From Microbiology and Tumorbiology Center (MTC)
Karolinska Institute (KI) and the Swedish Institute for Infectious Disease Control (SMI), Stockholm, Sweden

Evaluation of Reverse Transcriptase Assay for Viral Load Monitoring

Gary E Corrigan

Karolinska Institutet

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To the memory of my mother,
the arrival of my son,

…..and Jennifer.

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LIST OF ABBREVIATIONS

AIDS, Acquired immunodeficiency syndrome
ART, Anti-retroviral treatment
ARV, Anti-retroviral
ATP, adenosine triphosphate
AZT, Azidodeoxythymidine
bDNA, branched DNA
BrdUTP, Bromo-deoxyuridine triphosphate
CCR, CC chemokine receptor
CD4+ cells, Helper T lymphocytes
cDNA, complimentary DNA
CXCR, CXC chemokine receptor
ddI, Didanosine
DNA, Deoxy-ribonucleic acid
dNTP, Deoxy-nucleotide triphosphate
EB, External buffer (supplied by J Schüpbach)
ELISA, Enzyme linked immunosorbent assay
env, Envelope gene
FI, Fusion inhibitor
FIV, Feline immunodeficiency virus
gag, Group specific antigen gene
HIV, Human immunodeficiency virus
HRP, Horseradish peroxidase
LOD, Level of detection
LTR, Long terminal repeat
mAb, monoclonal antibody
mMuLV, Moloney murine leukemia virus
mRNA, messenger RNA
NASBA, Nucleic acid sequence-based amplification
NIBSC, National Institute for Biological Standards and Control
NNRTI, Non-nucleoside reverse transcriptase inhibitor
NRTI, Nucleoside/nucleotide reverse transcriptase inhibitor
odT, oligo-deoxythymidylic acid

PBMC, Peripheral blood mononuclear cell

PCR, Polymerase chain reaction

PERT, Product enhanced reverse transcriptase

PI, Protease inhibitor

PNPP, Para-nitrophenyl phosphate

pol, Polymerase gene

prA, poly ribocytidylic acid

RNA, Ribonucleic acid

RT, Reverse Transcriptase

RT-PCR, reverse transcriptase polymerase chain reaction

SIV, Simian immunodeficiency virus

SHIV, Simian-human immunodeficiency virus

SU, Outer surface protein

TAM, Thymidine analogue mutation

TM, Transmembrane protein

TP, Triphosphate

UK, United Kingdom

US, United States of America

VL, Viral load

VQA, Viral Quality Assessment

WHO, World Health Organisation

WT, Wild-type

LIST OF PUBLICATIONS

This thesis is based in the following communications, which are referred to in the text by their roman numerals:

I Corrigan GE, Al-Khalili L, Malmsten A, Thorstensson R, Fenyo EM, Kallander CF, Gronowitz JS. **Differences in reverse transcriptase activity versus p24 antigen detection in cell culture, when comparing a homogeneous group of HIV type 1 subtype B viruses with a heterogeneous group of divergent strains**. AIDS Res Hum Retroviruses. 1998 Mar 1;14(4):347-52.

II Greengrass VL, Turnbull SP, Hocking J, Dunne AL, Tachedjian G, Corrigan GE and Crowe SM. **Evaluation of a low cost reverse transcriptase assay for plasma HIV-1 viral load monitoring,** Curr HIV Res, 2005, 3, 183-90.

III Jennings C, Fiscus SA, Crowe SM, Danilovic AD, Morack RJ, Scianna S, Cachafeiro A, Brambilla DJ, Schüpbach J, Dunne A, Stevens W, Respess R, Oliviero E. Varnier OE, Corrigan GE, Gronowitz JS, Ussery MA, and Bremer JW**. Comparison of two human immunodeficiency virus (HIV) RNA surrogate assays to the standard HIV RNA assay**. J Clin Micro. 2005, 43 (12), 5950-6.

IV Corrigan GE, Olausson-Hansson E, Mörner A, Berry N, Källander CFR and Thorstensson R. **Reverse transcriptase viral load correlates with RNA in SIV/SHIV infected macaques.** AIDS Res Hum Retroviruses. 2006 Sept 1;14(4):347-52.

V Corrigan GE, Grutzmeier S, Albert J, Källander CFR and Thorstensson R**. Accumulation of NRTI resistance mutations in the HIV-1 genome in vivo results in an initial but transient reduction in reverse transcriptase fitness.** Submitted.

RELATED DOCUMENTATION, NOT INCLUDED IN THE THESIS

Cox SW, Corrigan G, Palmer S. **Inhibition of azidothymidine triphosphate of RT from paired AZT-susceptible and resistant isolates of HIV-1**. Antivir Chem Chemotherapy. 1995 6(2), 123-6.

Awad RJ, Corrigan GE, Ekstrand DH, Thorstensson R, Kallander CF, Gronowitz JS. **Measurement of levels of human immunodeficiency virus type 1 reverse transcriptase (RT) and RT activity-blocking antibody in human serum by a new standardized colorimetric assay**. J Clin Microbiol. 1997, 35 (5):1080-9

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Malmsten A, Shao XW, Aperia K, Corrigan GE, Sandstrom E, Kallander CF, Leitner T, Gronowitz JS. **HIV-1 viral load determination based on reverse transcriptase activity recovered from human plasma**. J Med Virol. 2003 Nov;71(3):347-59.

Tuaillion E, Gueudin M, Lemée V, Gueit I, Roques P, Corrigan GE, Plantier J-C, Simon F, Braun J. **Phenotypic susceptibility to nonnucleoside inhibitors of virion-associated reverse transcriptase from different HIV types and groups.** JAIDS, 2004, Vol 37, 5, 1543-9.

Lombart JP, Vray M, Kafando A, Lemée V, Ouédraogo-Traoré R, Corrigan GE, Plantier JC, Simon F and Braun J**. Evaluation of plasma virion reverse transcriptase activity and heat-dissociated-boosted p24 assay for HIV load monitoring in Burkina Faso, West Africa.** AIDS, 2005, 19: 1273-7.

ABSTRACT

Retaining an active reverse transcriptase (RT) enzyme is a fundamental requirement for all retroviruses to replicate. Bearing in mind that HIV has a very high propensity to mutate measuring RT activity to determine the level of retroviral replication has the capacity to eliminate problems associated with divergence, as the virus at all costs must retain an active RT enzyme. We show that two p24 antigen assays utilizing different p24 capture antibodies quantify HIV-1 replication inadequately. We also documented the development (version 1 and 2) and use of a RT assay for the quantification of viral load in HIV infected individuals and SIV/SHIV infected macaques. Finally, we defined RT-fitness as the ratio of HIV-1 RT activity/RNA (fg RT/1000 RNA copies) in an attempt to determine if mutations associated with ARV therapy alter the fitness of the RT enzyme.

Our results showed that the RT assay strongly correlated with conventional methods for viral load determination of both HIV in humans and SIV/SHIV in macaques. Trends in RT-load and RNA-load mirrored each other even under ARV therapy, indicating that both assays quantified the same fundamental process of viral replication, even though they measured two different replication markers. Regarding RT-fitness, the NRTI resistance mutation T215Y was associated with reduced RT-fitness, while acquisition of L74V in viruses already containing T215Y increased the RT-fitness. To conclude, the RT assay documented in this thesis should be considered as a viable alternative for viral load monitoring, particularly in regions where expensive and complex gene-based technologies are not a viable option. Furthermore, RT-load may have the potential to add a further virological fitness dimension to viral load measurement and should be further investigated.

INTRODUCTION

AIDS: The beginning

AIDS was first described in several cities in the US in the early part of the 1980's [1]. Initially, increasing numbers of cases of Pneumocystis *jiroveci* (formely know as *carinii*) pneumonia and Kaposi's sarcoma were documented in homosexual men and intravenous drug abusers. Remarkably such diseases were very uncommon in these populations and usually only seen in persons with immunodeficiency. In the wake of these discoveries similar cases of immunodeficiency were described in haemophiliacs and blood transfusion recipients [2]. All cases showed a similar presentation of clinical symptoms associated with reduced T-helper cell count, increasing severity of immunodeficiency, wasting and eventual death, usually due to the complications of opportunistic infections [3].

HIV: Origins

HIV, the causative agent of AIDS was discovered in 1983 [4-6]. After immense political and scientific arguing it was decided that the virus was co-discovered by both French and American scientists [7-9]. Later the same decade, in 1986, a new yet very similar virus was isolated from individuals displaying symptoms of immunodeficiency in West Africa. These individuals did not have antibodies towards the earlier isolated HIV, indicating that a different agent was causing their immunodeficiency [10]. This virus was later designated as HIV-2 and cases were discovered even outside of Africa [11]. Both HIV-1 and -2 are retroviruses and our closely related mammalian cousins, the monkeys, harbour similar viruses. In their natural host these simian retroviruses normally do not cause immunodeficiency. Only if the virus is transferred to a new susceptible host does the virus cause symptoms related to loss of immune function. These simian retroviruses are consequently called SIV, for simian immunodeficiency virus, and can be found in several monkey species [12]. Scientists use the SIV monkey AIDS model to study HIV and AIDS in humans, due to similarities between the species and disease symptomolgy [13, 14]. They have even genetically modified SIV to be more closely related to HIV. These chimeric

viruses contain both genetic material from the human and simian viruses and are called SHIV [15].

Due to the similarities between HIV and SIV, scientists believe that HIV was transferred from our primate cousins during cross species transmission [16]. In relation to HIV-1, the most widely spread human retrovirus, it was most likely transferred to humans on three different occasions, giving rise to three distinct genetic groups (M (main), N (non-M) and O (outlier)). HIV-1 is genetically very similar to SIV_{cpz}, the simian retrovirus found in chimpanzees [12]. While SIV_{sm} , found in sooty mangabeys is virtually identical to HIV-2 [17]. Transfer of SIV_{cpz}, to chimpanzees probably occurred during consumption of lower monkeys, as chimps do kill and eat smaller monkey species [18]. Consequently, transfer to humans has most likely taken place due to the hunting, butchering and eating of monkey meat.

HIV is a retrovirus (Latin: *retro*, backwards), so-called as it contains a RNA genome coding for a reverse transcriptase (RT) enzyme, which transcribes the viral RNA to DNA. This process is both unique and specific to retroviruses. Furthermore, this genetic flow of information is essentially backwards compared to all other organisms.

Over the past 20 years HIV/AIDS has become a worldwide pandemic involving millions of people [19-29]. Particularly affected are countries of the developing world [30], which make up over 80% of all infected. Alarmingly, some areas of sub-Saharan Africa, such as Botswana and South Africa are estimated to have over 30% HIV sero-positive prevalence rates [31-37]. These countries in recent times have introduced national treatment programs to combat HIV/AIDS. The reduction in price of antiretroviral drugs has certainly helped such endeavours [38-40]. Even so, the availability of drugs is only part of the solution. Structured patient care and monitoring also needs to be implemented and maintained. Currently used gene-based technologies such as RT-PCR, bDNA and NASBA [41-44] are typically used in the developed world to monitor the level of HIV in patients blood (viral load (VL) monitoring). Unfortunately, these techniques are often expensive, complex and require sophisticated laboratory infrastructure. Consequently their use in developing countries has been limited.

Structure and genomic organisation of HIV-1

HIV-1 is a spherical particle of approximately 100 nm. The outer envelope is composed of a phospholipid bilayer derived from the infected host cell, with viral proteins embedded and protruding from the surface. A schematic representation of HIV-1 is shown in Figure 1. HIV-1 is a *lentivirus* of the *retroviradae* 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 replicative 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.

