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ASSAYS FOR MONITORING HIV THERAPY IN LOW-MIDDLE INCOME COUNTRIES

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To my beloved family

ABSTRACT

The purpose of this thesis was to develop and/or evaluate assays for HIV viral load (VL) monitoring and HIV drug resistance testing of potential importance for research studies and clinical care in low- and middle income countries (LMICs). This was achieved using reverse transcriptase based (RT) assays and blood samples from Vietnamese and Swedish HIV-1 infected patients.

In **Paper I**, HIV-1 obtained from 63 treatment-naïve Vietnamese patients was analysed by population sequencing and phylogenetic analysis with regard to transmitted drug resistance (TDR), subtype and the time of the most recent common ancestor (tMRCA). All strains belonged to HIV-1 CRF01_AE and TDR was found in 6.3% of the patients, including Y181C, L74I, V75M and L210W mutations. tMRCA was found to be 1989.8 for a larger clade and 1997.5 for a smaller clade. Sequences from intravenous drug addicts were intermingled with sequences from sexually infected patients, indicating frequent exchange of virus between the transmission risk groups. Our data suggests that TDR and the transmission patterns between risk groups rate should be monitored regularly and prospectively.

In **Paper II**, we evaluated the feasibility, sensitivity and specificity of an RT-based assay for quantification of HIV. A high correlation ($r^2 = 0.97$), agreement (log difference = 0.34; 95% CI -0.35;1.03), sensitivity (98%) and specificity (100%) were found between the RT-based assay and the Roche Cobas TaqMan. Its feasibility was further confirmed in a clinical trial including 605 Vietnamese HIV-1 infected patients. Our results show that the RT-based assay is an attractive low-cost alternative for monitoring of efficacy of antiretroviral therapy programs in resource-limited settings.

In **Paper III**, a simple phenotypic RT-based assay was developed for the detection of drug resistance to the 2nd generation NNRTI etravirine (ETR) and cross-resistance patterns to the 1st generation NNRTIs. For all recombinant HIV-1 RTs, ETR displayed expected IC₅₀ values equivalent to previous reports. The test could detect ETR resistance in plasma samples (n=28) obtained from treatment-naïve and experienced Swedish HIV-1 infected patients associated with Y181C and L100I substitutions as well as discriminate between the impact of K103N on the IC₅₀ value of nevirapine but the lack of impact on the IC₅₀ value of ETR. In **Paper IV**, a further comparison was performed between our phenotypic ETR resistance assay and the genotype obtained by direct sequencing and ultra-deep pyrosequencing (UDPS) in 20 Swedish patients with past or ongoing failure on the 1st generation NNRTIs. Most of the strains from the patients had various degrees of decreased phenotypic ETR susceptibility despite absence of ETR resistance associated mutations (RAMs) according to direct sequencing. Additional resistance mutations corresponding to <20% of the viral populations were found by UDPS in 9 analysed patients. In four of these, the mutations are likely to have contributed to phenotypic resistance. The patient treatment histories and the UDPS data supported that our phenotypic assay may be more sensitive than direct sequencing in identifying minor quasispecies with resistance mutations. The degree and pattern of an increased assay sensitivity as well as the clinical relevance remains to be determined.

In **Summary**, since the evaluated RT-based assays are simple to perform, use basic laboratory equipment, and does not require complex interpretations, they could be a low cost alternative for both studying VL and drug resistance to 1st and 2nd generation NNRTIs in LMICs.

LIST OF PAPERS

- I. Bontell I, Cuong D.D, **Agneskog E**, Diwan V, Larsson M, Sönnernborg A. Transmitted drug resistance and phylogenetic analysis of HIV CRF01_AE in northern Vietnam. *Infection, Genetics and Evolution* 2012;12(2):448-452
- II. Cuong D.D, **Agneskog E**, Chuc N, Santacatterina M, Sönnernborg A, Larsson M. Monitoring the efficacy of antiretroviral therapy by a simple reverse transcriptase assay in HIV-infected adults in rural Vietnam. *Future Virology* 2012;7(9):923-931
- III. **Agneskog E**, Nowak P, Källander C.F.R, Sönnernborg A. Evaluation of etravirine resistance in clinical samples by a simple phenotypic test. *Journal of Medical Virology* 2013;85(4):703-708
- IV. **Agneskog E**, Nowak P, Casadellà M, Noguera M, Paredes R, Källander C.F.R, Sönnernborg A. Assessment of cross-resistance between NNRTI in clinical samples by a simple phenotypic test. *Manuscript submitted*.

The papers will be referred to in the text by their Roman numerals (**I – IV**).

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ART	Anti-retroviral therapy
ARV	Antiretroviral
ASPCR	Allele-specific real-time PCR
ATP	Adenosine triphosphate
BrdUTP	Bromo-deoxyuridine triphosphate
CRF	Circulating recombinant form
DHHS	Department of Health and Human services
DLV	Delavirdine
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DRM	Drug resistance mutation
dsDNA	Double stranded deoxyribonucleic acid
EFV	Efavirenz
ELISA	Enzyme linked immunosorbent assay
ETR	Etravirine
GRT	Genotypic resistance testing
HAART	Highly active anti-retroviral therapy
HTLV-III	Human lymphotropic virus type III
HIV	Human immunodeficiency virus
IAS-USA	International Antiviral Society-USA
IDU	Intravenous drug user
IC ₅₀	Half maximal inhibitory concentration
LAV	Lymphadenopathy-associated virus
LMICs	Low and middle income countries
MSM	Men who have sex with men
MTCT	Mother to child HIV transmission
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NGS	Next generation sequencing
NRTIs	Nucleoside reverse transcriptase inhibitors
NVP	Nevirapine
PCR	Polymerase chain reaction
PI	Protease inhibitor
PI/r	Ritonavir-boosted protease inhibitor
PLHIV	People living with HIV
PMTCT	Prevention of MTCT
<i>pol</i>	Polymerase gene
RAMs	Resistance associated mutations
RIBA	Recombinant immunoblot assay
RNA	Ribonucleic acid
RT	Reverse transcriptase
RPV	Rilpivirine
SIV	Simian immunodeficiency virus
TDR	Transmitted drug resistance
TDRMs	Transmitted drug resistance mutations
VL	Viral load
WHO	World Health Organization
WT	Wild-type

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1 THE HUMAN IMMUNODEFICIENCY VIRUS

In the beginning of 1981, men who have sex with men (MSM) in the US fell ill from opportunistic infections and Kaposi's sarcoma, a rare form of cancer. The symptoms demonstrated clear evidence that the patients suffered from immune suppression and these clustered symptoms were named acquired immunodeficiency syndrome (AIDS) [1]. Two years later in 1983 it was reported by a French research group [2] that the immunodeficiency was caused by a retrovirus, and that target groups were not limited to MSM, indeed it was a disease that could affect all. Soon after, an American research group also published their findings [3, 4]. Both the French and the American groups noted that the virus infected T-lymphocytes, why they named it LAV (lymphadenopathy-associated virus) and HTLV-III (human lymphotropic virus type III), respectively. In 1986, the virus was renamed human immunodeficiency virus (HIV) [5].

Today, almost thirty years after the start of the global HIV/AIDS epidemic, we are still unable to control the spread of the virus and during 2011 approximately 34.2 million people were estimated to be living with HIV (www.unaids.org).

There are two types of HIV; type 1 (HIV-1) that was first isolated and type 2 (HIV-2) that was discovered in 1986 [6]. The more pathogenic HIV-1 is spread worldwide and is responsible for the vast majority of cases of AIDS, whereas the less pathogenic HIV-2 is mostly found in the western parts of Africa [7]. HIV is thought to originate from the simian immunodeficiency virus (SIV) prevalent in African non-human primates and the passage to humans is thought to have taken place in the beginning of the 20th century [8, 9]. HIV-1 (from now on referred to as HIV) is divided into groups; major (M), outlier (O) and non-M non-O (N). The M group is further divided into subtypes A-K which have a distinct geographic distribution worldwide, although there is a rapid spread of different subtypes to new areas of the world. Occasionally, two viruses of different subtypes "meet" in the cell of an infected person and the genetic material from these viruses combine to create a hybrid virus. If this "new" strain survives long enough to infect at least two separate individuals it is referred to as a "circulating recombinant form", CRF.

1.1 STRUCTURE AND REPLICATION

HIV is a spherical particle of approximately 100 nm. The outer envelope is composed of a phospholipid bilayer derived from the infected host cell. A schematic presentation of HIV is shown in Figure 1. HIV is a *lentivirus* of the *retroviridae* family and as such contains its genetic information in the form of two single stranded RNA molecules. The viral particle also contains viral enzymes used during the replication process. The reverse transcriptase (RT) converts the viral RNA to DNA, while the integrase enzyme integrates the new viral DNA into the host cell DNA. Two cellular transfer RNA (tRNA) strands are also carried within the virion and these act as primers for the reverse transcription carried out by the RT. The genome encodes three major genes, *env*, *pol* and *gag* as well as several regulatory

and accessory proteins essential for the viral replication. The envelope (*env*) gene codes for the surface envelope glycoproteins (gp120 and gp41). The group specific antigen (*gag*) gene codes for the matrix (MA), nucleocapsid (NC) and capsid (CA) proteins, while the polymerase (*pol*) gene codes for the viral enzymes, reverse transcriptase, protease and integrase [10].

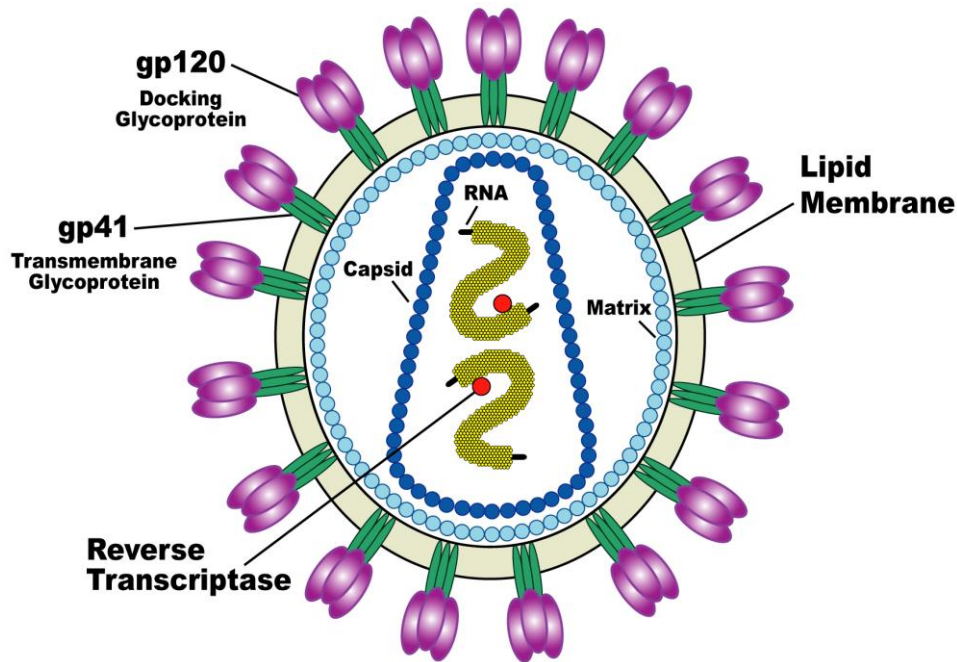


Figure 1. The HIV virion (published under commons, PD-USGov-HHS-NIH)

The viral life cycle (see Figure 2) begins by interactions between the viral gp120 and the CD4 receptor on the target cells; T-lymphocytes, macrophages, monocytes, dendritic cells and microglial cells [11-13]. In addition to the CD4 molecule on the host cell, the virus requires either of the co-receptors CCR5 or CXCR4 for entry [14], which triggers a fusion between the membranes of the virus and the cell, releasing the viral nucleocapsid into the cell.

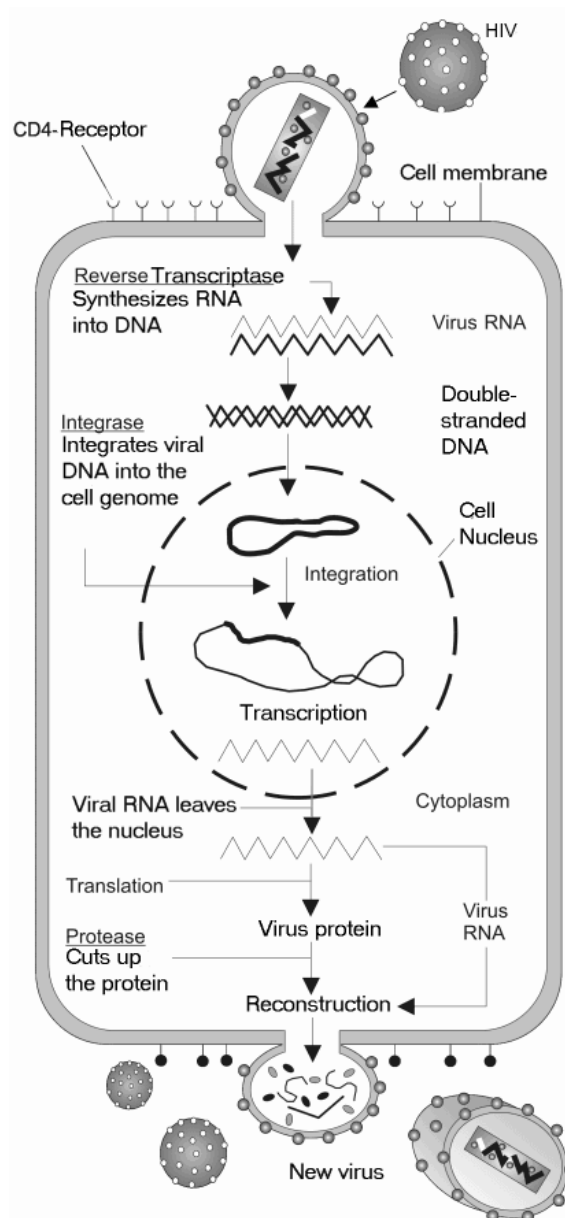


Figure 2. Schematic overview of the HIV replication cycle (published under commons, GFDL-en).

Once inside the cell, the viral nucleocapsid undergoes partial uncoating, releasing its contents into the cell cytoplasm. The two copies of viral positive single stranded RNA are exposed and reverse transcription of the viral RNA to DNA is performed by the reverse transcriptase (RT) enzyme. Through RT ribonuclease activity, the DNA strand is removed from the RNA strand and a second DNA strand is synthesised. The dsDNA is then transported from the cytoplasm into the nucleus of the cell whereafter it is ligated into the host chromosomal DNA by the viral enzyme integrase and becomes the proviral DNA [15]. However, the RT lacks proof reading, which consequently introduces mutations in the provirus and is the source of the high genetic variability of HIV [16, 17]. This is beneficial for the virus as it can quickly adapt to the environment in which it replicates and

thereby escape the immune system as well as drug pressure. During the reverse transcription, long terminal repeats (LTRs) are added to both ends of the DNA and LTRs are crucial for facilitating the subsequent transcription of the viral genome. Frequently, transcription of the integrated proviral DNA is initiated immediately but in some cells the proviral DNA remains latent for a variable length of time until host cell activation, which makes it very difficult to clear the viral infection [18]. The transcription of the provirus is initiated by cellular factors. Viral mRNA is produced from the provirus by action of a cellular RNA polymerase and the resulting mRNA is spliced, and are then transported out of the nucleus into the cytoplasm [19]. The mRNAs are translated in the cytoplasm to precursor proteins. The viral proteins assemble at the host cell surface where the Env proteins, necessary for budding from the host cell membrane, are inserted. Shortly after budding, the gag and gag-pol precursors are cleaved by viral protease and new infectious virions are produced, ready for the next round of infection [20-22].

