency
ENF	enfuvirtide
env	envelope
ETR	etravirine
FDA	Food and Drug Administartion
FPV	fosamprenavir
FTC	emtricitabine
gag	group specific antigene
HAART	highly active antiretroviral therapy
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IDV	indinavir
IN	integrase
INI	integrase inhibitor
IVDU	intravenous drug user
kbp	kilo base pair
LPV	lopinavir
LTR	long terminal repeat
MVC	maraviroc
NFV	nelfinavir
NJ	neighbors joining
NNRTI	non-nucleotide reverse transcriptase inhibitor
NRTI	nucleotide/nucleoside reverse transcriptase inhibitor

NVP	nevirapine
PBMC	peripherial blood mononuclear cell
PI	protease inhibitor
PR	protease
RAL	raltegravir
RT	reverse transcriptase
RTI	reverse transcriptase inhibitor
RTV	ritonavir
SIV_{CPZ}	simian immunodeficiency virus from chimpanzees
SQV	saquinavir
TAM	thymidine analogue mutation
TDF	tenofovir
TDR	transmitted drug resistance
TE	treatment experienced
TN	treatment naive
TPV	tipranavir
URF	unique recombinant form

1. INTRODUCTION

During last decades HIV infection has become one of the main problems of infectious diseases and presents a major challenge to the medicine worldwide. From the beginning of the HIV epidemic about 60 million subjects have been infected and 25 million have died. In 2009 it was estimated that 31–35 million persons live with HIV infection. Every year about 2.6 million new HIV infections and 1.8 million AIDS deaths are reported (www.who.int).

The AIDS and its causative agent HIV are relatively new. Recent studies suggest that virus started to spread in wider scale not before 1940s (Korber, Muldoon et al. 2000). However, it took more than 40 years when the AIDS was noticed and the syndrome causing virus got identified. Discovery of this virus had been awarded with the Nobel price in Medicine in 2009 which again refers that the management of HIV epidemics is one of the most important challenges in contemporary medicine and science.

For a long time HIV infection was untreatable slowly progressing to the death in about 7 years. The first treatments emerged in the 1980s but the real breakthrough was in 1995 when the first effective antiretroviral treatment – HAART was initiated. Today the HIV infection in countries with full access to medical care is life-long chronic disease which allows life-expectancy comparable to non-infected persons (Bhaskaran, Hamouda et al. 2008).

Unfortunately so far many drawbacks and questions are still associated with treatment of HIV infection. Firstly, there is a need for complex and well managed ARV therapy because of the rapidly developing drug resistance. Secondly, the emerging TDR compromises the choice of first line treatment among TN patients. Thirdly, the impact of the natural variability of HIV to the efficiency and outcome of HAART is not well described. These are the main questions of the worldwide scientific and medical community to be resolved before a full control over the HIV epidemic is achieved.

For Estonia, a country with the highest HIV-1 prevalence and incidence in Europe, the HIV epidemic possesses additional socio-economical hurdles, especially as the infected population consists of various minorities including Russian speaking immigrants and IVDUs community (Estonian Health Board, Tallinn, 2010 (cited 2010 February 16). Accessed at www.terviseamet.ee). In terms of HIV-1 epidemic Estonia is a country belonging to “new Eastern-European” HIV-1 epidemics. Compared to Western-Europe the knowledge about the HIV-1 molecular epidemiology and DR in Eastern-Europe and especially in Estonia is limited. Futhermore the evolving of the epidemic forces to pay more attention to the generaliation of epidemics, to its consequences of scaling-up the HAART and to the outcome of the disease caused by under-investigated non-B type viruses.

2. REVIEW OF LITERATURE

2.1 HIV-1 genomic structure and molecular epidemiology

HIV-1 is a retrovirus belonging to the family of lentiviruses (*Lentiviridae*). Its provirus is approximately 9800 nt in length (Muesing, Smith et al. 1985).

2.1.1 Genome structure

Similar to other retroviruses HIV-1 genome consists of central protein coding region which has both ends flanked by the LTR. Proteins encoded by HIV-1 are divided into major structural, regulatory and accessory proteins as shown in Figure 1 (Gallo, Wong-Staal et al. 1988).

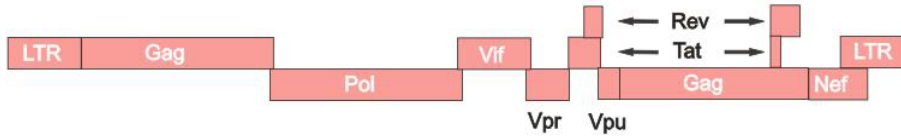

Figure 1. Structure of HIV-1 genome.

2.1.1.1 Long terminal repeats

LTRs are composed of three regions – U3, R and U5 (Starcich, Ratner et al. 1985; Hope and Trono 2000).

- U3 region consists of cellular transcripton factor binding sites
- R region starts with the transcriptional start site and ends with the mRNA polyadenylation sites
- U5 contains Tat binding site, packing signals for HIV-1 genomic RNA and lysyl-tRNA binding site. The lysyl-tRNA is a primer for the reverse transcription.

2.1.1.2 Major functional proteins

The HIV-1 possesses three major functional proteins. The gag protein is structural unit of the viral capsid. Gag contains the binding sites for viral genomic RNA and is responsible for incorporation of viral RNA into the capsid. Gag protein initiates the budding of viral particle from the cellular surface. Inside of the viral particle the gag polyprotein is cleaved to mature matrix, capsid and nucleocapsid proteins by viral PRs (Gottlinger, Sodroski et al. 1989).

The pol region of viral genome is always expressed as gag-pol fusion polyprotein as a consequence of ribosomal frame shift during the translation of

gag protein (Jacks, Power et al. 1988). Thereafter polyprotein is processed by viral PR to following functionally active viral enzymes: PR, RT, RNase H and IN (Hope and Trono 2000).

- PR – HIV-1 PR is a 99 aa long aspartyl PR which is active in dimeric form (Miller, Jaskolski et al. 1989). It is responsible for maturation of gag and gag-pol precursor polyproteins (Schneider and Kent 1988). Contrary to classic proteases it does not have consensus target sequence but it recognizes the specific asymmetric shape in substrate peptide sequences (Prabu-Jeyabalan, Nalivaika et al. 2002).
- RT – is a 440 aa protein having a RNA- and DNA dependent DNA polymerase activity. It is responsible for reverse transcription of viral RNA to DNA and incorporation with RNase H for the synthesis of the second chain of this DNA. Similarly to PR the RT active form is a dimer consisting of p65 and p50 subunits (p50 is active subunit consisting of RT sequence; p65 is inactive subunit consisting of RT and RNase H fusion sequences). The HIV-1 RT lacks proofreading activity causing its error-prone nature and therefore high intra- and interindividual viral sequence diversity (Roberts, Bebenek et al. 1988; Wensing, van de Vijver et al. 2005). In combination with high recombination rate and short half-life of HIV-1 particles this allows rapid selection of immune system escape as well as DRMs (Ho, Neumann et al. 1995; Michael 1999; Rollman, Hinkula et al. 2004).
- IN – is a 288 aa protein which is responsible for integration of viral genomic DNA into host chromosome. IN possesses at least three distinct functions – exonuclease activity for trimming viral genomic DNA, endonuclease activity for cleaving host genomic DNA for proviral integration and ligase activity for single stranded ligation of viral and host DNA (Bushman, Fujiwara et al. 1990).
- RNase H main function is the degradation of viral RNA in DNA:RNA duplex during the reverse transcription (Hope and Trono 2000).

The env is approximately 500 aa long fusion protein, which is translated into endoplasmatic reticulum and glycosylated in several aspartate residues. Thereafter it is cleaved to gp41 and gp120 proteins which, however, remain linked with non-covalent interaction. Such complex forms a homo-trimer that is responsible for HIV-1 binding and fusion to target cell (Bushman, Fujiwara et al. 1990). Functionally the gp120 is responsible for interactions between HIV-1 cellular receptor CD4 and cellular co-receptors CCR5 or CXCR4 (Landau, Warton et al. 1988; Hope and Trono 2000).

2.1.1.3 Regulatory proteins

HIV-1 has two regulatory proteins tat and rev. Tat is RNA binding protein activating transcription in 5´viral RNA terminus by binding to transactivation response element in LTR. It promotes the transcriptional elongation of HIV-1 RNA. Tat interacts with many cellular proteins to influence the expression of

several host genes. The second regulatory protein rev binds to viral RNA regulating the transition from early to late phase of HIV-1 gene expression (Kim, Byrn et al. 1989; Hope and Trono 2000).

2.1.1.4 Accessory proteins

HIV-1 has four accessory proteins – nef, vif, vpr and vpu. These proteins are not essential for in vitro replication but act as virulence factors in vivo. Most of these proteins have multiple functions. Nef downregulates the CD4 and MHC Class I molecule expression and modulates the T-cell activation (Roeth and Collins 2006). Vif is required in HIV-1 replication in PBMCs but not in other cell types (Gabuzda, Lawrence et al. 1992). Vpr is incorporated into viral particle and facilitates the nuclear transport of preintegration complex and modulates the cell cycle regulation (Romani and Engelbrecht 2009). Vpu is integral membrane phosphoprotein, which downregulates the CD4 and enhances the virion release from the cell surface (Schubert, Bour et al. 1996). Vpu releases the env protein from CD4+ cells in endoplamatic reticulum allowing the effective budding of new viral particles (Willey, Maldarelli et al. 1992).

2.1.2 HIV-1 taxonomy and phylogenetic distribution

The HIV in humans is diverse class of viruses. Phylogenetic analysis of similar viruses in other primates has demonstrated multiple-cross species transmissions of these viruses to human populations (Figure 2) (Buonaguro, Tornesello et al. 2007).

The HIV-1 is divided into three groups – M (major), O (outlier) and N (non-M/non-O) (Figure 2). It seems that these groups are not monophyletic and most probably originate from the strains of simian immunodeficiency viruses (SIV_{CPZ}) found in chimpanzees *Pan troglodytes troglodytes* in West-Equatorial Africa (Gao, Bailes et al. 1999). The most divergent set of HIV-1 taxons in the world have been described in that particular area. (Janssens, Nkengasong et al. 1994; Delaporte, Janssens et al. 1996; Peeters, Gaye et al. 1996; Vidal, Peeters et al. 2000; Mokili, Rogers et al. 2002; Buonaguro, Tornesello et al. 2007). Recently the HIV-1 group P has also been found in one Cameroonian woman, however, contrary to other HIV-1 groups this virus possesses the closest relative to gorilla simian immunodeficiency viruses (Figure 2) (Plantier, Leoz et al. 2009).

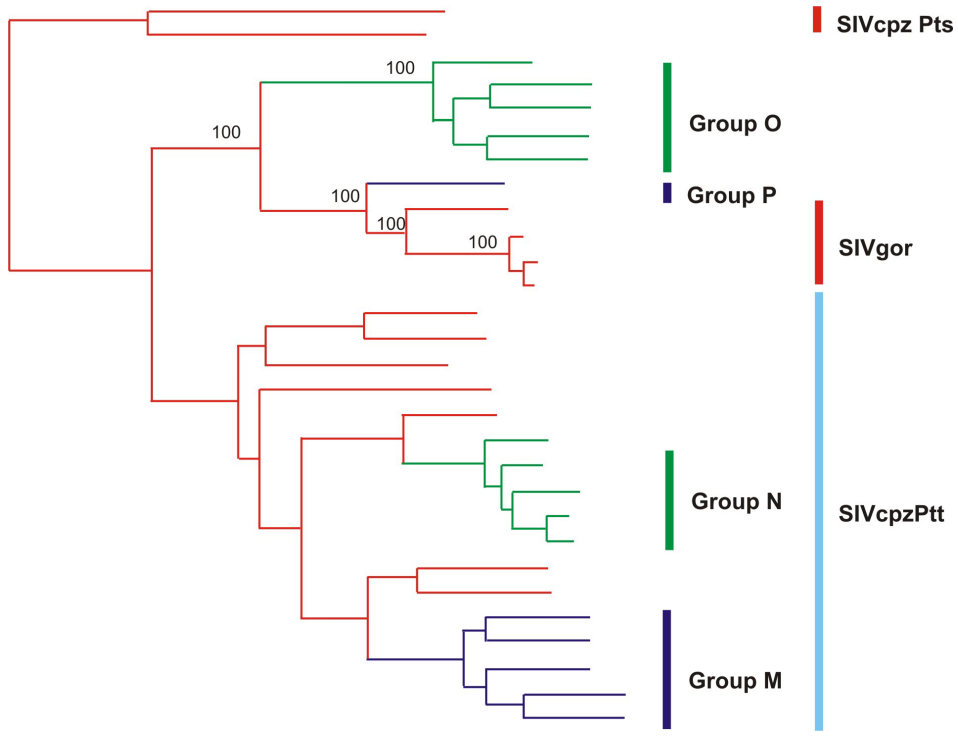

Figure 2. Phylogenetic tree representing HIV-1 group M, N, O and P and related SIV sequences. Figure is adapted from (Plantier, Leoz et al. 2009).

From these HIV-1 groups the M-group has by far the greatest clinical importance and it also demonstrates the largest sequence diversity. Group M is divided into 9 subtypes (A, B, C, D, F, G, H, J and K) and tens of recombinant forms (www.hiv.lanl.gov/content). The latter in turn are divided into CRFs and URFs. The CRFs are defined as viral strains which have been isolated from at least two epidemiologically unrelated persons, whereas URFs are viruses described only in one person or epidemiologically related persons (Robertson, Anderson et al. 2000). The largest abundance of different CRFs and URFs have been described in Central-Africa, in the region where several HIV-1 clades co-circulate (Konings, Haman et al. 2006). The recombinant forms have also been found between different HIV-1 groups and in single cases even between HIV-1 and HIV-2 strains (Butler, Pandrea et al. 2007; Yamaguchi, Vallari et al. 2008).

Recent detailed phylogenetic analysis of HIV-1 group M viruses showed that subtypes A and F can be further separated into sub-subtypes: A1, A2, A3 and F1, F2. Their phylogenetic diversity is comparable to that in other subtypes. In phylogenetical terms these viruses should be considered as independent subtypes (Triques, Bourgeois et al. 2000; Gao, Vidal et al. 2001; Ojesina, Sankale et al. 2006). On the contrary the HIV-1 subtypes B and D are phylogenetically so similar that they could be regarded as one subtype (Triques,

Bourgeois et al. 1999; Triques, Bourgeois et al. 2000; Gao, Vidal et al. 2001; McGrath, Hoffman et al. 2001; van der Auwera, Janssens et al. 2001).

The most prevalent HIV-1 subtype worldwide is subtype C causing approximately 50% of all infections (Buonaguro, Tornesello et al. 2007). It is spread mainly in Southern–African countries and India. In terms of frequency it is followed by the subtypes A and B, the former dominating in Central- and Eastern-Africa as well as in Eastern-Europe and the latter in Western- and Central-Europe, North- and South-America, Australia and several South-East-Asian and Northern-African countries (Figure 3). Other subtypes like F, G, H, J and K demonstrate much restricted spread and are localized mainly in Central-Africa. CRFs account for approximately 18% of all HIV-1 infections (Osmanov, Pattou et al. 2002; Hemelaar, Gouws et al. 2006). The most widespread of them are CRF01_AE and CRF02_AG spreading predominantly in South-East Asia and West-Africa, respectively (Osmanov, Pattou et al. 2002).

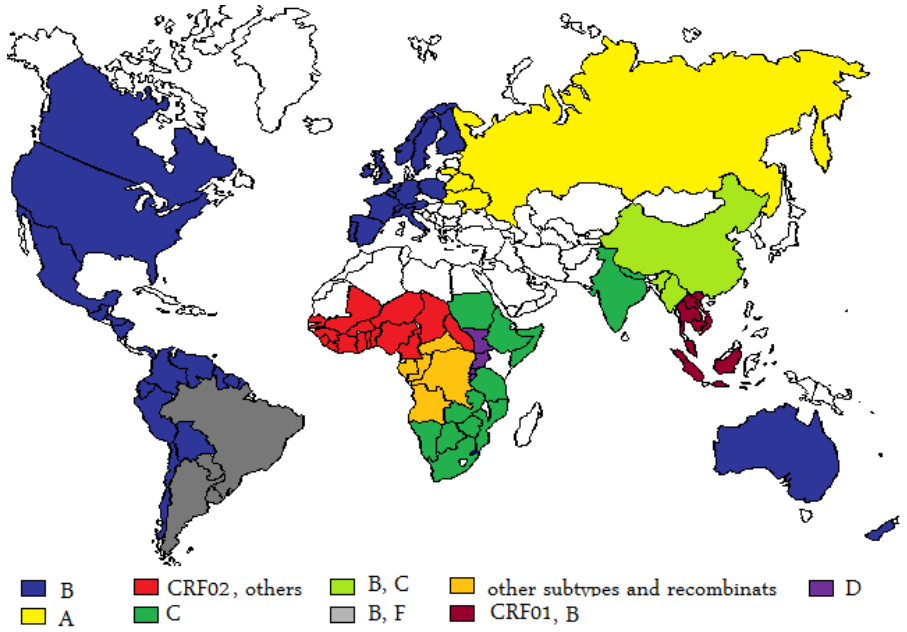

Figure 3. Distribution of HIV-1 subtypes and CRFs in the world. White represents areas with insufficient data.

