Nucleic Acid Sequence-Based Amplification: Relative Performance and Applications in HIV-1 Disease Monitoring and Patient Management

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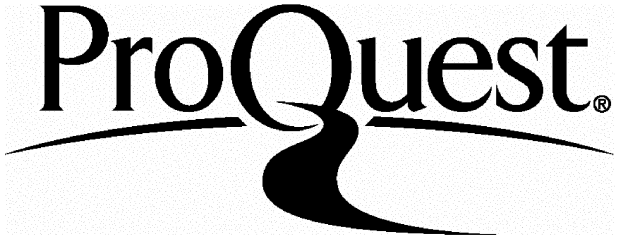

Abstract

In recent years there have been significant advances in the understanding of the pathogenesis of HIV-1 infection. Central to this progress has been the development of accurate and reproducible methods of measuring HIV-1 RNA in plasma. This has been pivotal in studies of viral dynamics, disease progression and antiretroviral drug efficacy. This thesis describes evaluations of the performance of Nucleic Acid Sequence-Based Amplification (NASBA) technologies in quantifying HIV-1 RNA, relative to other commercial systems, investigating reproducibility, sensitivity and reliability across subtypes using a number of panels designed with either plasma dilutions of laboratory strains of HIV-1, or by selected clinical viruses of interest.

Quantification of HIV-1 B and non B subtypes was investigated to study the impact of increasing diversity of HIV-1 subtypes in our clinic population. The evaluations described here involve close collaborations with assay manufacturers (as an alpha testing site) and clinical users, and have encouraged the continual improvement of viral load quantification systems, supporting the development of new versions of RT-PCR and NASBA assays. Further, attempts were made to develop an economic in house viral quantification system based on immunocapture and RT-PCR using an internal biological standard.

To investigate the versatility of viral load measurement in different clinical settings, studies were performed in primary HIV-1 infection and established HIV-1 infection. A well characterised cohort of patients undergoing primary HIV-1 infection (n = 47), were evaluated for the relative performance of NASBA quantification during the initial stages of infection and followed for up to 3 years. In addition, this enabled a comparison of virological responses to therapy in early HIV-1 disease and analysis for

antiretroviral resistance mutations to investigate the prevalence of resistance transmission in this recently infected group.

A clinical trial of patients with established infection was undertaken to determine the practical significance of virological measures relative to immunological responses and therapeutic drug monitoring.

The performance of NASBA in quantifying viral load in a wide range of studies was shown to be less sensitive than the bDNA and RT-PCR assays and also less able to amplify viruses of more diverse subtypes. In a number of studies the quantification of samples with a low viral load (< 5,000 copies/ml) by NASBA was shown have greater variance. However, at higher viral copy numbers (> 5,000 copies/ml), the reproducibility of results was equivalent to alternative viral load systems and other markers of disease monitoring and drug efficacy. NASBA performed adequately in characterising patients undergoing primary HIV-1 infection relative to other disease markers such as p24 antigen, anti-HIV-1 serology, and the conventional understanding of virological events during this early infection period. Further, NASBA viral load measures demonstrated that although antiretroviral therapy was very effective during this period, it was not significantly more effective than therapy initiated later. It was noted in this cohort that the frequency of resistance transmission was low for nucleoside (15%) and non-nucleoside (6%) inhibitor resistance, and absent for protease inhibitors.

The investigations into an in house system showed that a modified immunocapture using latex microparticle produced inconsistent results, and a variable high background in the detection system precluded further evaluation. By comparison, the reverse transcription and amplification step was modified successfully and further optimisation of this method was undertaken within the department to allow routine use.

Throughout this study, the objective has been to evaluate the current and future use of viral load measurement. This thesis has gone some way towards validating the

expanding use of viral load since the identification of HIV-1 RNA as a disease marker. As long as the reliability of viral quantification systems supports the prevailing clinical environment and is evaluated in studies such as this, it may continue to act as a principal tool in HIV-1 clinical research and patient care.