1.2 COURSE OF INFECTION

HIV may be transmitted by sexual contact, transfer of infected blood and from mother to child during pregnancy, birth or breastfeeding. Although the rate of disease progression is highly variable among HIV patients, most infections follow a typical course shown in Figure 3 that can be divided into three stages: primary infection, chronic infection, and AIDS [23]. The primary or acute infection starts shortly after HIV enters the body and the high replication of the virus initiates an immune response by producing HIV antibodies and cytotoxic T lymphocytes. This stage that occurs a few weeks after initial infection [24] may be accompanied by a flu-like illness. It is characterised by high viral loads [25], referred to as the acute phase viremia. After the acute stage of HIV infection the patient progresses to the chronic stage which may last for up to ten or more years in untreated patients before AIDS develops [26].

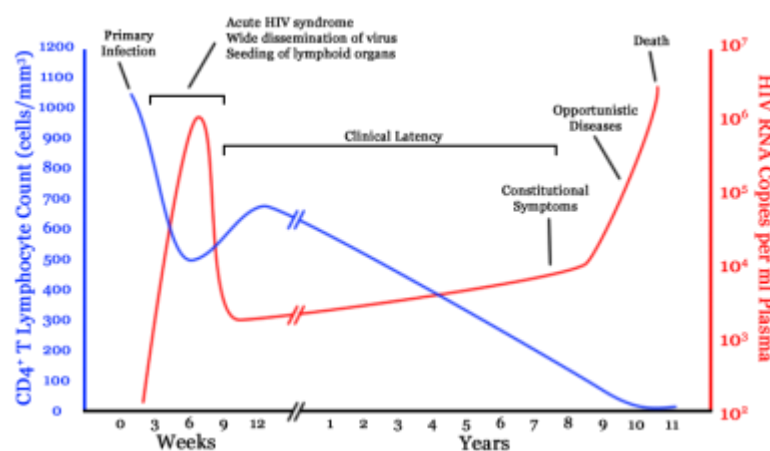


Figure 3. Course of HIV infection (published under commons, hiv-timecourse.png)

1.3 HIV SUBTYPES

HIV is divided into two different types: HIV-1 and HIV-2. HIV-1 is divided into three major groups: group M (main), group O (outlier) and group N (non-M, non-O) [27]. Group M dominates the world epidemic and has 10 subtypes (A to K). Sub-Saharan Africa is predominated by HIV-1 subtype C, which is causing >50% of the global HIV-1 epidemic (Figure 4). Subtype C was first described by our research group in 1990 [28].

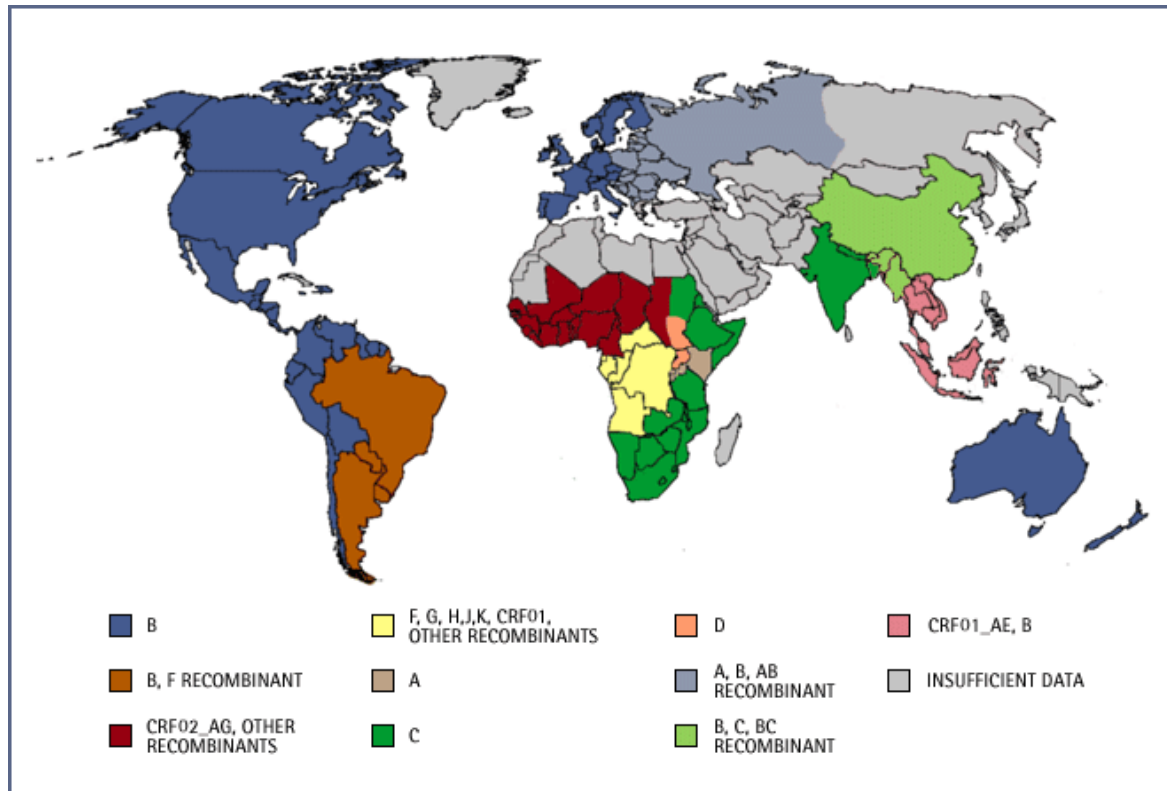


Figure 4. Global distribution of HIV-1 subtypes

(Source: <http://www.pbs.org/wgbh/pages/frontline/aids/atlas/clade.html>)

No strong evidence for differences in clinical outcome or transmission rates have been described between subtypes, although such differences have been suggested to be present in a few studies [29-31]. A major implication in practice of the genetic differences between subtypes is that the precision of molecular based assays such as PCR based assays for measuring VL and genotypic resistance testing may be influenced by this genetic variation [32]. In addition, when it comes to HIV drug resistance development, the pattern of resistance associated mutations may to some extent differ between subtypes although this occurs only for a limited number of mutations [33-36].

In Vietnam, which will be described further in **Papers I** and **II**, the first documented Vietnamese case detected was a subtype B virus [37], but since then the epidemic has been

dominated by the recombinant strain CRF01_AE, which is the predominant subtype in South-East Asia [38, 39].

1.4 MOLECULAR EPIDEMIOLOGY

Vietnam's HIV epidemic is considered to be one of the fastest growing in Asia and is one of the 10 leading causes of mortality in the country [40]. Since the first case was detected in 1990, over 50,000 people have died of AIDS and at the end of 2010, there were 254,000 people living with HIV (PLHIV) [41]. Some provinces have progressed to a generalised epidemic with more than 1 % of the adult population infected with HIV, such as Quang Ninh, Ho Chi Minh City and Hai Phong (UNAIDS, 2006).

HIV transmission in Vietnam has so far largely been driven by intravenous drug users (IDUs) and more recently the spread of HIV in Vietnam increasingly appears to occur through sexual transmission [42] which suggests that the epidemic may become more difficult to control. In **Paper I** phylogenetic analyses including molecular clock calculations was performed to investigate the HIV transmission patterns in Quang Ninh province, Northeastern Vietnam.

A phylogenetic tree is a branching diagram or “tree” showing the inferred evolutionary relationships among different species, organisms, or genes from a common ancestor. It can be used to study evolutionary relatedness of different organisms or relationship between strains of the same organism. Due to the fast evolution of HIV, it is possible to use phylogenetic trees for detailed evolutionary and epidemiological studies. The branching-tree is called the topology and the length of the branches describes their genetic distances, which is related their evolutionary time. Calculations of the time of the most recent common ancestor (tMRCA) can be done. In **Paper I**, our study of the Vietnamese samples were found to be clustered into two distinct groups; one small clade that had a tMRCA in year 1997.5 and a larger group with an estimated tMRCA in 1989.8.

There are different ways of assessing confidence of the branches in the tree. The traditional method is called bootstrap analysis, which was used to perform the phylogenetic analysis of the strains in Northeastern Vietnam in **Paper I**. This analysis showed that 100% of patients included in the study were infected with HIV-1 subtype CRF01_AE with $\geq 95\%$ bootstrap support.

2 DIAGNOSIS AND MONITORING OF HIV INFECTION

2.1 DIAGNOSIS

The standard way to diagnose a HIV infection is by the detection of antibodies against the virus. This test is performed by enzyme linked immunosorbent assay (ELISA), where the ability of antibodies present in plasma or serum to bind viral lysate or proteins is determined. To confirm that the person is infected, a Western blot or the similar recombinant immunoblot assay (RIBA) could be performed. Here, the reactivity of the antibodies is determined after separation of the viral proteins by electrophoresis. In the positive test the patient serum should react with two or more of p24, gp41, gp120 or gp160. In low-middle income countries (LMICs), the diagnosis is made instead by two complementary ELISAs, an approach which has a similar precision.

2.2 MONITORING

In high-income countries, disease progression is monitored by CD4+ T-cell counts, plasma HIV RNA levels (“viral load”, VL) and clinical symptoms. In untreated patients, CD4+ T-cell count is the most important marker indicating level of immunosuppression and hence the urgency of initiating ART. In treated patients, measuring HIV RNA levels in plasma is the key component for monitoring treatment outcome and/or adherence. A higher VL results in a more rapid disease progression in untreated patients [43, 44]. In practice in high income countries, both CD4 T-cell count and VL are frequently used for assessing patient prognosis [45]. At ART failure and when the virus becomes resistant, this is rapidly detected as an increase of the VL. The failure is usually defined as increasing HIV RNA levels while being on ART.

Presently (2013) in Sweden and in most high-income countries, Taqman PCR is the assay of choice for HIV RNA quantification which has a sensitivity of 20 copies/ml [46]. Monitoring is recommended every three to six months in treatment-naïve HIV-infected patients, around 4-6 weeks after treatment initiation, and thereafter every three to six months, according to the Swedish HIV guidelines [47]. Mostly, the efficacy of ART is high with 92% of Swedish patients reaching the aim of undetectable VL with the standard techniques in 2012 [48]. In the past a substantial number of patients in high income countries failed ART with HIV drug resistance development as a consequence. Presently, the number of patients failing ART is limited and hence resistance development is much more uncommon than in the past [49].

In LMICs, VL measurement can seldom be done due to high costs, lack of expertise and equipment in addition to logistic problems. Instead, the monitoring of ART in most LMICs is presently based on clinical parameters and CD4+ T-cell counts, see chapter 4. Therefore, there is a need of simpler methods for the assessment of VL in such contexts. In **Paper II**,

it was shown that an ELISA-based method from CaviDi (Uppsala, Sweden), measuring the activity of the HIV reverse transcriptase (RT), enzyme is proportional to the VL in the plasma [50, 51]. In **Paper II**, we also compared Roche Cobas TaqMan[®] with ExaVir Load and found a strong correlation ($r^2 = 0.97$; $p < 0.001$) between the two assays.

3 HIV THERAPY

3.1 ANTI-HIV DRUGS

The HIV lifecycle provides several potential opportunities to block viral replication. The first antiretroviral drug, *zidovudine* (ZDV), was introduced in 1987. During the early days of the HIV pandemic antiretroviral treatment (ART) was given as a mono-and/or dual therapy. This turned out to be a suboptimal strategy that soon resulted in a high degree of drug resistance [52-54]. The clinical benefits were not satisfactory and a more potent strategy was urgently needed. Today, three or more drugs from at least two different classes are used simultaneously to selectively reduce the level of viral replication and minimise the risk of developing drug resistance. Combination of ART has dramatically decreased mortality and increased the quality of life of HIV-infected individuals [55].

Today, there are five different classes of drugs used in ART; nucleoside analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside analogue reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors, and entry inhibitors, all with different modes of action.

3.1.1 NNRTI

In this thesis, the focus is on the NNRTI class and in particular *etravirine* (ETR). These drugs are blocking the activity of the RT by binding into a hydrophobic pocket, located close to but not in the active site of the enzyme (Figure 5). The steric interaction of the NNRTI makes the RT less flexible and thereby prevents it from further action [56, 57]. Five current drugs belong to the NNRTI; *efavirenz* (EFV), *nevirapine* (NVP), *delavirdine* (DLV, not approved in EU), *etravirine* (ETR) and *rilpivirine* (RPV).

The current Swedish guidelines (updated 2011) for first-line ART in treatment naïve patients recommend the use of two NRTIs together with one NNRTI, or two NRTIs and one ritonavir-boosted protease inhibitor PI/r (www.rav.nu), and are quite similar in the US (Department of health and human services (DHHS), International Antiviral Society-USA (IAS-USA) and *Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents*: <http://aidsinfo.nih.gov>, 2011) and in Europe (*The European AIDS Clinical Society*: www.european aids clinical society.org, 2011). A revised version of the Swedish guidelines will be launched in autumn 2013 and the combination of two NRTIs together with one NNRTI is likely to still be one of the alternatives for the first line therapy.

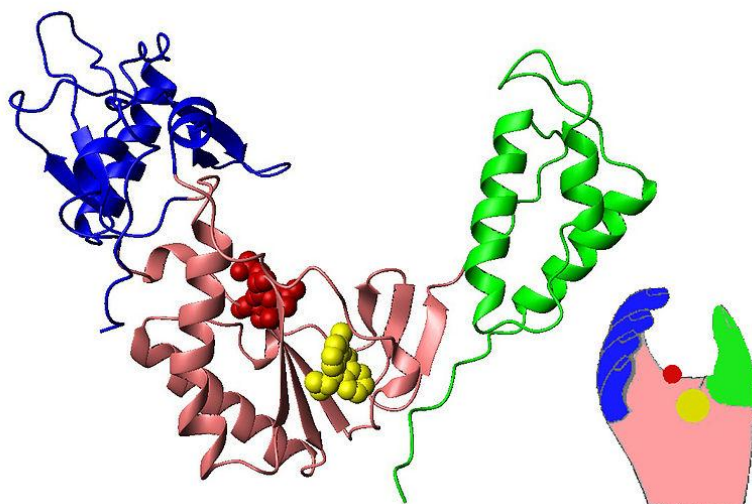


Figure 5. This ribbon representation of the RT active domain illustrates its hand-like structure, showing fingers (blue), palm (pink) and thumb (green). The active site (red atoms), where DNA is elongated, is in the palm region. Also shown is an NNRTI drug (yellow) in the pocket where it binds (published under commons, source <http://www.psc.edu/science/madrid2000.html>)

3.1.1.1 *First generation NNRTI*

NPV and EFV are the first generation NNRTIs that are approved for treatment of HIV infection. These drugs are still used frequently in high income countries and are the cornerstone in LMICs. In contrast, DLV was not approved in EU and has scarcely been used in USA because of side effects. The efficacies of NVP and EFV in combination with two NRTIs are good and the major problems are side effects and the low genetic barrier to development of HIV drug resistance (see below) [58]. The genetic barrier, defined as the number of mutations required to overcome drug-selective pressure, is an important factor for the development of HIV drug resistance. Frequently, NNRTIs are used in a fixed dose combination, together with NRTIs, as a single tablet regimen in order to enhance the convenience and the adherence to the treatment, both in high income countries and LMICs.