The most predominant subtype in North-America and Western-Europe is subtype B. The phylogenetic analysis indicates that these viruses have monophyletic origin dating back to 1960s in Haiti (Gilbert, Rambaut et al. 2007). Western-European viruses, however, do not form a single cluster of Western-World subtype B viruses suggesting that several independent introductions from USA or from Haiti have occurred (Paraskevis, Pybus et al. 2009).

Other HIV-1 subtypes have been spread in Western-Europe in lower extent. For example, the relatively high prevalence of HIV-1 subtype G viruses has been described in Portugal only (Esteves, Parreira et al. 2003).

However, during last decade many studies have demonstrated the increasing prevalence of non-B subtypes in Western-Europe mainly among those with potential of acquiring infection abroad (eg. recent immigrants from Africa) (Deroo, Robert et al. 2002; Chaix, Descamps et al. 2003; Snoeck, van Laethem et al. 2004; Descamps, Chaix et al. 2005; Lospitao, Alvarez et al. 2005).

In Estonia first cases of HIV-1 infection were reported in 1988 but the incidence (9 per million inhabitant in 1999) of it remained low until 2000. The main route of infection at that time was homo- or heterosexual transmission (Ustina, Zilmer et al. 2001; Laisaar, Avi et al. 2011). The molecular epidemiological studies have confirmed that the subtype distribution was similar to that seen in Western–Europe – unrelated subtype B viruses accompanied by a few cases of A, C, D, G and CRF02_AG subtypes (Liitsola, Laukkanen et al. 1996; Ustina, Zilmer et al. 2001).

In the early 1990s after the collapse of Soviet Union and together with the rapid increase of intravenous drug use the concentrated HIV-1 epidemics emerged in the Southern Ukraine, spreading rapidly to neighboring countries like Belarus and Russia (Bobkov, Cheingsong-Popov et al. 1997; Lukashov, Karamov et al. 1998; Nabatov, Kravchenko et al. 2002; Naganawa, Sato et al. 2002). By 1999 the expanding Eastern-European HIV-1 epidemic had reached to the surrounding regions of Estonia – Latvia and St. Petersburg area in Russia. Vast majority of infections were caused by monophyletic subtype A1 viruses (Bobkov, Cheingsong-Popov et al. 1997; Roudinskii, Sukhanova et al. 2004). In lesser extent the HIV positive subjects, mainly in the region of Kaliningrad oblast and Ukraine, were also infected by the new recombinant form CRF03_AB originating from the same subtype A1 and subtype B (Liitsola, Holm et al. 2000; Naganawa, Sato et al. 2002). Among these Eastern-European subtype A1 viruses the sub cluster labelled with protease mutation V77I was also described (Roudinskii, Sukhanova et al. 2004; Vazquez de Parga, Rakhmanova et al. 2005; Zarandia, Tsertsvadze et al. 2006).

In Estonia the concentrated HIV-1 epidemic burst in August 2000 when the number of HIV-1 infected subjects arose rapidly reaching the highest prevalence (1053 per million inhabitants) in European Union in 2001 (Uuskula, Kalikova et al. 2002). Since then the incidence has been gradually decreasing and was 307 per million inhabitants in 2009. Still Estonia is one of the three countries in Eastern-Europe and Central-Asia in which the estimated HIV-1 prevalence exceeds 1% of adult population (www.unaids.org). In contrast to population infected with HIV-1 prior to 2000 (eg. mainly hetero- or homosexually infected middle-aged persons) over two-thirds of affected subjects were male IVDUs under the age of 30 (Figure 4) (Estonian Health Board, Tallinn, 2010 (cited 2010 February 16). Accessed at www.terviseamet.ee) (Lõhmus, Rüütel et al. 2002; Talu, Abel-Ollo et al. 2008).

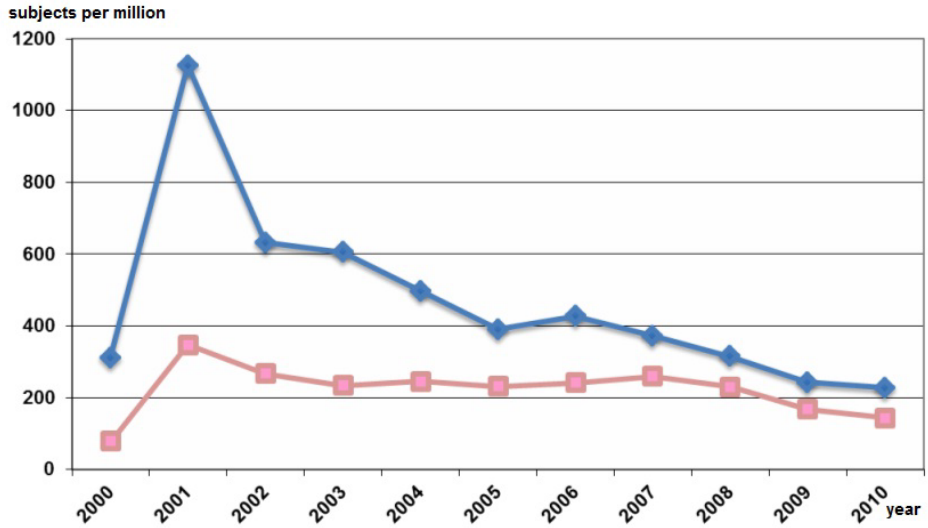

Figure 4. Newly diagnosed HIV-1 cases per million in Estonia 2000-2010. Blue squares indicate men, red squares indicate women (Estonian Health Board, Tallinn, 2010 (cited 2010 February 16). Accessed at www.terviseamet.ee; Statistics Estonia, Tallinn, 2010 (cited 2010 January 5, July 5, and Nov 8). Accessed at www.stat.ee).

Surprisingly and in contrast to other former Soviet Union countries the Estonian HIV-1 epidemic was not caused by the subtype A1 but it was predominated by the rare HIV-1 CRF06_cpx and in lesser extent by its recombinants with the subtype A1 (Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005).

The HIV-1 CRF06_cpx was first described in 1998-1999 in Mali and Burkina Faso as a CRF formed by subtypes A, G, K and J (Figure 5A) (Oelrichs, Workman et al. 1998; Montavon, Bibollet-Ruche et al. 1999; Triques, Bourgeois et al. 2000; Montavon, Toure-Kane et al. 2002). Prior to the Estonian outbreak the CRF06_cpx was spreading mainly in Mali, Niger and Burkina Faso and only single strains had been described outside of Africa (Oelrichs, Workman et al. 1998; Montavon, Bibollet-Ruche et al. 1999; Mamadou, Montavon et al. 2002; Montavon, Toure-Kane et al. 2002; Mamadou, Vidal et al. 2003).

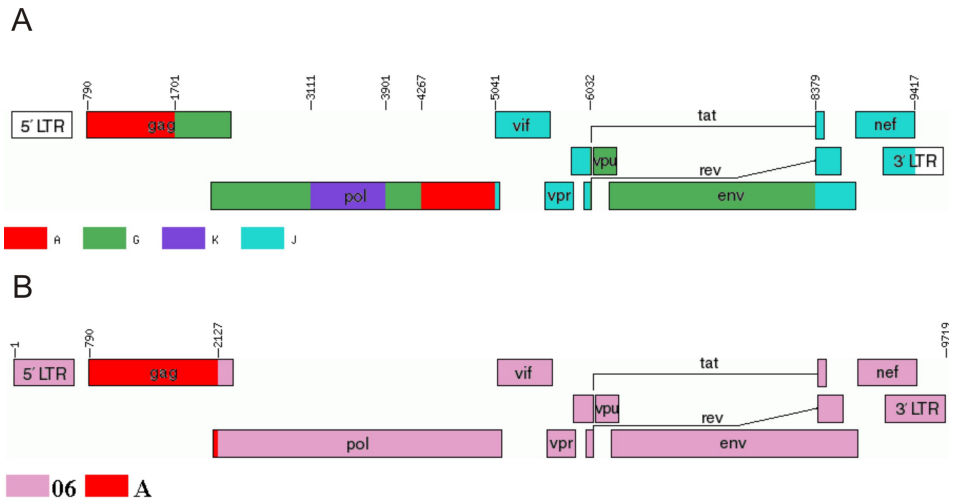

Figure 5. The structure of HIV-1 CRF06_cpx (panel A) and CRF32_06A1 (panel B). In panel A the strain contains sequences from subtypes A, G, K, J and of unknown origin (marked as white) and in panle B from CRF06_cpx and in lesser extent from subtype A1 (www.hiv.lanl.gov).

In early 2000 the Estonian HIV-1 epidemic was highly homogeneous – approximately 70% of infections were caused by the monophyletic CRF06_cpx strains containing highly similar or even identical sequences (Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005). This suggests the possibility of one source origin of Estonian HIV-1 epidemic. Still, about fifth of the viral strains were the second generation recombinants between CRF06_cpx and Eastern-European subtype A1 viruses designated also as CRF32_06A1 (Figure 5B) (Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005). Deeper analysis of these strains revealed a number of different URFs suggesting the high rate of superinfections in Estonian IVDU population (Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005). Furthermore, in rare cases the pure subtype A1 sequences as well as subtype B sequences have also been described (Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005).

2.2 Antiretroviral agents and HAART

The first ARV therapy was used in late 1980s when ZDV monotherapy was given. As a results of treatment the reduction in the incidence of opportunistic infections, increase in the CD4+ cell count and decrease of mortality was observed (Fischl, Richman et al. 1987; Volberding, Lagakos et al. 1990). Unfortunately due to the emerging resistance to ZDV the long term clinical outcome after monotherapy was not different of that seen in the placebo group (Concorde-Coordinating-Committee 1994). Therefore since early 1990s the

ZDV monotherapy was recommended only for patients with advanced disease (Concorde-Coordinating-Committee 1994; Volberding, Lagakos et al. 1995).

The greatest breakthrough in the treatment of HIV-1 infection was achieved in mid-1990s with the introduction of triple ARV therapy called HAART. This was made possible due to the discovery and introduction of two new classes of ARV agents – the PIs (SQV, RTV, IDV) and NNRTIs (NVP). New triple therapy combining the dual NRTI backbone with a PI demonstrated during treatment a remarkable increase of time to AIDS defining events and death (Hammer, Squires et al. 1997). For example, the introduction of HAART resulted about two fold decrease in new cases of AIDS and about three fold decrease in mortality within 4 years (Fleming, Ward et al. 1998; Murphy, Collier et al. 2001).

The usage of PIs also led to the introduction of boosted PI regimen – a low dose RTV was added to other PI-containing regimens. This in turn improved the treatment outcome by increasing the time left until the development of AIDS and death. This boosting mechanism was caused by the RTV ability to inhibit cytochrome P450 CYP 3A4 enzyme which results in the increase of drug's blood concentration and gives greater antiviral effect compared to unboosted PIs (Kempf, Marsh et al. 1997; Walmsley, Bernstein et al. 2002). In initial studies such regimen practically did not select out any DRMs which had become the main obstacle of long-term ARV therapy (Walmsley, Bernstein et al. 2002).

Despite successful HAART the growing number of ARV agents, the number of patients undergoing ARV treatment, especially in low income countries, and the prolonged periods during which patients have taken these substances has drawn our attention to the toxicities and the emergence of drug resistance to ARV agents. To overcome these problems new ARV classes CCR5 antagonists, integrase inhibitors and next generation PIs and NNRTI have been developed recently (De Clercq 2010).

2.2.1 ARV classes

By the year 2010 a total of 25 ARV agents consisting of 8 NRTIs, 4 NNRTIs, 10 PIs, 1 FI, 1 INI and 1 CCR5 receptor antagonist have been approved in US and in Europe (Table 1).

Table 1. ARV drugs approved by the Food and Drug Administration and by European Medicines Agency

NRTI		**PI**	
abacavir	ABC	amprenavir	APV
didanosine	ddI	atazanavir	ATV
emtricitabine	FTC	darunavir	DRV
lamivudine	3TC	fosamprenavir	FPV
stavudine	d4T	indinavir	IDV
tenofovir	TDF	lopinavir	LPV
zalcitabine	ddC	nelfinavir	NFV
zidovudine	ZDV or AZT	ritonavir	RTV
NNRTI		saquinavir	SQV
delavirdine	DLV	tipranavir	TPV
efavirenz	EFV	**FI**	
etravirine	ETR	enfuvirtide	ENF
nevirapine	NVP	**CCR5 inhibitors**	
INI		maraviroc	MVC
raltegravir	RAL		

2.2.1.1 Nucleotide/nucleoside reverse transcriptase inhibitors

NRTIs are nucleoside or nucleotide analogues which compete with the natural nucleotides for the incorporation to newly synthesized DNA. In case of incorporation into the proviral DNA they cause nucleotide chain termination and prevent further replication process (Shafer 2004).

All 7 NRTIs (Table 1) are pro-drugs which need phosphorylation for the activation. However, the different activity of intracellular kinases and expression of uptake transporters the intracellular concentration of triphosphorylated NRTI methabolites may greatly vary between cell types causing significant intra- and interindividual variability resulting in different inhibiting potential of these ARV drugs (Shafer 2002).

2.2.1.2 Non-nucleoside reverse transcriptase inhibitors

Contrary to NRTI, NNRTIs act as non-competitive RT inhibitors. Despite their diverse chemical structure they bind to the same small hydrophobic pocket near the active site of RT (Ghosn, Chaix et al. 2009). NNRTIs are able to inhibit only the HIV-1 group M viruses having no impact to the HIV-2 or HIV-1 group O (Witvrouw, Pannecouque et al. 1999; Shafer 2004). The first generation NNRTIs (DLV, EFV, NVP) are tightly fixed into the hydrophobic pocket of RT. Such interaction can be easily broken by the emergence of one DRM which makes the first generation NNRTIs of low genetic barrier drugs. In contrast, the second generation NNRTIs (ETR) are designed with more flexible chemical structure allowing several conformations within the hydrophobic pocket and

therefore require emergence of several DRMs before the agent loses its affinity to NNRTIs (Ghosn, Chaix et al. 2009).

2.2.1.3 Protease inhibitors

PIs are molecules which bind to HIV-1 PR with high affinity occupying larger space than enzyme natural substrate (polypeptide chain) itself. At the beginning of the PI usage many of these drugs had major problems with their bio-availability causing high bill burdens, low adherence and limited long term viral inhibition, therefore leading to the emergence of DRMs (van Heeswijk, Veldkamp et al. 2001). The change in PI efficiency took place due to the introduction of low dosage RTV as a pharmacokinetic booster as described before. Today almost all PIs have been administered with low-dose ritonavir.

2.2.1.4 Integrase inhibitors

The INIs block the HIV-1 integration complex insertion to the host cell genome. Lack of cross-resistance with other so far widely used PR and RT inhibitors has made it an essential component of ARV regimens against highly resistant viruses (Powderly 2010). A considerable advantage is also INI's potential of low toxicity comparing to other first line HAART components (Powderly 2010).

2.2.1.5 Entry inhibitors

Entry inhibitors are divided into two classes: CCR5 antagonists and fusion inhibitors. CCR5 antagonists bind to HIV-1 co-receptor CCR5 blocking the R5 tropic virus binding to its co-receptor and thus preventing the viral entry into the target cells (Pierson, Doms et al. 2004). The fusion inhibitors are short peptides consisting of aa sequences similar to HIV-1 env transmembrane C-terminal heptat repeat region (gp41). These drugs prevent the formation of a complex between gp41 N-terminal and C-terminal hepta-repeat regions required for the viral and host cell membrane fusion (Makinson and Reynes 2009). Albeit having closely related targets these two drugs have distinct DRM profiles (Shafer and Schapiro 2008).

2.2.2 HIV-1 DRM and their testing

The HIV-1 DRM can be tested by using genotypic and phenotypic assays. The genotypic assays determine the ARV DRMs in the viral nucleic acid sequences and interpret them according to previously composed algorithms (Shafer 2002). Phenotypic assays determine the DR directly measuring the viral replication in cell cultures in the presence of different consentration of ARV agents. The sensitivity of HIV-1 drug resistance tests compared to viral quantification

assays is approximately 20 times lower (25–50 copy/μl vs. 1000 copy/μl) as the drug resistance tests generally amplify about ten times longer viral genomic region (Shafer 2002).

In clinical practice the genotypic assays are more widely used than phenotypic assays because of their lower price, wider accessibility and shorter run-out time. The advantages of genotypic assays over phenotypic assays involve their ability to detect mutations from viral mixtures up to 20% of populations and transition mutations referring to archived drug resistance (Meynard, Vray et al. 2002; Shafer 2002). Phenotypic assays are mainly used for the evaluation of the new DRMs and for the determination of DR for heavily treated patients (Shafer 2002).

Both these assays are able to determine the DR only in actively replicating viruses and not in the viruses archived in the resting T-cells or other tissues. Therefore these tests represent the viral resistance only against the recent ARV therapies (Kozal, Shafer et al. 1993; Koch, Yahi et al. 1999).