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Chapter 1 Introduction

1.1 Emergence of HIV-1

In the early 1980s, reports first came from the USA describing admitted patients, previously in good health, who were suffering from chronic opportunistic infections associated with severe immunodeficiency[1]. In 1981, this syndrome had been officially recognised as acquired immune deficiency syndrome (AIDS)[2] with symptoms presenting as a consequence of declining CD4 T-lymphocytes. The epidemiology of the disease encompassed homosexual men, haemophiliacs, recipients of blood transfusions and intravenous drug users suggesting that the disease was transmitted *via* blood. The filtration and sterilisation of plasma factors for haemophiliacs, effectively removing bacteria and fungi, implied that the transmissible agent was a virus, infecting in a similar way to hepatitis B.

A breakthrough in the characterisation of the virus causing AIDS was made in 1983. Barré-Sinoussi, Chermann, Montagnier and associates at the Pasteur Institute isolated a virus from the lymph nodes of a patient with recurrent enlarged lymph glands. At the time, this lymphoadenopathy syndrome (LAS) was believed to precede an AIDS diagnosis. The isolated virus was later named Lymphadenopathy Associated Virus (LAV)[3]. Lymphadenopathy is associated with several viral infections. One group of researchers headed by Gallo and Popovic saw that the characteristics of the disease were similar to those reported for human T-cell Leukaemia Virus (HTLV), suggesting that the aetiological agent was a retrovirus. Progressive work by both groups described their isolates as being similar to HTLV. Each infected CD4 T cells and the replicative cycle involved the enzyme reverse transcriptase. However, the virus remained unique in its

pathogenesis of the immune system. It was this singular property which led to Gallo and colleagues to define it as HTLV-III[4]. The isolation of the two viruses, LAV and HTLV-III, and their suggested pathologies were published in the same issue of Science in 1983[3,5].

A further group of researchers isolated what they called AIDS Associated Retrovirus (ARV) from known risk groups with no prior clinical diagnosis. This virus showed cross-reactivity with LAV and importantly, it demonstrated that the virus was present before a clinical diagnosis, strongly suggesting it as the causal agent of AIDS[6]. Sequence analysis of the three viruses LAV, HTLV-III and ARV determined them as retroviruses with substantial sequence similarity. Thus in 1986 the International Committee on Taxonomy of Viruses adopted the term Human Immunodeficiency Virus (HIV-1)[7].

1.2 Transmission

HIV, although a blood borne virus, is primarily sexually transmitted. Other common routes of infection are percutaneous puncture and vertical transmission from infected mother to child. Unprotected homosexual sex is believed to be the most effective mode of sexual transmission[8] due to the increase risk of membrane lesions. The probability of transmitting the virus during heterosexual sex is substantially lower, with male-to-female transmission more efficient than female-to-male transmission[9]. Other risk factors for contracting the virus *via* sexual contact are genital ulcers, the presence of other sexually transmitted diseases (STDs) and viral load of the infecting partner[10,11]. Regardless of sexual practise, there is an increased risk of sexual transmission where either partner has a high number of immune cells in the genital fluid. This could be a

result of non-specific lesions or due to a prior STD. With the infective partner this expanded population of virally infected cells increases the chances of transfer. Whilst in the non-infective partner, a high number of immune cells will increase the likelihood of that individual becoming infected.

The transmission of HIV from mother to child ranges from around 12-40%[12-15] depending on various events before and after childbirth. Any event that increases the likelihood of maternal/foetal blood crossover is a significant risk factor. Possible transmission events include amniocentesis, birth and breast-feeding. High virus count[16] and advanced immunosuppression[12] increase the probability of virus transfer. Consequently, if the mother is taking antiretroviral therapy which is successful in suppressing viral replication, the probability of infection is significantly reduced[16].