3.1.1.2 *Etravirine*

ETR is a second generation NNRTI that has been approved for treatment of HIV infection in treatment experienced adult patients who are harbouring HIV strains resistant to the first generation NNRTIs (EFV and NVP). Thus, in high income countries ETR is frequently used together with a boosted protease inhibitor and one or two NRTIs in patients with limited treatment options due to HIV drug resistance. ETR shows good activity *in vitro* against most wild-type strains of HIV, as well as against several strains resistant to available first generation NNRTIs (EFV and NVP). Furthermore, ETR appears to present a higher barrier than first-generation NNRTIs to the development of drug resistance. Whereas the presence of a single mutation is sufficient to affect the virological response to EFV or NVP, the resistance profile of ETR is more complex. Importantly, the most

prevalent NNRTI-associated mutation K103N has been claimed not to affect the ETR response alone [59]. However, evidence of ETR cross-resistance is present when multiple NNRTI mutations are detected during ART failure [60, 61]. In a study from Thailand, approximately 60% of patients failing first generation NNRTI-based ART had a high-level of ETR resistance. The role of ETR in second-line therapy may thus be limited in late NNRTI failure settings [60] and there is a need for resistance testing in order to identify possible candidates for ETR therapy.

3.1.1.3 Rilpivirine

Rilpivirine (RPV) is also a second generation NNRTI that is approved for treatment of HIV infection in NNRTI-naïve patients in most high-income countries. In EU, but not in the USA, the indication is to use RPV in treatment-naïve patients with VL less than 100,000 HIV RNA copies per ml. Thus, RPV is not approved for the use of patients failing NVP, EFV or ETR. In addition, there is a very high degree of cross-resistance between RPV and ETR [62]. RPV is available as a fixed-dose combination of emtricitabine (FTC), rilpivirine (RPV) and tenofovir disoproxil fumarate (TDF). Resistance mutations appears at a very low frequency, however when they occurs it is often the E138K substitution in combination with the M184I substitution during RPV treatment.

4 HIV THERAPY IN LOW-MIDDLE INCOME COUNTRIES

At the end of 2011, 8 million people were receiving ART in LMICs. This is a 26-fold increase since 2003. Another 7 million people need to be enrolled in treatment to meet the target of providing ART to 15 million people in 2015 [63]. By the end of 2011, 54% of the people eligible for treatment were receiving ART. Coverage is highest in Latin America (70%) and the Caribbean (67%), followed by sub-Saharan Africa (56%), Asia (44%), Eastern Europe and Central Asia (23%) and lowest in the Middle-East and North Africa (13%) [64].

The transmission of HIV from an HIV-positive mother to her child is called vertical or mother-to-child transmission (MTCT) [65, 66]. In the absence of any interventions HIV transmission rates are between 20-45%. MTCT can be nearly fully prevented if both the mother and the child are provided with antiretroviral drugs throughout the stages when infection could occur. WHO recommends a range of options for prevention of MTCT (PMTCT), which includes providing ARVs to mothers and infants during pregnancy, labour and the post-natal period, or offering life-long treatment to HIV-positive pregnant women regardless of their CD4 count.

The widespread availability of ART increases the risk for transmission and acquisition of drug resistant HIV-1 variants that compromise ART. However, drug resistance testing and follow up of the patients in order to retain the disease in the chronic phase and with low VL is not equally expanded. Indeed, accumulating data suggest that there is a steady increase of HIV-1 drug resistant mutations in patients in LMICs, such as in sub-Saharan Africa [67]. A systematic literature review and pooled analysis of data from WHO surveys [67] indicate that there is an increasing levels of drug resistance, primarily to NNRTIs.

In LMICs, first generation NNRTIs, EFV or NVP are used as a first-line therapy, most frequently together with 2 NRTIs (presently tenofovir or zidovudine and lamivudine). In order to enhance adherence and convenience as well as save costs the treatment is frequently given as fixed dose regimen in single dose tablets. Resistance to first generation NNRTIs as well as to NRTIs is an increasing problem in the treatment of HIV-1 infected patients in LMICs [68-70]. Access to other drugs varies in different LMICs. Frequently alternatives to the first line regimen are limited to a few drugs although there is an increasing use of ritonavir-boosted protease inhibitors such as lopinavir/r. Presently, no studies on the use of second generation NNRTIs in LMICs have been published. However, studies from India and Thailand have shown that there is a risk for cross-resistance to ETR if several NNRTI mutations have developed [60, 61, 71].

To ensure the sustainability of ART programs in LMICs, it is of great importance to maintain patients on first-line regimens as long as possible. In high income countries VL

measurement is a standard for monitoring the effectiveness of ART. However, virological monitoring is not widely accessible among LMICs due to high processing cost and requirement of advanced equipped laboratory. LMICs have been encouraged by the WHO to increase access to VL testing. For clinical decision making related to switching drug regimen, virologic failure has been defined as $>5,000$ copies/ml [72]. In the absence of VL, the recommendations are to use clinical symptoms or CD4 cell count [73]. However, relying only on CD4 cell count assessment is neither sensitive nor specific for virologic failure [74-76], increasing the risk of viremia going unnoticed and the emergence of drug resistance. In high-income countries, viremic patients on treatment are assessed routinely for the presence of drug resistance mutations (DRMs) by genotypic or phenotypic resistance testing [77, 78]. A low-cost tool for assessing drug resistance and sustain use of the first-line regimen in LMICs is therefore needed [72, 79, 80]. In **Papers III** and **IV**, a technically simpler and cheaper cost method (see chapter 6) for monitoring resistance to ETR are described compared to the traditional drug resistance test. Other ways of maintaining an efficacious treatment could be to switch treatment at a pre-specified point based on the predicted pattern of drug resistance development [81].

5 HIV DRUG RESISTANCE

5.1 MECHANISM OF HIV DRUG RESISTANCE

HIV is characterised by its high genetic diversity. First, this high diversity is a result of the high levels of virus production and turnover. Second, HIV has a very high rate of nucleotide sequence evolution which in turn is due to the high error rate of the viral reverse transcriptase. This leads to the generation of many variants of HIV in a single infected patient. The swarm of genetic viral variants is called quasispecies, which may allow the virus to escape from the immune system as well from ART [82]. Viruses with mutations that result in a fitness advantage will outgrow other variants and become the dominant viral population among the quasispecies. Under continued drug pressure but with concomitant therapy failure, viral quasispecies with reduced drug susceptibility accumulate with time, but when the treatment stops, VL will increase and wild-type virus takes over due to its greater replicative capacity [83]. The drug resistant variants usually have reduced fitness compared to wild-type virus. This is especially true for viruses with single primary resistance mutations. In contrast, additional mutations, which may evolve over time during continued drug selective drug pressure, may compensate, thus restoring fitness to near wild-type levels. The rate of drug resistance depends on patient adherence to treatment, the genetic barrier, host genetics, and fitness of the drug resistant variant [84, 85].

5.2 ACQUIRED HIV DRUG RESISTANCE

In treated patients, drug resistance associated mutations can be acquired when virus suppression is not completely achieved and replication of the virus can continue at low levels. The genetic barrier, defined as the number of viral mutations required to escape from the selective pressure of the drug, is an important factor for the development of drug resistance [86-88]. Boosted PIs for example have a high genetic barrier as they require multiple (3-5) mutations to overcome the drug pressure [86, 88]. Conversely, several other drugs have a low genetic barrier as a single mutation is sufficient for viral breakthrough, including first generation NNRTIs (nevirapine, efavirenz), 3TC/FTC and first generation integrase inhibitors (raltegravir, elvitegravir) [86, 88]. Many mutations selected by the use of one drug also cause cross-resistance to other drugs of the same drug class, limiting further treatment options. Often, viruses with major resistance mutations have reduced replication rates. This can be compensated by compensatory mutations that emerge after the major mutations. They do not reduce drug susceptibility, but improve the replication of the virus.

5.3 TRANSMITTED HIV DRUG RESISTANCE

Viruses with resistance mutations can be transmitted to other individuals. Because wild-type virus is rarely co-transmitted together with the drug-resistant HIV, the quasispecies

have no “memory” of the wild-type [89]. There are three possible evolutionary pathways for this transmitted drug-resistant variants described. First, when there is a profound effect on the replication rate of the virus, the resistant variant may revert back to wild-type. Second, atypical variants may be observed when it results in higher replication rate than the original transmitted resistant variant. Finally, the resistant variant can persist. Mutations that induce only a limited decrease in the replication rate tend to persist. Furthermore, in patients experiencing treatment failure, multiple compensatory mutations may appear after the initial selection of resistance mutations that lower the replicative capacity. After transmission to a new host, evolution may be expected to occur in a stepwise manner. However, if all possible nucleotide changes would initially decrease the replicative capacity, reversion to wild-type will be blocked [90].

5.3.1 MINOR POPULATIONS

The non-dominating populations among the quasispecies are called minor populations. A possibility is that a minor population which evolves independently of the major population can eventually emerge as the dominating population, thereby serving as a reservoir of diversity and possibly accelerating the development of drug resistance [91, 92]. This could happen under drug pressure either because it had developed higher resistance or because of a change in drug pressure that gave the minor population a growth advantage over the major population. Possibly, minor populations can represent vestiges of previously dominant populations or earlier stages of HIV evolution or alternatively originate from tissue compartments with lower drug concentrations and consequently reduced drug pressure [92].

5.3.2 HIV DRUG RESISTANCE TESTING

Antiretroviral drug resistance testing has become an important tool in therapeutic management of HIV-1 infection in high-income countries. There are two categories of methods available for resistance testing, genotypic and phenotypic.

5.3.2.1 Genotypic assays

The genotypic way to determine resistance is to search the gene of interest for mutations known to be associated with reduced drug susceptibility [93]. Population based sequencing of the *pol* gene including regions encoding RT, protease and/or integrase is generally generated by in-house methods or by commercial assay such as ViroSeq from Abbott. The sequences can be used for online prediction at Stanford University HIV Drug Resistance Database (<http://hivdb.stanford.edu>). The cost of a genotypic assay is much less than the price of a typical phenotypic test but still expensive for LMICs. Furthermore, the genotypic assay is performed more rapidly, while for a phenotypic assay a longer time is needed. Therefore, the genotypic test is the preferred test in clinical practice and is recommended by the European HIV Drug Resistance Guidelines Panel [94] and the International AIDS Society-USA Panel [95]. However, genotypic testing is challenging due to the complexity

of interpreting the many different drug-resistant mutations and translating these mutations into treatment response. All interpretations of genotypic data are based on phenotypic data about which mutations give rise to reduced drug susceptibility. Several interpretation systems have been developed, providing rules to help physicians interpret genotypic results. Another more advanced approach has been developed based on large HIV clinical databases and bioinformatics methods. These databases contain information about clinical status, demographics, HIV RNA, CD4 T-cells levels in addition to viral sequences. By bioinformatics analysis an improved prediction of which drug combinations are best suited to use after treatment failure is obtained as compared to established interpretation systems based on genotype sequence analysis only [96-99].

Another problem with today's routine HIV genotyping methods, is that these techniques are not sensitive enough to detect viral populations that represent less than 20-25% of the total population [100-102], likely underestimating minor populations that may play a role in drug resistance development. Several methods to detect and quantify minor populations of drug resistant HIV have been described during recent years [103]. One of them is the allele-specific real-time PCR (ASPCR) [102-104], which allows detection of minority quasispecies with a sensitivity of down to 0.01% for certain mutations, however one or only a few mutations at a time can be analysed. Another alternative to be able to detect minor populations could be to use the next generation sequencing (NGS) technologies. The population based Sanger method [105] used today for routine HIV genotyping is considered as a "first generation" technology, and dominated the sequencing field for about two decades prior to the development of NGS [106].

5.3.2.1.1 Ultra-deep pyrosequencing (UDPS)

The high throughput of NGS technologies, generating million of sequence reads in a short time, makes them suitable for sequencing of whole genomes, such as human, bacteria and plants. In this sequencing, short sequence reads are generated from fragmented DNA. The reads are aligned to a known reference sequenced or assembled *de novo*. Due to the length of the reads, 454 sequencing has an advantage for deep sequencing projects, referred to as ultra-deep pyrosequencing (UDPS). UDPS allows identification of rare genetic variants, which are not detectable by population based Sanger sequencing [107-110]. The depth of the UDPS analysis is primarily determined by the number of templates that can be successfully extracted and amplified from the starting material and by the error rate of PCR and UDPS. In **Paper IV**, UDPS was used to identify additional NNRTI resistance associated mutations (RAMS) in minor populations.

5.3.2.2 Phenotypic assays

The phenotypic assays are based on determination of the ability of virus representing the patient's phenotype to grow in culture together with the drug at different concentrations [111, 112]. The classical approach was to isolate virus from patient cells, and infect a cell

culture with these in the presence of various concentrations of a drug and estimate the ability of the cultures to produce virus [113]. Except for the very long time required for such an assay, this handling is prone to introduce artefacts from the virus propagation, such as selection of irrelevant virus due to the artificial conditions in the culture.

Later recombinant methods have been developed [111, 112, 114], of which some are commercially available. These are based on PCR amplification of the patient *pol* gene (RT and PR) from virus isolated from a patient, and creation of a recombinant virus including the amplified *pol* gene. This recombination virus is then assayed for drug susceptibility in culture. The raw data output is the concentration of drug required to inhibit viral replication by 50% or 90% (IC_{50} or IC_{90} , respectively) relative to the control. Results are usually expressed as the IC_{50} of the drug being tested for the patient-derived virus divided by the IC_{50} for the reference virus. The value of this ratio is commonly referred to as a fold change in susceptibility used the drug concentration that inhibits the viral replication by 50%, compared to reference recombinant wild-type virus as demonstrated in Figure 6.