2.2.2.1 Genotypic drug resistance assays

The workflow of the genotypic assays generally begins with the viral RNA extraction, its reverse transcription to cDNA, amplification by PCR and Sanger sequencing in ARV target regions like PR, RT and recently also in IN and env ViroSeq version 2.0 HIV-1 Genotyping System (Abbott Laboratories, USA), Truegene HIV genotyping system (Bayer Health Care LLC, USA), (Lindstrom and Albert 2003; Steegen, Demecheleer et al. 2006; Grant and Zolopa 2008; He, Cheng et al. 2008; Paar, Palmetshofer et al. 2008; Saravanan, Vidya et al. 2009). Genotypics assay generally sequences almost entire PR and the 5´part of the RT (first 240 aa) region where the majority of DRMs are located. Acquired sequences are assembled and compared with subtype B reference sequences for the detection and interpretation of DRMs using different algorithms (see 2.2.2.2). The Sanger sequencing is the population sequencing which is able to detect indicates the DRMs for the most abundant quasispecies of the currently replicating viruses in blood.

Genotypic drug resistance testing can be conducted by commercial kits (commercial HIV-1 DR assays) or by protocols developed in non-commercial labs (so called in house assays). Recent study has indicated the high concordance between these two approaches (Saravanan, Vidya et al. 2009).

2.2.2.2 Genotypic test results

The genotypic test generates a list of mutations which has been associated with HIV-1 ARV DR in earlier studies. Based on these mutations the interpretation algorithms are used to calculate the viral susceptibility to different ARV agents most often by computerized expert systems. The independence of DR determination from the interpretation process makes possible the usage of different

interpretation systems on the same sequence information. The most commonly used list of DRMs, HIV-1 genotypic interpretation algorithms and interactive interpretation systems are as follows: Stanford University HIV Drug Resistance Database (hivdb.stanford.edu), ANRS drug resistance interpretation algorithm (www.hivfrenchresistance.org), Rega Institute Drug Resistance Interpretation Algorithm (www.rega.kuleuven.be) and International AIDS Society–USA drug resistance mutations list (www.iasusa.org) (van Laethem, De Luca et al. 2002).

2.2.2.3 Virtual phenotype

Alternative way to interpret HIV-1 DR sequence information is to use virtual phenotype. It is a commercial algorithm which uses large genotype-phenotype relation database, linking the query sequence to most similar DRM pattern possessing database entries. The corresponding database entry susceptibilities to different ARVs have been considered to calculate the clinical result about this query DR profile (Verbiest, Peeters et al. 2000; Shafer 2002).

2.2.2.4 Phenotypic resistance tests

In classical phenotypic assays the virus is isolated and cultivated for 10-14 days on healthy individuals PBMCs. This makes this test expensive and time consuming. On the contrary, modern commercial phenotypic assays reverse transcribe and amplify viral PR-RT regions and insert them to viral vectors. It allows to avoid the first viral replication in PBMCs, which increases the assay sensitivity and lowers the cost. In both cases the viruses or viral constructs' fitnesses have been compared to reference strains in cell culture conditions in the presence of different ARV drugs. The results have been reported as concentration of ARV agents which inhibit the viral replication by 50% or 90% (expressed as IC50 or IC90).

Recently clinical assays which determine the viral tropism to CCR5 or to CXCR4 expressing cells have also been introduced. The most widely used is a commercial Trofile co-receptor tropism assay (www.trofileassay.com). At the first stage it resembles other phenotypic assays consisting of target region (env) reverse transcription, amplification and insertion to viral vector lacking the env region. Finally the replication ability of the constructs will be tested on CCR5 or CXCR4 expressing cell lines.

2.2.3 Drug resistance mutations

According to mechanisms the HIV-1 DRMs can be divided into two categories – primary and secondary DRMs. The emergence of primary DRMs cause direct resistance to certain ARV agents and increases the viral load in vivo. As a cost of the emergence of these mutations viruses loose their replication fitness

compared to wild type viruses. The replication fitness can be restored by the emergence of other compensatory or secondary DRMs. Secondary DRMs usually do not cause DR, their impact to viral fitness emerges only together with primary mutations.

2.2.3.1 NRTI drug resistance mutations

NRTI mutations are divided into M184V, TAM, mutations selected by regimens lacking thymidine analogues (non-TAM), multi–nucleoside resistance mutations and non-polymorphic accessory mutations (Shafer and Schapiro 2008). According to resistance mechanism NRTI DRMs can be divided into two classes. The first one decreases the frequency of incorporation of NRTIs into viral genomic DNA and includes mutations M184V, non-TAMs K65R and L74V and multi–nucleoside resistance mutation Q151M (Deval, Selmi et al. 2002; Deval, White et al. 2004). The second mechanism removes once incorporated NRTIs from the growing polynucleotide chain thus leading to primer unblocking and includes TAMs, T69 insertions and many accessory mutations (Arion, Kaushik et al. 1998; Boyer, Sarafianos et al. 2001).

The most abundant NRTI mutation is M184V causing high level resistance to most common first line ARV agents like 3TC, FTC and low level resistance to ddI, ABC, and increases susceptibility to ZDV, d4T, TDF (Whitcomb, Parkin et al. 2003). The M184V is frequently accompanied by the mutation K65R and L74V (Moyle, DeJesus et al. 2005; Eron, Yeni et al. 2006; Gallant, DeJesus et al. 2006; Shafer and Schapiro 2008). Less common mutations occurring during virologic failure under the non-TA regimens are K65N, K70EG and Y115F (Shafer and Schapiro 2008).

TAMs have been selected by the thymidine analogues and are causing resistance to ZDV and d4T but in lesser extent also to ABC, ddI and TDF (Whitcomb, Parkin et al. 2003). These mutations have been selected out preferentially in persons who have received ARV therapy in pre-HAART era with incompletely suppressive thymidine analogue mono- or dual therapy. TAMs accumulate in two distinct but overlapping pathways (Marcelin, Delaugerre et al. 2004; Rhee, Liu et al. 2004; Cozzi-Lepri, Ruiz et al. 2005; Molina, Marcelin et al. 2005; Shafer and Schapiro 2008). The first (type I) pathway includes mutations M41L, L210W, T215Y, and the second (type II) the mutations D67N, K70R, T215F and K219QE. In the absence of drug pressure the T215YF mutation tend to revert to T215CDEISV but not back to its wild-type form, being therefore a marker for the presence of the original T215YF mutation (Yerly, Rakik et al. 1998; De Luca, Giambenedetto et al. 2007; Shafer and Schapiro 2008).

2.2.3.2 NNRTI drug resistance mutations

NNRTI DRMs are divided into primary, secondary, minor non-polymorphic and minor polymorphic accessory mutations. The NNRTI resistance is described as low genetic barrier resistance because only one primary DRM confers high-level cross-resistance against all first generation NNRTIs (Shafer and Schapiro 2008).

Primary NNRTI mutations in order of their frequency are K103NS, V106AM, Y181CIV, Y188LCH, G190ASE and are accompanied by the secondary mutations like L100I, K101P, P225H, F227L M230L and K238T (Bacheler, Jeffrey et al. 2001; Rhee, Taylor et al. 2006). Less commonly emerging mutations are V179F, F227C, L234I and L381F. The minor non-polymorphic NNRTI mutations A98G, K101E, V108I and V179DE occur with or without primary NNRTI DRMs and they confer only low level resistance against NNRTIs (Rhee, Gonzales et al. 2003). Contrary to the first generation NNRTIs the full scale resistance development against the second generation NNRTIs needs the selection of at least two or more primary DRMs **(**L100I, Y181CI, G190E, M230L and in rarer cases V179IF and Y318F (Scherrer, Hasse et al. 2009; Johnson, Brun-Vezinet et al. 2010).

2.2.3.3 PI drug resistance mutations

According to the impact on DR these mutations can be divided into major (primary) and accessory (secondary) PI DRMs. Based on their location PI DRM can also be divided into PR substrate cleft, PR flap and PR other conserved residues and polymorphic positions mutations. Recently the gag cleavage site mutations associated with PI drug resistance have been described (Shafer 2002).

Major or primary PI DRMs are localized in the 17 PR positions. Of them L23I, D30N, V32I, I47VA, G48V, I50VL, V82AFTS, I84V locate in PR substrate cleft region, M46IL and I54ML in the flap region and L76V, N88S and L90M in the interior of PR enzyme. Four PI DRMs (L24I, L33F, F53L and G73ST) possess antagonistic effect which means that by conferring resistance to one group of PIs they increase the susceptibility to the other group of PIs (Dauber, Ziermann et al. 2002; Vermeiren, van Craenenbroeck et al. 2007; Shafer and Schapiro 2008).

Accessory or secondary PI DRMs do not confer direct resistance but restore the viral replication capacity in the presence of primary PI mutations (Shafer and Schapiro 2008). Based on their natural presence mainly in non-B subtype viruses these mutations or in this case polymorphisms could be divided into two categories. The first one involves substitutions selected out mainly by the ARV therapy. They generally do not account naturally for more than 10% of viruses of different subtypes. Most well-known of them are L10IV and A71VT or L10FR and A71IL, which are present in TN or TE patients, respectively (Shafer and Schapiro 2005). In several recent studies rare naturally occurring substitutions like V11I, E34Q, E35G, K43T, K45I, K55R, Q58E, T74PAS, V75I,

N83D, P79AS, I85V, L89V, T91S, Q92K and C95F have been described. Many of them have been associated mainly with darunavir or tipranavir exposure (Wu, Schiffer et al. 2003; Rhee, Fessel et al. 2005; Scherer 2007; De Meyer, Vangeneugden et al. 2008; Meyer 2008). The second category of accessory PI DRMs are present in highly polymorphic positions like 20, 36 and 63 and have been discussed in chapter describing natural polymorphisms (see 2.3).

In addition several mutations selected during PI containing regimens have been localized in the protease cleavage site regions, especially into two cleavage sites: NC/p1 and p1/p6. The cleavages of these sites are potential rate limiting steps in viral protein maturation (Cote, Brumme et al. 2001; Dauber, Ziermann et al. 2002; Lambert-Niclot, Flandre et al. 2008).

2.2.3.4 INI drug resistance mutations

Similarly to NRTI DRMs the INI DRM can be divided into primary and secondary DRMs (Metifiot, Marchand et al. 2010). Majority of them situate in the binding pocket of INIs. Four DRM pathways have been associated with INI DR. The two most frequent are pathways initiated by the primary mutations N155H and Q148HRK which are accompanied by one or more additional secondary mutations like the L74M, E138A, E138K, G140S and the L74M, E92Q, T97A, Y143H, G163KR, V151I, or D232N, respectively (Malet, Delelis et al. 2008; Johnson, Brun-Vezinet et al. 2010). The pathways initiated by primary mutations Y143RHC and E92Q have been found less frequently (Malet, Delelis et al. 2008; Johnson, Brun-Vezinet et al. 2010).

2.2.3.5 HIV-1 tropism and drug resistance mutations against entry inhibitors

The HIV-1 possesses two DR mechanisms against viral entry inhibitors. Firstly, both ARV agents are able to inhibit mainly R5 tropic viruses (use only CCR5 receptor for the cell entry). Therefore the virologic failure against these drugs can emerge by outgrow of X4 or dual tropic viruses (Reeves, Miamidian et al. 2004; Xu, Pozniak et al. 2005). The changes in viral tropism have been correlated mainly with env V3 positions 11 and 25 charge changes (De Jong, De Ronde et al. 1992).

Additionally, both drugs are able to select out classical DRMs independently from viral tropism. In case of fusion inhibitor ENF such resistance is associated primarily with mutations G36DS, I37V, V38AME, Q39R, Q40H, N42T, or N43D in the first heptad repeat (HR1) region of the gp41 envelope gene (Reeves, Miamidian et al. 2004; Xu, Pozniak et al. 2005). However, mutations or polymorphisms in other regions of the envelope (e.g. the HR2 region) may affect susceptibility as well (Reeves, Miamidian et al. 2004; Xu, Pozniak et al. 2005). With maraviroc the DRMs which allow virus binding to drug-CCR5 receptor complex have been described. Majority of these mutations locate in V3

loop or gp41 region, however there is no consensus about specific signature of these mutations (Johnson, Brun-Vezinet et al. 2010; Seclen, Gonzalez Mdel et al. 2010).

2.2.4 Transmitted drug resistance mutations

TDR is HIV-1 ARV resistance which has been aquired by transmission of drug resistant viruses. TDR mutation has the potential to limit therapeutic options in newly infected patients undergoing ARV therapy. The worldwide levels of TDR vary greatly and change over time (Wensing and Boucher 2003). The highest levels of TDR have been observed in developed countries of Western-Europe and North-America, the areas with wide access to ARV agents and with relatively long history of ARV monotherapy. The highest rates (22%) of TDR mutations are seen in Los Angeles (Booth, Garcia-Diaz et al. 2007; Hurt, McCoy et al. 2009). The rates in Western-Europe are somewhat lower remaining at the level of 10 – 20% of newly diagnosed patients (Wensing, van de Vijver et al. 2005; Spread programme 2008; Vercauteren, Wensing et al. 2009).

The rate of TDR mutations in many European countries has followed specific trends. In 1990s the level of TDR was rapidly increasing until the early years of 2000 most likely as a consequence of mono- or dual therapy. Since then the level of TDR mutations has stabilized or even decreased below 10% (Spread programme 2008; Yerly, Junier et al. 2009; Descamps, Chaix et al. 2010). Similar dynamics have been described in US, however, the established stabilisation levels are about twice as high as in Europe (Booth, Garcia-Diaz et al. 2007; Hurt, McCoy et al. 2009).

Giving deeper insight into the pattern of TDR mutations it is evident that the prevalence of TAMs and T215YF or its revertants was higher during the mono- and dual therapy time as these mutations were selected out by the extensive use of non-suppressive regimens with thymidine analogues (ZDV and D4T). Introduction of HAART in mid-1990s, however, lead to preferred emergence of low genetic barrier mutations (e.g. M184V) and mutations to NNRTIs and PIs. For the same reasons the TDR mutations pattern in Western countries has moved from high genetic barrier DRMs to low genetic barrier DRMs (Wensing, van de Vijver et al. 2005; Vercauteren, Wensing et al. 2009). In terms of ARV classes the SPREAD study demonstrated that at present time the most common DRMs are NRTIs (5%) followed by PIs (3%) and NNRTIs (3%) mutations (Spread programme 2008; Chilton, Castro et al. 2010). The results of other studies conducted in individual Western-European countries (United Kingdom, France) confirm these findings (Paraskevis, Pybus et al. 2009; Chilton, Castro et al. 2010; Descamps, Chaix et al. 2010). Furthermore the Western-European studies have observed that TDR mutations is more frequently found in subtype B as compared to non-B subtype viruses (Spread programme 2008; Chilton, Castro et al. 2010).

2.2.4.1 Transmitted drug resistance mutations in Eastern–Europe

The data about TDR in Eastern-Europe, especially in countries of rapidly developing IVDUs HIV-1 epidemics are limited (Zarandia, Tsertsvadze et al. 2006; Santoro, Ciccozzi et al. 2008; Rumyantseva, Olkhovskiy et al. 2009). The most comprehensive data come from Eastern-European countries were the so-called Western-European type of HIV-1 epidemics is spreading especially from Hungary and Poland (Wensing, van de Vijver et al. 2005). In these countries the prevalence of TDR has fluctuated between 4% and 29%, however in most cases it has remained below the 10% (Juhasz, Ghidan et al. 2008; Kanizsai, Ghidan et al. 2010; Stanczak, Stanczak et al. 2011). In lesser extent the TDR has been investigated also in Romania, Slovenia and Bulgaria were the TDR remained in the range of 0%-10% (Babic, Seme et al. 2006; Paraschiv, Otelea et al. 2007; Santoro, Ciccozzi et al. 2008).

In the area of so called Eastern-Europe IVDU epidemics the most thoroughly investigated region is Latvia, showing the TDR prevalence of 3.4 % – 28% (Kolupajeva, Aldins et al. 2008; Balode, Westman et al. 2010). Some studies have also been conducted in Russia, Moldova and Georgia indicating the levels between 0-4% (Pandrea, Descamps et al. 2001; Smolskaya, Liitsola et al. 2006; Zarandia, Tsertsvadze et al. 2006; Kolupajeva, Aldins et al. 2008; Rumyantseva, Olkhovskiy et al. 2009; Balode, Westman et al. 2010; Vinogradova, Gafurova et al. 2010). However, one should bear in mind that most Eastern-European studies are small in sample size, analyse together TN and TE populations and/or include patients infected with various viral subtypes (Paraschiv, Otelea et al. 2007; Kolupajeva, Aldins et al. 2008; Santoro, Ciccozzi et al. 2008; Vinogradova, Gafurova et al. 2010).

2.3 Naturally occurring polymorphisms

HIV-1 possesses several natural polymorphisms in ARV target regions especially in PR sequences (van de Vijver, Wensing et al. 2006). By definition natural polymorphisms have been described as aa difference of HIV-1 subtype B consensus sequences that are found withmore than 1% prevalence in certain HIV-1 population. Natural polymorphisms usually locate in non-active site regions allowing therefore variations by the genetic drift or by immune selection (Kantor and Katzenstein 2003). Interestingly many of these natural polymorphisms which are spread mainly in non-B viruses are selected out by ARV therapy in subtype B referring to their potential impact on DR. The prevalence of natural polymorphisms have been extensively characterized in widely spread HIV-1 subtypes such as A, B, C, CRF01_AE and CRF02_AG (Geretti 2006).