Transmission *via* contaminated blood products was the most efficient way of transmitting multiple copies of HIV. The comparatively large volumes involved make the probability of transmitting a viable virus quite high. Groups particularly at risk were those who received blood products on a regular basis such as haemophiliacs and those undergoing renal dialysis. Those who were exposed to infected blood on one single occasion were transplant recipients and recipients of donated blood. HIV infection became evident relatively quickly depending on the pre-existing immunosuppression of the patient receiving the blood. While this route of transmission has been largely removed by efficient blood screening in the western world, it almost certainly still occurs in developing countries[17].

Intravenous drug use (IVDU) remains a high risk factor for HIV transmission and the practise of sharing hypodermic syringes in the use of heroin has contributed greatly to the prevalence of HIV in the drug-user community[18]. Similarly, percutaneous puncture through needle-stick injuries, particularly in health workers working with IVDU or HIV positive communities, has been shown to transmit HIV infection effectively. The IVDU

population is difficult to monitor and hard to reach with health education. The disease is less detectable within the IVDU population due to symptom similarity with the clinical effects of drug abuse. This might explain why the trend in rising IVDU transmitted AIDS cases is harder to predict accurately.

The most frequent method of transmission largely depends on the country and the region. For example, in developed countries homosexual sex is the predominant route of transmission, versus heterosexual transmission in developing countries. This can be broken down into individual countries and regions. For example, Spain has the highest rates of HIV transmission *via* IVDU in Europe[19] so now the rate of transmissions in Spain is related to behaviour in this risk group.

Heterosexual sex was at first mainly confined to transmission *via* an individual from a high-risk country, or from a high risk population i.e. IVDU. Since then, due to successful health education strategies and the responsiveness of the target population, homosexual transmission and IVDU rates in the western world are falling[20], in the UK the percentage of homosexual transmitted new infections dropped below 50% of total new infections in 1998 and continues to decrease[21]. However, heterosexual transmission is rising, especially in US and European minority groups[20], where culture, language and socio-economic factors may present a barrier to the sexual health message.

The distribution and prevalence of HIV varies from country to country. In developing countries the disease has a high incidence in all sections of the population. This is because the most frequent methods of transmission are sexually amongst heterosexuals and vertical transmission, i.e. from mother to baby. This is in contrast to Europe and North America where the disease still has a higher incidence in the sections of the population who have a high risk exposure to blood borne diseases, i.e. homosexual men, prostitutes and intravenous drug users. Haemophiliacs and recipients of blood

transfusions make up a considerable group in most countries; this is as a result of infected blood products received before there was effective screening of donations. However, current incidence of HIV transmission in this group is very low, with very few new transmissions, resulting in an overall decline of HIV infection in this section of the population. Unfortunately, the screening processes in the developing world are at an early stage, so there is still a risk of infection from contaminated blood products.

1.3 Epidemiology

In 1986 a new human retrovirus, distinct from LAV/HTLV-III, was isolated from two AIDS patients from West Africa[22]. Molecular cloning confirmed two divergent retroviruses categorised as HIV-1 and HIV-2[23]. In 1990 it was discovered that HIV-2 is pathogenically distinct[24] and it is now known that HIV-2 infection progresses to AIDS at a slower rate than HIV-1[25,26]. HIV-2 is endemic to West Africa[26,27]and is relatively confined to the African continent or immigrants of African origin[28].

Phylogenetic analysis of the HIV-1 gene *env*, in 1988[29] demonstrated distinct genetic differences between HIV-1 from different patients, particularly patients from Sub-Saharan Africa. The divergent strains of HIV-1 were characterised as subtypes and a nomenclature for assigning subtypes was drawn up[30,31]. Recently the classification was revised[32] to encompass three groups of viruses; M, N and O (full article is available from the Los Alamos website: http://hiv-web.lanl.gov:80/HTML/reviews/nomenclature/Nomen.html)[33]. Group M contains the main subtypes that dominate the pandemic: A to D, F and H-J and the newly discovered K. Group N contains non-M and non-O viruses, and group O viruses are outliers, too genetically different to belong to either group. In the mid-1990s

research into global subtype distribution showed A-D present across sub-Saharan Africa with low numbers of F and G[27] whilst subtype C was the predominant subtype in the North and Southern African countries[34].