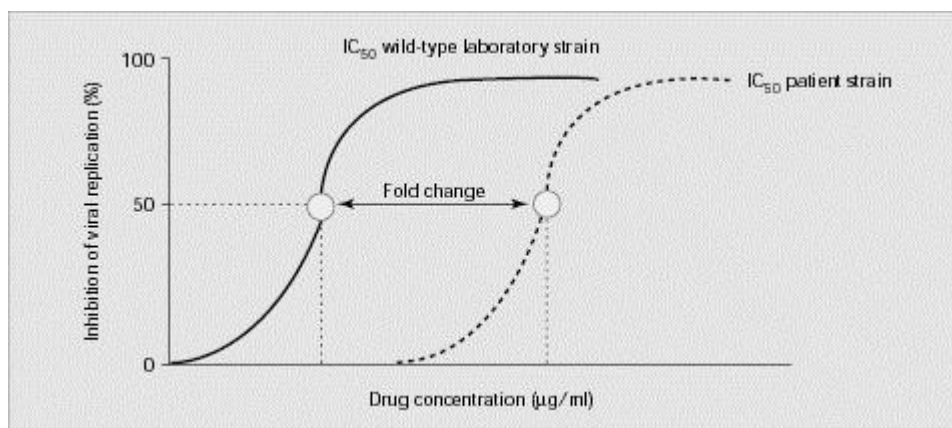


Figure 6. Phenotypic drug susceptibility curves. The continuous curve represents a wild-type drug-susceptibility virus. The shift to the right of the dashed curve, representing a strain isolated from a patient, indicates a reduction in drug susceptibility to a higher IC_{50} value. (Published under public domain, source: <http://www.ncbi.nlm.nih.gov/books/NBK2254/#A403>).

The interpretation of phenotypic data is based on the measurement of the fold change for each antiretroviral drug tested against pre-defined cut-offs. The first important issue related to phenotype interpretation is determining the appropriated cut-offs for defining a clinical isolate as either drug susceptible or drug resistant. Until, recently technical cut-offs were in use, based on the reproducibility of the assay on repeat testing. One improvement over technical cut-offs was the introduction of biological cut-offs, which are based on the distribution of the drug susceptibility of isolate from thousands of treatment-naïve patients [115].

For interpretation of phenotypic data based on biological cut-off, the clinical isolate is usually scored as susceptible to a certain drug if the fold change falls within the mean fold

change observed with samples from treatment-naïve patients, plus two standard deviations. A test result falling above cut-off can be said to be above the normal susceptible range. This provides a reference for comparison of the test with viruses circulating in the drug-naïve population, although it does not provide information about the likelihood that the virus tested will respond to treatment with a particular drug. Thus, neither technical cut-offs nor biological cut-offs provide a link between drug susceptibility measured *in vitro* and the virological response observed *in vivo*.

From a clinical perspective, the most relevant method of interpretation of phenotypic data is based on the use of clinical cut-offs. These are derived from clinical response data from treatment-experienced patients by determining the relationship between fold changes measured at baseline and the reduction in viral load after a defined period of treatment. Reliable determination of a clinical cut-off requires large sets of clinical data. This, in addition to the difficulty in extrapolating the activity of individual drugs within the context of combination therapies, are important obstacles to the determination of clinical cut-offs. Despite these difficulties, a number of clinical cut-offs have been proposed and are currently in use in commercially available phenotypic assays [116-118].

Because these phenotypic assays are time consuming, expensive and require specialised laboratory facilities, they are not widely used as clinical assays in Europe or in LMICs. Neither are genotypic resistance assays an alternative to use in LMICs because of the high cost and needs for expensive equipment and expert clinicians to interpret genotypic results.

Another alternative could therefore be to determine the phenotypic virus drug susceptibility at the RT enzyme level by using a phenotypic method [119-121]. Drug susceptibility testing on RT offers advantages compared with traditional phenotypic susceptibility tests as they are fast, technically simple and the results are not affected by the metabolism of cells used for virus culture in traditional phenotypic assays. In contrast with the results from genotypic assays, phenotypic methods do not require complex interpretations.

6 RT ASSAYS FOR VL QUANTIFICATION AND RESISTANCE TESTING

RT activity is a unique characteristic of all retroviruses since they need to convert their RNA genome to DNA in order to be incorporated into the host genome. Therefore, measurement of RT activity has the ability to provide an analytic tool to determine the viral replication in HIV. The RT uses the viral RNA genome as template to produce viral DNA prior to integration in to the host cell genome. This process can be measured *in vitro* using RNA template and a dNTP analogue (such as BrdUTP) together with colorimetric product [122-124], see Figure 7.

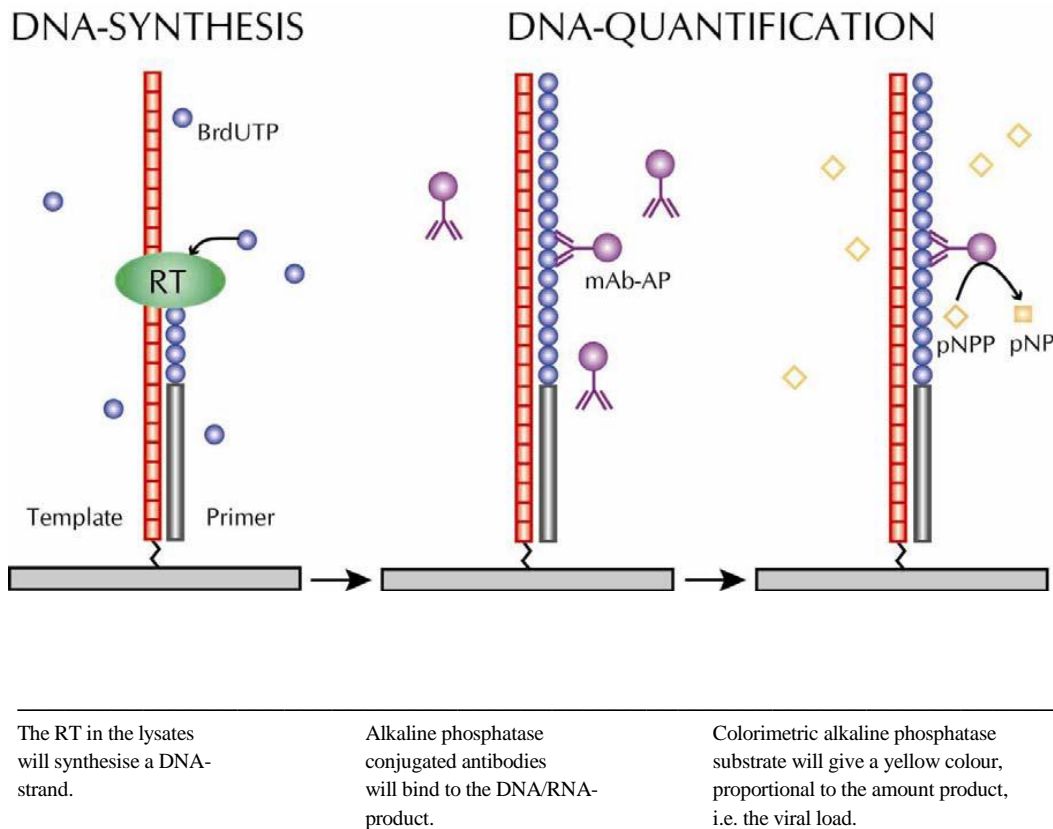


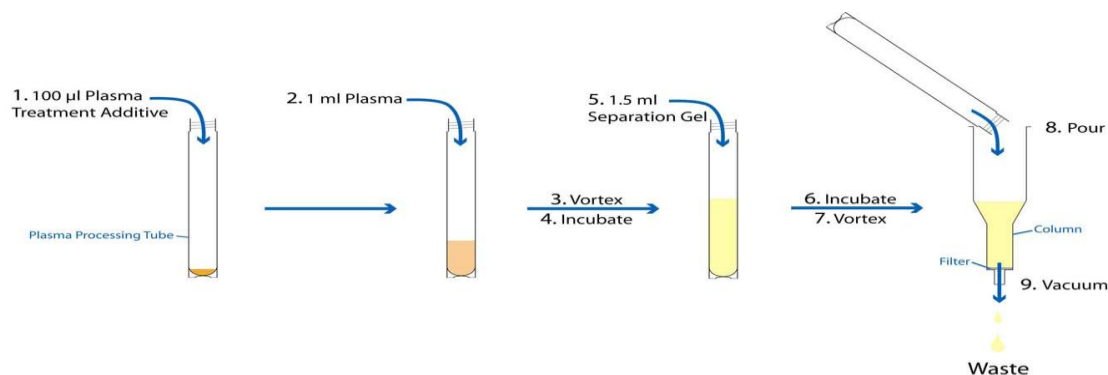
Figure 7. Schematic representation of the RT reaction and product detection. (Permission to reprint from Cavioli).

6.1 RT QUANTIFICATION

A methodology for quantification of HIV load based on ELISA methodology has been developed [125, 126]. It uses the HIV RT enzyme purified from patient plasma samples to catalyse the conversion of RNA to cDNA. The procedure consists of two main parts: the separation step for viral RT isolation (Figure 8a and 8b) and the reverse transcription step for quantification of the RT (Figure 7). First, the plasma is treated to inactivate cellular enzymes and the virus particles are then separated from the plasma by using a gel that

binds the virions. The immobilised virions are washed to remove inhibitors, including ARV drugs or RT-blocking antibodies. Virions are lysed to obtain the RT and the lysates are then transferred to a 96-well plate for assay of RT activity. In an overnight incubation, RT enzyme in the lysate incorporates BrdUTP into DNA strand complementary to the polyA template (bound to the wells) (Figure 7). Subsequently, an anti-BrdU antibody conjugated to alkaline phosphatase is added and the amount of incorporated BrdU is detected using a colorimetric substrate. The reaction plate is read at three time points by a standard plate reader at wavelength 405 nm. The first reading is the zero reading at 10 minutes, the second at 2 to 3 hours, and the third on the following day (16 to 24 hours) to ensure that small amounts of RT enzyme can be detected. Results are compared to a standard curve and the ExaVir Load Analyser software version 3.0 automatically converts the amount of RT in femtograms per milliliter (fg/ml) plasma to equivalent RNA copies per milliliter of plasma (copies/ml). The analytical sensitivity is 1 fg/ml. The measuring range is dependent on the duration of the RT assay and the performance of the plate reader used, but in these studies (**Papers III and IV**) it was typically 1 to 3,000 fg/ml, equivalent to 200 to 410,000 copies/ml.

Separation of RT



The plasma treatment Additive is a reducing agent that will inactivate any cellular polymerases present in the plasma.

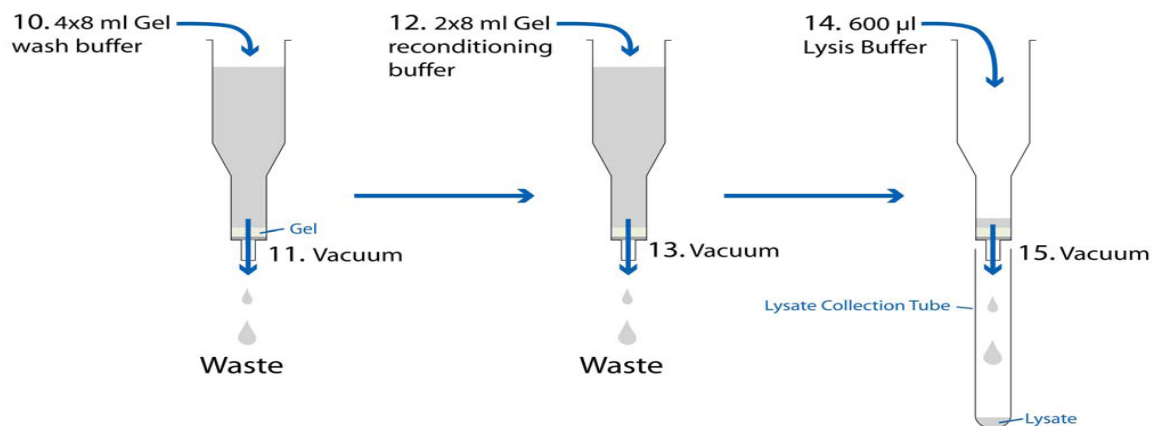
The Separation Gel is an ion-exchange gel that immobilises the HIV particle by binding to the lipid membrane of the virion.

As vacuum is created the plasma remainings will pass through the column. The filter will stop the gel, with the virions bound to it, from going through.

Illustration: Cavid AB

Figure 8a. Procedure for viral RT isolation in the ExaVir® Load. (Permission to reprint from Cavid).

Separation of RT (cont.)



Gel Buffer 1 removes agents that can disturb the RT-assay, like antibodies and ARVs.

Gel Buffer 2 creates a suitable environment for the RT.

The Lysis Buffer breaks the virion open and the contents (RT) is released. The lysate is collected for an analysis in the RT assay.

Illustration: CaviDi AB

Figure 8b. Separation of reverse transcriptase (continued). (Permission to reprint from CaviDi).

6.2 VIRAL PHENOTYPIC RESISTANCE TESTING

With available lysate containing purified RT from the patient plasma, the possibility for direct characterisation of RT activity arises. This allows for assessment of acquired or transmitted HIV drug resistance. Therefore a phenotypic resistance testing assay based on the RT enzyme was developed [127]. The principle of drug susceptibility determination is very similar to the above mentioned procedure of RT quantification described in chapter 6 and 6.1. The RT purified by the above described separation step is assayed together with a serial dilution of the ARV drug in the RT-assay described above. The enzymes incorporate BrdUMP to different extents depending on the susceptibility to the drug. From the inhibition obtained from the different drug concentrations, a profile of susceptibility of the RT is obtained, and an IC_{50} value is calculated. By comparison of the profile with those of wild-type and resistant standard RT, the level of resistance in the sample can be assessed. The methodology has earlier been evaluated for the NNRTIs EFV and NVP [127, 128].

7 NNRTI RESISTANCE ASSOCIATED MUTATIONS

Resistance to the first generation NNRTIs generally results from a single amino acid substitution such as the key mutations K103N or Y181C [129, 130]; this is referred to as low genetic barrier. These NNRTI resistance mutations frequently cause cross-resistance between EFV and NVP. Single nucleotide changes associated with NNRTI resistance can result in high-level resistance with only a slight loss of fitness [131, 132]. In practice a full cross-resistance is expected between NVP and EFV after failing therapy with concomitant resistance development. Table 1 demonstrates mutations associated with NNRTI drug resistance.

ETR appears to present a higher genetic barrier than the first-generation NNRTIs against the development of drug resistance. Results from selection experiments with wild-type HIV performed at high and low multiplicity of infection showed that at least two mutations are required for the development of ETR resistance compared with only a single mutation for a first-generation NNRTI [133]. The selection experiments identified the known NNRTI resistance-associated mutations (RAMs) L100I, Y181C, G190E, M230L and Y318F and the novel mutations V179I and V179F to be associated with development of ETR resistance [133]. Furthermore, the impact of individual mutations on resistance was highly dependent on the presence of specific co-existing mutations.

Importantly, the presence of the mutation K103N, commonly conferring resistance to the first-generation NNRTIs, has been claimed not to cause a loss of virological response to ETR. Full cross-resistance is however expected between the second generation NNRTIs, ETR and RPV.