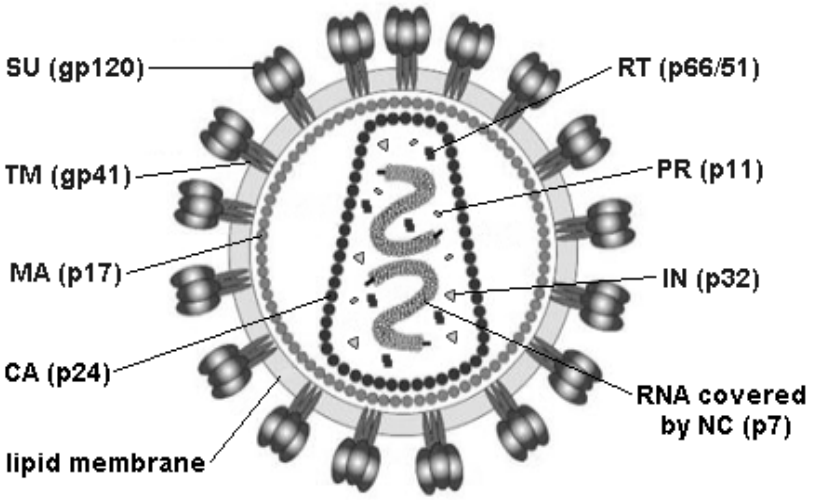

Figure 1: Structure of the HIV-1 virion (courtesy of Anders Malmsten).

Of the *retroviradae* the HIV-1 genome is rather complex (9.3 kb long) (see Figure 2), consisting of the standard *env*, *pol* and *gag* gene format with a host of other accessory genes (*vif*, *vpr*, *vpu*, *nef*, *tat* and *rev*) that are involved in regulation of viral replication [45].

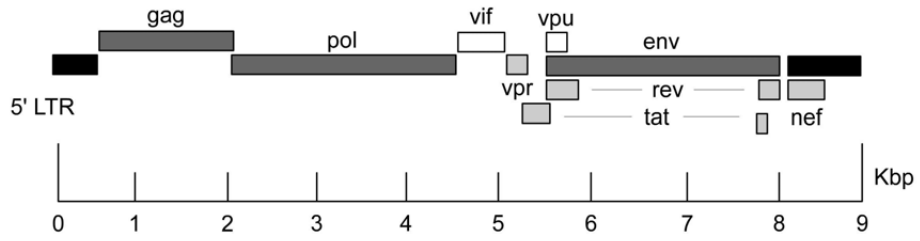

Figure 2: Schematic representation of the HIV-1 genome

Table 1 describes the basic functions of the HIV-1 genes, however, a more detailed description can be found at Frankel *et al* [46]. The genes are flanked at both sides by LTRs, which are integrally involved in the transcription of the RNA [47]. The envelope (*env*) gene codes for the glycosylated surface proteins, gp120 (SU) and gp41 (TM). The gp120 protein protrudes from the envelope surface and is anchored to the virus membrane via the gp41 trans-membrane protein. 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.

Table 1: Genes encoded by HIV-1 and their function

Gene	Functional protein(s)	Function(s)
env	SU (gp120) TM (gp41)	Binds to CD4 on host cell membrane Involved in fusion with host cell membrane
gag	MA (p17) CA (p24) NC (p7) P6	Matrix protein associated to viral envelope Viral capsid structural protein Forms complexes with RNA Involved in viral packing of Vpr
pol	RT (p66/51) IN (p32) PR (p11)	Reverse transcription and RNase-H activity Integration of provirus in to host cell genome Protelytic cleavage of precursor viral proteins
nef	Nef (p27)	Increases infectivity, CD4 degradation and proviral DNA synthesis, packed inside virion
rev	Rev (p19)	Regulates processing of viral mRNA
tat	Tat (p14)	Transcriptional transactivator and enhancer
vif	Vif (p23)	Enhances infectivity, packed inside virion
vpr	Vpr (p15)	Mediates transport of viral proteins to nucleus, arrests cell cycle, packed inside virion
vpu	Vpu (p16)	Enhances CD4 degradation and particle release

HIV-1 Life Cycle

Like all other exogenous viruses HIV-1 requires a host cell for replication. The major host cell target for HIV-1 is cells expressing the CD4 surface antigen [48]. However, some other cell surface molecules have been demonstrated to mediate HIV-1 binding and internalisation [49]. The viral ligand for the CD4 receptor is the gp120 molecule. Binding of gp120 to CD4 leads to conformational changes in the gp120 molecule essential for facilitating viral entry. Other co-receptors such as CCR5 and CXCR4 are also involved with viral entry and have been shown to determine syncytium inducing capacity and host cell tropism [50, 51]. The CCR5 receptor is most often utilised by non-syncytium inducing macrophage tropic viruses while CXCR4 is mainly used by syncytium inducing T-cell tropic strains. Interestingly, complete absence of the CCR5 receptor on cells results in strong protection from HIV-1 infection both *in vitro* and *in vivo*, while decreased expression seen in heterozygosis Δ32-CCR5 allele expression has been linked to reduced disease progression in infected individuals [52, 53].

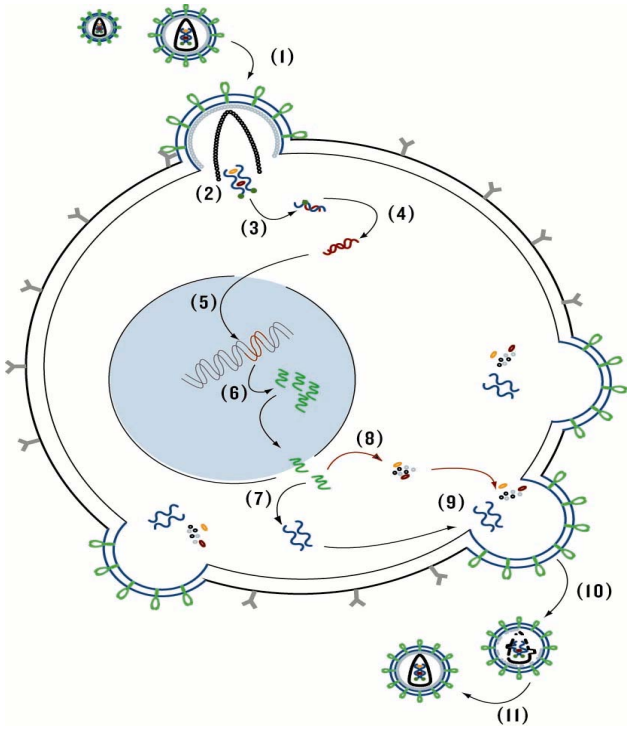

Figure 3: HIV-1 replication cycle

The HIV-1 replication process (see Figure 3) begins with the binding of the virus gp120 to the host cell CD4 receptor (1) [54]. This initiates a conformational change in the gp120 [55, 56] allowing binding to the adjacent co-receptor, either CCR5 or CXCR4. This process enables the TM peptide gp41 to assist in the fusion of the host cell and viral membranes [57] and facilitates viral entry. Following entry, uncoating occurs in the cell cytoplasm (2). Production of cDNA from the viral RNA is carried out by the reverse transcriptase enzyme (3) [58]. The resulting cDNA is then transported to the cell nucleus with help of the vpr protein (4) and integrated in the host cell DNA by the viral integrase enzyme (5), a process which may not be entirely random [59, 60]. Once this has occurred the viral DNA remains a part of the cell DNA and will be transcribed as a cellular gene. Although the HIV-1 replication is at this stage fully reliant on cellular transcription and translation (6) the virus does carry additional genes, which have the capacity to regulate the level of replication [61-64]. The cellular RNA polymerase produces the viral mRNA, initially only coding for the accessory proteins takes place, but later under their influence full-length transcripts of the other proteins appear (7-8). Translation of large precursor polyproteins occurs in the cytoplasm by the ribosomes. The precursors are cleaved by the viral protease enzyme prior to assembly (9). The newly assembled viral particles are released by budding from the cell membrane (10). Additional proteolytic cleavage of the gag and gag-pol polyproteins occurs inside the virus after budding (11). Finally the virus is mature and fully functional, ready to infect a new CD4+ cell.

Course of HIV-1 infection

HIV-1 infection can be divided in to three distinct stages; primary infection, clinical latency and clinically apparent disease (see Figure 4). Primary infection constitutes the initial stage of disease directly after infection and is associated with the dissemination of virus throughout the body of the newly infected individual. The clinical presentation of primary infection is rather diffuse, ranging from no obvious symptoms to a distinct and recognisable clinical syndrome, often characterised by fever, lymphadenopathy, soar throat, rash and mucocutaneous ulcers [65, 66]. During primary infection the levels of virus in the blood is usually very high. Only after the induction of HIV-specific immune responses, which normally initially involves

cytotoxic T-lymphocytes and antibodies towards exterior viral glycoproteins, but also RT-blocking antibodies [67], does the level of viraemia decline. The initial appearance of specific antibodies towards the virus is often termed seroconversion and these antibodies are often used to diagnose the presence of HIV-1 infection. Primary infection is usually followed by a period of clinical latency where the infected individual usually shows few or no clinical signs of HIV-1 infection. The time-span for clinical latency has increased over the last 15 years due to the introduction of ART and better treatments for HIV related diseases [68, 69]. Although the patient may show no clinical signs or symptoms there is still considerable levels of viral replication occurring. The level of viremia or viral load is usually quite stable during clinical latency, and this is thought to be due to a constant level of infection of new cells and removal of infected cells by the immune system [70]. However, this on going process causes a gradual but inevitable decline in CD4+ cells that results in loss of immune function and eventual immunodeficiency. The decline in immune function usually coincides with the appearance of HIV-related illnesses or opportunistic infections. Individuals with CD4+ T-cell counts below 200 cell/mm^3 are at great risk of AIDS-defining illnesses such as severe opportunistic infections and neoplasms. Thankfully, with the advent of potent ART even patients with very low CD4+ T-cell counts (<50 cell/mm^3) can reconstitute their immune system, however, usually not to normal levels above 500 cell/mm^3.

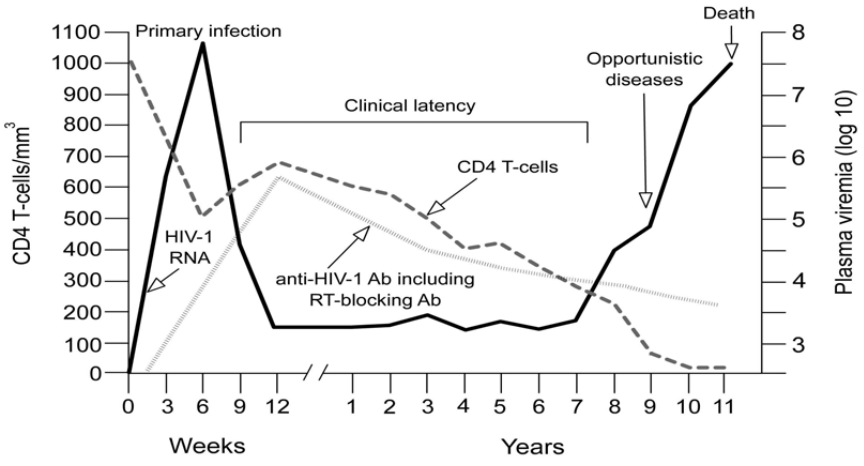

Figure 4: Course of HIV-1 infection

HIV types and subtypes

HIV can be further classified into two types, HIV-1 and HIV-2, two distinct but similar viruses [16, 71]. Both cause AIDS but HIV-2 seems to be less pathogenic [72] and possibly less transmissible through heterosexual intercourse [73]. Furthermore, reports indicate that dual HIV-1/-2 infection resembles the course of HIV-1 infection only [74-76] and that infection with HIV-2 does not seem to offer protection against future infection with HIV-1 [77]. Consequently, HIV-1 is responsible for the world AIDS pandemic while HIV-2 is usually only found in areas of West Africa [78] and countries with past socio-economical links to this area [79].