The recombination of two or more HIV-1 genome sequences has lead to the generation of recombinant subtypes. In 1996 it was demonstrated that HIV-1 subtype E viruses (epidemic in Southeast Asia[35]) were predominantly recombinants of subtype A viruses, sequence analysis established that subtype E viruses possessed a *gag* gene from subtype A and the envelope from subtype E; the parental subtype E strain was not found[36,37]. Later the nomenclature was changed and subtype E was classified as recombinant A[32](CRF01). Recombinant A/G has also now been discovered in West Africa[38].

In the 1990s subtype B virus was detected in all countries but was most prevalent in Western Europe and the US[34]. Over the last few years subtype distribution has shifted so that subtype diversity has increased outside Africa and particularly in Europe[39,40]. Mapping the geographical distribution of subtypes has enabled epidemiologists to associate subtype with different routes of infection. For example it was demonstrated that subtype recombinant A, the predominant subtype in Thailand, had a higher association with heterosexual transmission[41]. It was then possible to predict a rise in heterosexual transmission and infection events in Thailand. However, there have also been heterosexual epidemics of subtype B, a clade associated with homosexual/bisexual transmission. As yet there is no substantial evidence to link subtype patterns with biological or behavioural factors. Nevertheless, subtype analysis remains an investigative tool in epidemiological research. Figure 1-1 shows HIV-1 subtype diversity in the global pandemic.

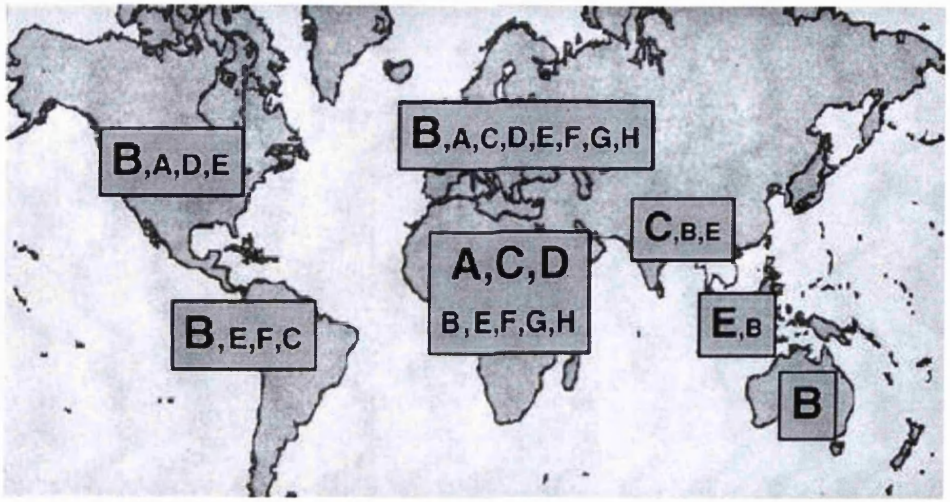


Figure 1-1 Global distribution of group M subtypes 1997[42]. Letters reflect which M subtypes had (in 1997) been isolated from that continent. The letter in bold reflects the predominant subtype in that area.

1.4 Virology

1.4.1 Structure

Electron microscopy has shown HIV-1 to be an icosahedron of approximately 100nm diameter, the surface of which is studded with glycoproteins[43]. The outer layer is formed when the virus buds from the host cell membrane, coating itself in the host phospholipid bilayer, through which virus proteins 'spike' outward. These envelope glycoproteins (gp) are named according to their molecular mass. The entire protein has a mass of around 160 kDa and comprises a transmembrane domain, gp41, and an external region, gp120. It is the gp120 which is closely involved in the binding of the virus to the CD4 receptor of the uninfected cell. The binding of this protein to CD4 and accessory molecules allows fusion of HIV-1 to the cell membrane, and initiates

integration into the cell. Beneath the lipid bilayer of HIV-1 is a cone shaped core comprised of viral capsid protein, predominantly p24. Contained within are two identical RNA strands and multiple copies of the enzyme reverse transcriptase (RT). Figure 1-2 shows HIV-1 virion composition including the major structural proteins.