			L	K	K	V	V		Y	Y	G	P		
Efavirenz			100	101	103	106	108		181	188	190	225		
			I	P	N	M	I		C	L	S	H		
					S				I		A			
	V	A	L	K		V		E	V	Y	G		M	
Etravirine	90	98	100	101		106		138	179	181		190	230	
	I	G	I*	E		I		A	D	C*		S	L	
				H				G	F	I*		A		
				P*				K	T	V*				
								Q						
			L	K	K	V	V		Y	Y	G			
Nevirapine			100	101	103	106	108		181	188	190			
			I	P	N	A	I		C	C	A			
					S	M			I	L				
										H				
				K				E	V	Y		H	F	M
Rilpivirine				101				138	179	181		221	227	230
				E				A	L	C		Y	C	I
				P				G		I				L
								K*		V				
								Q						
								R						

Table 1. Mutations associated with NNRTI drug resistance. Amino acid abbreviations: A, alanine; C cysteine; D, aspartet; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, Proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Gene positions and corresponding amino acid substitutions marked in **bold** type indicate a high impact on susceptibility and mutations with a lesser impact on susceptibility are represented in plain (non-bold) type. Figure adapted from [134].

8 AIMS OF THE STUDY

The aims of this thesis were to develop and/or evaluate assays for monitoring HIV treatment of relevance for the situation in low-middle income countries. More specifically, I studied simple reverse-transcriptase based assays for the determination of the HIV load and drug resistance to non-nucleoside RT inhibitors (NNRTI). These aims were achieved by:

- analysing HIV-1 from treatment-naïve Vietnamese patients by direct sequencing and phylogenetic analysis (**Paper I**)
- evaluating and implementing a simple RT-based assay for quantification of HIV in a Vietnamese cohort (**Paper II**)
- developing a simple phenotypic assay for detection of resistance to the new NNRTI etravirine (ETR) and to describe cross-resistance patterns between ETR and the first generation NNRTIs (**Paper III**)
- evaluating the phenotypic resistance assay in relation to sequence data obtained by direct sequencing and ultra-deep sequencing (**Papers III and IV**)

9 MATERIAL AND METHODS

For detailed information about material and methods as well as statistical analyses used in this thesis, see the respective papers. In brief, the following methods were used:

In **Paper I**, viral RNA was isolated using QIAamp ViralRNA kit (Qiagen). cDNA was synthesised using Superscript III First-Strand Synthesis Supermix (Invitrogen). A product spanning protease and the first two-thirds of reverse transcriptase gene of HIV-1 *pol*-gene was amplified. PCR-products were purified using QIAquick PCR-purification kit (Qiagen) and sent to Eurofins MVG Operon, Ebersberg, Germany for sequencing. Sequences were aligned and edited using the BioEdit and ReCall software. Genotypic resistance analyses of all sequences were performed using the Stanford HIVdb Sequence Analysis. Subtype classification was done using the REGA HIV Subtyping tool. Phylogenetic analyses were performed in BEAST v1.6.1 and tMRCA calculations were done.

In **Paper II**, quantification of HIV was done using ExaVir[®] Load version 3 and the principle behind this assay are described in chapter 6. Quantification of HIV RNA was performed by Cobas[®] AmpliPrep/Cobas[®] TaqMan[®]. A Spearman's rank correlation coefficient (r) was calculated, along with 95% confidence intervals for the correlation between HIV RT activity and HIV RNA. Bland-Altman plot was used to calculate the agreement of these two assays.

In **Paper III**, RT mutants were produced using the QuikChange site directed mutagenesis method (Stratagene). Purification of HIV-1 RT from plasma was done by using ExaVir[®] Load version 3 followed by determination of drug susceptibility of RTs towards ETR, both described in chapter 6. The genotype was determined by standard GRT.

In **Paper IV**, purification of HIV-1 RT from plasma was done by using ExaVir[®] Load version 3 followed by determination of drug susceptibility of RTs towards ETR, both described in chapter 6. The genotype was determined by standard GRT and UDPS was performed in nine of the samples.

9.1 ETHICAL CLEARANCE

The studies included in thesis were performed after approval from the Regional Ethical Committees at Karolinska Institutet. For **Papers I and II**, Dnr: 2006/1367-31/4, Hanoi Medical University Review Board (HMURB) in Bio-medical Research (No. 59/HMURB) and the Hanoi Medical Institutional Review Board (IRB) in Bio-medical research Ethics (No. 26/IRB). For **Papers III and IV**, Dnr. 2005/1167-31/3 and 2005/772-31/4. All subjects included gave their informed consent to prior study.

10 RESULTS AND DISCUSSION

The focus of this thesis was to develop and/or evaluate assays for monitoring HIV treatment of relevance for the situation in low-middle income countries.

10.1 PAPER I

Study background

In this study, baseline samples from 63 ART-naïve Vietnamese HIV-patients were analysed to assess the prevalence of transmitted drug resistance mutations (TDRM). Phylogenetic analyses were also performed including molecular clock calculations in order to investigate HIV transmission patterns in Northern Vietnam. All patients belonged to the cluster randomised controlled trial “Directly Observed Therapy for Antiretrovirals” (DOTARV), registration number NCT01433601, including 640 patients from the following districts/cities: Ha Long, Uong Bi, Dong Trieu, Yen Hung. This trial was conducted between July 2007 and November 2011, with two years of patient recruitment and two years of follow up.

The samples used were collected between December 2008 and January 2009 and genotypic analyses of 63 *pol*-gene sequences were performed using Stanford HIVdb Sequence Analysis, (<http://sierra2.stanford.edu/sierra/servlet/JSierra?action=sequenceInput>) [135]. The detected resistance mutations were compared against the TDRM surveillance list [136] as well as the IAS-USA 2010 update [137]. Subtype classification was performed using REGA HIV Subtyping tool [138].

Results and discussion

Drug resistance mutations in ART-naïve patients

All patients were found to be infected with HIV-1 subtype CRF01_AE with $\geq 95\%$ bootstrap support. In the 63 ART-naïve individuals, most viruses were found to be fully susceptible to all protease and reverse transcriptase inhibitors: in 39 (61.9%) sequences we found no resistance associated mutations at all, while 20 sequences (31.7%) had one or two polymorphic mutations that frequently occur in untreated patients. Four patients were, however, infected with viruses carrying resistance mutations, giving a TDRM prevalence of 6.3%. An overview of all detected resistance associated mutations is shown in Table 2.

Number of patients (%)	NRTI mutations	NNRTI mutations	PI mutations
1 (1.6)	L210W	None	None
1 (1.6)	L74W	None	L10I
1 (1.6)	V75M	None	None
1 (1.6)	None	Y181C	L10I
1 (1.6)	None	A98G	None
2 (3.2)	None	V179D	None
2 (3.2)	None	V106I	None
7 (11.1)	None	V106I	L101I/V
9 (14.3)	None	None	L101I/V
38 (60.3)	None	None	None
63 (100)			

Table 2. Number of patients with different resistance associated mutations. Mutations on the TDRM list (Bennett, 2009) are shown in bold text. Minor resistance mutations present on the IAS-USA list are shown in regular format.

Three of the TDRMs present in the analysed samples confer reduced susceptibility to NRTIs; L74I (n=1) and V75M (n=1) both confer low-level resistance to ddI (both), d4T (V75M) and ABC (L74I), while L210W (n=1) causes a low-level of resistance to all NRTIs except 3TC and FTC. The fourth TDRM was Y181C (n=1), which provides intermediate to high level of resistance to all NNRTIs. Minor mutations found for reverse transcriptase were: A98G (n=1), V179D (n=2), V106I (n=9), while L10I/V was found in the protease region of 18 sequences. No clinically significant resistance mutation for protease inhibitors were found.

Phylogenetic relationships and tMRCA calculations

The 63 *pol*-sequences were aligned with 190 CRF01_AE and four subtype B sequences retrieved from public and local databases. The initial analysis in BEAST revealed three clearly demarcated clades which all had a posterior probability support = 1. These defined three taxons that were used for the subsequent the most recent common ancestor (tMRCA) calculations; ‘CRF01_AE’ (which included all the Vietnamese samples plus the 190 CRF01_AE reference sequences), ‘Vietnam large clade’ (60/63 Vietnamese strains in this study), and ‘Vietnam small clade’ (three Vietnamese samples that clustered separately from the others) (Figure 9).

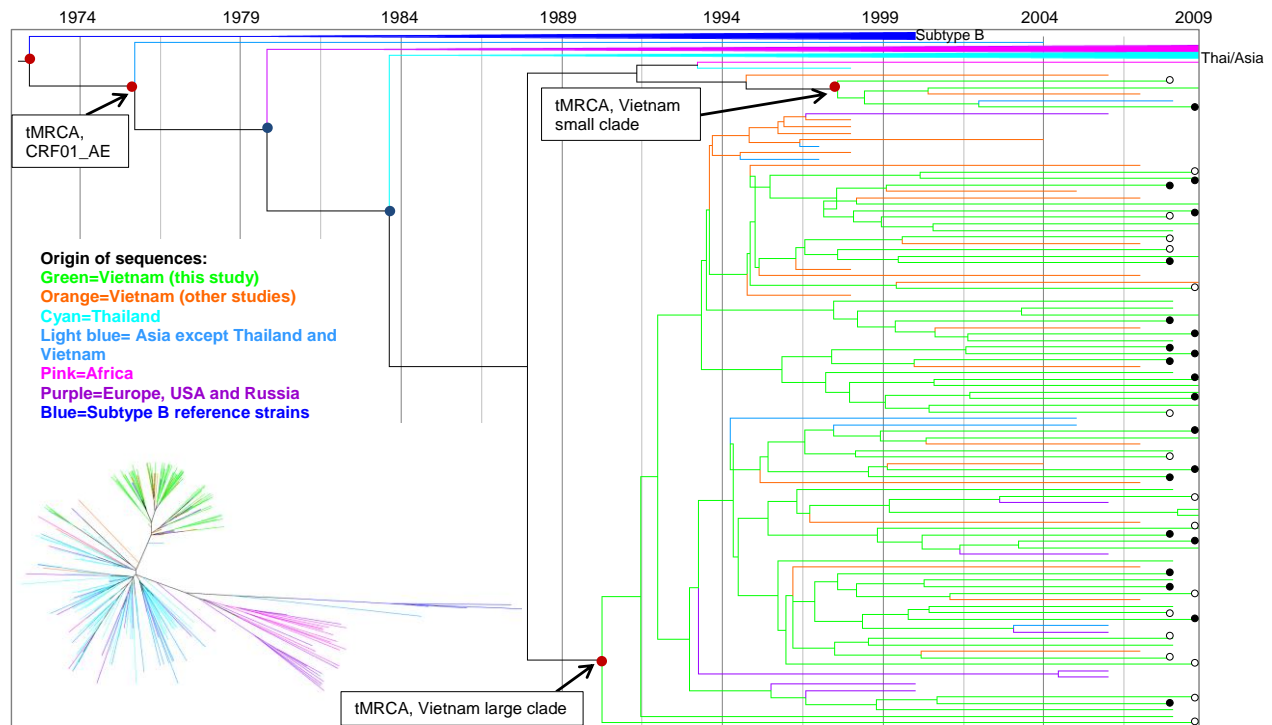


Figure 9. Phylogenetic trees showing the nodes used for tMRCA calculations and the mixture of strains from intravenous drug users and sexually infected patients in Northern Vietnam. The small inset tree shows all 257 strains with the Vietnam large and small clades encircled. In the larger tree some clades have been collapsed for clarity. The branch length corresponds to the year of sampling. Node markings: red circles, posterior probability > 0.99; blue circle, posterior probability > 0.90. Tip markings: filled circles, intravenous drug users; open circles, sexually infected patients. No tip marking, unknown mode of transmission.

Previous studies of the CRF01_AE epidemiology in Vietnam have shown that HIV was first introduced in the southern part of the country. By 1993, over 950 infections had been diagnosed in Vietnam, of which only three cases were found in the north [139]. The introduction of HIV-1 CRF01_AE in Vietnam has been estimated to have occurred at least a decade prior to the first detections of clinical cases and by the late 1980's the disease is believed to have spread among IDUs in South Vietnam and thereafter to IDUs in the northern part of the country around 1993-1994 [140]. Our results from the clade currently spreading through sexual and intravenous transmission in Northern Vietnam date the tMRCA a few years prior to this, around 1990. Vietnam large clade includes samples from Ha long, Uong Bi, Dong Trieu and Yen Hung from the current study (n=60), as well as sequences from Hai Phong [141], Bac Giang and Hai Duong [140] also located in the coastal North-Eastern part of Vietnam (n=22), plus a number of intermixed strains from China and the Czech Republic (n=13). The tMRCA for the North Vietnam cluster calculated by Liao et al (2010) [142] was based on a smaller number of samples (8 Vietnamese + 2 Chinese samples), which explains discrepancy between these studies. Indeed, six of these strains were included in the current study and the tMRCA of these strains fell around 1993-1994 (Figure 9, Vietnamese strains sampled 1998). It is therefore

likely that larger sampling rather than methodological differences accounts for the different time estimates, and that HIV first spread to Northern Vietnam around 1990 or earlier.

The Vietnam small clade has an estimated tMRCA around 1997, but since the number of strains is small it is difficult to say whether they represent an emerging cluster in the north or if the three infections were unrelated. BLAST searches confirmed that these strains were more similar to samples from southern Vietnam (Ho Chi Minh City, An Giang) and Thailand than to North Vietnamese and Chinese CRF01_AE strains. One of these samples originated from a truck driver, who had travelled widely throughout Vietnam in this job, and the other two samples came from women who were/had been married to drivers. It is therefore possible that these strains were independently introduced from the southern part of the country. None of these genetically divergent strains carried TDRMs.

The Vietnam samples analysed in this study originated from four clinics in the Quang Ninh province in Northeastern Vietnam, near the border to China. These clinics are all located within a radius of approximately 35 km, and no local clustering was found for the respective sites. Twenty-nine samples originated from patients with a history of intravenous drug use, 27 individuals were infected through sexual transmission and the mode of transmission for the remaining seven patients was unknown. Samples from patients with different modes of infection were completely intermixed in the phylogeny (Figure 9) indicating that HIV-transmission frequently occurs between intravenous drug users and non-drug users in northern Vietnam.

10.2 PAPER II

Study background

In this study we analysed the feasibility of the RT-based ELISA method for quantification of HIV in monitoring virologic outcome and ART efficacy. We also compared the RT-based method with Cobas TaqMan PCR. 605 ART-naïve patients from the study cohort for directly observed therapy with antiretrovirals (DOTARV) were included. The details of the Vietnamese cohort and the clinical outcome of the treatment are described in detail in **Paper I**.

From the total number of patients (640) in the cohort, 35 (6%) patients were excluded due to being non-naïve. Intention to treat analysis was applied to estimate treatment outcomes (mortality, virologic suppression rate, and virologic failure rate). Survival analysis was used to study the time from the start of ART to “virologic failure”, defined as VL >1000 copies/ml, Kaplan-Meier estimations of the survival curve and Log-rank tests are presented, stratified by baseline VL.