2.3.1 Protease polymorphisms

A total of 30 PR aa and 99 RT aa positions are described as polymorphic in group M HIV-1 subtypes and CRFs (Kantor and Katzenstein 2003). Most widely spread of them locate in PR positions 20, 36, 63, 71, 77 and 93 (Shafer, Kantor et al. 2000; Kantor and Katzenstein 2003). Similarly to secondary DRMs these polymorphisms do not cause direct resistance to ARVs but rather compensate or restore the DRM possessing virus replication capacity (Martinez-Picado, Savara et al. 1999; Nijhuis, Schuurman et al. 1999; van Maarseveen, de Jong et al. 2006). Their direct impact to the emergence of DR is unclear. Only once it has been described in vivo that PR M36I facilitates the emergence of PR resistance (Perno, Cozzi-Lepri et al. 2001). Naturally the most widely spread PR polymorphisms that have been associated with the ARV therapy are M36I, R41K, H69K, L89M; the well known consensus sequences in all so far investigated non-B subtypes (hivdb.stanford.edu) (Rhee, Kantor et al. 2006). The sole exception is subtype D which does not possess polymorphisms H69K and L89M most likely because of its higher similarity to subtype B. Other polymorphisms have been presented as consensus aa in a lesser extent – L10IV in subtypes A, G, CRF01_AE and CRF02_AG; K20I in subtypes F, G and CRF02_AG, E35D in subtypes A, F and CRF01_AE. In other subtyes these PR associated polymorphisms have been presented less frequently (hivdb.stanford.edu) (Rhee, Kantor et al. 2006).

2.3.2 Reverse transcriptase polymorphisms

Of the first 240 RT aa positions 41 have been indicated as polymorphic in different subtypes and CRFs (Shafer and Shapiro 2008). Of the 41 the aa codons 69 (in subtype F), 75 (in CRF01_AE), and 118 (in subtypes A, B, D) have been associated with NRTI and positions 98 (in subtypes B, C, and G), 106 (subtypes G, AE and CRF02_AG) and 179 (subtypes B, C, D, F, G, CRF02_AG) with NNRTI resistance (Hirsch, Brun-Vezinet et al. 2000; Shafer 2002). In rare cases two accessory mutations E44DA and V118I belonging to type I TAM pathway have been reported also as natural polymorphisms because of their occurrence in more than 1% of ARV naive viruses (Kantor and Katzenstein 2003). Additionally, the polymorphism F214L is described to increase the genetic barrier against type I TAMs (Shafer and Schapiro 2008; Spread programme 2008).

2.3.3 Integrase polymorphisms

Of 288 IN aa positions 115 have been described as polymorphic in different subtypes (Rhee, Liu et al. 2008). From them the substitutions S153A and E157Q are directly associated with DR and presented in about 1% of different

HIV-1 subtype sequences (Rhee, Liu et al. 2008). Other polymorphisms indirectly associated with DR such as I72V, L74M, T97A, S119GR, V151I, K156N, E157Q, G163KR, V165I, V201I, I203M, T206S and S230N have been found in much greater frequency. From them the substitutions V201I, I72V and T206S are most widely spread. V201I is consensus aa in all non-B subtypes, I72V in subtypes A, D, F, CRF01_AE, and T206S in subtype G and CRF02AG (Myers and Pillay 2008; Rhee, Liu et al. 2008; Ceccherini-Silberstein, Malet et al. 2009; Eshleman, Hudelson et al. 2009; Garrido, Geretti et al. 2010).

Recent phenotypic DRM tests have demonstrated that in the absence of primary DRMs the role of the abovementioned polymorphisms in INI susceptibility is minimal in B as well as in non- B subtype viruses (van Baelen, van Eygen et al. 2008; Low, Prada et al. 2009).

2.4 The subtype specific differences in drug resistance development

Several studies have demonstrated that in general most DRMs are similar and equally selected by all HIV-1 subtypes and CRFs under the same ARV treatmen (van de Vijver, Wensing et al. 2010). However, the growing number of investigations has shown that the subtype specific development of DRMs in different subtypes exists.

In PR region the most thoroughly described is the preferred selection of L90M mutation in subtypes C, G, F and CRF01_AE compared to D30N in subtype B (Cane, de Ruiter et al. 2001; Sugiura, Matsuda et al. 2002; Grossman, Paxinos et al. 2004) during treatment with PIs. The preferred emergence of L90M has been associated with presence of M89IV (a widely described polymorphism in non-B subtype viruses) and with polymorphisms N83T and 74S specifically in subtype G viruses (Abecasis, Deforche et al. 2005; Calazans, Brindeiro et al. 2005; Martinez-Cajas, Pant-Pai et al. 2008). All these polymorphisms are able to restore the replication capacity of L90M carring viruses (Abecasis, Deforche et al. 2005).

Another subtype specific response to ARV therapy has been found in CRF01_AE viruses exposed to NFV. In these cases the non-active site mutation N88S develops instead of D30N and N88D as described in subtype B viruses (Ariyoshi, Matsuda et al. 2003; Bandaranayake, Kolli et al. 2010).

Most widely described suptype specific difference among NRTI DRMs is the selection of K65R. This mutation is rare in subtype A compared to subtype B and C viruses (Gupta, Chrystie et al. 2005). However, in studies using single dose of NVP K65R emerges less likely in subtype D and C than in subtype A viruses (Eshleman, Hoover et al. 2005). In cell culture studies the faster selection of K65R was also found in subtype C compared to subtype B viruses (Brenner, Oliveira et al. 2006).

The subtype specific effect of NNRTI DRM emerges most strongly in RT position 106. In subtype B viruses mainly the V106A and in subtype C the

V106M will be selected (Brenner, Turner et al. 2003; Morris, Pillay et al. 2003; Grossman, Istomin et al. 2004). The likely explanation of preferred selection of V106M in subtype C is the change of a single nucleotide (from GTG to ATG) while in subtype B the emergence of the same DRM would require at least two bp substitutions (Brenner, Turner et al. 2003). Clinically the V106A confers resistance only to NVP but V106M is able to cause resistance to all first generation NNRTIs (Martinez-Cajas, Pant-Pai et al. 2008).

2.5 Literature summary

Within the last 15 years the HIV-1 epidemic has emerged in Eastern-Europe; mainly IVDUs are affected and the epidemics are caused mostly by the monophyletic HIV-1 strains originating from subtype A1. In Estonia, however, the HIV-1 epidemic is similar to that described in Eastern-Europe, except that the infection is caused by the rare recombinant form CRF06_cpx instead.

Recent studies have revealed that different subtypes and CRFs possess many polymorphisms in ARV agents target regions. However, mainly there have been investigated the widely spread subtypes and CRFs. Therefore there is very limited information about the subtype specific substitutions and DRM in Eastern-European HIV-1 strains and especially about the CRF06_cpx strain circulating in Estonia. The last one has been described so far only in single studies from resource poor-settings in Central-Africa.

In addition, the management of HIV-1 infection may further be complicated by the development of TDR. In Western-Europe where ARV therapy (including mono- and dual therapy) has been used for almost two decades about 10% of newly infected subjects carry DR viruses. In Eastern-Europe, including Estonia the situation has been poorly described so far. It is likely that it differs from mature epidemics because here the mono- and dual therapy has hardly been in use. On the other hand within last five years the consumption of ARV agents has strikingly increased in Estonia.

So far the HIV-1 scientific investigations in Estonia have been conducted mainly by prof. Mart Ustav and by prof. Anneli Uusküla groups. In both groups there have been defended two Ph.D thesis by Maarja Adojaan and Kristi Rüütel. Maarja Adojaan's works concentrate mainly on Estonian HIV-1 infections molecular epidemiology, host genetic factors and some aspects of the development of HIV-1 vaccine (Adojaan, Kivisild et al. 2005; Adojaan, Molder et al. 2007; Martinon, Kaldma et al. 2009; Molder, Adojaan et al. 2009). Kristi Rüütel´s studies investigated the epidemiological aspects of Estonian HIV-1 infection including the risk-behaviour (IVDU) and quality of life of people living with HIV-1 (Platt, Bobrova et al. 2006; Ruutel, Uuskula et al. 2008; Ruutel, Pisarev et al. 2009).

3. AIMS OF THE RESEARCH

The general aim of the research was to describe ARV resistance profile of the HIV-1 viruses circulating among the TN and TE subjects in Estonia during the concentrated HIV-1 epidemic.

The study had following specific aims:

1. To describe the V3, PR, RT and IN regions of HIV-1 viruses circulating in Estonia in 2005 to 2006 and 2008.
2. To describe natural polymorphisms in PR, RT and IN regions of HIV-1 viruses among TN subjects in Estonia.
3. To describe the association between the primary DRM in the RT region and IN polymorphisms in Estonia.
4. To characterize trends of TDR among HIV-1 positive subjects in 2005-2006 and 2008 in Estonia.
5. To characterize primary DRMs in HIV-1 CRF06_cpx viruses in TE patients who have failed at least one ARV regimen.

4. PATIENTS AND METHODS

The studies and analyses forming the basis of this thesis are presented in Table 2.

4.1 Study design

Current study involves five different populations (Table 2).

The TN PR-RT and TN IN studies monitored naturally occurring DRMs and polymorphisms in PR-RT and IN regions in TN patients. The TE IN study aimed to find out the PR-RT DRM associated substitutions in IN region among TE patients infected with viruses possessing at least one DRM in RT region. The TDR mutations study investigated the prevalence of TDR mutations among newly diagnosed TN patients three years after the scale-up of ARV therapy in Estonia and the TE PR-RT study described the DRM among TE patients who have failed ARV therapy.

Subjects of the first two studies were invited by their treating physician to donate 8-16 ml blood for HIV-1 genotyping and fill in a questionnaire including demographic (date of birth, sex, nationality, risk behaviours, possible date of infection and possible route of transmission) and clinical characteristics (viral load and CD4+ cell count). The route of transmission was considered as intravenous if the intravenous drug use episodes were reported regardless of the sexual contact with HIV positive subject; heterosexual if the subject had been in sexual contact with the HIV positive person and had no other risk factors; or unknown if the subject denied having known risk factors of HIV-1 infection. The TN PR-RT and TN IN studies consisted of subjects originating from Ida-Viru County (epicentre of the epidemic) and Tartu prison.

All populations were representative selection of Estonian HIV positive subjects who are mainly young male IVDUs originating from Tallinn, the capital of Estonia, or Ida-Viru County (Laisaar, Avi et al. 2011). All patients had been tested HIV positive during the concentrated HIV-1 epidemic between 2000 and 2010.

In all populations the presence of HIV-1 infection was first tested with the fourth generation enzyme-linked immunoassay (Vironistica HIV Uniform II Ag/Ab, BioMerieux, Marcy Etoile, France) at local HIV testing laboratories and then verified by the immunoblotting assay (INNO LIA HIV I/II Score Western-blot, Microgen Bioproducts Ltd, Surrey, UK) in the HIV Reference Laboratory of the West Tallinn Central Hospital.

Table 2. Description of studies incorporated into this thesis

Study design	Sampling period	Study population	Primary aim	Publi-cation
1/Evaluation of natural DRMs and polymorphisms among TN subjects in PR-RT region (**TN PR-RT population)**	September 2005– October 2006	115 TN sub-jects diagnosed HIV positive in 2000-2005 from Ida-Viru region and Tartu Prison	To determine natural DRM and poly-morphisms in Estonian HIV-1 CRF06_cpx viruses in PR and RT region	1
2/ Evaluation of natural DRMs and polymorphisms among TN subjects in IN region **(TN IN population)**	September 2005– October 2006, April– May 2007	104* same as study 1	To determine the natural DRM and polymorphisms spreading in Estonian HIV-1 CRF06_cpx viruses in IN region	2
3/Monitoring of IN region polymorphisms among TE population **(TE IN population)**	October 2006 – May 2007	10**	To describe the association between IN region polymorphisms and RT region primary DRMs in HIV-1 CRF06_cpx	2
4/TDR mutations monitoring after scale-up of ARV therapy **(TDR population)**	April 2008 – November 2008	201*** TN subjects with newly diagnosed HIV infection	To describe the prevalence of TDR among newly diagnosed HIV-1 positive subjects in 2008	3
5/Monitoring of DRMs in PR-RT region among TE subjects **(TE PR-RT population)**	September 2006 – November 2010	97**	To describe the DRM in TE subjects who failed at least one ARV regimen	–

* 77 subjects of this population overlap with TN PR-RT study subjects; the additional 27 samples were collected in the syringe exchange programs from Ida-Viru County and Tallinn in April-May 2007

** TE IN and TE PR-RT study populations consisted of patients in Ida-Viru Central Hospital who had been diagnosed HIV-1 positive in 2000–2007 and 2000–2010, respectively. Their treating physician had diagnosed their ARV treatment failure and their blood samples were sent to Tartu University Clinics for HIV-1 drug resistance testing. In case of TE IN study population subjects whose viruses possessed at least one DRM in HIV-1 RT or PR region were involved.

*** Samples consisted of leftovers of plasma samples sent from the first level HIV screening centers, anonymous cabinets and by physisians suspecting HIV-1 infection to the HIV Reference Laboratory of the West Tallinn Central Hospital for the HIV confirmatory testing.

4.2 Ethical considerations

The protocols of TN PR-RT and TN IN studies have been approved by the Ethics Review Committee on Human Research of the University of Tartu (142/8, 24th of October, 2005) and subjects signed informed consents under the supervision of their treating physicians. The results of HIV-1 PR-RT genotyping and their interpretations have been made available for patients and their physicians. In TE IN, TDR and TE PR-RT studies the approval of the Ethics Review Committee was considered unnecessary as surplus of non-personalized sera were used.

4.3 Blood sample collection and processing

In all studies except the TDR study appoximately 20 ml blood was taken via venepuncture into BD Vacutainer Cell Preparation Tubes (BD Diagnostics, Becton Dickinson, USA) and centrifuged at 3000 rpm for 30 min in the laboratory of Institute of Microbiology within 4 hours after collection. In TDR study at least 400 µl of plasma was obtained after the HIV confirmatory testing in the HIV Reference Laboratory. All samples were stored at the – 80°C until further analysis but no longer than for 6 months. The viral genomic RNA was extracted from 0.4 ml of plasma using the QIAamp Viral RNA Mini Kits (Qiagen, Germany) as recommended by the manufacturer and stored at – 80°C for a maximum of 6 months.

4.4 Reverse transcription and PCR

The HIV-1 genotyping in different regions was conducted using modified protocols published elsewhere (Liitsola, Laukkanen et al. 1996; Nijhuis, Schuurman et al. 1999; Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005; Lataillade, Chiarella et al. 2010). The PR-RT protocol was originally developed by prof. Jan Albert (Karolinska Institutet, Stockholm, Sweden)

The viral genomic RNA was reverse transcribed, amplified and sequenced in following regions:

- PR region (2,139-2,647 bp in HXB2) – in TN PR-RT population
- RT region (2,533- 3,331 bp in HXB2) – in TN PR-RT population
- *env* region (6,962-7,371 bp in HXB2) – in TN PR-RT population
- IN region (4,141- 5,219 bp in HXB2) – in TN IN and TE IN population
- PR-RT region (2,242- 3,333 bp in HXB2) – in TE IN, TDR and TE PR-RT population

4.4.1 PR, RT and env region amplification in TN PR-RT population

In PR, RT, and env regions reverse transcription and the first round PCR was performed using 4 µl of viral genomic RNA, Illustra Ready-to-Go RT-PCR Beads (GE Healthcare, UK) and corresponding primers for PR, RT and env regions as shown in Supplemental Table 1 (Article 1). Then 2 µl of the first round PCR product was transferred to the second round PCR. One unit of 6:1 mixture of Taq and Pfu (Fermentas, Lithuania) DNA polymerase and 0.3 µM primers (Supplemental Table 1 in Article 1) were used in the second round PCRs.

4.4.2 PR-RT region amplification in TE IN and TDR populations

In PR-RT region the HIV-1 RNA was reverse transcriptased into cDNA using the following conditions: 1x reaction buffer, 1mM dNTP, 16U M-MuLV RT (Fermentas, Lithuania), 16U RNase inhibitor (Invitrogen, USA), and 0.4 µM antisense primer JA272degen (5′-GGATAAATMTGACTTGCCCART-3′). PR-RT region was amplified in a first round PCR using the following conditions: 1x HotStart Buffer, 1.5 mM MgCl2, 0.2 µM dNTP, 0.4 µM primer JA269degen (5′-AGGAAGGMCACARATGAARGA-3′) and JA272degen. Second round PCR was carried out in the same conditions as the first round using primers JA270 (5′-GCTTCCCTCARATCACTCTT-3′) and JA271 (5′-CCACTAAYT TCTGTATRTCATTGAC-3′). One unit of 6:1 mixture of Taq and Pfu DNA polymerase (Fermentas, Lithuania) was used in both PCR rounds.