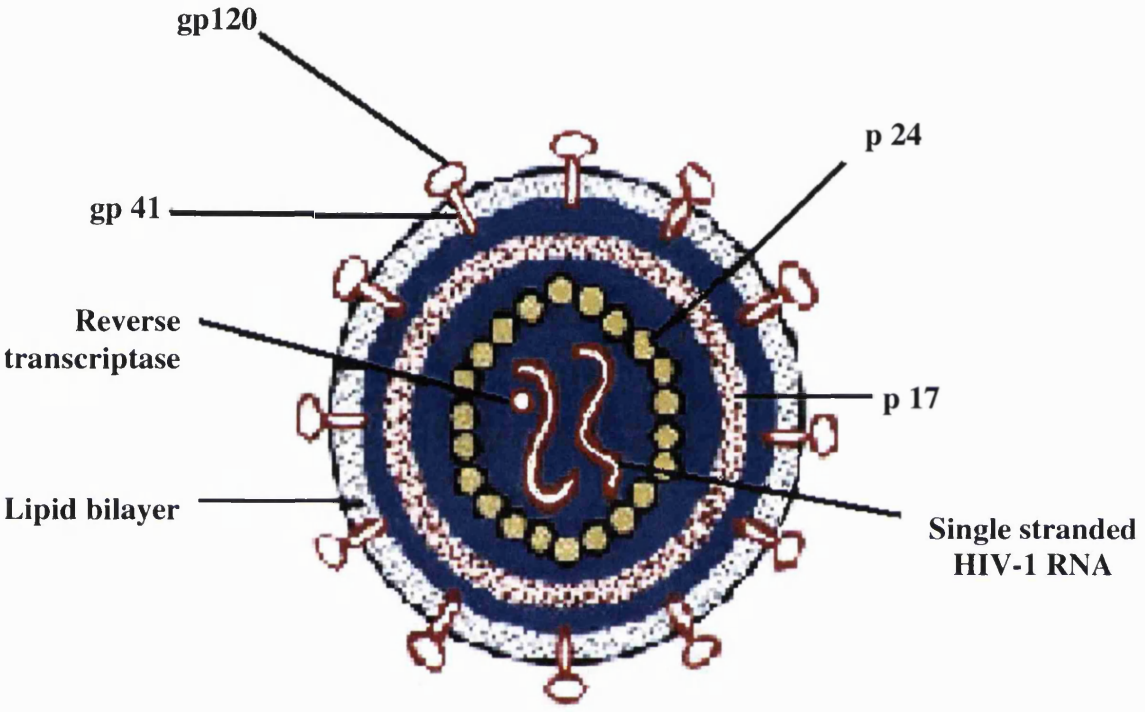


Figure 1-2 Virion composition[42] including major structural proteins gp41, gp120, p17 and p24. The diagram shows a single copy of reverse transcriptase (present in multiple copies) and two copies of single stranded genomic RNA. The lipid bilayer (human cell membrane) covers the virion when the virus buds from the host cell.

1.4.2 Genome

The HIV-1 genome is around 9.8 Kb and the mRNA transcript encodes three main genes common to all retroviruses; *gag, pol* and *env*. These code for large precursor proteins that are proteolytically broken down into smaller subunits. Cleavage of the *gag*

polyprotein gives four structural and core protein molecules including p24. The precursor *pol* incorporates viral enzymes reverse transcriptase (RT), protease (Pr) and integrase. *Env* encodes the envelope protein gp160, divided into gp41 and gp120. Messenger RNA products are spliced to give various regulatory and accessory proteins. Of those, *tat, rev* and *nef* are best defined as predominantly involved in the activation or regulation of virus replication. Gene products *vpr, vpu* and *vif* are accessory molecules, thought to be involved in virus assembly, budding, and infectivity in early disease. At either end of the genome there are base repetitions known as long terminal repeat (LTR) sequences, these encode transcription activator binding sites. The number and complexity of its regulatory pathways allows HIV-1 to moderate its replication according to host factors, reproducing itself rapidly when required.