Also a total of sixty plasma samples were randomly selected for a comparative study and quantified with both ExaVir Load version 3.0 (described in chapter 6) and Cobas TaqMan

PCR version 2.0 (detection limit <40 copies/ml). ExaVir Load was performed at Uong Bi General hospital according to the manufacturer's instructions and Roche Cobas TaqMan PCR was performed at the Bach Mai Hospital in Hanoi according to manufacturer's instructions.

A Spearman's rank correlation coefficient (r^2), along with 95% CIs was calculated for the correlation between HIV RT activity and HIV RNA. In addition, we used a Bland-Atman plot to calculate the agreement of these two assays.

Results and discussion

Evaluating the RT assay in the Vietnamese cohort

A key component in the study was the use of VL quantifications through ExaVir Load. Since our study was the largest prospective study in which this methodology was used we needed to evaluate the precision in the Vietnamese setting.

Initially, we quantified 60 samples with both ExaVir Load and TaqMan PCR of which 44 (73%) had detectable virus. A good concordance was found between the methods. The median VL was 36,025 (IQR 200-165,770) copies/ml by ExaVir and 74,900 (IQR 41-208,000) copies/ml by Taqman. There were 15 samples (25%) with undetectable VL by both assays, from 16 treated patients. One sample showed a VL of 45 copies/ml by TaqMan but an undetectable VL by ExaVir Load. Thus, the sensitivity of the ExaVir Load assay relative to the TaqMan PCR was 98% (44/45) and the specificity was 100% (15 of 15 patients with TaqMan VL <40 copies/ml had undetectable RT activity).

The Spearman coefficient of correlation was $r^2 = 0.97$ [95% CI (0.95 – 0.98); $p < 0.0001$], (Figure 10). There was a good agreement between two assays with a mean of difference in log VL of 0.34 [95% CI (-0.35; 1.03)] (Figure 11).

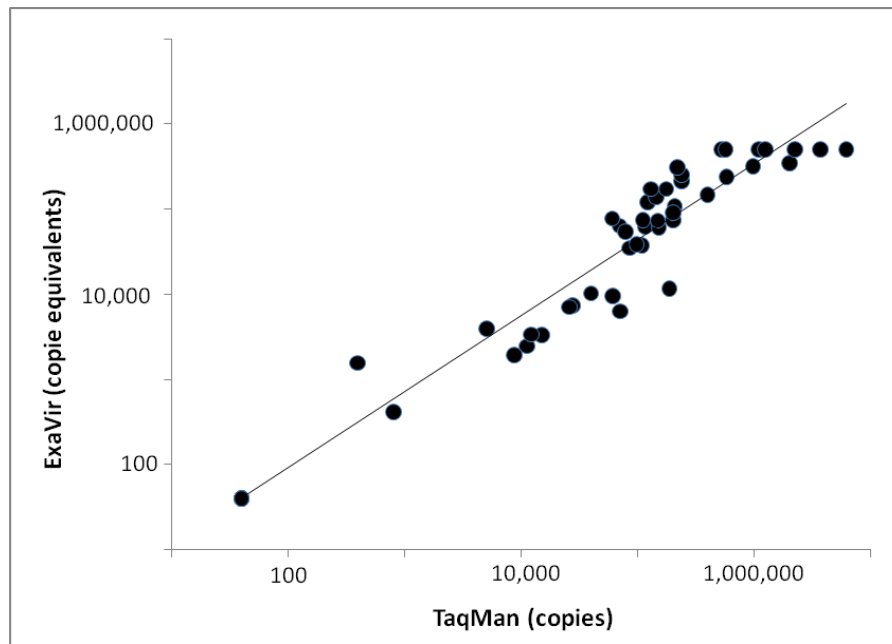


Figure 10. Correlation between Roche TaqMan and CaviD ExaVir Load assays. Undetectable values are scored as 40 copies corresponding to the lower limit of RNA quantification. The Spearman correlation coefficient was $r^2 = 0.97$ (95% CI 0.95–0.98, $p < 0.0001$). The equation for the regression line is $\log \text{ExaVir Load} = 0.8931 \log \text{TaqMan} + 0.1773$

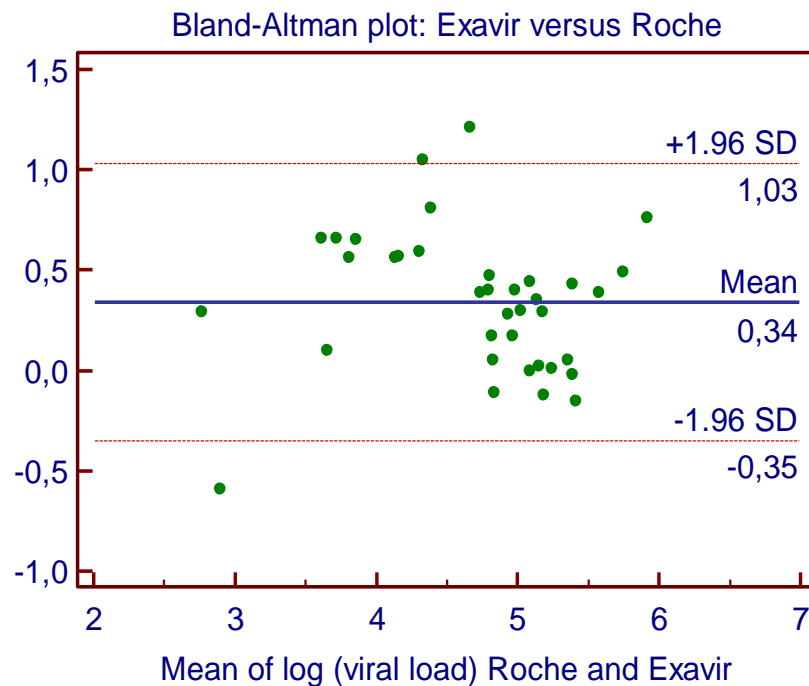


Figure 11. Bland – Altman plot analysis to compare between Roche TaqMan and CaviD ExaVir Load assays. The mean of difference in log VL results between two assays was 0.34 [95% CI (-0.35 ; 1.03)].

Results of VL testing in the clinical study

Analysis of the VL using ExaVir Load was carried out in a total of 2408 samples of 605 patients. A detailed description of the virological treatment results are described in **Paper I**. When analysing the 605 ART-naïve patients, after 24 months, 35 (5.8%) patients developed virologic failure, of which 15 (43%) were primary virologic failure (VL did not become undetectable after 6 months of ART). The cumulative virologic failure rate among samples assessed with VL during 24 months was 6.8% (95% CI 4.9-9.3). In patients with a high VL at baseline ($\geq 100,000$ copies/ml) virologic failure was more likely to be developed than in those with baseline VL $< 100,000$ copies/ml (Kaplan-Meier failure estimates Log-rank $p < 0.001$ (Figure 12)). Virological suppression rates were analysed at months 6, 12, 18, 24 among all patients according to intention-to-treat analysis which were 76%, 72%, 67%, 64% and among patients on treatment 93%, 93%, 94%, 94%, respectively.

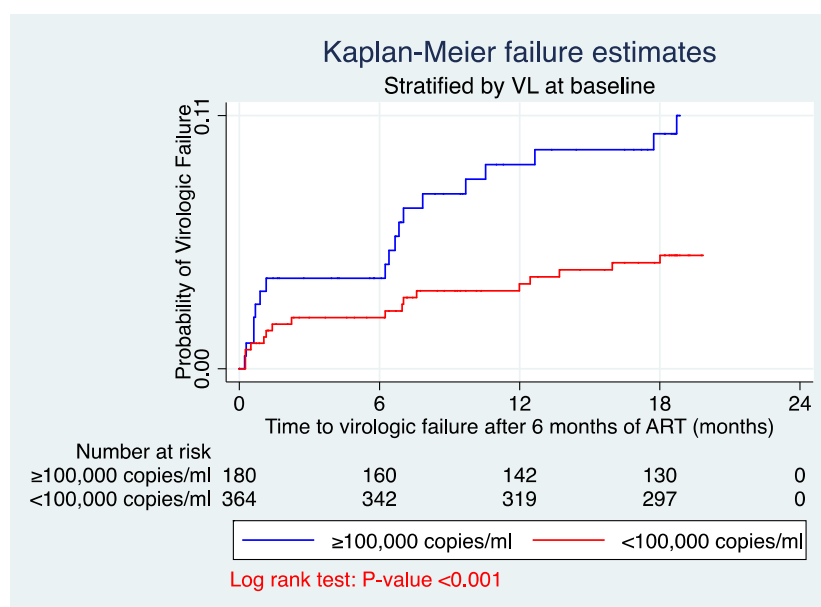


Figure 12. Kaplan-Meier showed VL at baseline as a risk factor for virologic failure (Log-rank p -value < 0.001).

10.3 PAPER III

Study background

The aim of this paper was to adapt a phenotypic drug susceptibility assay, earlier developed for measurement of resistance to the first generation NNRTIs, nevirapine, and efavirenz, for the detection of resistance to the second generation NNRTI etravirine (ETR).

The method was first optimised through titrations of the concentrations of ETR and other reagents in the assay. To evaluate the assay, five NNRTI resistant RT mutants were

produced (L100I, K103N, L100I/K103N, V179D, Y181C. As controls, T215Y and M41L/T69-SG/L210W/T215Y NRTI mutants were also included in the evaluation of the assay. All mutants were also tested for phenotypic susceptibility towards NVP to be able to confirm our previously published results on ExaVir Drug and NVP [127, 128, 143].

In order to correlate phenotype with genotype, 28 plasma samples from HIV-1 infected patients at Karolinska University Hospital were also analysed for further clinical evaluation of the assay. Plasma from 15 newly diagnosed presumably treatment-naïve patients and from 13 patients who had failed NNRTI containing therapy with ≥ 1400 HIV-1 RNA copies/ml were selected. The ETR phenotype obtained using the ExaVir Drug assays was compared to the predicted ETR phenotype obtained by routine genotypic analyses.

Inter- and intra-assay variability of the assay was also tested. The variation of the IC_{50} value for ETR was measured by testing two plasma samples (3 and 7) from the Karolinska HIV cohort at three different occasions (run 1 to run 3), each time in quadruplicates. Intra-assay means and SD (standard deviation) were calculated. Inter-assay SDs were calculated using the modified SD formula, $[(SD \text{ of the assay means})^2 - (\text{mean intra-assay SD})^2 / r]^{1/2}$ where r is the number of replicates. The SD achieved was used to calculate the CV (coefficient of variation) by using the formula $CV = (SD/\text{mean}) \times 100$. The effect of variation in the amount of RT on the IC_{50} value was also tested by measuring the ETR susceptibility on two samples serially diluted in 2.5 fold steps ranging the RT amount of 40-828 fg RT/ml plasma.

Results and discussion

Effects of NVP and ETR on recombinant HIV-1 RTs

We found that the recombinant RTs had in general the expected drug susceptibility (Table 3). The RT with Y181C, L100I and L100I + K103N mutations, respectively, exhibited pronounced decreased susceptibility to the drugs. The K103N mutation yielded increased IC_{50} towards NVP, but not ETR. The RT with Y179D had a very slightly increased IC_{50} , as compared to the controls. The wild-type strain HXB2 and T69S-SG mutations showed a similar susceptibility pattern for NVP and ETR. The RT mutant with T215Y showed a slight increase in the IC_{50} for NVP but not for ETR.

In this paper we showed that both NVP and ETR displayed expected IC_{50} values for all recombinant HIV-1 RTs equivalent to previous reports [59, 127] and the test could plausibly detect ETR resistance associated with Y181C and L100I substitutions as well as discriminate between the impact of K103N on the IC_{50} value of NVP but the lack of impact on the IC_{50} value of ETR [59, 144].

RT analysed	<u>IC₅₀ (μM)</u>	
	NVP	ETR
<i>NNRTI substitution panel</i>		
BH10*	1.1 ± 0.1	0.9 ± 0.2
BH10 E478Q	3.6 ± 1.6	0.9 ± 0.2
BH10 V179D, E478Q	8.2 ± 1.1	3.8 ± 1.0
BH10 Y181C, E478Q	>100	35.9 ± 9.8
BH10 L100I, E478Q**	37.9 ± 11.9	13.4 ± 2.7
BH10 K103N, E478Q	166.7 ± 11.7	1.4 ± 0.2
BH10 L100I, K103N, E478Q	237.3 ± 8.3	50.1 ± 20.6
<i>NRTI substitution panel</i>		
HXB2***	3.3 ± 1.5	2.5 ± 0.4
HXB2 T215Y	8.4 ± 0.3	1.4 ± 0.1
HXB2 M41L, T69S-SG, L210W, T215Y	2.9 ± 1.8	1.5 ± 0.7

Table 3. Effects of nevirapine and etravirine on recombinant HIV-1 RTs. Data are presented as mean IC₅₀ values and SD; IC₅₀, 50% inhibitory concentration; NNRTI, non-nucleoside RT inhibitors; NRTI, nucleoside RT inhibitors; NVP, nevirapine; ETR, etravirine. Data are based on three experiments for ETR susceptibility, except 10 experiments for BH10*, L100I** and HXB2***. NVP susceptibility was repeated at least three times (BH10, HXB2 and L100I, were repeated seven, four and five times, respectively), except for Y181C that was estimated at one occasion.

Reproducibility of ETR susceptibility and variation effect

The reproducibility data for the two samples tested at three different occasions in four replicates showed inter assay variation (CVs) of 9.4 and 11.1% (Table 4). Data also showed that the IC₅₀ values for ETR (mean ± SD: 1.6 ± 0.03 μM and 3.1 ± 0.04) were not influenced by the RT amount within the 40-828 fg/ml RT range (Figure 13).

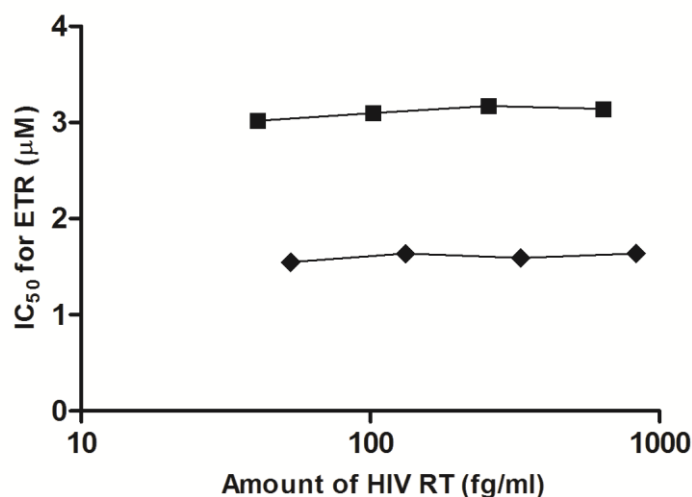


Figure 13. Presents the effects of variations in the amount of RT on the IC₅₀ value for ETR. ETR susceptibility was determined on two HIV- RTs isolated from patient sample 9 ■ and 4 ◆ and diluted to the indicated concentrations, ranging from 40 to 828 fg RT/ml.