HIV is an extremely diverse virus with an immense capacity to mutate [16, 80]. HIV-1 can be divided in to several different subtypes or clades, based on the genetic characteristics of the *env* and/or *gag* genes. The largest and most common group, group M (for main) contains subtypes A to K. Additionally, recent thought puts subtype E as a recombinant A/D virus, rather than a distinct subtype. The spread of different subtypes on a global scale has been rather distinctive and is of great importance with regards to the success of future vaccines against HIV [81, 82]. HIV-1 subtype B is the dominant viral subtype in the western world, while subtype C has been responsible for most infections in sub-Saharan Africa [83], where most cases of HIV-1 are found. With the increase in air and road travel, dissemination of HIV-1 subtypes that were initially confined to particular geographic areas, has now been seen [84-87]. In addition, recombination between subtypes is quite common [88-94], particularly in endemic areas with high infection rates [83].

Groups that do not belong to group M also exist but are rather uncommon and usually only found in central Africa, in and adjacent to Cameroon [95, 96]. These groups have been designated group O (outliers) and Group N (for non-M, non-O). These viruses are extremely divergent and are widely distanced phylogenetically from group M and even from viruses in their own group. Consequently, RNA based VL tests using conserved regions of the viral genome as primer sites have been shown to inadequately amplify the more divergent strains of HIV-1 [97-103]. This problem is a

major Achilles-heel for all HIV molecular assays based on the use of specific primers, due to the incredible diversity of HIV-1.

Reverse Transcriptase (RT)

RT is the enzyme found in all retroviruses that catalyses the conversion of viral RNA to DNA, an essential step in the viral replication cycle. The RT enzyme is unique to retroviruses and is not found in virtually any other organism. This makes RT a prime target for ARV drugs and also an ideal candidate for measuring the presence of retroviruses in a sample. As a matter of historical interest, the detection of RT activity in virions isolated from chickens led to the discovery of the first retroviruses, Rous sarcoma virus in 1970 [104].

The RT enzymes of different retroviruses have been shown to have different reaction requirements for optimal activity. The C-type γ-retroviruses, such as mMuLV requires Mn^{2+} as cation, while the lentiviruses, such as HIV-1, -2, SIV and FIV all have optimal activity with Mg^{2+} [105]. Bearing this in mind, developing an assay for the measurement of HIV RT also has the added bonus of being able to quantify the activity of all other lentiviruses. Therefore a lentiviral RT activity assay has the potential to be used as a universal tool for the detection and quantification of all lentiviruses, and should not be affected by viral type or subtype.

Treatment of HIV

The HIV lifecycle provides several potential opportunities to block viral replication. The four classes of drugs currently approved or in clinical trials for treatment of HIV infection act on one of the following replication steps; (i) viral adsorption, by binding and blocking viral gp120, (ii) viral entry, through blocking usage of the viral co-receptors CCR5/CXCR4, (iii) virus-cell fusion, by binding and blocking the viral gp41, (iv) reverse transcription, by inhibiting the catalytic activity of the viral reverse transcriptase enzyme, (v) proviral DNA integration, by inhibiting the catalytic activity of the viral integrase enzyme, (vi) viral mRNA transcription process and (vii) protein maturation and cleavage, by inhibiting the viral protease enzyme.

Since the introduction of AZT (zidovudine) to treat HIV infection in 1987, several new drugs have followed (see Table 2). Todays state-of-the-art treatment involves the use of combinations of several drugs to target different areas of the viral life cycle to selectively reduce the level of viral replication.

The four classes of drugs approved for antiretroviral (ARV) therapy belong to:-

1) Nucleoside/nucleotide Reverse Transcriptase Inhibitors (NRTI)
2) Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI)
3) Protease Inhibitors (PI)
4) Fusion Inhibitors (FI)

New drugs targeting other parts of the viral lifecycle, such as viral integration, are in different stages of pre- and clinical development [106-111]. The NRTI class of drugs mimic the natural dNTP substrate (either ATP, GTP, CTP or TTP). Incorporation of the drug in to the elongating DNA strand causes chain termination, as an additional dNTP cannot be added to the 5' end. NNRTI drugs bind directly to specific regions of the RT enzyme. Binding either blocks the functionality of the enzyme or interferes with the uptake of the dNTP substrate in to the active site. PIs block the proteolytic cleavage of the translated viral polyproteins by the viral protease enzyme. This results in the production of immature non-infectious viral particles.

In addition, FIs are a recent introduction and the only approved drug is T20 (Fuseon), a twice-daily injection, which binds to the viral gp41 and thus blocks viral entry into the host cell. T20 is extremely expensive and only used for the treatment of ARV-experienced patients when conventional ARV drugs no longer have effect due to multi-drug resistance. Finally, three new drugs, etravirin (NNRTI), raltegravir (integrase inhibitor) and maraviroc (CCR5 inhibitor) have entered phase III clinical trials and should be released soon for general use depending on final trial outcomes.

Table 2: Antiretroviral drugs used for the treatment of HIV-1 infection

NRTIs	NNRTIs	PIs	Fusion inhibitor
zidovudine (AZT)	nevirapine (NVP)	ritonavir	enfuvirtide (T-20)
lamivudine (3TC)	efavirenz (EFV)	indinavir	
didanosine (ddI)	delavirdine (DLV)	saquinavir	
zalcitabine (ddC)		nelfinavir	
stavudine (d4T)		amprenavir	
abacavir (ABC)		lopinavir	
emtricitabine (FTC)		atazanavir	
tenofovir (TDF)		fosamprenavir	

Drug Resistance to Reverse Transcriptase inhibitors

HIV-1 has a tremendous capacity to mutate [112-116]. This mutation rate is due to HIV-1's high replication rate, the RTs lack of proof-reading capacity and that the enzyme is rather error prone [117-125]. As a consequence ARV therapy ultimately selects for virus with mutations that can by-pass the effect of the drug. All drugs currently being used to treat HIV infection have been shown to give rise to genomic mutations associated with drug resistance. Resistance to the NNRTI class of drugs involves the substitution of a single amino-acid in the reverse transcriptase, which blocks the drug from annealing to its binding pocket on the enzyme. These mutations, e.g. Y181C, L100I and K103N, arise very quickly if the virus is treated with NNRTI mono-therapy or sub-optimal multi-drug combinations [126, 127], and cross-resistance between the different NNRTI drugs is common.[128-132]. NRTI resistance on the other hand is rather complex and involves a gradual build-up of mutations. Two biochemical mechanisms for NRTI resistance have been documented. The first involves mutations that allow the RT to discriminate between NRTIs and the true dNTP substrate, thus preventing chain termination of the growing DNA strand [133, 134]. The second is caused by nucleotide excision mutations (NEMs), such as mutations at codons 41, 67, 70, 210, 215 and 219, which allow the mutated RT to remove the dideoxy-nucleotide monophosphate (ddNTP) from a terminated cDNA chain, by ATP-dependent hydrolysis.

HIV Fitness

ARV treatment in some patients results in incomplete suppression of viral replication, which ultimately gives rise to the immergence of resistant virus variants. The associated changes that occur in the viral genome give rise to structural changes in the viral proteins that allow the virus to replicate in the presence of the drug [135-147]. However, the virus also has to pay a replicative price for this ability, which is associated with a reduced level of fitness or decreased replicative capacity [139, 148-156]. Upon the removal of drug pressure, the resistant virus variants, in the majority of instances are replaced by WT virus [157-161], with a fully reinstated replicative capacity [139, 162, 163]. As such, virus with ARV associated mutations tend to be in the majority of cases less fit than their WT counterparts, present before the initiation of ARV therapy [164]. The reduction in viral fitness associated with resistance to ARV therapy could have underlying clinical relevance, particularly in terms of designing optimal therapies for individual patients based on their treatment histories and virus phenotype, together with attempts to reduce clinical progression and efforts to model the epidemiology of transmission of drug resistant viruses [165-169].

Several groups have studied the area of viral fitness in relation to drug resistance [137, 162, 163, 170-184]. Even so, a lot of the data accumulated on the fitness of drug-resistant HIV-1 has been hampered by cumbersome and expensive assays. This has unfortunately made large scale investigations and comparison of different studies rather difficult [185]. The RT is an essential enzyme involved in the production of DNA from viral RNA for all retroviruses, and an active RT is a fundamental requirement for HIV to be infectious. Many ARV drugs target the RT enzyme and development of resistance to drugs against the RT enzyme results in genomic mutations that alter the physical structure of the enzyme, rendering the RT less susceptible to inhibition. These changes may also have an effect on the catalytic activity or the fitness of the RT enzyme.

MONITORING OF HIV-1 VIRAL LOAD

RNA-based Assays

The two most routinely used RNA VL tests for monitoring ARV therapy are; Roche Molecular Systems RT-PCR (Amplicor, version 1.5) and Bayer Healthcare Diagnostics bDNA (Versant, version 3.0). Both tests are complex and require a high level of operator skill. The fact that they are molecular-based requires the need for separate RNA extraction and amplification rooms, a rare commodity in resource poor setting. The Roche RT-PCR method initially released as version 1.0 has been superseded by version 1.5, with a better primer mix, due to poor performance with non-B subtype HIV-1 [186-188]. The method can be carried out entirely manually or fully automated, with both sample extraction (using Ampliprep) [43, 189-191] and amplification (using the Cobas workstation, see Figure 5).

Figure 5: Roche Amplicor RT-PCR COBAS workstation.

The LOD is determined by the sample volume used, and whether an ultracentrifugation step is involved. The ultra-sensitive method has a level of detection of 50 RNA copies/ml blood plasma [192]. The Versant bDNA method is based on the amplification of the signal achieved after binding of the viral RNA rather than amplification and later visualisation of the gene product, as is the case for RT-

PCR. Versant bDNA is fully automated during the amplification stage but relies on manual RNA extraction. Like RT-PCR the level of detection is 50 RNA copies/ml blood plasma [193], however, the methodology requires an overnight incubation step, making the procedure somewhat longer than the 6 hours required for RT-PCR.

p24 Assay

Assays for p24 antigen are used predominantly for monitoring HIV-1 replication in cell culture supernatants of the virus. The assay normally has a sandwich format, with a binding antibody towards the p24 antigen coupled to a solid phase, such as the bottom of a 96-well microtiter plate. The p24 antigen if present in the sample will bind to the antibody on the solid phase. An additional anti-p24 antibody is added which is coupled to an enzyme for colorimetric or fluorimetric detection (see Figure 6 for a schematic representation). Assays can be found as in-house versions [194] or as commercially available kits. Detection of p24 antigen has also been used in attempts to measure VL in plasma from infected individuals, as in paper **III** and [195, 196], usually with poor or low correlation with RNA-based techniques. Measuring VL based on p24 antigen has some drawbacks. Firstly, p24 assays are based on specific capture of the p24 antigen using antibodies. These antibodies are often produced using p24 antigen from subtype B HIV-1. Considering HIV-1's high propensity to mutate, non-B viruses will potentially be quantified at a lower level due to inadequate capture of non-B p24 antigen, as was shown in paper **I**. Newer p24 assays used for measuring viral replication in cell culture, however, have been developed which address this issue and have been shown to quantify different HIV-1 subtypes and in some cases even HIV-2. Secondly and probably most importantly, cells infected with HIV-1 secrete non-virion-associated p24 in to the bloodstream. The biological relevance of this p24 in HIV-1 infection and response to therapy is not fully understood and according to Jorg Schüpbach and his colleagues should be further studied [197-202]. Even so, p24 detection in paediatric diagnosis of HIV-1 infection has shown some promise [197].