4.4.3 IN region amplification in TN IN and TE IN populations

In IN region the HIV-1 RNA was reverse transcriptased into cDNA using the following conditions: 1x reaction buffer, 1mM dNTP (Fermentas, Lithuania), 16U M-MuLV RT (Fermentas, Lithuania), 16U RNase inhibitor (Invitrogen, USA), and 0.4 µM antisense primer INREV-Idegen (5′-TCTCCTGTATGCA GACCCCAATAT-3′). IN region was amplified in a first round PCR using the following conditions: 1x HotStart Buffer, 1.5 mM MgCl2, 0.2 µM dNTP, 0.4 µM primer INFORIdegen (5′-GGAATYATTCARGCACAACCAGA-3′) and 0.4 µM primer INREV-Idegen. Second round PCR was carried out in the same conditions as the first round using primers HIV+4141degen (5′-TCTA YCTGKCATGGGTACCA-3′) and INREVII (5′-CCTAGTGGGATGTGTACT TCTGA-3′). One unit of 6:1 mixture of Taq and Pfu DNA polymerase (Fermentas, Lithuania) was used in both PCR rounds.

4.5 Sequencing

All second round PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Germany) and directly sequenced using the ABI Prism Big Dye 3.0 fluorescent terminator sequencing chemistry (Applied Biosystems,

USA) with the second round PCR primers and in the PR-RT region in additional sequencing primers JA273 (5′-CCCTCAAATCACTCTTTGGC-3′), JA274 (5′-AAAATCCATACAATACTCCA-3′), JA275 (5′-TTATTGAGTT CTCTGAAATC-3′) and JA276 (5′-TGTATATCATTGACAGTCCA-3′).

4.6 Sequence analysis

Sequences were assembled to contiques using the Vector NTI software (Invitrogen, UK). Alignments were conducted by the MEGA 4.0 software packages and phylogenetic trees were constructed using the (NJ method as described elsewhere (Naganawa, Sato et al. 2002). All reference sequences were taken from the Los-Alamos HIV Sequence Database (www.hiv.lanl.gov). The statistical robustness of NJ trees and reliability of branching pattern were confirmed by bootstrapping (1000 replicates). Consensus sequences were constructed using MEGA 4.0 software package.

For the aa frequency analysis the studied PR-RT and IN sequences were translated into aa sequences and were analysed by the MEGA 4.0 software packages comparing the aa composition in corresponding positions with subtype B reference sequence (HXB2 from Los Alamos HIV sequence database (www.hiv.lanl.gov)).

Phylogenetic trees were constructed for TN PR-RT (in env and RT regions), TN IN and TE IN, and TDR population sequences using the following subtype and CRF reference sequences:

For TN PR-RT population: AF004885, U51190, AF069670, AF484509, AF286238, AF286237, K03455, AY423387, AY173951, AY331295, U52953, U46016, AF067155, AY772699, K03454, AY371157, AY253311, U88824, AF077336, AF005494, AF075703, AJ249238, AY371158, AJ249236, AJ249237, AF377956, AF084936, AF061641, U88826, AF061642, AF190127, AF190128, AF005496, AF082394, AJ249235, AJ249239, U54771, L39106, AF193276, AF193253, AF064699, L20587, AY535659, DQ400856, AY500393, AB286851, AJ245481, AJ288982, AF064699 and AJ288981.

For TN IN and TE IN population: AF004885, AF286238, K03455, U52953, K03454, AF077336, AY371158, AF084936, AF190127, AF082394, AJ249235, U54771, L39106, AF193276, L20587, AJ245481, AJ288981, AB286851, AF064699, AY535660, AY500393, DQ167216, DQ400856, and AY535659.

For TDR population: AF004885, DQ676872, AF286238, K03455, U52953, K03454, AF077336, AY371158, AF084936, AF190127, EF614151, AJ249235, U54771, AY271690, AF414006, AB286851, AJ245481, AF064699, AY500393, AY535659, DQ400856 and L20587.

4.7 Simplot analysis

For determination the recombination structure of the putative recombinant forms sequences were analysed with the Simplot software using bootscanning method and following parameters: Window 200 bp, Step 20 bp, GapStrip: on, Reps 100, Kimura (2- parameter), T/t 2.0 and the NJ. The region similarity to certain subtype or CRF was confirmed if permutated tree value was higher than 70%. Additionally, in TDR study population putative recombinant sequences were reanalysed with the Simplot bootscanning analysis using the window size of 400bp. The HIV-1 subtype reference sequences used in the Simplot analysis in corresponding regions were as follows:
For TN PR-RT population: AF004885, AF286238, K03455, U52953, K03454, AF077336, AY371158, AF084936, AF190127, AF082394, AJ249235, L20587, AY500393 and AY535659.
For TN IN and TE IN population: AF004885, AF286238, K03455, U52953, K03454, AF077336, AY371158, AF082394, AJ249235, AY500393 and AY535659.
For TDR population: AF004885, AF286238, K03455, U52953, K03454, AF077336, AY371158, AF414006, AF084936, AF190127, EF614151, AJ249235, U54771, AY271690, AY500393 and AY535659.

4.8 Determination of drug resistance mutations and polymorphisms

4.8.1 For PR-RT region

DR associated polymorphisms, DRM and DR interpretations were conducted in TN PR-RT population for PR and RT sequences using the International AIDS Society-USA database (www.iasusa.org) or Stanford University HIV Drug Resistance Database Genotypic Resistance Interpretation Algorithm (Version 4.3.1) and for IN and TDR populations using Stanford University HIV Drug Resistance Database Genotypic Resistance Interpretation Algorithm (Version 4.3.1).

4.8.2 For IN region

The aa polymorphisms in every IN position were analyzed using subtype B consensus sequence from Los Alamos HIV sequence database (www.hiv.lanl.gov). The functionally conserved motifs (DDE, HHCC), extended active site residues (Q62, C65, T66, H67, E92, N120, F121, I141, P142, Y143, Q148, V151, N155, K156, K159) and LEGP/p75 site residues (R166, D167, Q168, A169, E179 and H171) were evaluated (Rhee, Liu et al. 2008). INI drug resistance associated polymorphisms and INI DRM list were used as described by Myers and Pillay (Myers and Pillay 2008). The

substitutions were then characterized in three groups according to their influence on viral replication in the presence of INIs used in clinical trials or in vitro experiments as follows: the N155H, Q148KRH and E92Q were considered as primary INI DRMs, substitutions H51Y, T66I, L74MAI, T97A, F121Y, E138K, G140SA, Y143RC, S147G, V151I, S153Y, E157Q, G163R, I203M, S230R, and R263K were classified as accessory mutations and T125K, A128T, Q146K, S153A, M154I, N155S, K156N, K160D, V165I, V201I, T206S, S230N, V249I, and C280Y were considered to be associated with non-clinical INI drug resistance (Myers and Pillay 2008; Rhee, Liu et al. 2008; Lataillade, Chiarella et al. 2010).

5. RESULTS AND DISCUSSION

5.1 Study populations predominantly consisted of intravenously infected subjects

As presented in Table 3 all study populations for which demographic data were available consisted predominantly of young male IVDUs.

Table 3. Demographic and clinical characteristics of the study populations

Study No	Study population	Median age (IQR)	Percentage of IVDUs	N of subjects – total/%male	Median CD4 cells/ml (SD)	Median VL copies/ml (SD)
1	TN PR-RT	27(24;30)	65	115/70	417 (+-284)	190,271 (+-395,994)
2	TN IN	27 (23;30)	66	104/84	414 (+-297)	175,200 (+-349,866)
3	TE IN	no data	no data	10/no data	174 (+-82)	63,163 (+-55,781)
4	TDR	no data	no data	201/61	no data	no data
5	TE PR-RT	no data	no data	83/no data	178 (+-160)	45,013 (+-762,338)

5.2 The HIV-1 CRF06_cpx has been the most predominant subtype during the last 7 years

Regardless of the investigated population or the year of the sample collection all sequenced regions demonstrated that CRF06_cpx was by far the most predominant subtype accounting for about 70% or more of cases (Table 4, Figure 6, Figure 1 in Article 1, Figure 1 in Article 2 and Figure 1 in Article 3).

Table 4. The distribution of Estonian HIV-1 subtypes and recombinant forms in different genomic regions based on the phylogenetic tree and Simplot analysis

Study name	Total number of subjects	Number of sequenced samples in corresponding region	HIV-1 subtypes n (%)				
			CRF06_cpx	recomb **	A1	B	CRF02_AG
TN PR-RT	115	PR - 87	nd*	nd*	nd*	nd*	nd*
		RT - 56	49 (88)	6 (11)	1 (2)	1 (2)	0
		env - 67	64 (96)	0	3 (4)	0	0
TN IN	104	IN - 104	89 (86)	14 (13)	3 (3)	0	0
TE IN	10	IN -10	7 (70)	1 (10)	0	1 (10)	1
TDR	201	PR-RT - 145	123(85)	17 (12)	2 (1)	3 (2)	0
TE PR-RT	83	PR-RT - 59	57 (97)	0	0	2 (3)	0

* Sequenced PR genomic region was too short for phylogenetic tree analysis
nd – no data
**- recombinants between CRF06_cpx and subtype A1 or some other subtypes

The second largest group in almost all regions was a recombinant form of CRF06_cpx and subtypes A1 or other subtypes; the only exception was env region where the recombinant sequences were absent (Table 4). In phylogenetic analysis these recombinant sequences clustered outside other subtypes or CRFs reference sequences (bootstrap cut off <70%) or formed sub-clusters with long root branches inside other monophyletic clusters (Figure 1 in Article 1).

Additionally in almost all analysed regions only single sequences (less than 3%) belonged to subtype A1, subtype B or CRF02_AG clades (Table 4, Figure 6, Figure 1 in Article 1, Figure 1 in Article 2 and Figure 1 in Article 3).

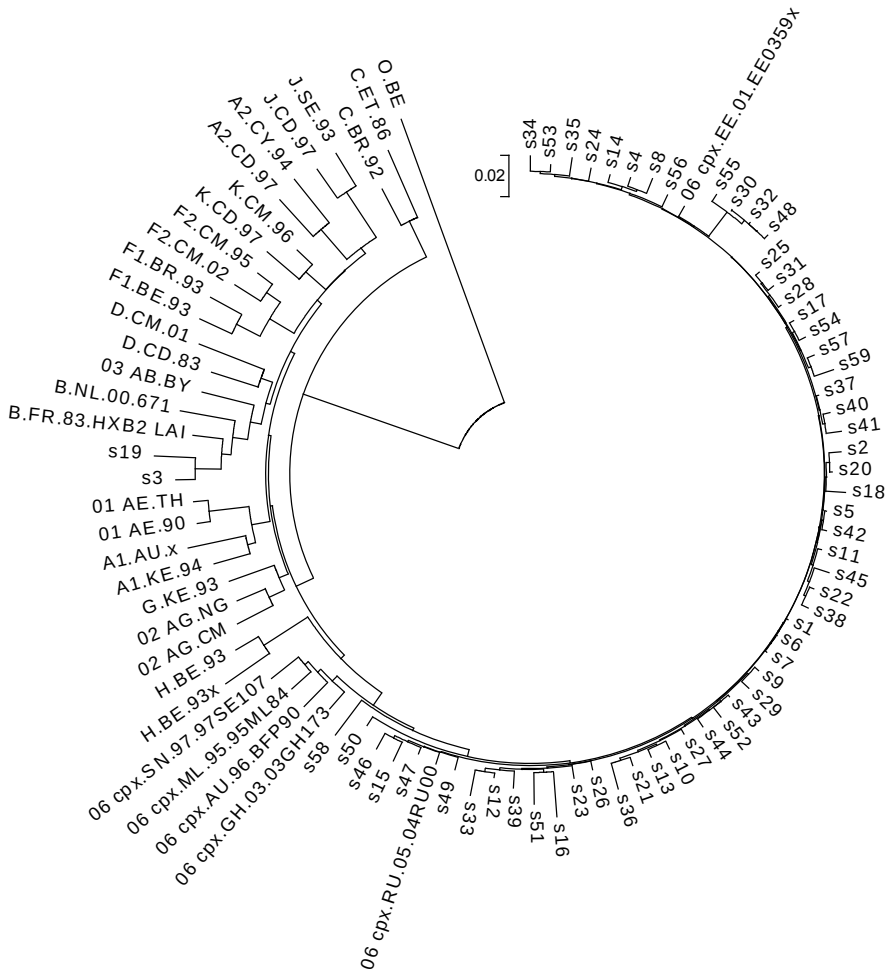

Figure 6. NJ tree of PR-RT sequences of the TE PR-RT population including reference sequences of all subtypes, CRF01_AE, CRF02_AG, and CRF06_cpx. The tree is rooted by the HIV-1 group O reference sequence. A genetic distance scale of 0.02 is indicated. Majority of Estonian TE PR-RT sequences cluster together with CRF06_cpx sequences.

The subtype distribution in the present studies was in good concordance with the earlier results in Estonia in 2000-2003 (Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005). These studies found that the phylogenetic distribution in gag/pol region consisted of 77% of CRF06_cpx and 10-16% of recombinant forms between CRF06_cpx and subtype A1. Moreover similar to us they did not found any CRF06_cpx and subtype A1 recombinants in env region (Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005).

These results suggest that recombination in *env* region of Estonian HIV-1 viruses might be impeded. One potential explanation could be the greater diversity of *env* compared to gag or pol sequences. However, to our best knowledge such preferred recombination in certain HIV-1 genomic regions has not been described before; rather there have been indications to equal distribution of recombination breakpoint throughout the entire HIV-1 genome (www.hiv.lanl.gov).

As presented in Figure 1 in Article 1, Figure 1 in Article 2 and Figure 1 in Article 3 and similarly in Adojaan and Zetterberg the subtype A1 viruses clustered together with Eastern-European subtype A1 reference sequences suggesting that Estonian subtype A1 infected patients are epidemiologically most likely linked to Eastern-European subtype A1 HIV-1 epidemics (Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005).

The overwhelming predominance of CRF06_cpx strains for almost 10 years demonstrates the stability of the Estonian HIV-1 phylogenetic structure despite being potentially exposed to the HIV-1 subtype A1 and lesser extent CRF03_AB epidemics in surrounding countries like Latvia and Russia (Lukashov, Huismans et al. 1999; Smolskaya, Liitsola et al. 2006; Thomson, de Parga et al. 2007; Thomson, Vinogradova et al. 2009). At the same time the Estonian CRF06_cpx viral strains have also been restricted to Estonia. Only single cases of similar CRF06_cpx viruses have been reported in Russia, Sweden and Latvia; all emerged after the CRF06_cpx outbreak in Estonia (Skar, Sylvan et al. 2008; Rumyantseva, Olkhovskiy et al. 2009; Balode, Westman et al. 2010; Vinogradova, Gafurova et al. 2010). In the phylogenetic analysis these CRF06_cpx sequences cluster inside the Estonian CRF06_cpx viral clade suggesting their potential links to Estonian CRF06_cpx epidemic (Skar, Sylvan et al. 2008).

This all refers that the Estonian HIV-1 CRF06_cpx epidemic is most likely seeded independently of other HIV-1 epidemics in former Soviet Union or neighboring countries and most probably originates directly from the West- or Central Africa, more specifically from Mali or Burkina Faso where the prevalence and diversity of CRF06_cpx viruses is the highest in the world (Montavon, Toure-Kane et al. 2002; Ouedraogo-Traore, Montavon et al. 2003; Tebit, Ganame et al. 2006). Furthermore the Estonian HIV-1 epidemic has not been influenced by our Nordic neighbors – Finland and Sweden as CRF06_cpx and its next generation recombinants in these countries are mostly not found (Liitsola, Holmstrom et al. 2000; Liitsola, Ristola et al. 2000; Kivela, Krol et al. 2005; Kivela, Krol et al. 2007; Skar, Sylvan et al. 2008; Skar, Axelsson et al. 2011).

5.3 Molecular characteristics of Estonian CRF06_cpx viruses

In phylogenetic tree analysis all Estonian CRF06_cpx sequences in all genomic regions of current study form a single monophyletic cluster inside the CRF06_cpx sequences circulating in other countries (Figure 6, Figure 1 in Article 1, Figure 1 in Article 2 and Figure 1 in Article 3). Furthermore, in more conserved genomic regions (e.g. RT) the viral sequence homogeneity is so high that some viruses indicate the same or nearly identical sequences (Figure 1 in Article 1 and Figure 1 in Article 2). Therefore one can speculate that the PCR contamination can be the cause of such high similarity. However, highly similar or identical clusters have also been found recently in Estonia independent from our group (Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005). In addition nearly identical sequences among the Eastern-European HIV-1 subtype A1 infection have also been demonstrated within few years after the HIV-1 outbreak in Ukraine (Novitsky, Montano et al. 1998; Nabatov, Kravchenko et al. 2002; Saad, Shcherbinskaya et al. 2006; Thomson, de Parga et al. 2007).

5.4 Simplot analysis of putative recombinant forms between CRF06_cpx and other HIV-1 strains

In TN PR-RT population six putative recombinant forms in RT region were analysed by Simplot software. All these sequences revealed similar recombination structure starting with the CRF06_cpx and ending with the subtype A1 sequences.