1.4.3 Life cycle

The binding of gp120 to one or more cell surface receptors is essential for the infection of a cell. Following HIV-1 attachment there is a conformational change in gp120 that allows it to attach another region to a co-receptor. This brings gp41 into closer contact with the cell surface, allowing it to bind to a fusion receptor, initiating the interaction of the two membranes. The HIV-1 plasma membrane is integrated into the host cell membrane and the HIV-1 core enters the cytoplasm. Reverse transcription of viral RNA produces cDNA and this is duplicated into a double stranded structure. The DNA is transported to the nucleus of the cell where it is integrated into chromosomal DNA of the host cell. Production of viral RNA from integrated proviral DNA depends on the expression of HIV-1 regulatory proteins, and host cell activation status. Once HIV-1 replication has been initiated, the cellular machinery transcribes viral proteins from spliced viral mRNA. Capsid proteins are assembled to enclose genomic RNA and the core migrates to the cell surface. Processing of the *gag* and *pol* polyproteins assembles

envelope proteins, which are incorporated into the plasma membrane as the virus buds from the cell.

1.4.4 Host genetic mechanisms

The most frequent route of HIV-1 transmission is *via* sexual intercourse. Infected cells or free virus in genital fluids must be transmitted across the mucosal lining. Thus, the integrity of the epithelium is a crucial factor in transmission of the virus. As described previously, the risk increases if either partner has high numbers of immune cells at the site of infection. Cell types at the site of sexual transmission are most likely to be antigen presenting cells including macrophages and dendritic cells, or activated memory T lymphocytes. This last group acts as a preliminary defence mechanism and is localised to any site of inflammation. Either group is susceptible to HIV-1 infection and, because of their localisation, these cells are the most likely to be the first infected by HIV-1[44].

The virus binds to a number of cell surface accessory molecules which promote the fusion of gp120 with CD4. These are signal transduction molecules typically involved with the binding of the cell to chemokines, which normally initiate an intracellular response. As many as six receptors are known to act as co-receptors for HIV-1. However, the two most frequently targeted are known as CCR5 and CXCR4. The natural ligands for the CCR5 molecule are the β-chemokines RANTES, and macrophage inflammatory proteins MIP-1α and MIP-1β. These chemokines have been shown to block HIV-1 entry into cells *in vitro*, demonstrating effective competitive inhibition for the receptor[45]. The receptor CXCR4 binds stromal-cell derived factor 1, SDF-1, a chemo-attractant recruiting to the site of inflammation. Co-receptor usage seems essential to ensure the binding of virus to cells expressing low levels of CD4, or