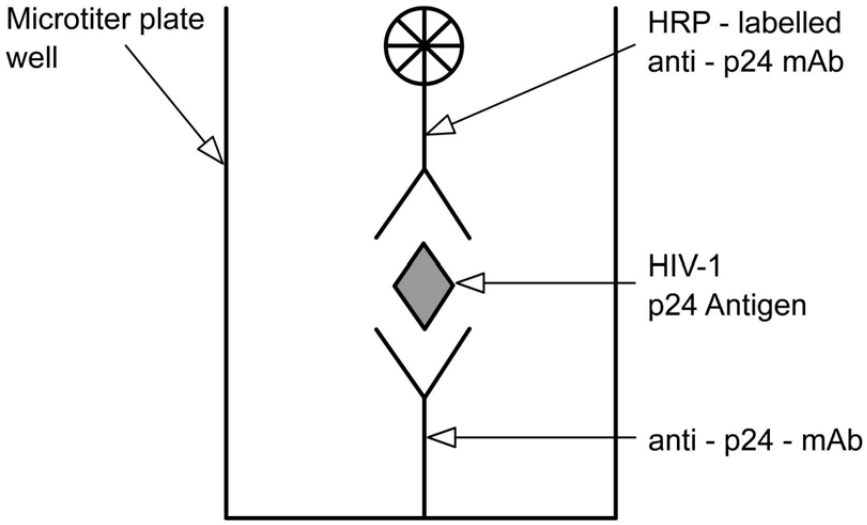

Figure 6: Schematic representation of a p24 antigen assay in a microtiter plate.

RT-based Assays

RT activity is a fundamental and conserved activity of all retroviruses. For viral replication to take place HIV-1 must retain a fundamental level of RT activity, otherwise no new viral particles will be produced. Therefore there is an evolutionary brake or mutational threshold on the extent to which the RT enzyme can mutate.

RT activity is a unique characteristic of all retroviruses. Therefore measurement of RT activity has the ability to provide a very promising analytic tool to determine viral replication in HIV-1. 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 templates (such as prA) and either radioactive dNTP substrate or a dNTP analogue (such as BrdUTP) together with colorimetric or fluorimetric product detection [67, 203-206], see Figure 7.

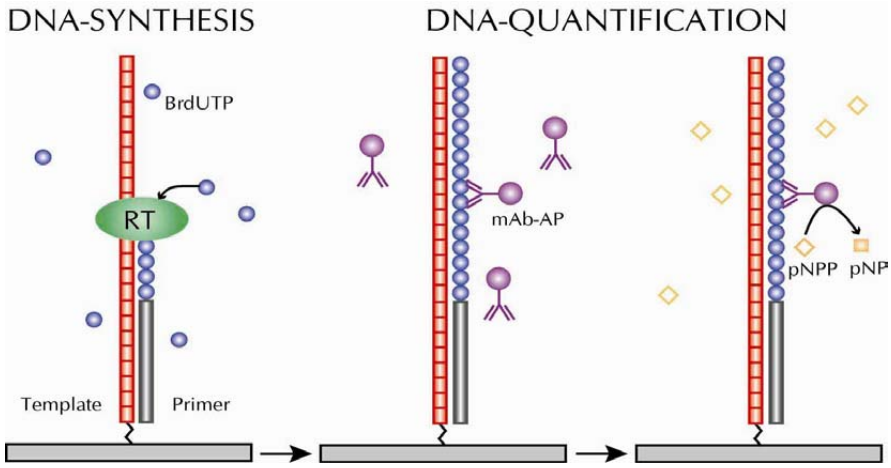

Figure 7: Schematic representation of the RT reaction and product detection in a 96-well microtiter plate

Initially RT assays were only useful for quantifying HIV in cell culture, as in paper **I**. The two major problems hampering the use of standard RT assays for measuring VL in plasma from infected individuals are; the presence of RT-blocking antibodies [67] and the use of NNRTI drugs in the treatment of HIV-1 infection. The RT is a large protein of >100 kD and as such is immunogenic. This promotes the development of anti-RT IgG antibodies with the capacity to bind and block the catalytic activity of the enzyme, so-called RT-blocking antibodies [67, 207, 208]. The NNRTI class of ARV drugs, such as efavirenz and nevirapine, bind directly to the RT enzyme and block the catalytic activity. Therefore trying to measure RT activity in plasma from infected individuals in the presence of RT-blocking antibodies and/or NNRTI drugs would result in a dramatic underestimation of the RT VL (RT-load).

Other RT based techniques, such as the PERT [209] and AMP-RT [210] assays, have also been proposed as potential HIV-1 VL tests. Both have demonstrated reasonable correlation with gold standard RNA-based technologies, and even longitudinal studies have shown impressive covariance with time [195, 211]. AMP-RT has also been successfully used for infant diagnosis of HIV-1 infection [212], as well as for subtype and type studies [213]. Both methods are, however, sensitive to RT-blocking antibody disturbance. Considering that virtually all HIV-1 infected individuals develop RT-

blocking antibodies at different levels [67], the degree of disturbance would therefore be different for each sample tested. This would have the effect that VL levels would not just be based on the amount of RT present in the samples but also reflect the level of inhibition by the patients RT-blocking antibodies. Both Bürgisser *et al* [195] and Garcia-Lerma *et al* [211] tried to circumvent this problem by assessing the level of inhibition by the RT-blocking antibodies. This was carried out by assaying the sample together with a recombinant RT enzyme. The level of RT-blocking antibody inhibition was taken in to account when the VL was reported. One major problem with this approach is that the RT enzyme used for the inhibition experiments may not give equal blocking capacity with all patient samples due to antigenic differences. Another problem with these techniques is the necessity for the use of PCR, a requirement that renders both assays less attractive when looking for simpler and less costly tests for use in resource-limited settings.

ExaVir® Load, RT VL test

Our research group has worked upon the problems outlined above. We subsequently developed and launched an RT VL test kit (ExaVir® Load) at the Xth International AIDS conference in Barcelona in 2002. This test minimizes the effects of RT-blocking antibodies and NNRTI drugs by first purifying the viral particles from the plasma by binding the virus to a gel followed by washing away the disturbing substances. The RT remains protected inside the virus and can be later released into an antibody/NNRTI free environment for determination of RT activity, as in **II**, **III**, **IV**, **V** and [214-218]. The RT VL test, ExaVir® Load uses HIV RT enzyme purified from plasma samples to catalyse the conversion of RNA to cDNA. Using a virion-binding gel, the viral particles are purified from the plasma. Bound virions are washed to remove inhibitors, including ARV drugs or RT-blocking antibodies. Virions are then lysed and lysates transferred to a 96-well plate for assay of RT activity. In an overnight incubation, RT enzyme in the lysate incorporates BrdUTP into a DNA strand complementary to the polyA template (bound to the wells). Subsequently an anti-BrdU antibody conjugated to alkaline phosphatase is added and the amount of incorporated BrdU detected using a colorimetric substrate. The color intensity of each well is read using a standard plate reader (wavelength 405 nm). Results are compared to a standard curve and HIV RT activity is determined and expressed as femtogram

(fg) HIV RT activity/ml plasma (fg/ml). Sample results are also converted to RNA copies/ml equivalents using the internal software conversion factor, provided from the manufacturer. The equipment used and the standard procedure for the RT VL assay is outlined in Figures 8 and 9, respectively.

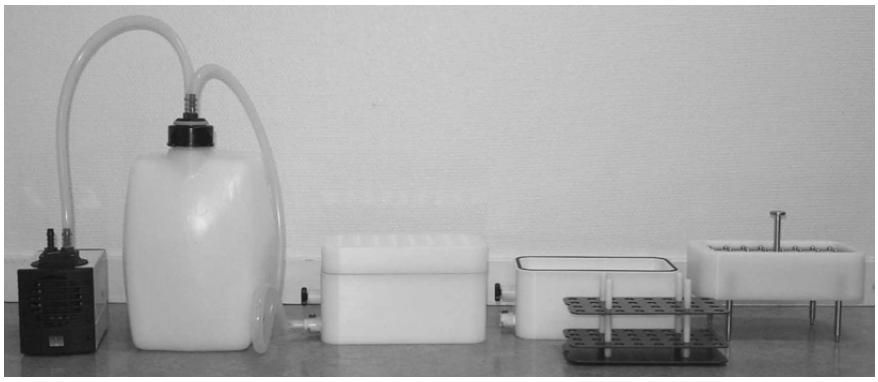

Figure 8: Equipment used for the RT VL test

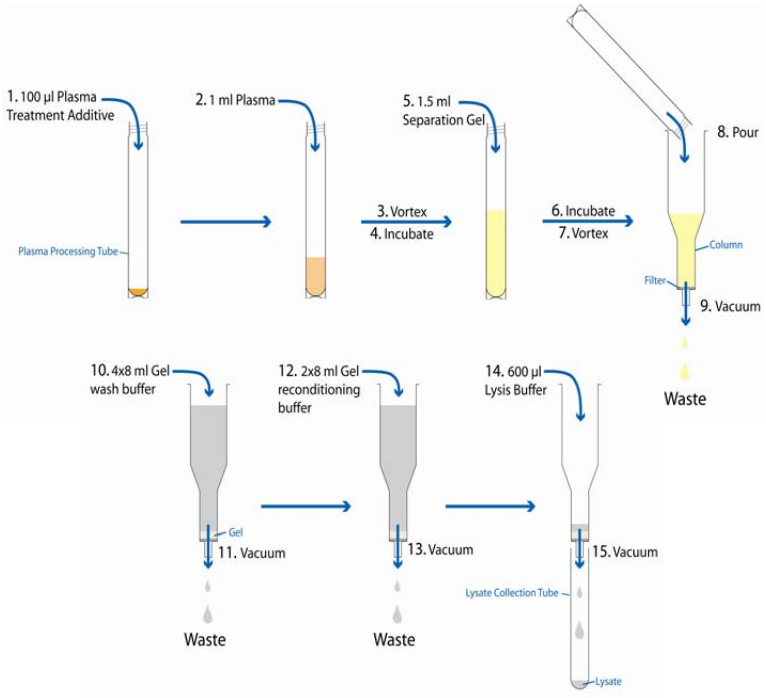

Figure 9: Step-by step procedure for viral RT isolation in the RT VL test

Problems facing new, simpler and less costly technologies for VL determination

RNA VL is the accepted "gold-standard" technique for measuring the amount of HIV-1 in blood plasma from infected individuals. HIV-1 RNA VL is performed using state-of-the-art technologies that are often fully automated and strictly quality controlled to maintain a high level of precision and accuracy. New techniques are usually spin-offs from university laboratories or start-up companies and often lack automation and rigorous quality control measures to insure reliability. Consequently and quite understandably the acceptance of new technologies to monitor HIV VL faces extensive scientific evaluation and scrutiny before they can possibly be considered for use. With the increasing availability of ART in resource-limited settings there is a growing need for cheaper and simpler tests. However, these new technologies will only make a meaningful impact if both scientific and governmental organisations give financial backing to promising technologies.