In TN IN and TE IN sequences 14 and 1 putative recombinant forms, respectively, represented complex structures between CRF06_cpx, subtype A1 and CRF03_AB sequences. The recombination breakpoint analysis of these viruses revealed two similar sequence clusters consisting of five and three strains, respectively, and seven URFs.

In the TDR population Simplot analysis was carried out using two window sizes (200 and 400 bp) for 17 putative recombinant PR-RT sequences. In both analyses all sequences turned out to be URFs between subtype A1, CRF06_cpx and CRF02_AG.

Complex recombinant structure at Estonian HIV-1 viruses has also been described before by Adojaan et al (Adojaan, Kivisild et al. 2005). They sequenced two viral strains from these viruses and designated it as a new "Estonian born" HIV-1 circulating recombinant form CRF32_A106 (Figure 5B) (www.hiv.lanl.gov) (Adojaan, Kivisild et al. 2005).

5.5 Drug resistance mutations and polymorphisms in TN PR-RT sequences

The samples of TN PR-RT population were collected before scaling-up of HAART in Estonia. There were no primary DRMs or accessory DRMs in PR or RT region. Thus all viruses in TN PR-RT population were susceptible to currently available RTIs and PIs.

These results were largely expected for two reasons. Firstly, the use of ARV therapy in Estonia was very limited until 2006 (Figure 7). Secondly, the predominant CRF06_cpx viruses began to circulate in Estonian population with the emergence of concentrated epidemic in 2000 – that is after the mono- and dual therapy period which was the main factor to induce TDR in developed countries (Salminen, Nykanen et al. 1993; Liitsola, Laukkanen et al. 1996; Ustina, Zilmer et al. 2001; Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005; Laisaar, Avi et al. 2011).

These data suggest that absence of DRMs in CRF06_cpx viruses is similar to other HIV-1 subtype B and non-B viruses from the pre-ARV treatment period (Shafer 2002; Vergne, Diagbouga et al. 2006).

On the contrary to the absence of primary and accessory DRMs the CRF06_cpx viruses are rich of well conserved drug resistance associated PR polymorphisms in TN PR-RT population (I13V, K14R, F17E, K20I, E35D, M36I, R41K, L63H, H69K and L89M). The same results were obtained in TDR population (Table 5).

Table 5. The rate (%) of most frequent natural PR polymorphisms in Estonian HIV-1 viruses

PR polymorphisms / Study populations	I13V	K14R	G17E	K20I	E35D	M36I	R41K	L63H	H69K	L89M
TDR all n=139	97	93	81	91	82	97	95	77	100	98
TDR CRF06_cpx n=123	99	94	89	98	80	99	93	85	100	97
TN PR-RT all	94	96	74	87	90	100	92	92	99	100

According to the frequency of these polymorphisms in HIV-1 CRFs they can be divided into three categories.

The first category consists of PR polymorphisms M36I, H69K and L89M presented in more than 95% of strains circulating in Estonia. These substitutions are described in more than 90% of sequences of almost all non-B subtype viruses (Kantor and Katzenstein 2003; Kantor and Katzenstein 2004; Kantor, Katzenstein et al. 2005; Rhee, Kantor et al. 2006). The high prevalence of

M36I, H69K and L89M polymorphisms among CRF06_cpx viruses has also been found in West-African studies from Burkina Faso, Mali and Hungary (Tebit, Sangare et al. 2009).

The second category of PR polymorphisms includes I13V, K14R, G17E, K20I, E35D, L63H, which have been found in 70 to 99% of strains in current studies (Table 5). In non-B subtypes these substitutions are consensus aa in majority, but not all subtypes and CRFs (Kantor and Katzenstein 2003; Kantor and Katzenstein 2004; Kantor, Katzenstein et al. 2005; Rhee, Kantor et al. 2006). In CRF06_cpx viruses from other countries these polymorphisms have been presented in almost all cases except the L63H which is found only in few viruses from Burkono Faso and Mali (hivdb.stanford.edu) (Rhee, Kantor et al. 2006; van de Vijver, Wensing et al. 2006; Tebit, Sangare et al. 2009). This lets us suggest that the highly conserved L63H is unique to viruses circulating in Estonia.

The third group consists of PR polymorphisms L10I, I15V, G17D, K43R, L63P, I64M, K70R, I72V, V82I, and I93M; all of them were rare (<10%) in CRF06_cpx in current study as well as in many other subtypes (Rhee, Kantor et al. 2006). From them only substitutions I15V, L63P and V82I are consensus sequences in subtypes C and F; B, C and D; and subtype G, respectively (Rhee, Kantor et al. 2006) (hivdb.stanford.edu).

Two subtype A1 strains possessed polymorphisms E35D, M36I, N37D, H69K, L89M and I93L. These polymorphisms, except N37D and I93L, are also consensus sequences in other subtype A viruses (hivdb.stanford.edu). However, it is worth to mention that Estonian subtype A1 viruses did not carry the polymorphism V77I characteristic to Eastern-European subtype A1 subcluster and are shown to spread in several countries of former Soviet Union (Roudinskii, Sukhanova et al. 2004; Zarandia, Tsertsvadze et al. 2006; Thomson, Vinogradova et al. 2009; Vinogradova, Gafurova et al. 2010).

5.6 Integrase region drug resistance mutations and polymorphisms

Similarly to PR and RT region no primary DRM were identified in IN region suggesting that all Estonian HIV-1 strains are susceptible to currently available INIs. These data are in good concordance with the results obtained in B and non-B subtypes indicating absence or very low prevalence of primary DRMs (<1%) in TN HIV-1 viruses (Rhee, Gonzales et al. 2003). However, because INIs have been introduced to the market only very recently (in 2007) it is not suprising that the IN region is less extensively investigated than the PR-RT region (Rhee, Liu et al. 2008; Ceccherini-Silberstein, Malet et al. 2009; Low, Prada et al. 2009; Passaes, Guimaraes et al. 2009; Ceccherini-Silberstein, van Baelen et al. 2010; Fish, Hewer et al. 2010; Lataillade, Chiarella et al. 2010). The sole exception among INI DRMs in our study is E157Q, found only in one strain. E157Q confers low level *in vitro* resistance (5-10x) to raltegravir and

elvitegravir (Ghosn, Mazet et al. 2009; Low, Prada et al. 2009; Varghese, Liu et al. 2010) and seems to be presented as natural polymorphisms in B and non-B subtypes with the prevalence rate of 1–2% of TN viruses. However, a higher frequency of this mutation (4%) was reported in a study in South-Africa invloving subtype C (Rhee, Liu et al. 2008; van Laethem, Schrooten et al. 2008; Eshleman, Hudelson et al. 2009; Passaes, Guimaraes et al. 2009; Fish, Hewer et al. 2010).

Similar to PR region several accessory mutations and polymorphisms like 72I[1], L74I, V201I and T206S were found in more than 95% of Estonian HIV-1 viruses in IN region. The most unique character was the absolute conservation of the IN polymorphisms L74I. The prevalence of L74I in other subtypes (e.g. B-D, F, G, CRF01_AE and CRF02_AG) is less than 20% (Rhee, Liu et al. 2008). Substitution 72I was presented in 92% (105/114) of all viruses and in 96% (85/89) of CRF06_cpx viruses. Different studies have demonstrated that this mutation is a consensus aa in the subtype A, B, D and CRF02-AG viruses (Rhee, Liu et al. 2008; Lataillade, Chiarella et al. 2010).

From the substitutions associated with INI DR in vitro only the V201I and T206S were widely spread being presented in 97% (110/114) and 96% (109/114) of the entire population and 98% (87/89) and 99% (88/89) among CRF06_cpx viruses, respectively. Of these V201I is consensus aa in all non-B viruses, and in subtype B viruses it is presented in about 10–20% of cases (Rhee, Liu et al. 2008; Fish, Hewer et al. 2010; Lataillade, Chiarella et al. 2010). The T206S is less prevalent being consensus aa only in CRF02_AG and subtype G viruses and in other subtypes including subtype B being presented approximately in 10% of cases. (Rhee, Liu et al. 2008).

5.7 No compensatory integrase mutation was found in reverse transcriptase drug resistance possessing viruses

Among 10 TE patients the association between HIV-1 RTI DRMs and possible substitutions in IN region was evaluated. No IN region polymorphisms associated with RTI DRMs were identified. This contrasts with the results of Ceccherini-Silberstein, who demonstrated the co-variation of RTI DRM F227L and T215Y with IN substitutions M154I and V165I (Ceccherini-Silberstein, Malet et al. 2009; Ceccherini-Silberstein, van Baelen et al. 2010). Several other studies, however, have not found these associations (Roquebert, Blum et al. 2008; Passaes, Guimaraes et al. 2009). We have to mention that the absence of the association in the current study need to be treated with caution because of low sample size of our analysed population.

[1] Isoleutcine in IN position 72 is consensus aa in majority of subtype B viruses, however among INI treated subtype B viruses this aa is overrepresented compared to INI naïve viral strains and therefore 72I have been considered as aa which is associated with INI treatment experience.

5.8 Transmitted drug resistance in Estonia is low but increasing

As presented above (see 5.5) no primary DRMs in PR-RT were identified in viruses sampled in 2005-2006. However, two years later in 2008 among newly diagnosed HIV-1 positive subjects the prevalence of primary DRM was found in 8 out of 145 subjects (5.5%; 95% CI 1.8 – 9.24). The dual class resistance was detected in three cases (2.1%; 95% CI 0 – 4.39). This indicates that the prevalence of TDR mutations is rapidly increasing, especially in the light of corresponding figures from Western-Europe as well as other developed countries where the prevalence of TDR has been rather stable (Wensing, van de Vijver et al. 2005; Vercauteren, Wensing et al. 2009).

However, despite of the rapid increase of TDR in Estonia its level at 5.5% is still lower than in developed countries – about 9% in Western-Europe and 15–20% in North-America (Wensing, van de Vijver et al. 2005; Booth, Garcia-Diaz et al. 2007; Hurt, McCoy et al. 2009; Vercauteren, Wensing et al. 2009). This situation is most likely triggered by different history of ARV use in the Western world in comparison to Eastern-Europe including Estonia. Firstly, the Estonian CRF06_cpx viruses have never been exposed to mono- or dual therapy which was the leading cause of emerging TDR in Western-European subtype B epidemics. Secondly, the HIV-1 subtype B strains which circulated in Estonia at the time of mono- and dual therapy are not epidemiologically linked to viruses causing current concentrated HIV-1 epidemic among IVDU (Ustina, Zilmer et al. 2001).

Comparing TDR mutations figures of other regions where the CRF06_cpx viruses spread (e.g. Burkina Faso, Mali) it appears that their level of TDR is higher than in Estonia (about 11%) and this is despite of restricted use of ARVs and short duration of HAART in these regions (Tebit, Sangare et al. 2009). This suggests that not only the availability of ARV agents, but also socio-economic and demographic factors are likely to contribute to the development of ARV resistance (Vergne, Diagbouga et al. 2006; Derache, Traore et al. 2007).

Amongst other Eastern-European countries the prospectively and systematically collected data on TDR are available only about Poland and Hungary. The TDR mutations rates in these countries are 4-14% and follow Western-European rather than Eastern-European trends. This is not surprising as these countries are not affected by the so called new Eastern-European type IVDU HIV-1 epidemics (Kanizsai, Ghidan et al. 2010; Stanczak, Stanczak et al. 2011).

Some information about TDR in Eastern-European IVDUs epidemic has been published in Latvia indicating that the TDR ranges from 3.4% to 7% and resembles very much the situation in Estonia (Kolupajeva, Aldins et al. 2008; Balode, Westman et al. 2010). In other Eastern-European countries affected by the IVDUs epidemics the monitoring of TDR has been sporadic and thus does not allow to follow time trends or draw conclusions on its rates. The studies are usually very small in size and the level of TDR is 0-4% (Zarandia, Tsertsvadze et al. 2006; Vinogradova, Gafurova et al. 2010).

When analysing rapid increase of TDR in Estonia between 2005-2006 and 2008 one should bear in mind the differences in study designs (TN PR-RT and TDR study, Table 2). The TN PR-RT study was cross-sectional and consisted of patients diagnosed HIV positive between 2000 and 2005, whereas the TDR study conducted in 2008 invloved only newly diagnosed patients. The fact that the TN PR-RT study contains mostly chronically infected subjects may lead to the underestimation of TDR in this population, because viruses might lose their DRMs in actively replicating viral population without the lasting ARV pressure.

5.8.1 The distribution of transmitted drug resistance mutations

Among TDR populations NRTI, NNRTI and PI resistance mutations were found in similar frequency – in 4 (2.8%), 3 (2.1%), 4 (2.8%) cases, respectively.

The most commonly observed mutations were RT K103N, PR M46I and PR L90M (each seen in three cases, 2.1%) and RT M41L and PR I85V (both seen in two viruses, 1.3%). The NRTI mutations M184V, M184I, T215C, T215D and NNRTI mutation P225H were all seen in a single case. Other substitutions associated with DR like the RT V108I and V179E were described each in two viral strains. As HIV-1 is a quickly mutating virus, not all described DRMs from these were presented in population as pure aa. Polymorphic positions from aforementioned mutations located in the RT positions K103KN and T215DN and PR positions T74ST and L90LM, each found in one case.

The relatively equal distribution of different DRM classes as well as low proportion of TAMs (M41L, T215C and T215D), seen in 4 out of 18 described DRMs only, refers to a modern pattern of DRMs and is similar to the HIV-1 DRMs accumulating in developed countries today (Bartmeyer, Kuecherer et al. 2010). These findings are not surprising as in Estonia large scale ARV therapy started after the introduction of HAART and only a tiny minority of HIV positive subjects have received mono- and/or dual therapy, but this happened before year 2000 when most inpatients were infected with subtype B viruses (Laisaar, Avi et al. 2011). Furthermore the pattern of TDR mutations also corresponds to the profile of ARV agents used in Estonia during last years (see 5.9).

5.8.2 The clusters of transmitted drug resistance mutations

According to the analysis of the phylogenetic relations of TDR mutations possessing viruses no monophyletic clusters carrying the same pattern of TDR mutations were found (Figure 1 in Article 3). This was actually expected as the DRM carrying viruses have been circulating in the Estonian population for a relatively short period of time.

The only possible TDR mutations transmission cluster might have been formed by two subtype B viruses as they possessed nearly identical DRM

patterns (PR M46I, I85V, L90M, RT M41L, T215C and PR M46I, I85V, L90M, RT M41L, T215DN) (Figure 1 in Article 3). However, the low number of the Estonian subtype B viruses in the current phylogenetic analysis does not allow to confirm the existence of significance of this cluster.

Corresponding studies in Western-Europe have described that 20% of TDR mutations carring viruses cluster to TDR clusters (Brenner, Roger et al. 2008; Hughes, Fearnhill et al. 2009).

5.9 CRF06_cpx drug resistance mutations in treatment experienced population

During 2001 to 2008 the ARV consumption has constantly risen from 0.077 DDD /1000/per day in 2001 to 1.481 DDD in 2008 (Figure 7). The rate of subjects receiving ARV therapy has also increased (from 1.7% of all HIV positive subjects in 2001 to 14.9% in 2008) (Estonian Health Board, Tallinn, 2010 (cited 2010 February 16). Accessed at www.terviseamet.ee). This reflects the situation characteristic to new HIV-1 epidemics. The most commonly used ARV agents in 2008 were 3TC (0.565 DDD), ddI (0.311 DDD), ZDV (0.217 DDD), EFV (DDD 0.196) and LPV/r (0.147 DDD). It is in accordance with Estonian and international HIV treatment guidelines (aidsinfo.nih.gov).

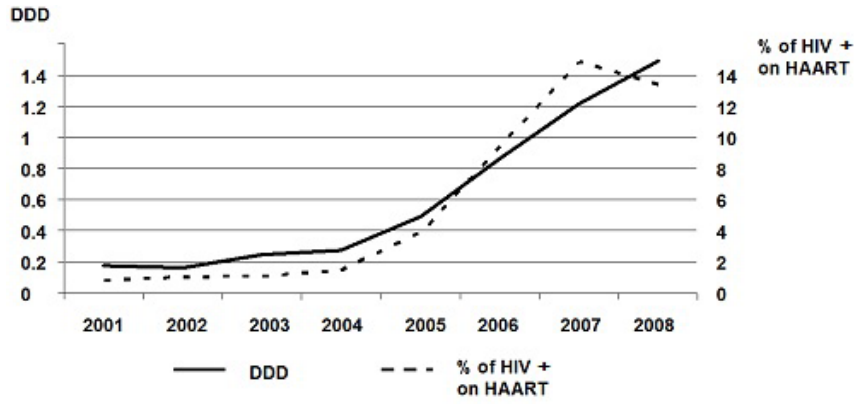

Figure 7. The consumption of ARV agents (dotted line) and percentage of HIV positive persons on HAART (solid line) in Estonia 2001–2008 as reported by the Estonian State Agency of Medicines, Tartu 2010 (cited 2010 February 16) Accessed at www.sam.ee) and Estonian Health Board, Tallinn, 2010 (cited 2010 February 16). Accessed at www.terviseamet.ee)

Between September 2006 and November 2010 83 treatment experienced patients with ARV failure have been tested for genotypic HIV-1 drug resistance (78 of them have failed their first HAART regimen) in the Institute of Microbiology of University of Tartu. The viral genomic RNA was successfully sequenced in PR and RT regions in total of 59 cases. At least one DRM was detected in 34 (58%) cases – NRTI, NNRTI and PI resistance was detected in 23 (39%), 31(53%) and 3(5%) cases, respectively. The predominant NRTI mutations were M184V, L74V, L74I, K70E and K219E found in 22, 8, 3, 2 and 2 cases, respectively (Table 6). NNRTI DRMs were K103N, Y188L, L100I and P225H presented in 28, 4, 3 and 3 cases, respectively. In PI treated population the most frequent PI DRM was V82A in 2 cases.