Patient tested	<u>Intra-assay variation in IC₅₀ (µM)</u>			<u>Inter-assay variation</u>		
	Run 1	Run 2	Run 3	Mean	SD ^a	CV (%)
3	4.6 (±0.5)	5.5 (±0.4)	5.0 (±0.4)	5.2	0.48	9.4
7	2.1 (±0.2)	2.5(±0.1)	2.1 (±0.2)	2.2	0.25	11.1

Table 4. Reproducibility data of ETR susceptibility. Two samples were tested on three independent occasions (run 1 to 3).

^a Inter-assay SDs were calculated using the modified SD formula, $[(SD \text{ of the assay means})^2 - (\text{mean intra-assay SD})^2 / r]^{1/2}$ where r is the number of replicates. The SD achieved was used to calculate the CV by using the formula $CV = (SD/\text{mean}) \times 100$.

Effects of ETR on patients HIV-1 RT

The potential clinical value of the method was determined by analysis of plasma samples. In all of the 15 samples from presumably treatment-naïve patients (Table 4), low IC₅₀ values (mean ± SD: 2.5 ± 1.0 µM) were found for RT activity in the presence of ETR. In patient 3, however, sequencing revealed the presence of K103N most likely due to previous drug exposure or infection with a resistant variant. Also, in 13 NNRTI-experienced patients who were failing ART we found a concordant result. Seven samples (16, 17, 23, 25, 26, 27 and 28) had low IC₅₀ values (Table 4). The sequence analysis was consistent with the phenotypic results in three cases which lacked NNRTI mutations (25, 27 and 28). In four cases (16, 17, 23 and 26), sequence analysis showed mutations which are not clearly associated with ETR resistance (16: K103N; 17: A98G+V179I; 23:

V179I; 26: K103N+V108I). The A98G has been reported to be associated with decreased ETR response *in vivo* but has little, if any, effect on ETR susceptibility.

The remaining six samples had high (similar or higher than the IC₅₀ value of the mutant control) IC₅₀ (Table 4). The result concordantly showed high IC₅₀ values in plasma RT with Y181C (samples 20, 21 and 22) and intermediate IC₅₀ values were associated other known ETR associated mutations, confirming the reliability of our assay. However, for plasma RT which had intermediate IC₅₀ values a few discordant results were found in comparison with genotypic outcome. E.g. in a patient whose subtype C virus exhibited a K103N mutation, a slightly increased IC₅₀ was obtained at several repeated analyses. Since the standard sequencing only validates the major viral population, the possibility cannot be excluded that the genotypic assay failed to detect minor quasispecies contributing to the slightly increased IC₅₀ values to ETR detected here. However, an in-house allele-specific PCR could not identify any Y181C minor quasispecies.

Patient	NNRTI	fg RT/ml	ETR IC ₅₀ (μM)	Mutations at amino acid									Antiviral score according to			RNA copies/ml	HIV subtype
				98 A	100 L	101 K	103 K	108 V	179 V	181 Y	188 Y	190 G	Stanford	Tibotec	Monogram		
BH10	NA	NA	0.9±0.16										ND	ND	ND	NA	B
L100I	NA	NA	13.0±2.9		I								ND	ND	ND	NA	B
1	None	16	1.8										0	0	0	1800	C
2	None	159	3.4										0	0	0	66000	C
3	None	682	5.2±0.54				N						0	0	0	n/a	C
4	None	828	1.6±0.04										0	0	0	n/a	C
5	None	28	1.4										0	0	0	n/a	C
6	None	357	1.8										0	0	0	n/a	C
7	None	32	2.3±0.32										0	0	0	n/a	C
8	None	70	1.4										0	0	0	n/a	C
9	None	628	3.1±0.07										0	0	0	n/a	C
10	None	21	2.8										0	0	0	7900	CRF01 AE
11	None	91	2.4										0	0	0	14000	A
12	None	106	2.3										0	0	0	16700	B
13	None	105	2.5										0	0	0	27700	A
14	None	437	3.4										0	0	0	56800	B
15	None	181	2.4										0	0	0	18100	CRF01 AG
16	EFV	45	1.2				N						0	0	0	12000	B
17	NVP	276	3.9	G					I				5	1	0	62000	A
18	EFV	98	14.5	G		E	N	I					15	2	2	17000	B
19	EFV	33	8.5	G			N				F		5	1	0	4200	B
20	NVP	203	>100						E	C			35	2.5	7	44000	CRF06 CPX
21	NVP	201	>100	G						C			35	3.5	4	24000	A
22	NVP	6	20.0	G					I	C			35	3.5	4	6300	A
23	NVP	329	2.5						I				0	0	0	183000	A
24	EFV	25	17.6			KE	N						15	0	0	11000	B
25	EFV	731	4.1										0	0	0	261000	B
26	EFV	3	4.9				N	I					5	0	0	1500	C
27	EFV	574	4.1										0	0	0	29000	C
28	EFV	434	2.8										0	0	0	54000	C

Table 4. Effects of etravirine on HIV-1 RT recovered from plasma of HIV-1 infected patients. Genotypic and phenotypic characterisation was performed on HIV-1 (expressed as fg RT/ml) from patients without treatment (1-15) or with NNRTI containing treatment (16-28). If no standard deviation is indicated, experiments were run at single occasions. The two reference recombinants RT, (BH10)-wild-type (WT) and its mutant form L100I, were tested in 14 experiments. Three genotypic scoring systems (Stanford, Monogram, Tibotec) defining predicted ETR susceptibility are presented. HIV-1 subtypes were defined by the pol gene. Abbreviations: NA, not applicable; n/a, not available; IC₅₀, inhibitory effect; NNRTI, non-nucleoside RT inhibitors; ETR etravirine; NVP, nevirapine; EFV efavirenz.

The correlation between the scoring systems (Tibotec ($r=0.60$ $p < 0.001$), Monogram ($r=0.63$ $p < 0.0004$), and Stanford ($r=0.80$; $p < 0.0001$)) and outcome of the method was significant for all algorithms despite that known ETR RAMs were detected in only 54% of patient isolate (7/13) by direct sequencing. As these scores are mostly directed to balance the major ETR mutations the differences between phenotypic and genotypic assays are not unexpected [145].

To summarise this paper, as general finding, the changes in IC_{50} values for ETR (and NVP) correlated well with the predicted results from direct sequencing of the *pol* region. The ExaVir Drug approach has previously been applied for NVP with success [127, 128] and its usefulness was confirmed in this paper and extended to ETR. In order to describe a more precise laboratory cut-off for decreased susceptibility and a clinically relevant cut-off, a more extensive evaluation has to be performed. However, we believe that this phenotypic RT drug susceptibility assay could be a low cost alternative for to genotyping or conventional phenotyping in limited resource settings in studying resistance to first and second generation NNRTIs.

10.4 PAPER IV

Study background

The aim of this paper was to perform a further clinical evaluation of our newly adapted RT-based assay for assessment of resistance to ETR in patients with past or ongoing failure on the first generation NNRTIs. We compare RT phenotype with the genotype obtained with standard direct sequencing. In those cases where there was a discrepancy between the major genotype and the RT phenotype, ultra-deep pyrosequencing (UDPS) was also performed to identify any minor sequence variants in the HIV-1 RT gene. Two reference recombinants RT's, BH10-wild-type and its mutant form L100I were included in the analyses of the RT-based phenotypic assay.

Altogether, 25 plasma EDTA samples of 20 HIV-1 infected patients (Table 5) were retrospectively included from the HIV cohort at Department of Infectious Diseases, Karolinska University Hospital, Stockholm, Sweden and analysed for ETR resistance by a reverse-transcriptase based phenotypic assay.

Of these, 15 treatment-experienced patients were randomly selected among subjects with ART failure. For eight samples the failing regimen contained an NNRTI. For 12 samples, the NNRTI had been stopped earlier and the failing regimen contained now antiretroviral drugs from other categories. Four treatment naïve patients were also included since they had been infected with NNRTI-resistant strains.

Genotypic resistance test, GRT, had been performed within the clinical care by direct sequencing of the *pol* gene in all of these individuals and one or more NNRTI mutations

had been found in all of them, except in one patient (patient 7). One NNRTI-naïve patient (patient 4), who had a K103R mutation, was also chosen as a negative control since this mutation is reported not to affect ETR susceptibility.

Pat.*	Sex*	Age*	Sub-type	Ongoing NNRTI treatment**	Earlier NNRTI treatment***	Other ongoing drugs****	HIV load (copies/ml)	CD4 count (cells/μl)
1	m	37	D	no	EFV (-134), NVP (-6)	no	17800	200
2	m	43	B	no	no	no	292000	100
3a	m	41	B	no	EFV (-472)	no	161000	10
3b				ETR (111)	EFV (-585)	DRV/r	117000	10
4	m	52	B	no	no	no	63000	373
5a	m	61	B	EFV (236)	-	LPV/r, T20	21800	300
5b				EFV (248)	-	LPV/r, T20	47000	335
6	f	48	C	no	EFV (-250), NVP (-60)	TDF, FTC, T20	6700	155
7	m	56	C	no	EFV (-286)	ATV,TDF,ABC,3TC	1500000	22
8a	m	46	B	NVP (337)	EFV (-418)	ABC, 3TC, TDF	13100	126
8b				NVP (339)	EFV (-420),	ABC, 3TC, TDF	3920	126
9a	m	61	B	no	EFV (-422), NVP (-192)	LPV/r, 3TC, RAL	8400	209
9b				no	EFV (-448), NVP (-218)	LPV/r, 3TC, RAL	20000	246
10	m	49	B	NVP (262)	EFV (-286)	ABC,3TC,TDF,T20	10400	662
11a	f	49	A	EFV (180)	-	ABC, 3TC	2600	260
11b				no	EFV (-308)	no	12200	219
12	m	42	C	no	NVP (-18)	ATV/r, ABC, 3TC	1100	380
13	f	40	C	no	no	no	151000	9
14	m	53	B	no	EFV(-172)	no	100001	420
15	m	51	B	no	EFV (-308)	DRV/r, TDF, FTC	910000	40
16	f	36	A	no	no	no	4420	303
17	f	42	D	no	NVP (-19)	ABC, 3TC, ZDV	1900	159
18	m	49	D	NVP (11)	EFV (-220)	ABC, 3TC, ZDV	12000	345
19	m	49	B	no	no	no	29000	234
20	f	49	B	no	EFV (-8)	no	4700	400

Table 5. Characteristics of 20 HIV-1 infected patients with antiretroviral treatment failure at the time of plasma sampling. *Patients were selected due to a failing ART with the exception of patients no 2, 13, 16, 19 who were infected with an NNRTI-resistant strain; a and b indicate a first and a second sample; m: male; f: female; age: years;**Figure within brackets indicate the number of weeks from the start of the last ongoing NNRTI-treatment to the sampling date. ***Figure within brackets indicates the number of weeks from cessation of the prior NNRTI-containing treatment to the sampling date.****ABC: abacavir; 3TC: lamivudine; FTC: emtricitabine; TDF: tenofovir; ZDV: zidovudine; LPV/r: lopinavir/ritonavir; ATV/r: atazanavir/ritonavir; DRV/r: darunavir/ritonavir; RAL: raltegravir; T20: enfuvirtide.

To be able to predict ETR susceptibility, three genotypic scoring systems were used, Stanford University, Monogram Weighted Score, Tibotec Weighted Genotype Score. The correlation between Monogram/Tibotec/Stanford scorings and IC₅₀ determined by our phenotypic RT assay was assessed by Spearman's rank test using software in GraphPad Prism version 5 (San Diego, California, USA).

Results and discussion

Phenotypic assay results in relation to mutational patterns

RT was isolated from 20 plasma samples from 15 patients with treatment failure (Table 6). In ten samples (3a, 4, 7, 9a, 11b, 12, 15, 16, 17, 18) with the lowest IC₅₀ values (mean \pm SD: 3.1 \pm 1.3; range: 0.7 - 4.5 μ M), there was a good concordance with the GRT. Thus, sequence analysis showed no mutations or non-ETR RAMs.

Six samples had IC₅₀ values which according to our earlier evaluation [146] can be considered as slightly increased (range: 6.4 – 13.6 μ M; 1: 11.8 μ M, 9b: 9.2 μ M, 10: 13.6 μ M, 11a: 7.3 μ M, 19: 6.4 μ M, 20: 7.2 μ M). In all of them only non-ETR RAMs were found. Four samples of three patients showed increased IC₅₀ values (5a: 45.8 μ M; 5b: 71.4 μ M; 13: 68.8 μ M 14: 29.1 μ M), while direct sequencing showed mutations which are not known to be predictive for decreased sensitivity for ETR (5a and 5b: K101I/R+V106G; 13: K103N+G190A; 14: A98S+K103N+E138A). Five samples (2, 3b, 6, 8a, 8b) had strongly increased IC₅₀ (>100 μ M). The mutational patterns were, to varying degree, predictive of a decreased sensitivity to ETR, including Y181C (3b, 8a, 8b), V901+L100I+K103N (6), and A98S+E138E/Q+K238T (2). The correlation between the Stanford scoring system and the phenotypic assay results ($r = 0.70$ $p < 0.0001$) was significant. The Monogram and Tibotec scoring systems also correlated with the IC₅₀ values ($r=0.65$ $p<0.0005$; $r=0.64$ $p<0.0005$, respectively).

One possible explanation for the discrepancy between the phenotypic and the genotypic test results obtained by direct sequencing is resistance in minor viral variants. Therefore, UDPS was used in nine samples in which a discrepancy was found.

Patient/ Sample	ETR IC ₅₀ (μM)	Mutations at amino acid													Antiviral score according to		
		90 V	98 A	100 L	101 K	103 K	106 V	108 V	138 E	179 V	181 Y	188 Y	190 G	238 K	Stanford	Tibotec	Monogram
BH10*	1.0±0.18														NA	NA	NA
L100I*	12.0±2.3														15	2.5	4
1	11.8		S				A								0	0	2
2	>100					N			Q					T	10	1	2
3a	4.5		S							D					10	1	1
3b	>100	I	S							D	C				45	4.5	6
4	3.9					R									0	0	1
5a	45.8				I/R		G								0	0	0
5b	71.4				I/R		G								0	0	0
6	>100	I		I		N									20	3.5	5
7	0.7														0	0	0
8a	>100					N					C				30	2.5	4
8b	>100					N					C				30	2.5	4
9a	4.6		S			N		I						T	0	0	1
9b	9.2		S			N		I						T	0	0	1
10	13.6					N		I							0	0	0
11a	7.3					N				I					0	0	1
11b	4.1									I					0	0	1
12	2.0					N									0	0	0
13	68.8					N							A		10	1	1
14	29.1		S			N			A						5	1.5	3
15	2.9					N									0	0	0
16	1.7					N									0	0	0
17	2.9					N									0	0	0
18	4.1	I				N									0	1	1
19	6.4					N									0	0	0
20	7.2					N									0	0	0

Table 6. Effects of etravirine on HIV-1 reverse transcriptase, recovered from plasma of HIV-1 infected patients. * Two recombinant reverse transcriptase, (BH10)-wild-type (WT) and its mutant form L100I, were used as references. Abbreviations: NA, not applicable; IC₅₀, inhibitory effect; ETR etravirine.**Genotype result was obtained six months earlier. The results of three genotypic scoring systems (Stanford, Monogram Weighted Score, Tibotec Weighted Genotype Score) predicting ETR susceptibility are presented. A Stanford score of 0-<15, ≥15-<60 and ≥60 are defined as susceptible, intermediate and resistant. Tibotec weighted genotypic score of 0-2, 2.5-3.5 and ≥4 are predictive of susceptible, intermediate and reduced response. Monogram defines a weighted score of 0-3 as susceptible and ≥4 as resistant.