by viruses that bind weakly to CD4[46]. Throughout the course of disease HIV-1 uses one or more co-receptors which have been linked to various HIV-1 phenotypes and pathogenic effects. HIV-1 which binds to a CCR5 receptor binding site tends to display a tropism for macrophages, dendritic cells and other antigen presenting cells. Whilst HIV-1 that predominantly binds to CXCR4 usually infects T lymphocytes. Although the HIV-1 receptor binding site generally corresponds to cell type preference, there are exceptions. Some virus strains have dual receptor usage and infect either cell[47]: other viruses use CCR5 but do not infect macrophages[48]. Dendritic cells and macrophages are among the first cells to be infected, usually because they are the most likely to be at the site of infection. There is strong evidence for the initial transmission of HIV-1 *via* CCR5 binding viruses. This is characterised by an observed increase in macrophage tropic virus following infection, and a subsequent decrease in that cell population. Host genetic factors influence the ability of HIV-1 to enter cells, and therefore also influence disease progression. Around 1% of the Caucasian population are homozygous for a 32bp nucleotide deletion in the DNA region coding for CCR5, causing a lack of gene expression and the absence of this functional receptor[45]. One study has shown this deletion to strongly protect against HIV-1 transmission[49]. However, another study showed that although patients who progressed to AIDS at a relatively slow rate had a higher frequency of CCR5 absence, it was not possible to link disease progression with CCR5 genotype when looking at individual patients within the same group[50]. Another membrane protein, CCR2, has been shown to affect disease progression. Although its exact role is not known, it has been shown to be an HIV-1 cofactor *in vivo*. A V641I change in the coding region for CCR2 has shown strong correlation with slowed disease progression[51]. Another host factor to have an impact on disease progression is HLA type, in that HLA class I alleles rather than class II, have considerable effects on disease progression. The HLA class 1 genes A29 and B22 were

shown to be associated with rapid disease progression, whilst genes B14 and C8 were associated with non-progression[52]. It has been suggested that the heterozygosity of HLA loci also plays a major role in delaying HIV-1 progression. One study cites that the extended survival of 28 to 40 percent of HIV-1 infected Caucasian patients who did not progress to AIDS for ten or more years could be attributed to their being fully heterozygous at HLA class I loci[53].

Chemokines which compete with HIV-1 for binding to cellular co-receptors have been shown to block HIV-1 entry into cells[54]. Therefore it may be that an individual's chemokine level can have an effect on HIV-1 disease progression. Genetic characteristics of chemokines have also been linked to progression. A homozygous genotype for SDF-1 known as SDF1-3'A/3'A has been associated with lower HIV-1 replication than the heterozygous allele[55].

1.4.5 Primary HIV-1 Infection

Primary HIV-1 infection (PHI) is the stage of disease following exposure to and consequent infection by the virus. Generally 2 to 6 weeks after exposure to HIV-1 the infected individual develops high levels of plasma HIV-1 RNA and mounts an HIV-1 specific immune response. At this time 20–90% of such individuals develop acute symptoms, similar to those of influenza, with varying severity. When first infected an individual produces antibody type IgM, this is the statutory primary response to any infection: as the immune response is refined the predominant antibody shifts to become IgG, a more specific antibody targeted to various HIV epitopes. The production of antibody (or HIV RNA if measured prior to antibodies) defines primary HIV-1 infection. As the level of antibody in the serum rises, the individual is said to 'seroconvert'. There are around nine antibody epitopes which are produced at varying time points following infection. Antibodies most commonly found respond to p17, p24,

p31, p51, p55, p66, gp41, gp120 and gp160 HIV-1 antigens. The technique known as western blot analysis can demonstrate which antibodies are being produced and how strong the response is. In the majority of individuals infected for more than a year, all the antibodies are present and the bands are relatively strong. Weak bands and an incomplete antibody repertoire can be an indication of recent infection, but time periods are difficult to define due to the individual variability of immune response. Antibodies to gp41 and p24 are measured by ELISA assay to confirm HIV-1infection, but it can take up to three months for the antibody levels to rise to ELISA detectable levels. The appearance of p24 antibodies is a direct result of increasing levels of HIV-1 p24 antigenaemia, and this has been suggested as an early detector of HIV-1 infection. But again, levels differ according to the individual, and once the immune response is generated, p24 antigen is cleared and may be no longer detectable. The period following transmission before an antibody response is detectable is known as the 'window period', during which time an individual is unsure of their HIV-1 status, this length of time can vary from within two months to, in some cases, longer than six months[56]. During this time it is possible to perform viral load quantification or a PCR test to directly measure HIV-1 RNA/DNA which is detected before the antibody response[56-58].