Table 6. The distribution of DRMs in TE PR-RT population.

Class	Mutation	Abs. number	%
NRTI	M184V	22	39
	L74V	8	14
	L74I	3	5
	K70E	2	4
	K219E	2	4
	M41L	1	2
	D67N	1	2
	K70R	1	2
	M184I	1	2
	T215F	1	2
NNRTI	K103N	28	49
	Y188L	4	7
	L100I	3	5
	P225H	3	5
	K101E	1	2
	K103S	1	2
	V106M	1	2
	G190A	1	2
	G190S	1	2
PI	V82A	2	4
	M46I	1	2
	I47V	1	2
	F53L	1	2
	I54V	1	2
	L90M	1	2

As expected the most common mutations (M184IV, L74IV and K103N) confer resistance to ARV drugs with low-genetic barrier – 3TC, ZDV and NVP. These three mutations have been widely spread also among the other B and non-B subtypes in NRTI and/or NNRTI treated persons (hivdb.stanford.edu).

The diversity of the DRM in Estonian TE patients, however, is narrower than that in the Western- and Northern-Europe (Wensing, van de Vijver et al. 2005; Vercauteren, Wensing et al. 2009). The most likely reason is the fact that majority of HAART failing patients have failed only their first treatment regimen, and due to the limited use of mono- and dual therapy in 1990s the number of patients with multiresistant HIV-1 strains is very low. In contrast, the CRF06_cpx viruses in other countries (Burkina Faso and Mali) possess more diverse DRM profile, including high prevalence of TAMs which in turn suggests higher proportion of subjects on suboptimal therapy or wider use of thymidine analogues (Tebit, Sangare et al. 2008).

6. GENERAL DISCUSSION

The current study investigating the genetic structure of Estonian HIV-1 viruses has provided one of the most comprehensive molecular insights into the new HIV-1 epidemics in Eastern-Europe, the area poorly characterized thus far. In contrast to Western-European predominantly homo- and heterosexually transmitted infections the new epidemics, including the Estonian one, mostly include young male IVDUs and are usually caused by very homogeneous monophyletic non-B subtype viruses (Bobkov, Cheingsong-Popov et al. 1997; Zetterberg, Ustina et al. 2004).

The distribution of HIV-1 subtypes in various parts of the world is likely the most widely studied area in the molecular epidemiology of infectious diseases. The main reason for such an intense investigation is the urgent need to improve prevention including development of vaccines. This requires comprehensive knowledge of molecular diversity of viral subtypes and their associations with other epidemiological factors (e.g. superinfections with other HIV-1 strains, co-infections with other viruses like HCV, HBV, HSV etc). The importance of HIV-1 subtypes lies also in their impact on transmissibility, disease progression, and the response to ARV therapy (Geretti 2006).

The current study demonstrated that throughout the concentrated Estonian HIV-1 epidemic the subtype distribution has undergone only minor changes; the CRF06_cpx viruses have predominated since year 2000 (Zetterberg, Ustina et al. 2004; Adojaan, Kivisild et al. 2005). These findings are concordant with those in other Eastern-European IVDU HIV-1 epidemics that have demonstrated a stable distribution of subtype A1 viruses (Rumyantseva, Olkhovskiy et al. 2009).

Interstingly during last 10 years the spread of CRF06_cpx viruses has been restricted mainly to Estonia and only single cases have been described in neighboring countries in Latvia, Sweden and St. Petersburg area of Russia (Skar, Sylvan et al. 2008; Masharsky, Dukhovlinova et al. 2010). On the same token the low prevalence of subtype A1 in Estonia is also surprising as this strain has caused massive outbreaks in many former Soviet Union countries that became affected by the IVDU epidemics just a year before the Estonian CRF06_cpx outbreak (Rumyantseva, Olkhovskiy et al. 2009).

It is noteworthy to mention that despite of very high incidence of HIV-1 (27.8 per 100,000 persons) the epidemic has not spread across the country and is still restricted to the North and North-East Estonia. However the spread from IVDU male to heterosexually infected women has occurred (Estonian Health Board, Tallinn, 2010 (cited 2010 February 16). Accessed at www.terviseamet.ee). Therefore one could hypothesize that HIV-1 strains that once have infected the subjects, could saturate a certain population of IVDUs and thus possess a sufficient protection against other HIV-1 strains.

Another feature characteristic to new IVDU epidemics is the monophyletic virus population as shown in the current study as well as in other Eastern-European countries and IVDU populations (Tovanabutra, Beyrer et al. 2004;

Thomson, de Parga et al. 2007). The spread of monophyletic and homogeneous viral strains among this risk group is potentially facilitated by several factors. Firstly, the IVDU transmission lacks the mucosal barrier and therefore enables virus to replicate for relatively long periods without a specific immune response. Secondly, many Estonian IVDUs abuse opiates, which at least in in vitro studies have been shown to be inducers of co-receptor CCR5 expression, therefore creating improved opportunities for the viral amplification (Guo, Li et al. 2002; Talu, Rajaleid et al. 2010). In clinical context such homogeneous viral epidemics may mean similar disease progression and response to ARV therapy. On the other hand, the latter one could also explain the homogeneous DRM pattern in TE population in Estonia described by us.

An important practical finding of the current study is that the Estonian CRF06_cpx or its recombinants with subtype A1 viruses do not possess naturally occurring primary or accessory DRMs in the PR, RT or IN region indicating that all circulating viruses should be susceptible to currently available ARV agents. For the practicing physicians this means that all first line ARV regimens suggested by the international HIV treatment guidelines including older agents are potentially appropriate choice for the treatment of HIV-1 infection in Estonia. However, so far no clinical trials have been conducted to prove that this is true in clinical practice. The absence of naturally occurring DRMs is also characteristic to all other HIV-1 group M subtypes (including subtype B) (Kantor 2005, Kantor 2006).

The current study was one of the few that genotyped the PR region and the first one genotyping the IN region in a rare HIV-1 clade like CRF06_cpx. A high frequency of natural polymorphisms in PR and IN region was demonstrated affirming once again that non-B subtype viruses are rich in polymorphism in PR and IN region (Rhee, Gonzales et al. 2003; Rhee, Liu et al. 2008). The clinical relevance of these polymorphisms is still unclear. One could speculate that they facilitate resistance development to ARV agents. Although studies on this are still limited the ones that have pooled many different non-B subtype viruses indicate that the subtype does not play a role in response to HAART. However, more recently the number of patients infected and treated with the non-B subtypes has risen worldwide. As a result studies demonstrating subtype specific pathways in DRM development and even subtype dependent efficiency of ART regimens have been published (Perno, Cozzi-Lepri et al. 2001; Brenner, Turner et al. 2003; Abecasis, Deforche et al. 2005; Kantor, Katzenstein et al. 2005). There is clearly a need for appropriately designed studies, especially in areas of new IVDUs epidemics to resolve the issue of influence of subtypes on the treatment response.

We also demonstrated the rapid rise of TDR from 0% to 5% in two years (2006-2008) among Estonian treatment naïve HIV-1 subjects. Although such rapid rise is of concern it is still about two times lower than recently reported in the homo- and heterosexually transmitted Western-European and North-American epidemics where the rates have stabilized to about 9% or even show decreasing trends in some regions (Wensing, van de Vijver et al. 2005;

Vercauteren, Wensing et al. 2009). The initial absence of TDR in Estonia was expected as majority of the Estonian HIV-1 patients have received HAART instead of mono- or dual therapy; the latter being the main cause of the rapid increase of TDR in Western-Europe in 1980-90s (Schuurman, Nijhuis et al. 1995; Wensing, van de Vijver et al. 2005; Vercauteren, Wensing et al. 2009). Furthermore, although a population of mainly homosexual subjects undergoing mono- or dual therapy in 1990s existed in Estonia, it was very small and had probably no epidemiological links to the IVDUs forming the predominant population in the current concentrated epidemic (Ustina, Zilmer et al. 2001). The rapid increase in TDR by 2008 is more difficult to explain. One could speculate that the potential reasons are a dramatic scaling up of the HAART (seven fold from 2004 to 2007) and poor adherence of ARVs likely to occur in the IVDUs population. The wide fluctuations of TDR levels have been suggested among IVDUs in the territory of new Eastern-European epidemics, however, data are still very limited (Vazquez de Parga, Rakhmanova et al. 2005; Zarandia, Tsertsvadze et al. 2006; Vinogradova, Gafurova et al. 2010).

Pointing out the main limitations of the current study one should notice the cross-sectional study designs and the inability of Sanger sequencing to detect DRMs in minority viral quasi-species. More recently new and very sensitive technologies like real-time PCR or deep and ultra-deep sequencing have been developed and introduced; they have a potential to describe additional minority DRMs (Metzner, Leemann et al. 2011; Mild, Hedskog et al. 2011). The second limiting factor is the underestimation of TDR mutations due to the studied population which consisted of newly diagnosed rather than newly infected subjects. Some US and European studies have noted that newly diagnosed persons might posses lower level of TDR because of longer replication time without the ARV pressure, which leads to the disappearance of mutation possessing viruses from the actively replicating population (Weinstock, Zaidi et al. 2004; Wensing, van de Vijver et al. 2005). Thirdly, the current study could not report some DRM mutations which recently have been described to accumulate in viral gag cleavage sites or RNase H region, because these mutation locate in viral genomic region that remained out of the scope of the conducted analyses (Weinstock, Zaidi et al. 2004; Knops, Kemper et al. 2010; Michels, Staszewski et al. 2010).

7. CONCLUSIONS

- The HIV-1 subtype distribution in Estonia after the commencement of the concentrated outbreak in early 2000s has remained stable at least until 2008. The vast majority of viruses according to the sequenced regions (V3, PR, RT and IN) belong to the monophyletic CRF06_cpx cluster. In fewer cases the CRF06_cpx has formed local recombinants mainly with the Eastern-European subtype A1 viruses. These recombinant sequences were found in all genomic regions except the V3. At the same time the subtype B viruses, widely spread in Western-Europe and subtype A1 circulating in Latvia and Russia were found only in single cases. All this suggests that the Estonian HIV-1 epidemic has developed independently and even now is minimally influenced by foreign or neighboring HIV-1 epidemics.
- Estonian CRF06_cpx and its recombinant viruses do not possess any naturally occurring PR, RT and IN DRMs suggesting their similarity to other HIV-1 group M subtypes and CRFs. In practical terms this means that viruses circulating in Estonia should be susceptible to all available ARV agents. Thus the ARV treatment guidelines developed mainly for subtype B viruses are applicable in the Estonian settings. However, similar to other non-subtype B viruses almost all Estonian CRF06_cpx and recombinant viruses possess numerous DR associated natural polymorphisms in PR and IN region. The clinical relevance of these polymorphisms is still largely unknown. Recent studies have shown their association with the development of subtype specific DRM pattern in different HIV-1 subtypes. Thus, in the circumstances of increasing use of ARV therapy clinical studies to estimate the influence of natural polymorphisms or subtype on the response to ARV therapy are urgently needed, especially in case of new epidemics of Eastern-Europe.
- There is no correlation between RT region DRMs and IN region polymorphisms among Estonian TE and DRM possessing HIV-1 CRF06_cpx viruses.
- The level of TDR in 2008 in Estonia is still low (5.5%). However, after scaling up ARV treatment a rapid increase has been observed. All this indicates that there is a need to monitor patients for DRM prior the beginning of ARV therapy. In any case a plasma sample should be collected for future DR testing.
- The pattern of DRMs in HIV-1 CRF06_cpx among TE patients failing ARV therapy is largely similar to that seen in Western-Europe among subtype B viruses. The predominant presentation of NNRTI and NRTI DRMs reflects rather the character of consumed ARV agents than the specifics of viral subtype.

8. SUMMARY IN ESTONIAN

Eestis ringlevate HIV-1 CRF06_cpx ja tema rekombinantsete viiruste looduslikud polümorfismid ja ülekanduv ravimresistentsus

Viimastel kümnenditel on HIV-1 infektsioon muutunud maailma üheks olulisemaks nakkushaiguseks. 2009 aastal oli maailmas umbes 31-35 miljonit HIV positiivset, AIDS-i suri 1,8 miljonit inimest ning uusi nakkusjuhte oli 2,6 miljonit.

Geneetilise mitmekesisuse alusel jagatakse HIV-1 viirused kolme suurde gruppi, millest ülemaailmset epideemiat põhjustavad peamiselt M-grupi viirused. Need jagunevad omakorda 9 subtüübiks (A, B, C, D, F, G, H, K, J) ning mitmekümneks nendevaheliseks rekombinantseks vormiks (CRF01_AE, CRF02_AG jne.).

Alates 1995. aastast on HIV-1 vastases ravis kasutusel HAART (*highly active antiretroviral therapy*) – kombinatsioon vähemalt kolmest retroviiruse vastasest ravimist. Taolise ravi kasutuselevõtuga kaasnes põhimõtteline murrang – raskest surmaga lõppevast tõvest on saanud krooniline suhteliselt kõrget elukvaliteeti lubav haigus. Paraku on HAARTiga eotud ka mitmed probleemid, sealhulgas ravimresistentsuse teke. Seda tingib viiruse vigaderohke replikatsiooniprotsess, mis ravirežiimi rikkumise korral põhjustab kiire ravimresistentsusmutatsioonide (RRM) ilmumise.

HIV-1 RRM-id võib jagada primaarseteks ehk otseselt ravimresistentsust tekitavateks ning sekundaarseteks ehk RRM kandvate viiruste replikatsioonivõimet taastavateks mutatsioonideks. Viimased võivad esineda erinevatel HIV-1 subtüüpidel ka looduslike polümorfismidena eriti viiruse proteaasi (PR) ja integraasi (IN) piirkonnas. Siiani on üksikud uuringud näidanud, et need polümorfismid ei mõjuta otseselt ARV-ravi efektiivsust, kuigi neil võib olla mõju ARV käigus kujuneval RRM mustrile.

Üheks oluliseks ARV-ravi edukust mõjutavaks teguriks on ülekanduv ravimresistentsus. See omandatakse koos ülekanduva viirusega varem ravi saanud patsientidelt. Paljudes, eriti pikaaegse ARV-ravi ajalooga riikides (nt USA) ulatub selle tase kuni 20 protsendini, Euroopas on see aga viimastel aastatel püsinud 9 protsendi piires.

Eestis puhkes konsentreeritud HIV-1 epideemia 2000. aastal, mis viis Eesti nii HIV-1 uute juhtude kui ka üldise levimuse osas Euroopa juhtivate riikide hulka. Oma iseloomult kuulub Eesti epideemia nn Ida-Euroopa uut tüüpi HIV-1 epideemia piirkonda, mida iseloomustab väga kõrge HIV-1 ja HCV levimus peamiselt noorte meessoost süstivate narkomaanide hulgas. Nakkust põhjustavad seda tüüpi epideemiates molekulaarselt väga sarnased monofüleetilist päritolu HIV-1 tüved. Peaaegu kogu Ida-Euroopas on selleks subtüüpi A1 kuuluv tüvi, vähesel määral aga ka CRF03_AB. Eesti on siinkohal erandlik, kuna nakkust põhjustab peamiselt HIV-1 CRF06_cpx viirus. Nimetatud

rekombinantse vormi laiemat levikut on seni kirjeldatud vaid mõnes Lääne-Aafrika riigis.

HIV-1 viiruse looduslikke polümorfismide, RRM-de ning ülekanduva ravimresistentsuse levikut on Ida-Euroopas siiani uuritud suhteliselt vähe. Üksikud ilmunud tööd käsitlevad sageli väikesi populatsioone ning läbilõike uuringutena suudavad anda infot vaid piiratud aastate kohta. Enne käesoleva töö läbiviimist polnud avaldatud ühtegi uuringut Eestis levinud HIV-1 CRF06_cpx viiruste ravimresistentsusega seotud polümorfismide, RRM-de ning ülekanduva ravimresistentsuse kohta.