Challenges of HIV VL monitoring in resource limited settings

Routine VL testing to monitor ART is well established in resource-rich countries [219, 220]. However, implementation of these techniques in resource-limited nations has faced major economic and infrastructural difficulties. In 2003 the WHO guidelines for ARV monitoring in developing countries called for simpler monitoring practices and designated VL as an optional component, which should only be attempted if all the basic criteria for carrying out the tests are in place. Simpler VL assays have also been developed to address VL in less developed laboratory environments. One such assay, ExaVir® Load, is the cornerstone of this thesis. Even so, new tests are very often met with some degree of scepticism and are usually required to undergo long evaluation trials by the scientific community. This is all well and good, but the need for such tests is urgent in developing nations and they may decide not to wait for scientific evaluations (often carried out in resource rich countries) to be completed but rather opt for the cheaper and less technically demanding routines to monitor their patients. Meanwhile, not using VL risks the development of drug resistance, which can undermine a well-intentioned ARV program based on a limited number of drugs. This type of practise can and ultimately

will promote an epidemic of drug resistant virus, which will certainly hamper future possibilities of adequately controlling HIV in developing countries.

Much time and effort has been put into addressing the price of ARV drugs. This is certainly understandable where drugs are not available, however, with the current climate of falling drug prices and greater availability, price issues associated with VL testing needs to be brought to the forefront. Pricing of VL tests has in recent times been drastically reduced in certain parts of the world (17US$ in Botswana). However, this has not been made available to all resource poor nations (80-100 US$ in Kenya). Additionally, the consumable costs per test are not included in the discounted test price. Furthermore, lack of access and availability of both kits and consumables hamper the current efforts of many nations in providing adequate monitoring to their HIV patients.

One side of the equation that is sometimes forgotten is the requirement of skilled technical staff to perform the tests. Viral load tests are complex and technically demanding and as such require highly trained staff. Such people are often not available in resource-poor nations, or if they are, these individuals choose not to work in state run laboratories that pay a fraction of what they would earn working in industry.

AIMS

- To use a RT assay to compare the performance of two different p24 antigen assays to quantify HIV-1 replication in cell culture from different HIV-1 subtypes, (paper **I**).

- To determine if RT assay can be used to monitor HIV-1 VL, (papers **II** & **III**).

- To evaluate the ability of RT assay to quantify VL in SIV/SHIV infected macaques, (paper **IV**).

- To determine if drug resistant mutations in the viral genome are associated with alteration in RT-fitness, (paper **V**).

PATIENT SAMPLES AND VIRAL ISOLATES

HIV-1 isolates (paper I)

The HIV-1 isolates used in paper **I** consisted of a homogeneous group of viruses isolated from HIV-1 infected Italian mothers and their children. The heterogenous group of isolates were supplied as a multi-subtype panel (subtypes A to F) from NIBSC in the UK. These viruses were collected from different regions of the world and were obtained through the WHO network for HIV isolation and characterisation. Two subtype O isolates and an additional subtype E isolate were also included in the heterogeneous panel. All isolates were cultured using PBMCs from the same two donors under identical growth conditions and in the same laboratory. Samples of the culture supernatants were harvested on days 7 and 10 post-infection and stored at –70°C.

HIV-1 plasma samples (paper II)

Blood plasma was collected from HIV seropositive patients attending the Infectious Diseases Clinic of The Alfred Hospital in Melbourne, Australia between 1997 and 2004. Plasma was separated by centrifugation within 24 hours of collection and stored below –70°C. HIV RNA levels were determined using thawed plasma within 10 days of sample collection. Samples not initially tested using the ultra-sensitive RT-PCR assay or the bDNA version 3.0 assay were retested at the time of the study by RT-PCR. All samples were retrospectively tested using the RT assay, ExaVir® Load v1 or v2. Samples that were above assay cut-off (>100,000 RNA copies/ml by RT-PCR) were diluted 1:10 with HIV seronegative human plasma and HIV RNA quantification was repeated at the time of study. Samples were not tested for HIV-1 subtype. However HIV-1 subtype B is the predominant subtype circulating in Australia [221, 222].

HIV-1 Specimens (paper III)

The Virology Quality Assessment (VQA) laboratory created three panels to evaluate the RT (ExaVir® Load v1 and v2) and the Ultrasensitive p24 (Perkin Elmer) assays, see Table 3. The first panel consisted of well-characterized VQA HIV-1 viral stock (subtype B HIV) that was seeded into HIV-seronegative plasma at defined

concentrations (0-750,000 HIV RNA copies/ml). Samples were coded and tested in replicates by two testing laboratories (VQA Laboratory, Chicago, IL and University of North Carolina at Chapel Hill, UNC). Testing was performed using the RT assay (ExaVir® Load v1 and v2), the Ultrasensitive p24 kit (Perkin Elmer, using kit lysis buffer), a "boosted" version of the p24 kit utilizing an external lysis buffer (EB, kindly provided by Dr. Jorg Schüpbach) and the HIV RT-PCR assay (Roche). The VL for each member of this panel was determined by averaging the data obtained by using the HIV RT-PCR assay (Roche). Each laboratory ran each panel multiple times to produce 96 results for each kit.

The second panel consisted of plasma from HIV-infected donors enrolled in the VQA donor program (subtype B HIV-1) and cultured VQA viral stock seeded into HIV-seronegative plasma (HIV-1 subtypes A, C, D, E, and F). Eight laboratories tested the second panel one to five-times using one or more assays. The VLs for this panel ranged from 2.34 – 5.46 $\log_{10}$ HIV RNA copies/ml as defined by triplicate testing performed by the VQA Lab using the HIV RT-PCR assay (Roche).

The third panel produced by the VQA laboratory consisted of a spiked panel (subtype B HIV-1) designed to evaluate the improved sensitivity in the newly enhanced RT assay (ExaVir® Load v2). The range in VL for this panel was 0-10,000 HIV RNA copies/ml with an emphasis on replicates at the low end (100-6,000 HIV RNA copies/ml). Nominal concentrations were used to evaluate this panel since HIV RNA testing was not performed. Table 3 shows the sample configuration of all 3 panels created for testing of the two VL surrogate assays.

Table 3: Panel Configuration. Spiked panels consist of stock virus seeded into seronegative plasma. Clinical samples consist of plasma samples collected from HIV infected donors.

PANEL ID	HIV Subtype	Nominal[1] Concentration (RNA copies/ml)	Sample Type	# Replicates per RT/p24 Panels[2]	# Replicates per RT-PCR Panel[3]
001	B	0	Seronegative Plasma	2	2
001	B	500	Spiked	5	4
001	B	1000	Spiked	5	4
001	B	2000	Spiked	5	3
001	B	10000	Spiked	4	3
001	B	50000	Spiked	4	2
001	B	250000	Spiked	3	3
001	B	750000	Spiked	4	3
PANEL ID	**HIV Subtype**	**Nominal Concentration (RNA copies/ml)**	**Sample Type**	**# Replicates per RT/p24 Panels**	**# Replicates/RT-PCR Panel**
002	A	20000	Spiked	2	2
002	B	NA	Clinical[4]	20	20
002	C	20000	Spiked	2	2
002	D	20000	Spiked	1	1
002	E	20000	Spiked	3	3
002	F	20000	Spiked	1	1
002	NA	0	Seronegative Plasma	3	3
PANEL ID	**HIV Subtype**	**Nominal Concentration (RNA copies/ml)**	**Sample Type**	**# Replicates per RT/p24 Panels**	**# Replicates per RT-PCR Panel**
003	B	0	Seronegative Plasma	4	NA
003	B	100	Spiked	6	NA
003	B	400	Spiked	6	NA
003	B	1000	Spiked	5	NA
003	B	2000	Spiked	5	NA
003	B	6000	Spiked	4	NA
003	B	10000	Spiked	2	NA

[1]The expected value obtained from serial dilution of a stock virus into HIV-seronegative plasma.
[2]RT = ExaVir® Load v1 and v2); p24 = Ultrasensitive p24 assays (Perkin Elmer and Perkin Elmer + EB)
[3]Roche panels had fewer replicates to accommodate smaller assay run size.
[4]Clinical samples were collected from 18 different donors (replicate specimens were collected from 2 donors) and spiked samples were generated from viral stocks.

Animals and SIV/SHIV plasma samples (paper IV)

The monkeys used in paper **IV** were female cynomolgus macaques (*Macaca fascicularis*) of Chinese origin. The animals were housed in the Astrid Fagraeus laboratory at the Swedish Institute for Infectious Disease Control. Housing and care procedures were in compliance with the provisions and general guidelines of the Swedish Animal Welfare Agency and all procedures were approved by the Local Ethical Committee on Animal Experiments. Fourteen macaques were challenged intrarectally (i.r.) with 50 MID_{50} of SIV_{mac251}. All macaques became infected and

plasma VL was monitored for 12 weeks.

An additional twenty-eight macaques were challenged i.r. with 10 MID_{50} of the R5 virus-SHIV-Bx08. All macaques, except one, became detectably infected and plasma VL was monitored for 8 weeks. Both groups of monkeys were tested negative for SIV, simian T-cell lymphotropic virus type 1 (STLV-1) and simian retrovirus (SRV) prior to the study.

HIV-1 plasma samples (paper V)

Two hundred and two samples from HIV-1 infected individuals attending the out-patients clinic at Venhälsan, South Hospital in Stockholm over an eleven year period (from 1995 to 2006) were included in the study. Samples were supplied from antiretroviral treated and untreated patients with known RNA-load and viral genome sequence. Samples were included based on the criteria that a genomic sequence had been carried out and that the RNA VL was >2000 RNA copies/ml. Treatment histories and CD4+ T-cell counts for some patients at time of sampling were also supplied. All samples were stored at –70°C prior to testing for RT-load.

METHODS

RT assay (paper I)

The RT assay used in paper **I** was a commercially available ELISA supplied by Cavidi Tech AB. The test kit was based on the technology developed by Ekstrand *et al* [205]. Each sample was analyzed at three different dilutions (1:5, 1:25 and 1:125) to enable accurate determination of RT activity. Poly(rA) coupled to the bottom of the 96-well microtiter plate acts as template for the RT enzyme, while oligo$(dT)_{22}$ and bromodeoxyuridine 5'-triphosphate (BrdUTP) act as primer and substrate, respectively. Incorporated BrdU is measured using an anti-BrdU monoclonal antibody conjugated to alkaline phospahtase (AP). Colorimetric determination of the bound antibody is carried out using para-nitrophenyl phosphate (pNpp). The amount of RT activity in each sample was then calculated relative to a serially diluted reference enzyme standard of known concentration.