Ultra-deep pyrosequencing (UDPS) results

UDPS was performed on nine samples (1, 2, 5b, 10, 11a, 13, 14, 19, 20) for which there seemed to be a discrepancy between the major genotype and the RT phenotype (Table 7). When direct sequencing was compared with UDPS, all mutations corresponding to >20% were detected and no mutations corresponding to <20% of the viral population. Altogether eleven mutations were detected by UDPS, but not by direct sequencing, ranging from 0.54% to 19.56%. There was a concordance between the direct sequencing and the UDPS for mutations consisting of >20% of the viral population. Also, eleven additional RAMs were found by UDPS, in all cases <20% of the viral population, which is well in line with earlier results on the detection levels of direct sequencing [107, 108, 110, 147].

In four of the samples (1, 11a, 13, 14), the UDPS detected minor variants including such which are associated with decreased ETR susceptibility and may have contributed to the phenotypic resistance explaining the discrepancy with the genotype obtained by direct sequencing.

In three samples (2, 5b, 20), identical mutations were found with the two sequencing techniques. Thus, the UDPS did not revealed any further minor variants that could explain the increased IC₅₀ values of 100 µM, 71.4 µM and 7.2 µM, respectively.

In the remaining two samples (10, 19), the UDPS showed additional minor mutations not known to be associated with decreased ETR susceptibility.

Patient/ Sample	ETR IC ₅₀ (μM)	Mutations at amino acid*												Antiviral score according to		
		90 V	98 A	100 L	101 K	103 K	106 V	108 V	138 E	179 V	181 Y	190 G	238 K	Stan	Tibo	Mon
1	11.8	Direct UDPS **2409(1127-3244)	S S *68.63%				A A 18.8%	I I 0.54%		I I 0.57%	C C 6.88%			0 30	0 2.5	2 7
2	>100	Direct UDPS 1958 (1108-2870)							Q Q 32.47%				T T 98.64%	10 10	1 1	2 2
5b	71.4	Direct UDPS 2305(1201-3397)			I/R I/R 65.64%/ 31.09%		G G 99.83%							0 0	0 0	0 0
10	13.6	Direct UDPS 1704(876-2325)				N N 98.85%		I I 100%		I I 3.24%				0 0	0 0	0 1
11a	7.3	Direct UDPS 1613(873-1887)				N N 97.44%				I I 100%		A A 3.59%		0 10	0 1	1 2
13	68.8	Direct UDPS 2506(1546-2696)	I I 2.26%		I I 0.61%	N N 99.41%						A A 95.57%		10 25	1 6	1 4.5
14	29.1	Direct UDPS 1855(1118-2536)		S S 96.77%		E E 0.81%	N N 22.1%		A A 100%				T T 19.56%	5 15	1.5 2.5	3 6
19	6.4	Direct UDPS 1499(832-2325)				N N/S 95.07%/ 3.05%	I I 1.09%							0 0	0 1.5	0 2
20	7.2	Direct UDPS 2412(1228-2953)				N N 97.37%								0 0	0 0	0 0

Table 7. NNRTI-resistance results obtained through phenotypic testing, direct sequencing and ultra-deep sequencing (UDPS). ETR = etravirine; Direct = Direct Sanger sequencing; UDPS = Ultra-Deep Pyrosequencing; Stan = Stanford; Tibo = Tibotec; Mono = Monogram. *figure beneath amino acid indicate percentage consisting of a mutated population as determined by UDPS. ** Median (IQR) nucleotide coverage per patient.

Clinical interpretation of the resistance results:

Resistance results in patients who had stopped NNRTI. Twelve samples (1, 3a, 6, 7, 9a, 9b, 11b, 12, 14, 15, 17, 20) of eleven patients were drawn after that an NNRTI containing regimen had been terminated (Tables 5). In patients 1 and 20, NVP and EFV had been stopped six and eight weeks earlier, respectively. The IC₅₀ values (Table 6) were slightly increased (11.8 µM and 7.2 µM, respectively) but the Stanford scoring predicted full sensitivity (A98S+V106A; K103N, respectively). However, in patient 1 UDPS revealed additional minor mutations (K103T+V108I+V179I+Y181C) which possibly could explain this difference (Table 7). In patients 6 and 14, a clear decreased phenotypic sensitivity against ETR (6: >100 µM; 14: 28.8 µM) was found despite that >1 year and > 3 years, respectively, had passed since the cessation of an NNRTI. The genotyping further supported this finding for patient 6 (direct sequencing: V90I+L100I+K103N) and patient 14 (UDPS: A98S+K101E+K103N+E138A+K238T).

In four patients, several years had passed since their NNRTI treatment was terminated (7, 11b, 15: >5.5 years; 9a: 3.5 years). In patient 12, 18 weeks had passed. No phenotypic resistance was found in these patients although non-ETR RAMs persisted (11b: V179I; 12 and 15: K103N; 9a: A98S+K103N+V108I+K238T). However, a second sample of patient 9 drawn four months later showed a slightly decreased phenotypic sensitivity (9.2 µM) with the same mutational pattern with a concomitant increase in plasma viral load. UDPS could not be performed due to lack of plasma.

Resistance results in patients with ongoing NNRTI failure. Eight samples (3b, 5a, 5b, 8a, 8b, 10, 11a, 18) of six patients were drawn during failure of an NNRTI containing regimen. In four samples (3b, 8a, 8b, 18), a concordance was seen between the pheno- and genotype. Thus, patient 3b exhibited a high ETR resistance with both methods (IC₅₀: >100 µM; genotype: V90I+A98S+V179D+Y181C). In both samples of patient 8, phenotypic resistance (>100 µM) and the mutations K103N+Y181C were found. Patient 18 had a sensitive phenotype (4.1 µM) and was devoid of mutations other than K103N+V90I after 10 weeks failure.

In four samples, discordance was seen between the RT-based phenotype and the genotype. In patient 5, two samples were drawn with three months interval during failure with EFV-containing regimen. An increasing IC₅₀ of 45.8 µM and 71.4 µM, respectively, was seen despite that direct sequencing as well as UDPS showed only mutations (K101I/R+V106G) which are not known to be ETR-associated. Patient 10 exhibited an increased IC₅₀ (13.6 µM), but the identified mutations (direct sequencing: K103N+V108I; UDPS: K103N+V108I+V179I) predicted an ETR sensitive virus. In patient 11 a sample drawn early after failure showed a slightly increased IC₅₀ (7.3 µM), while the direct

sequencing showed K103N+V179I. However UDPS identified additionally G190A giving support for that resistance was developing.

Effects of ETR on patients with transmitted NNRTI resistance. Four patients (no 2, 13, 16, 19), who had been infected with an NNRTI resistant strain, were also analysed. Patients 2 and 13 had a strongly increased IC₅₀ value (>100 µM; 68.8 µM) despite that the sequence analysis predicted only a slightly decreased sensitivity (2: K103N+E138Q, K238T; 13: K103N+G190A) (Table 2). The remaining two samples had the K103N only, and a low 1.7 µM (16) and a slightly increased 6.4 µM (19) IC₅₀ value, respectively, was found.

To summarise this paper, our RT-based phenotypic assay showed decreased ETR susceptibility in patients where direct sequencing predicted ETR-sensitive virus. The clinical treatment history was concordant with that our phenotypic results corresponded to a true decreased susceptibility for ETR. Thus, during early ART failure and before the NNRTI was stopped, an increase of the IC₅₀ was seen in four of eight samples despite that a sensitive virus was predicted by the genotype. Also, in two patients who stopped NNRTI some weeks before the sampling, an increased IC₅₀ but not ETR-resistance mutations was found. For subjects who had stopped the first generation NNRTI-containing regimen for one or more years, a good concordance between the methods was seen. In addition, in two subjects (7 and 16) with very poor adherence, no phenotypic resistance was identified and only K103N in one of them. These patient histories and the UDPS comparison indicate that our phenotypic method may detect resistance to ETR despite that the direct sequencing predicts a sensitive phenotype.

The clinical utility of the phenotypic method remains to be established. It is clear that there is a strong correlation between the results of our phenotypic method and the predicted antiviral scores according to three genotypic scoring systems. A high IC₅₀ was found in all samples with key-mutations, Y181C and L100I, which are associated to ETR resistance. However, in a substantial number of samples there was a phenotypic decreased susceptibility but no known ETR-RAMs. The clinical relevance of these findings and clinical cut off of our method can only be studied on larger patient-populations. Even so, this study showed that the adaptation of the RT based phenotypic test for detection of ETR resistance in plasma is possible. The assay is simple to perform, uses basic laboratory equipment, and does not require complex interpretations. This phenotypic RT drug susceptibility assay could therefore be a low cost alternative for studying resistance to first and second generation NNRTI and useful for studies on the kinetics of NNRTI resistance during ART failure.

11 CONCLUSIONS AND FUTURE PERSPECTIVES

Although ART has reduced HIV-1 associated morbidity and mortality, development of drug resistance has become a major problem. Ideally, when ART is rolled out in resource-limited settings, it should be coupled to virological monitoring. However, due to a high cost and lack of technical equipment and expertise, this is frequently not available. This increases the risk of undetected treatment failure and the development of widespread drug resistance.

The prevalence of transmitted drug resistance (TDR) is of importance for determining the choice of 1st line ART, which in Vietnam and other LMICs consists of NNRTI-based ART. In **Paper I** the prevalence of TDR was 6.3%, which is slightly higher compared to other recent studies from South-East Asia. Nonetheless, apart from one patient whose virus had Y181C mutation, the TDR detected are of limited clinical importance and do not rule out the use of the standard first-line treatment regimen. However, in view of the increasing use of different antiretroviral drugs in Vietnam it is important to regularly monitored prospectively in Vietnam.

In **Paper II** we showed that the RT VL assay was a useful tool for monitoring VL and feasible for monitoring virological outcome and assess ART efficacy in Vietnam. The RT VL assay also displayed a strong correlation with Cobas TaqMan PCR and showed similar sensitivity to the PCR-based method. This, together with the fact that the RT assay requires only basic laboratory equipment, makes the test an alternative technique for developing countries. Having access to VL can facilitate early detection of drug failure and aid in enhancing early adherence support and the choice of new therapy regimes, thereby preventing the emergence of more advanced drug resistance patterns. Keeping in mind that most ART programs in LMICs are presently based on a limited number of drugs, implementing these programs without simultaneous implementation of VL testing is risky, as initial treatment gains will be ultimately lost to future drug failures and spread of resistant viral strains.

Assays using RT purified from the virus particles of a patient sample allow both for VL quantification and assessment of phenotypic drug susceptibility, the latter being important not only at initiation of ART but also at therapy switch after treatment failure. The RT-based methodology has earlier been described for measuring phenotypic drug susceptibility to the first generation NNRTIs (EFV and NVP) [127, 128, 143]. In **Paper III** we showed that the RT-based phenotypic resistance assay can be used for detection of resistance also to the second generation NNRTI ETR, including cross-resistance to other NNRTIs, in clinical samples. Low IC₅₀ values were shown in treatment-naïve patients. Furthermore, a good reproducibility was demonstrated, with the outcome of the test being independent on the amount of plasma HIV RNA. Also, in treatment naïve-patients, the result concordantly showed high IC₅₀ values in plasma RT where Y181C was detected by direct sequencing. Intermediate IC₅₀ values were associated with other known ETR associated mutations, confirming the reliability of our assay.

In **Paper IV** a further thorough clinical evaluation of the RT-based resistance assay was performed in patients with past or ongoing failure with first generation NNRTIs. Most of the strains had a various degree of decreased ETR susceptibility as measured by the phenotypic test. However, the mutational patterns based on direct sequencing did not always predict ETR resistance, potentially indicating that the assay may overestimate the presence of decreased drug susceptibility. Nevertheless, in **Paper III** ETR gained expected changes in IC₅₀ values for all recombinant HIV-1 RTs equivalent to previous report [59, 127]. Thus, it is clear that the method can discriminate between mutants with or without ETR RAMs. One possible explanation for the discrepancy between the phenotype and the genotypic test results obtained by direct sequencing can be resistance in minor viral variants. In **Paper IV** we analysed patient samples with unexpectedly high IC₅₀ values with UDPS, and found minor viral populations with ETR RAMs in four of the nine samples. In at least three of them, the proportion of mutated virus was sufficiently high to provide a potential explanation for the discrepancy between phenotype and the genotype obtained by direct sequencing. This indicates that our phenotypic method may in fact be more sensitive than direct sequencing in identifying minor quasispecies with RAMs in the RT gene. Also, the clinical treatment history for the patient included in **Paper IV** was concordant with that our phenotypic results corresponded to a true decreased susceptibility for ETR. The patient histories and the UDPS comparison indicate that our phenotypic method may detect resistance to ETR despite that the direct sequencing predicts sensitive phenotype.

The cause of the discrepancies between the phenotype and the genotype results is not known presently. The mutations predictive for cross-resistance to ETR-resistance have been mainly identified in clinical studies using direct sequencing and it cannot be excluded that not-yet described mutations exist which may influence the ETR sensitivity. The effects of defined mutations may also vary in different genetic environments. Our phenotypic method uses lysates originating from intact virions. The genotypic and phenotypic method based on recombinant viruses use HIV RNA which partly may represent defective virus [148], therefore it can be speculated that our phenotypic assay is more representative for the ongoing viral replication.

A less costly and technically simple test such as the RT assay presented in this thesis could be considered for VL monitoring and resistance testing as alternatives to conventional HIV RNA quantification by Roche Cobas TaqMan PCR and direct sequencing, in regions where expensive molecular-based methods are not a viable alternative. ExaVir Load was successfully used in a Uong Bi General hospital in northern Vietnam, and its implementation in other settings should still be possible provided basic laboratory equipment such as a spectrophotometer and an incubator are available. Regarding, the phenotypic assay for detection to ETR resistance, to describe a more precise laboratory (technical) IC₅₀ cut-off for decreased drug susceptibility an extensive evaluation has to be carried out. Although, access to ETR in LMICs still is limited, ETR may be of relevance for LMICs since resistance to the first generation

NNRTIs as well as to NRTIs is an increasing problem in treatment of HIV-1 infected patients in resource-limited settings.

Although, a further evaluation is needed to define clinical cut-offs we believe that the RT-based assay may be an alternative to more costly HIV drug resistance tests, especially in LMICs. The possible use for measuring resistance towards NRTIs as well as the other second generation NNRTI, RPV, may also be of interest since RT inhibitors are widely used both in high- and low-income countries.

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