Uurimistöö eesmärgid

Töö üldine eesmärk oli kirjeldada Eestis ringlevate HIV-1 viiruste ravimresistentsuse mustrit nii ARV-ravi mitte saanud kui ka saanud isikutel.
Uuringu alaeesmärgid olid:

- Kirjeldada Eestis ringlevate viiruse V3, PR, RT ja IN genoomsete piirkondade genotüüpe aastatel 2005 kuni 2006 ja 2008
- Kirjeldada ravinaiivsete viiruste PR, RT ja IN genoomsete piirkondade looduslikke polümorfisme Eestis ringlevatel HIV-1 CRF06_cpx ja tema järgmise põlvkonna rekombinantidel
- Kirjeldada seoseid Eestis ringlevate HIV-1 CRF06_cpx ja tema järgmise põlvkonna rekombinantsete viiruste RT regiooni primaarsete RRM-de ja IN regiooni polümorfismide vahel
- Võrrelda HIV-1 ülekanduvat ravimresistentsust 2005-2006 ja 2008 aastal
- Kirjeldada primaarseid RRM Eestis ringlevatel HIV-1 CRF06_cpx ja tema järgmise põlvkonna rekombinantidel ravi saanud patsientidel.

Uuritavad ja metoodika

Uuring viidi läbi järgnevates osades:

- Esimene uuring määras HIV-1 viiruse PR, RT polümorfisme. Uuritavateks olid 2005–2006 aastal Ida-Viru Keskhaigla ning Tartu vanglas HIV-positiivsed ravinaiivsed patsiendid. Uuritavate demograafilised näitajad olid üldjoontes sarnased Eesti HIV-1 positiivsete vastavate näitajatega (65% olid süstivad narkomaanid, 70% mehed ja keskmine vanus 27 aastat).
- Teine uuring keskendus IN piirkonna polümorfismide ja RRM-ide uurimisele. Uuritav populatsioon koosnes valdavalt eelmainitud populatsiooni patsientidest.
- Kolmas uuring vaatles RT piirkonna RRM-de mõju IN piirkonna polümorfismidele aastatel 2006–2007 Tartu Ülikooli Mikrobioloogia Instituuti saadetud ARV ravi läbikukkunute viirustel.

- Neljanda uuringu populatsiooni moodustasid ülekanduva ravimresistentsuse määramiseks 2008. aastal HIV positiivseks diagnoositud ravinaiivsed isikud.
- Viienda uuringu populatsiooniks olid HIV-1 CRF06_cpx viiruste RRM mustri uurimiseks aastatel 2006–2010 Tartu Ülikooli Mikrobioloogia Instituuti saadetud ARV ravi läbikukkunute koeproovid.

Kõikides uuringutes sekveneeriti patsientide vereplasmast eraldatud viiruse genoomne RNA, mis oli konverteeritud pöörd-transkriptsiooniga cDNA-ks ja paljundatud nested-PCR-iga. Saadud järjestusted assambleeriti kontiigideks, mida kasutati fülogeneetilisteks analüüsideks, rekombinatsioonide määramiseks, ravimresistentsusmutatsioonide ning geneetiliste polümorfismide detekteerimiseks.

Peamised tulemused ja arutelu

Kooskõlas varasemate Eestis läbiviidud uuringutega on alates 2000. aastast jäänud Eesti HIV-1 subtüübiline jaotuvus muutumatuks. Kõikides sekveneeritud HIV-1 piirkondades moodustasid enamuse (umbes 85%) CRF06_cpx viirused, millele järgnesid (umbes 12%) Eesti kohalikku päritolu rekombinantsed vormid. Üksikjuhtudel kirjeldati ka A, B, CRF02_AG ja CRF03_AB subtüüpidesse kuuluvaid viiruseid. Looduslikke ravimresistentsusmutatsioone Eestis ringlevatel viirustel ei leitud ei PR-RT ega ka IN piirkonnas. Seega peaksid Eestis levivad HIV-1 viirused olema tundlikud kõigile olemasolevatele ARV ravimitele. Seevastu leidus hulgaliselt mitte-B subtüüpidele omaseid ARV-raviga seotud polümorfisme, millest paljud olid tugevalt konserveerunud ning esinesid rohkem kui 90% viirustest – PR regioonis K14R, M36I, H69K ja L89M ning IN regioonis V72I, L74I, V201I ja T206S. Ilmselt ei mõjuta nimetatud polümorfismid HAART efektiivsust otseselt, küll aga võivad nad põhjustada subtüübi spetsiifilist RRM kujunemist.

2008. aastal läbi viidud uuring näitas värskelt HIV-1 positiivseks diagnoositute ravinaiivsete isikute hulgas ravimresistentsuse tõusu 5%-ni. Mitmetes HIV-1 ravijuhistes on nimetatud tase määraks, millest alates soovitatakse läbi viia ravieelset genotüüpilist HIV-1 ravimresistentsustesti. Leitud ülekanduvatest RRM-idest olid suhteliselt võrdselt levinud kõik peamised ARV ravimklasside mutatsioonid (NRTI – 2,8%, NNRTI – 2,1%, PI – 2,8%). Kuigi Eesti ülekandunud ravimresistentsus jääb hetkel veel madalamaks kui Euroopa vastav näitaja, ei pruugi edasine olukord taolisena püsida. Sellele viitab Eesti HIV positiivsete isikute erinev demograafiline stuktuur – erinevalt Lääne-Euroopa HIV positiivsetest on nad reeglina noored mehed, nakatunud intravenoosselt ning nad pole kunagi saanud HIV-1 vastast monoteraapiat, mis on olnud Lääne-Euroopas üks põhilisi ülekanduva ravimresistentsuse põhjustajaid. Samuti võib edasisele ülekanduvale ravimresistentsuse tõusule oma mõju avaldada ka Ida-Euroopa HIV-1 epideemiale omane kiirelt kasvav ARV-ravimite tarvitamine ja süstivatele narkomaanidele omased adherentsi probleemid.

Töö viimases osas analüüsitud ravi saanute populatsioonis leiti RRM-d 34 viirusel (58%). Levinumad olid NRTI mutatsioonid M184IV, L74IV, K70ERQ, K219E, L74I/V ja T215YF, NNRTI mutatsioonid K103N, V179E, V108I, K101EN ja G190AS ning PI mutatsioonid L90M, M46I, I54V ja V82SA. Nimetatud mutatsioonide esinemine korreleerub hästi Eestis kasutatavate peamiste ARV ravi režiimidega. Samuti on RRM muster suhteliselt sarnane teiste arenenud riikide mutatsioonide mustriga. Ainsaks oluliseks erinevuseks on subtüübispetsiifilise V179E suur esinemissagedus CRF06_cpx viirustel võrreldes subtüüp B viirusega.

Järeldused

- Eesti HIV-1 viiruse subtüübilises struktuuris on viimasel aastkümnel olnud stabiilselt valitsevaks tüveks CRF06_cpx ja vähesemal määral tema rekombinantsed vormid teiste subtüüpide, eelkõige subtüüp A1 viirustega. Taoline olukord viitab Eesti HIV-1 populatsiooni suhtelisele resistentsusele teiste naaberpiirkondades levinud subtüüpide leviku ees.
- Eestis CRF06_cpx viirustel ei esine looduslikult RRM-e peamistes ARV sihtmärk regioonides (PR, RT ja IN). See näitab, et Eesti HIV-1 on tundlik kõikidele laiemalt levinud ARV ravimite klassidele ja et rahvusvahelised HIV-1 ravijuhised sobivad ka Eesti HIV-1 infektsiooni raviks.
- Eestis levivatel HIV-1 viirustel leiti PR ja IN regioonis mitmeid kõrge esinemissagedusega ARV-raviga seotud looduslikke polümorfisme. Nende otsene mõju ravi edukusele pole veel teada ning vajab edasisi kliinilisi uuringuid.
- 2008. aastal HIV positiivseks diagnoositute hulgas leiti, et ülekandunud ravimresistentsus on tõusnud 5%-ni. Nimetatud tase on piiriks, millest alates on rahvusvahelistes ravijuhistes soovituslikuks ravieelne HIV-1 geno-tüüpilisustestide läbiviimine.
- Ebaõnnestunult ravitud patsientide viiruste sagedasemate RRM jaotus on CRF06_cpx viiruste korral sarnane subtüüp B viirustega. Nimetatu viitab, et kuigi CRF06_cpx viirused vastavad ARV-ravile sarnaselt subtüüp B viiruste-ga, võib neil esineda subtüübispetsiifilisi ravimresistentsusmutatsioone ning sellest lähtuvad ka subtüübispetsiifilised eripärad ARV-ravile allumises.

9. ACKNOWLEDGEMENTS

This work was carried out in the University of Tartu, Institute of Microbiology. Studies were supported by the Estonian Science Foundation (grant 8004, 8415 and 8856), Center of Excellence of Translational Research of Neuro-immunology (project SFOS WP1-NeuroAIDS), Basic Financing and Target Financing of Estonian Ministry of Education and Research (Grant number: SF0182726s06), European Union through the European Regional Development Fund and by the Archimedes Foundation and EU 6. Frame Project (Concentrated Action on Seroconversion to AIDS and Death in Europe (CASCADE) (MARMB06151R)). Different studies were performed in collaboration with West-Tallinn Central Hospital, Ida-Viru Central Hospital and Tartu Prison.

Studies conducted in thesis are based on teamwork with the contribution of many persons whom I wish to express my deepest gratitude. I would like to aknowledge the following persons:

- Professor Irja Lutsar, my dissertation supervisor and true mentorship throughout the process. She introduced me with the details in the fascinating field of clinical research. I highly appreciate her constructive criticism and her way of always setting the highest targets.
- My collaborators Kristi Huik, Merit Pauskar, Tõnis Karki, Maarja Sadam, Tõnu Krispin in our group and professor Marika Mikelsaar, Epp Sepp and Reet Mändar in Microbiology Institute for their help, support and friendship.
- Our collaborators from other institutions Valentina Ustina, Svetlana Semjonova, Ülle Valvar, Piret Paap, Külliki Ainsalu, Mika Salminen, Kirsi Liitsola, Jelena Schmidt and Natalia Nikitina.
- All patients, who participated in the study for their cooperation.
- I would like to cherish people who have afforded me the personal background that has supported me throughout this work: my biology teachers Aivo Tamm, Anneli Veegen, Urmas Kokassaar and my former supervisors Maido Remm and Jaanus Remme.

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PUBLICATIONS

CURRICULUM VITAE

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Born:	October 15, 1980, Tartu, Estonia
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Education

1996–1999	Hugo Treffner Highscool, Tartu, Estonia
2000–2004	University of Tartu, genetechnology, molecular diagnostics and transgene technology, B. Sc
2004–2006	University of Tartu, biomedicine, M.Sc
2006–2011	PhD student, Faculty of Medicine, University of Tartu

Professional employment

2007–...	University of Tartu, Faculty of Medicine, Department of Microbiology, researcher
2005–2006	University of Tartu, Faculty of Medicine, Department of Microbiology, technician

Academic interests

Research fields: HIV-1 infection, host related genetic and epigenetic factors (CCR5, CCL3L1) associated with HIV-1 disease progression and transmission, HIV-1 drug resistance, natural diversity and evolution.

List of publications

1. Avi, R; Huik, K; Pauskar, M; Ustina, V; Karki, T; Krispin, T; Ainsalu, K; Paap, P; Schmidt, J; Nikitina, N; Lutsar, I. (2010). Emerging Transmitted Drug Resistance in Treatment Naive Human Immunodeficiency Virus – 1 CRF06_cpx Infected Patients in Estonia. Scandinavian Journal of Infectious Diseases, 122–8.
2. Avi, R.; Huik, K.; Sadam, M.; Karki, T.; Krispin, T.; Ainsalu, K.; Paap, P.; Schmidt, J.; Nikitina, N.; Lutsar, I. (2010). Characterization of Integrase Region Polymorphisms in HIV-1 CRF06_cpx Viruses in Treatment Naïve Patients in Estonia. Aids Research and Human Retroviruses, 1109–13
3. Huik, K.; Sadam, M.; Karki, T.; Avi, R.; Krispin, T.; Paap, P.; Rüütel, K.; Uusküla, A.; Talu, A.; Abel-Ollo, K.; Lutsar, I. (2010). CCL3L1 copy number is a strong genetic determinant of HIV seropositivity in Caucasian intravenous drug users. The Journal of Infectious Diseases, 730–739.

4. Avi, R; Huik, K; Sadam, M; Karki, T; Krispin, T; Ainsalu, K; Paap, P; Schmidt, J; Nikitina, N; Lutsar, I. (2009). Absence of Genotypic Drug Resistance and Presence of Several Naturally Occurring Polymorphisms of Human Immunodeficiency Virus-1 CRF06_cpx in Treatment-Naive Patients in Estonia. Journal of Medical Virology, 81, 953–958.
5. Huik, K.; Avi, R.; Sadam, M.; Karki, T.; Denks, K.; Krispin, T.; Ustina, V.; Rüütel, K.; Uusküla, A.; Murphy, G.; Lutsar, I. (2009). Performance of the BED-CEIA in population infected with HIV1 recombinant viruses CRF06cpx and CRF06A. In: Clinical Microbiology and Infection: 19th European Congress of Clinical Microbiology and Infectious Diseases Helsinki, Finland, 16–19 May 2009, 2009.
6. Tamman, S; Denks, K; Huik, K; Parm, Ü; Avi, R; Lutsar, I. (2008). Klebsiella pneumoniae genotüpiseerimine multiensüüm-multipleks amplified fragment length polymorphism PCR (AFLP-PCR) meetodil. In: Eesti Arst: 2008.
7. Huik, K.; Avi, R.; Sadam, M.; Karki, T.; Albert, J.; Lutsar, I. (2008). Drug Resistance Mutations in HIV-1 CRF06_cpx Viruses Isolated from Treatment Naïve and Experienced Patients. In: European Journal of Medical Research: 19th European Students' Conference; Berlin; 28 September - 3 October 2008, 2008.
8. Avi, R.; Huik, K.; Karki, T.; Sadam, M.; Krispin, T.; Ainsalu, K.; Paap, P.; Schmidt, J.; Nikitina, N.; Lutsar, I. (2008). Drug Resistance Mutations and Genetic Barrier for Drug Resistance Development in Estonian HIV-1 CRF06_cpx Integrase Sequences. 14th International Bioinformatics Workshop on Virus Evolution and Molecular Epidemiology; Cape Town, South Africa; 1–5 September 2008, 2008.
9. Avi, R.; Huik, K.; Sadam, M.; Karki, T.; Krispin, T.; Paap, P.; Ainsalu, K.; Schmidt, J.; Nikitina, N.; Lutsar, I. (2008). Natural polymorphisms associated with integrase inhibitor drug resistance in Estonian HIV-1 CRF06_cpx strains. 18th European Congress of Clinical Microbiology and Infectious Diseases; Barcelona; 19.04–22.04.2008, 2008.
10. Sadam, M.; Karki, T.; Huik, K.; Avi, R.; Rüütel, K.; Uusküla, A.; Lutsar, I (2008). CCL3L1 Variable Gene Copy Number Influence on the Susceptibility to HIV-1/AIDS Among Estonian Intravenous Drug User. The 15th Conference on Retroviruses and Opportunistic Infections (CROI), Boston, 03.02–06.02.2008, 2008.
11. Denks, K.; Huik, K.; Avi, R.; Sadam, M.; Krispin, T.; Lutsar, I. (2007). GB virus C infection among HIV-positive patients in Estonia. International Journal of Antimicrobial Agents, 29, S155–S155.
12. Avi, R.; Huik, K.; Karki, T.; Sadam, M.; Paap, P.; Smidt, J.; Ainsalu, K.; Krispin, T.; Lutsar, I (2007). The coexistence of secondary PR mutations M36I, K20I and L63H predominates in CRF06_cpx and its next generation recombinant viruses circulating in Estonian treatment naive patients. In: INTERNATIONAL JOURNAL OF ANTIMICROBIAL AGENTS:, 2007, S549–S549.

ELULOOKIRJELDUS

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2004–2006	Tartu Ülikool, biomeditsiin, M.Sc
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Teenistuskäik

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2005–2006	Tartu Ülikool, Mikrobioloogia Instituut, laborant

Teadustegevus

Uurimisvaldkonnad: HIV-1 infektsioon, peremehe poolsed faktorid HIV-1 infektsiooni progressioonil ja nakatumisel (CCR5, CCL3L1), HIV-1 ravim-resistentsus, looduslik mitmekesisus ja molekulaarne evolutsioon.

Publikatsioonid

1. Avi, R; Huik, K; Pauskar, M; Ustina, V; Karki, T; Krispin, T; Ainsalu, K; Paap, P; Schmidt, J; Nikitina, N; Lutsar, I. (2010). Emerging Transmitted Drug Resistance in Treatment Naive Human Immunodeficiency Virus-1 CRF06_cpx Infected Patients in Estonia. Scandinavian Journal of Infectious Diseases, 122–8.
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3. Huik, K.; Sadam, M.; Karki, T.; Avi, R.; Krispin, T.; Paap, P.; Rüütel, K.; Uusküla, A.; Talu, A.; Abel-Ollo, K.; Lutsar, I. (2010). CCL3L1 copy number is a strong genetic determinant of HIV seropositivity in Caucasian intravenous drug users. The Journal of Infectious Diseases, 730–739.
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Resistance and Presence of Several Naturally Occurring Polymorphisms of Human Immunodeficiency Virus-1 CRF06_cpx in Treatment-Naive Patients in Estonia. Journal of Medical Virology, 81, 953–958.

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