HIV-1 p24 Assays (paper I)

Two different p24 peptide ELISAs were used in paper **I** to determine p24 antigen concentration in the cell supernatants of different HIV-1 isolates. To determine if there was a difference in these assays we choose a commercially available kit from Abbott based on monoclonal anti-p24 antibodies and an in-house ELISA developed at Karolinska Institute using both poly- and monoclonal anti-p24 antibodies [194].

p24 assay for HIV-1 VL determination: Perkin Elmer HIV-1 p24 ELISA plus the ELAST® ELISA Amplification System (paper III)

The Ultrasensitive p24 assay (Perkin Elmer) was performed according to the manufacturers' instructions using the kit lysis buffer. Briefly, 50µl of plasma sample was lysed with pre-diluted kit lysis buffer, heated at 100°C for 5 minutes, and then cooled to room temperature. A volume of 250µl of specimen, control or standard was then added to the respective wells of a 96-well plate. The plate was incubated overnight then washed. The captured p24 antigen was labelled with a biotinylated-anti-p24-antibody followed by a streptavidin-HRP (horseradish peroxidase) step. The biotinlylated tyramide reagent (provided in the ELAST kit) was used to "amplify" the streptavidin-coated bound antigen and was followed by a second streptavidin-HRP

step that was then developed colorimetrically and read kinetically for 10 minutes followed by a final reading after 30 minutes when the reaction was "stopped" with concentrated acid. Quantitative results were reported based on the algorithms incorporated into the p24 assay software.

The boosted version (Perkin Elmer + External Buffer (EB, provided by Jorg Schüpbach)) of this assay was performed according to the manufacturer's instructions, with one deviation. The specimen (50µl) was first incubated with the EB at room temperature for 10 minutes. This sample was then lysed with pre-diluted kit lysis buffer and heat denatured in the same manner as described above. Quantitative results were also reported based on the algorithms incorporated into the p24 assay software.

HIV-1 RNA VL assays (papers II, III, IV and V)

HIV RNA testing was performed using the COBAS Amplicor HIV-1 Monitor™ assay version 1.5 ultra-sensitive preparation (RT-PCR; Roche Diagnostics, Branchburg, NJ, USA) or the Quantiplex™ HIV RNA assay, version 3.0 (bDNA; Bayer Diagnostics, Emeryville, CA, USA). Both assays were performed according to manufacturers' instructions. All laboratories involved in the different studies participate in internationally recognised quality assurance programs or in the case of the VQA laboratory in paper **III**, actually provide such a service. The LOD for both RNA VL methods was 50 RNA copies/ml.

SIV QC RT-PCR (paper IV)

The LOD of the in-house QC RT-PCR method used to determine SIV and SHIV RNA VL was 40 RNA copy equivalents/ml. Experimental procedures were carried out according to the methodology described by Ten Haaft *et al* [223]. As target sequence, a conserved 267 base-pair region in the SIV *gag* gene with primer and probe regions homologous for SIV_{mac}, SIV_{sm} and chimeric SHIV viruses, was used. An internal standard was also used based on the same 267 base-pair sequence but with an additional 26 base-pair probe region. The reported upper detection limit of the QC-RT-PCR method is 4×10^7 RNA copy equivalents/ml.

RT VL assays (papers II, III, IV and V)

RT VL was quantified using two versions of the ExaVir® Load kit (v1 and v2, Cavidi Tech AB, Uppsala, Sweden), as recommended by the manufacturer. In most instances 1 ml of plasma sample was used, however, in some cases lower volumes were only available and the remaining volume (up to 1 ml) was made up using HIV-seronegative plasma. For these samples the kit software carried out the mathematical calculation of the true VL based on the level of dilution.

Two different versions of the ExaVir® Load kit were tested, v1 and v2. The v2 kit differed from the v1 kit on several key steps, which increased the LOD.

1) Increased addition of sample lysate to the wells from a volume of 75/30µl to 150/30µl.
2) Increasing the RT polymerisation time from approximately 20 to 40 hours.
3) Addition of new detergents to the Gel Wash and the Lysis Buffer.

The improvements made from the initial v1 assay brought the LOD in the v2 assay down to levels seen in currently used standard RNA VL tests. This new capability made the v2 kit an interesting possibility for use in settings were RNA VL determinations are either impossible or impractical.

Sequencing of HIV-1 (paper V)

The method used is described in Lindström *et al* [224]. Nested primers were used to amplify a region of the *pol* gene corresponding to the protease and first half of the RT. The primers were optimally designed to anneal to conserved regions of the HIV-1 genome, even those of non-subtype B. After PCR amplification and extraction, the purified amplicons were sequenced using the ABI PRISM BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer). The resulting sequencings were assembled and translated to amino-acids. Phylogenetic tree analysis was carried out to determine subtype based on the *pol* gene and also rule out possible PCR contamination or sample mix-up.

RESULTS AND DISCUSSIONS

RT activity versus p24 antigen

Maintaining an active RT enzyme is a basic requirement for replication for all retroviruses. Therefore in paper **I** we chose to use an RT activity test as "gold-standard" assay to compare the ability of two different p24 antigen assays to monitor HIV-1 replication in cell culture. The p24 protein of HIV-1 has the capacity to diverge with changes in the *gag* gene. With the knowledge that most p24 assays at the time of this study were based on the use of anti-p24 antibodies produced towards HIV-1 subtype B p24 antigen, we decided to determine the quality of two such assays to quantify HIV-1 replication in cell culture from both a heterogeneous group of subtype B viruses and a group of divergent stains made up of multiple subtypes.

All viruses were cultured identically to minimise growth variations. Samples were measured for RT activity using a commercially available RT activity test kit and two p24 ELISAs, one an in-house test and the other a commercial assay from Abbott. The ratios between amount of RT activity and p24 antigen (p24/RT ratio) were calculated for each virus. The majority of isolates gave similar ratios, however, 5 viruses from the heterogeneous panel (1 subtype A, 1 subtype B, 1 subtype C and both subtype O viruses) gave very low ratios indicating lower levels of p24 antigen detection per unit of RT, or that the p24 antigen binding capacity of the antibodies used in the ELISA did not adequately detect p24 antigen in these viruses. Furthermore, when we directly compared the ability of the p24 assays to detect p24 antigen we could show clear specificity differences between the anti-p24 antibodies used in the two kits to detect p24 antigen in the different viral isolates.

RT VL versus RNA VL

In papers **II** and **III** we tested the ability of two different versions (v1 and v2) of the RT VL assay (ExaVir® Load) to monitor HIV-1 VL in plasma compared to currently used techniques (RT-PCR and/or bDNA). Both versions of the RT assay correlated strongly ($r > 0.85$, $p < 0.0001$) with both RT-PCR and bDNA from a range of different samples and subtypes (see figure 10, an example from Braun *et al* [225], the first published paper using v1 of the RT assay).

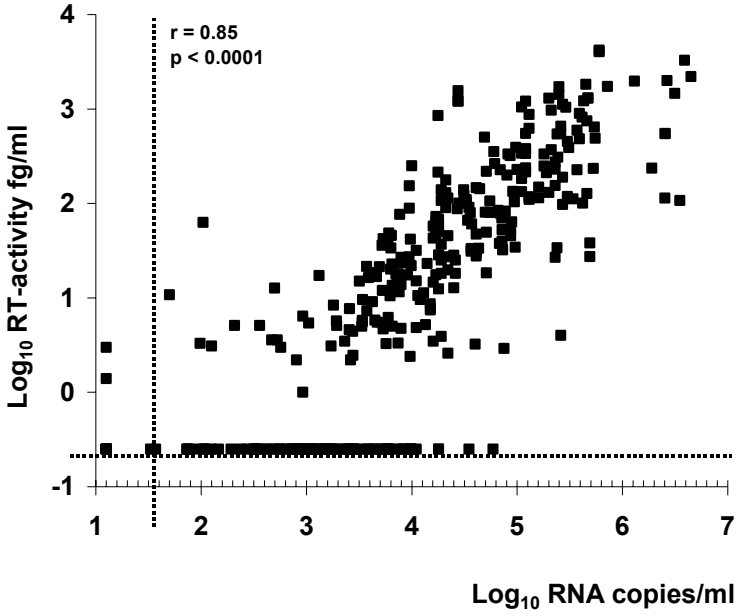

Figure 10: Correlation of HIV-load based on RT activity and RNA assay (log_{10} values)

Version 1 (v1) of the RT assay was used in both papers **II** and **III** and gave approximate 95% detection rates for samples containing >10,000 RNA copies/ml. Furthermore, samples selected for patient follow up showed virtually identical VL trends over time (see figure 11, from paper **II**) and were also independent of change of therapy (see figure 12, an example from Braun *et al* [225]).

This indicated that both RT and RNA-load, although measuring different replication markers, are measuring the same fundamental process of viral replication, and that RT activity is not affected by the presence or change of antiretroviral therapy. Additionally, in Braun *et al* [225], samples from HIV-1 subtype O and HIV-2 were quantified, something which cannot be achieved with standard HIV-1 VL technologies.

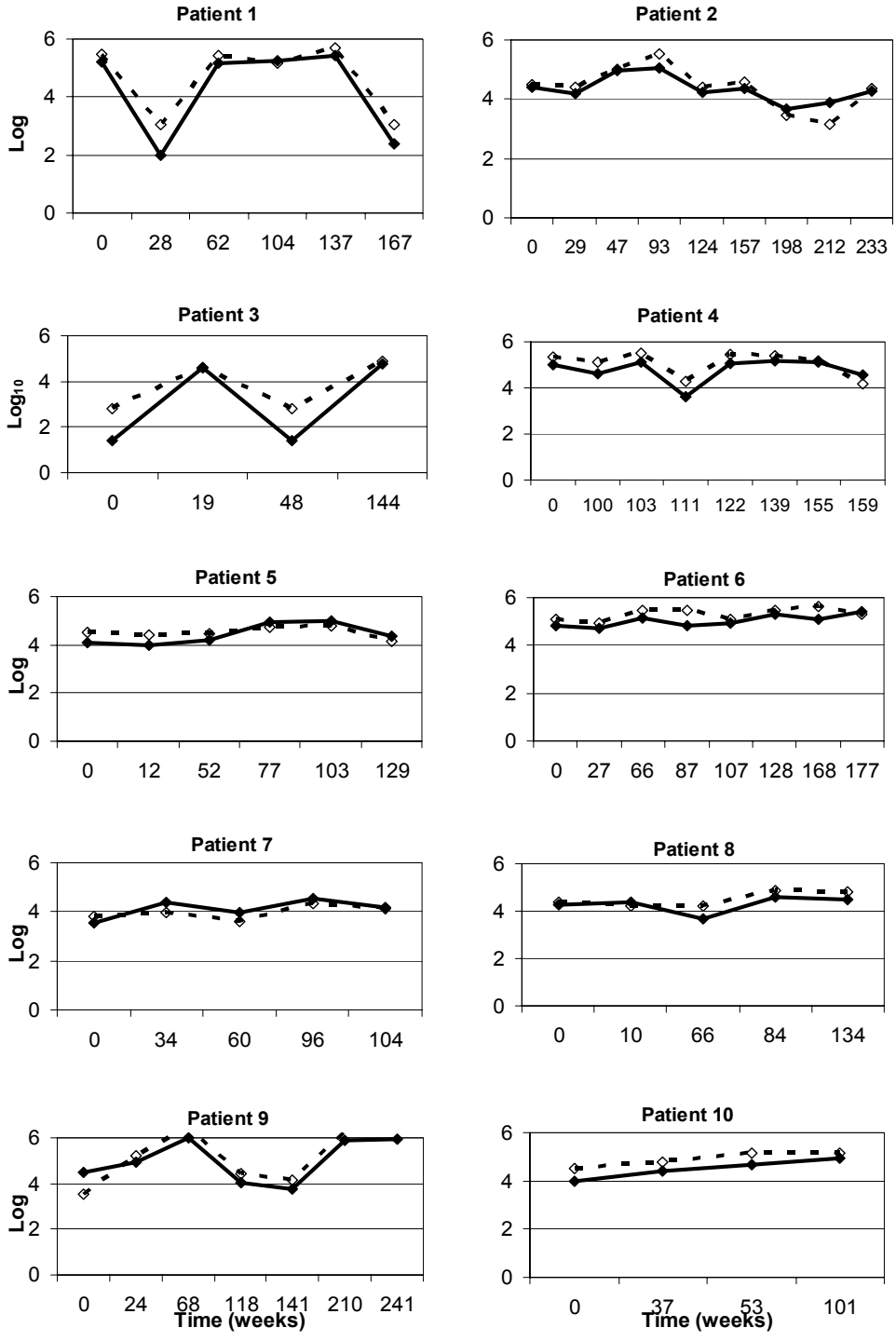

Figure 11: Trends in VL levels over time measured by RT assay and RT-PCR.

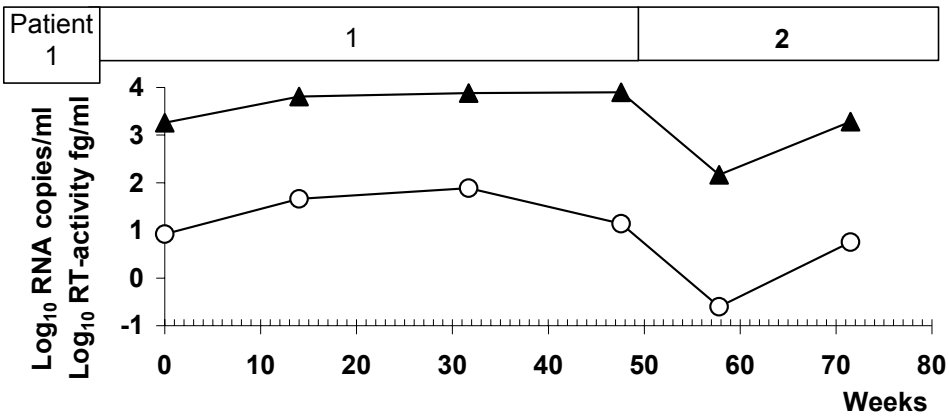

Figure 12: Viral load levels over time measured by RT assay and RT-PCR. 1 and 2 indicates different ART regimes.

The RT VL version 2 (v2) assay was first tested in paper **II** and the LOD was improved substantially compared to v1 (see Table 4). In paper **II**, 95% of all samples with >1000 RNA copies/ml were detected. Additionally, samples selected to determine if previous or current efavirenz therapy could possibly cause reduced RT-load showed no such tendencies. This finding was very important for use of RT-load to monitor ART, and was rather reassuring as efavirenz, in contrast to the other NNRTI drugs such as delavirdine and nevirapine is characterized as a tight binder of the HIV-1 RT, due to its rapid RT binding and very slow dissociation rate [226]. This showed that ARV drugs used to treat patients did not interfere with the measurement of RT-load by the RT assay.

Table 4: Sensitivity of the RT assay in comparison to HIV RNA

	Version 1 assay		Version 2 assay	
HIV RNA (copies/ml)	**% detectable by HIV RT**	**Number of samples**	**% detectable by HIV RT**	**Number of samples**
<50	0	3	-	0
50-1,000	7	14	69	16
1,001-5,000	42	24	88	16
5,001-10,000	76	21	91	11
10,001-50,000	98	56	100	26
50,001-100,000	97	33	100	12
>100,000	100	38	100	4

HIV RNA tested using: COBAS Amplicor HIV-1 Monitor assay™ version 1.5 ultra-sensitive preparation; Roche Diagnostics, Branchburg, NJ, USA or Quantiplex™ HIV RNA assay version 3.0; Bayer Diagnostics, Emeryville, CA, USA

HIV RT (reverse transcriptase) tested using ExaVir® Load version 1.0 assay kit and ExaVir® Load kit version 2; Cavidi Tech AB, Uppsala, Sweden

Paper **III** was a multi-site evaluation of both the RT VL assay and an additional assay based on the measurement of HIV-1 p24 antigen as surrogate markers for VL determination. A summary of the technical and performance characteristics of the three assays used in this study is shown in Table 5.

Table 5: Technical and performance characteristics of the three HIV-1 VL tests used in paper **III**

Assay Characteristic	RT-PCR	RT Assay	p24 Assay
Detects	HIV-1 RNA	Lentivirus RT activity	HIV-1 p24 antigen
Cost per test	~100€	~25€	~10€
Sensitivity (copies/ml)	<50 (ultra) or <400 (standard)	<400	<50000
Expertise	High	Moderate	Moderate
Laboratory standard	High	Moderate	Moderate
Equipment	Molecular based	ELISA based	ELISA based
Sample turn around time	1 day	3 days	1 day
Technique	Automated	Manual	Manual
Subtype problems	Yes	No	Yes
Quantifies HIV-2	No	Yes	No
Quantifies SIV and SHIV	No	Yes	No

Viral isolate and patient plasma panels were created by the Viral Quality Assessment (VQA) laboratory at Rush Medical College in Chicago in order to determine LOD and reproducibility of both assays compared to RT-PCR (Roche). The samples tested in the RT and p24 assays consisted of a subtype B (Panel 1) and a multi-subtype panel (Panel 2). In addition, v2 of the RT assay, due to its increased LOD, was also tested with a panel consisting of a serial dilution of a sample ranging from 100 to 10,000 RNA copies/ml (Panel 3). Eight different sites from different regions of the

Table 6: Observed Detection Rates for Panels 1, 2 and 3 - # positive results / total (%), pooled across laboratories and runs.

Panel	HIV Subtype	Nominal Concentration (HIV RNA copies/mL)	Median Log_{10} HIV RNA copies/mL (RT PCR)[1]	Cavidi v1 %detected(n)	Cavidi v2 %detected(n)	PE %detected(n)	PE + EB %detected(n)	Roche %detected(n)
001	B	0	Undetectable	0 (12)	0 (6)	0 (12)	0 (12)	0 (16)
001	B	500	732	37 (30)	100 (15)	0 (30)	0 (30)	100 (32)
001	B	1000	1185	33 (30)	100 (15)	0 (30)	0 (30)	100 (32)
001	B	2000	2182	60 (30)	100 (15)	0 (30)	0 (30)	100 (23)[2]
001	B	10000	12394	92 (24)	100 (12)	0 (24)	0 (24)	100 (24)
001	B	50000	52394	100 (24)	100 (12)	0 (24)	100 (24)	100 (16)
001	B	250000	275861	100(18)	100 (9)	100 (18)	100 (18))	100 (24)
001	B	750000	817569	100 (24)	100 (12)	100 (24)	100 (24)	100 (22)[3]
Panel	**HIV Subtype**	**Nominal concentration (HIV RNA copies/mL)**	**Median Log_{10} HIV RNA copies/mL (RT PCR)**	**Cavidi v1 %detected(n)**	**Cavidi v2 %detected(n)**	**PE %detected(n)**	**PE + EB %detected(n)**	**Roche %detected(n)**
002	A	20000	30768 - 40585	100 (26)	100 (6)	0 (16)	86 (14)	100 (6)
002	B	NA	219 - 983	9 (65)	27 (15)	18 (40)	69 (35)	93 (15)
002	B	NA	1241 - 8061	65 (65)	93 (15)	35 (40)	66 (35)	100 (15)
002	B	NA	36332 - 288850	100 (143)	100 (33)	86 (88)	99 (77)	100 (33)
002	C	20000	14740 - 43701	100 (26)	100 (6)	0 (16)	79 (14)	100 (6)
002	D	20000	18230	54 (13)	100 (3)	0 (8)	71 (7)	100 (3)
002	E	20000	32245 - 50167	79 (39)	100 (9)	0 (24)	86 (21)	100 (9)
002	F	20000	33910	100 (13)	100 (3)	0 (8)	86 (7)	100 (3)
002	NA	NA	0	0 (39)	0 (8)[2]	0 (24)	0 (21)	0 (9)
Panel	**HIV Subtype**	**Nominal Concentration (HIV RNA copies/mL)**	**Median Log_{10} HIV RNA copies/mL (RT PCR)**	**Cavidi v1 %detected(n)**	**Cavidi v2 %detected(n)**	**PE %detected(n)**	**PE + EB %detected(n)**	**Roche %detected(n)**
003	B	0	ND[4]	ND	0 (12)	ND	ND	ND
003	B	100	ND	ND	11 (18)	ND	ND	ND
003	B	400	ND	ND	100 (18)	ND	ND	ND
003	B	1000	ND	ND	100 (13)[5]	ND	ND	ND
003	B	2000	ND	ND	100 (15)	ND	ND	ND
003	B	6000	ND	ND	100 (15)	ND	ND	ND
003	B	10000	ND	ND	100 (6)	ND	ND	ND

[1]Determined by replicate testing using the Standard Roche Monitor Test, v1.5.
[2]One invalid sample result was excluded.

world were included in the study to evaluate assay robustness in different laboratory settings. A summary of the evaluation and assay performance of the RT and p24 assays tested is shown in Table 6.

The RT (v1) assay detected 98% of the specimens in panel 1 with VL of 10,000 RNA copies/ml or greater, while the improved RT (v2) assay detected 100% of the specimens in panel 1 with VL of 500 RNA copies/ml or more. For panel 2, the v2 RT assay also out-performed the v1 assay. For panel 3 with the lower level VL samples, the RT assay (v2) detected 100% of the specimens with VL of 400 copies/ml or greater. This brought the RT assay to a LOD in the region of the standard molecular based techniques currently used in the developed world for monitoring HIV-1 VL.

For panel 1, the other assay tested, the p24 assay detected 100% of specimens with VL of >250,000 copies/ml. The addition of the external buffer provided by Dr Schüpbach increased the detection rate to 100% for samples with VL of >50,000 copies/ml. Detection rates for the Ultrasensitive p24 (Perkin Elmer) assay were also improved in panel 2, when the external buffer was included. Detection of samples from panel 2 ranged from 0 - 86% for the Ultrasensitive p24 assay, but improved to 66-99% when the external buffer was used in the procedure. Better sensitivity was observed in clinical specimens than in spiked samples, regardless of the buffer used, which further emphasises the potential limitations of the use of p24 as a marker for VL due to the detection of non-virion associated p24 shed from infected cells.

RT VL assay versus QC RT-PCR for monitoring VL in SIV/SHIV infected macaques

The monitoring of VL in macaques has usually been carried out using non-standardised in-house PCR based methods. Use of these methods has often created problems with interpretation of data between different experimental sites, due to differences in assay set up and calibration. The RT VL assay is optimised for the detection and quantification of RT activity from lentiviruses. The assay was shown to correlate strongly with other commercial assays for HIV-1 VL monitoring in papers **II-III**. The SIV being a lentivirus should have an RT enzyme with similar reaction requirements to HIV. Therefore the RT assay should hypothetically function for SIV

and SHIV RT also. To determine this we chose to compare the RT VL assay with an in-house QC RT-PCR method established in our laboratory.

In paper **IV**, a strong correlation was shown between RT and RNA VL for both SIV (Spearman, r =0.92 and p <0.0001) and SHIV (Spearman, r =0.95 and p <0.0001). Additionally, trends in VL over time gave consistently similar results for both viruses, as shown for SIV in Figure 13. The VLs for the SIV infected animals tended to be higher and remain high compared to those infected with SHIV. Interestingly, the average SIV and SHIV VL by QC RT-PCR was consistently 3 – 5 times less than the RT assay. Upon further testing using a standardised serially diluted sample of SIV from NIBSC in the UK, we could conclude that the difference in VL between the assays was probably not a biological difference between the different viruses in relation to the amount of RT and RNA, but rather to a difference in calibration of the two assays. The standardised samples from NIBSC correlated more closely to the VL values established with the RT assay, indicating that the QC RT-PCR used in the study was probably not optimally calibrated.

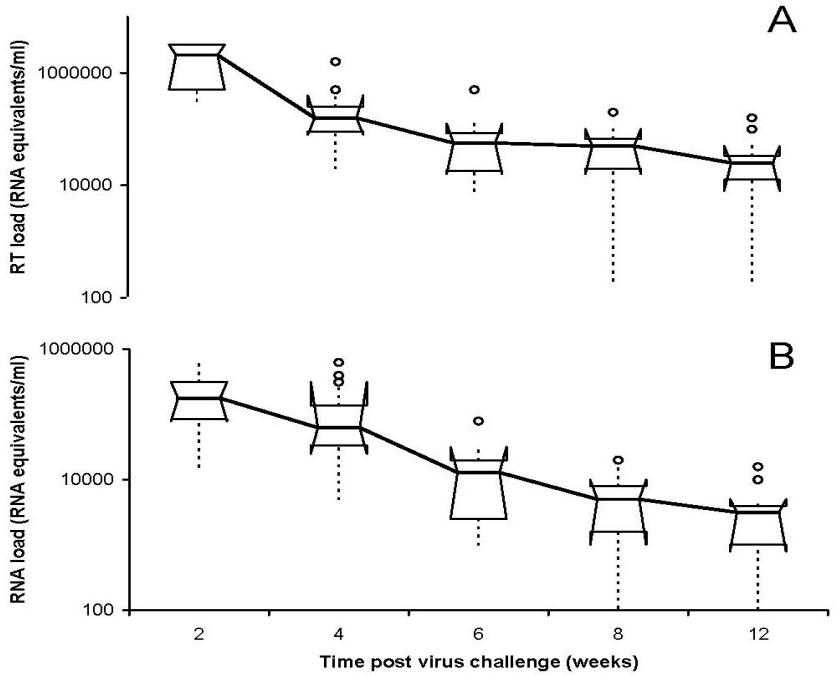

Figure 13: Trends in SIV VL measured with RT assay (A) and QC RT-PCR (B)

To address issues of reproducibility we tested twenty one plasma samples in duplicate for SIV RT activity using RT assay. Six samples had RT-load greater than the upper detection limit and were excluded from the statistical analysis. Results from each sample duplicate were transformed to $\log_{10}$ RNA equivalents/ml (paper **IV**). At all VL levels tested the RT assay gave a very low level of sample variation (% coefficient of variation range, 0.3 – 4.25), which corresponds to less than 0.2 $\log_{10}$ difference, which is better than the variation seen in current commercial VL assays for HIV-1.

Transient reduction in RT-fitness with accumulation of NRTI resistance mutations

In paper **V** we analysed 202 HIV-1 infected patients to determine if ART related mutations in the viral genome were associated with reduced RT-fitness. We defined RT-fitness as the ratio of HIV-1 RT activity/RNA (fg RT/1000 RNA copies) in an attempt to determine if mutations associated with ART altered the fitness of the RT enzyme. Patient samples were divided into groups based on their mutation profiles, (1) wild-type (WT), (2) containing NNRTI resistance mutations, (3) containing NRTI resistance mutations and (4) containing PI resistance mutations. Samples with PI or NNRTI resistance mutations showed similar HIV-1 RT activity/RNA ratios as samples with WT profile (Mann-Whitney test, WT vs. PI, p =0.173, n =196 and WT vs. NNRTI, p =0.338, n =202). This indicated that both PI and NNRTI resistance mutations generally did not alter the level of RT-fitness. In contrast, samples with NRTI resistance mutations (with 1 up to 11 mutations) showed altered HIV-1 RT activity/RNA ratios compared to the WT group (Mann-Whitney test, WT vs. NRTI, p =0.006, n =202), indicating that some of the samples containing NRTI resistance mutations did have significantly altered levels of RT-fitness.

Table 7 shows the effect of individual NRTI mutations on RT-fitness. The mutations at M41L, E44D, T215Y and K219N showed highest significance related to altered RT-fitness (Mann-Whitney 2-tailed, p <0.01). Other mutations V118I, Q151M, M184V and L210W showed lower significance (p <0.05), while the remaining mutations were non-significant. In our NRTI resistant sample group, M41L and T215Y usually appeared first and often together. Of the 91 viruses with M41L only 6 did not contain T215Y, all viruses with E44D contained T215Y and only 1 virus with

K219N did not contain T215Y. This indicated that T215Y seemed to be the major mutation involved in the reduction of RT-fitness in these viruses.

Table 7: Effect of NRTI mutations on HIV-1 RT activity/RNA ratio

Mutation at position	Number of samples	HIV-1 RT/RNA ratio Median	HIV-1 RT/RNA ratio Average	Difference towards WT group
none	64	2.35	4.53	
M41L	**91**	**1.85**	**3.00**	**p<0.01[a]**
E44D	**37**	**1.44**	**3.41**	**p<0.01**
A62V	6	1.70	1.65	Ns
K65E	1	8.4	8.4	Nd
D67N	79	1.84	2.74	p<0.05
T69D	18	3.32	4.06	Ns
K70G/R	37	1.98	2.53	Ns
L74V	10	3.01	3.73	Ns
V75M	23	1.75	3.01	Ns
F77L	9	3.43	3.76	Ns
Y115F	4	1.13	2.70	Ns
F116Y	6	1,70	2,67	Ns
V118I	37	1.96	2.92	p<0.05
Q151M	7	1.81	3.05	p<0.05
M184V	103	1.95	3.07	p<0.05
L210W	60	1.78	3.36	p<0.05
T215Y	**108**	**1.84**	**2.88**	**p<0.01**
K219N	**45**	**1.66**	**2.44**	**p<0.01**

Two tailed probability in Mann-Whitney test
Ns = not significant
Nd = Not determined

Considering the T215Y involvement in reducing RT-fitness we choose to evaluate if additional mutations in association with T215Y were involved in either further reduction or possibly restoration of RT-fitness. Analysis of additional NRTI mutations on RT enzymes containing T215Y showed that only mutation at L74V showed a significant increase in the HIV-1 RT activity/RNA ratio (median =3.08, p <0.05, n =10), compared to the median ratio for RT enzymes containing T215Y (median = 1.84). None of the other NRTI mutations in association with T215Y had a significant impact on RT-fitness.

CONCLUSIONS

Retaining an active RT enzyme is a fundamental requirement for all retroviruses to replicate. Hence the gene coding for the RT enzyme is the most conserved in the HIV genome. Remembering that HIV-1 has a very high propensity to mutate, determining viral replication based on the measurement of viral antigens or RNA brings a degree of uncertainty related to the use of specific capture antibodies or primers/probes used in these test methods. As such, measuring RT activity to determine the level of retroviral replication has the capacity to eliminate the problems associated with divergence, as the virus at all costs must retain an active RT enzyme.

In paper **I** we showed that assays using antibodies towards specific antigenic sites on the p24 protein could result in under-estimation of the level of viral replication in cell culture. Quantitative differences in the ability to determine the level of p24 antigen by the two p24 assays tested was also demonstrated. This study was carried out in 1998 and since then the detection capabilities of p24 antigen assays have been improved to detect different HIV-1 subtypes.

In papers **II, III, IV** we showed that RT VL assay was a useful tool for monitoring VL in HIV, SIV and SHIV infections. The RT-load assay displayed a strong correlation with all molecular assays used and the version 2 assay even showed similar sensitivity to the currently used PCR-based methods. Bearing this in mind, and the fact that the RT assay requires only basic laboratory equipment and infrastructure, this makes the test a very promising technique for use in developing countries where the use of PCR-based molecular techniques are not feasible. In addition, the RT VL assay, due to the conserved nature of the RT enzymes found in the different lentiviruses, has the potential to be used as a general tool for the monitoring of viral replication for all lentiviruses. Furthermore, with some modifications to the test conditions in the RT reaction step, the RT assay could possibly be extended to all retroviruses.

In paper **V** we showed that mutations in the HIV-1 genome associated with resistance towards NRTI drugs gave an initial but transient reduction in RT-fitness. Our data indicated an alteration in the level of RT-fitness in viruses harbouring resistance

mutations towards NRTI but not PI and NNRTI drugs. Of all NRTI mutations evaluated T215Y was most strongly involved with reduction in RT-fitness. The T215Y mutation is found during the initial development of resistance towards AZT, and is associated with introducing a proof-reading capability on the RT through an ATP-dependent phosphorolytic activity on chain terminated primers. Interestingly, addition of mutation at L74V was shown to increase RT-fitness in samples already possessing T215Y. Mutation at L74V is associated with ddI resistance and resensitisation of AZT-resistant viruses to AZT inhibition, when present with one or more thymidine analogue mutations (TAMS). The mechanism behind this process is not entirely clear but is thought to be connected with a reduced ATP-dependent phosphorolytic activity of RT enzymes with TAMs. In essence, our data indicates an initial reduction in RT-fitness with the acquisition of T215Y, while addition of L74V seems to restore RT-fitness. The potential ability of RT-load to give additional biological information on the virus, not available with RNA-load alone, has the potential to add an additional virological fitness dimension to VL measurement and should be further investigated.

Finally, on a more personal note, working to install VL testing in Africa and Asia over the past 5 years has given me many challenges, some bordering on extreme frustration and others of overwhelming joy at bringing this type of technology to areas which have been deemed impossible by our scientific peers. Many see VL testing as a luxury that cannot be afforded or implemented in resource limited settings. I understand the statement but do not entirely agree with it. VL assays cost money and require expertise that is often lacking in resource poor countries. However, characterising VL as a luxury is somewhat of a misconception, as failure to implement this technology in countries providing ARV therapy will ultimately undermine treatment efforts due to the immergence of drug resistance. Having access to VL can indicate potential drug failure and aid in the choice of therapy regimes, thereby helping prevent the emergence of drug resistant viral strains. Bearing in mind that most ART programs in Africa are based on a limited number of drugs, implementing these programs without advocating VL testing is “playing with fire”, as initial treatment gains will be ultimately lost to future drug failures and spread of resistant viral strains. Therefore VL tests should not be deemed a luxury but a necessity for future treatment success. A less costly and technically simple test such

as the RT assay presented in this thesis should be considered for VL monitoring in regions where expensive molecular-based methods are not a viable alternative.

FUTURE PLANS

1) The RT VL assay is currently being used for clinical use to monitor patients accessing ARV therapy in different sites around the world.
2) An international multi-site evaluation of methods used for monitoring VL in SIV/SHIV infected macaques has been initiated.
3) Further studies will be carried out to determine the relevance of NRTI resistance mutations on the level of RT-fitness. Reduction in RT-fitness associated with ARV therapy should be further evaluated to aid in the planning and maintenance of ARV treatment regimes. This may be of particular use in resource limited setting where NRTI treatment regimes, absent of a PI drug, are the backbone of nearly all ARV programs.
4) A faster and more sensitive version 3 of the ExaVir® VL test is currently being beta-tested in Melbourne, Australia.

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