Once infected, the virions which were transmitted undergo rapid replication leading to very high plasma HIV-1 RNA levels. At this stage of disease the expansion of the virus population has a common ancestral source (the transmitted virions) so the population is genetically similar[59]. Hence the number of significantly different viruses or quasispecies is limited.

The high level of viraemia allows dissemination of virus throughout the body. It is now established that infection of the CNS and of resting CD4 T cells occurs very early in the course of primary infection[60,61]. The high viraemia also greatly increases an individual's

infectious capability, with large quantities of virus present in genital secretions. Evidence suggests that the epidemic of new HIV-1 infections is partially driven by recently infected individuals[62].

The early immune response involves the production of neutralising antibodies and the anti-HIV-1 cytotoxic T lymphocyte (CTL) response. CTLs become sensitised to HIV-1 structural proteins, predominantly those encoded by *gag* and *env*. In PHI, CTL numbers and the severity of response correlate with HIV-1 viral load and inversely with CD4 cell counts[63]. As the antiviral immune response evolves, follicular dendritic cells trap HIV-1 virions as antigen-antibody complexes in lymphoid tissue. As a consequence, plasma HIV-1 RNA concentrations drop by around 2 or 3 $\log_{10}$[64]. Antibody responses to p24 antigen are more rapid and they clear circulating p24 to undetectable levels within a few weeks. This transient period of p24 antigenaemia may be used to confirm a seroconversion diagnosis.

In contrast, viral load may fluctuate for around six months before levels stabilise. Some regard this stabilisation level or 'set-point' as a prognostic marker for disease progression[65-67]. Further research demonstrated not a set-point, but rather an inflection point or nadir, the point at which the viral load decrease stopped, shortly before gradually increasing[68].

Current opinion is that viral replication does not stop or stabilise, but is ongoing, and this is demonstrated by slight increases in viral load over time. Data from a cohort of HIV-1 infected haemophiliacs followed for seventeen years, demonstrated that $\log_{10}$ RNA levels increased linearly over time, with a median yearly rate of increase of 0.12 $\log_{10}$ copies/ml[69].

The efficacy of the immune response in reducing circulating virus, characterised by a low viral load nadir at around four months, has important consequences for disease progression and predicting CD4 cell decline[68]. The drop in CD4 cell count during acute

infection is strongly prognostic of immune cell competency in later disease[70]. A large fall in CD4 cell counts early in primary infection can reduce the likelihood of immune reconstitution and CD4 cell counts may not return to pre-infection levels.

The factors that govern the rate of viral load decline, and to what level, are varied and unclear. Most agree that host factors, genetic and physical, can have a decisive outcome on HIV-1 pathogenesis and the time to development of AIDS. Genetic differences in immune cell activity and antiviral response as well as cell susceptibility to infection, can determine direct pathogenic effects of HIV-1. Clinical health upon infection should also be considered as potentially prognostic. The role of other viruses, foreign antigens, and cytokines in heightening immune activation (and thereby increasing the ability of HIV-1 to replicate in host cells), may have a profound effect on disease progression. Following acute infection there is a clinically latent period that remains asymptomatic as long as viral replication can be contained by the immune system. HIV-1 infected lymphoid tissue causes progressive impairment of the thymus, characterised by a decreased production of cells. Assessing markers of thymic emigrant alphabeta T cells complements viral load and CD4 cell counts in predicting disease progression[71]. Once the immune system becomes compromised by irreversible cell loss it can no longer effectively respond to further challenges, allowing opportunistic infections to cause disease and eventually death. The reasons behind immune system decline are varied and inconclusive. What is apparent is that HIV-1 is able to cause immune cell death directly, by cytopathicity and indirectly, by immune dysregulation.

1.4.6 Immunopathogenesis

After the initial infection event virus is sequestered in the lymph nodes by antigen presenting cells[72]. Here the virus establishes a chronic infection, replicating continuously in lymphoreticular tissues[73,74], causing irreparable structural damage as the disease progresses. Once an infected cell is activated its cell cycle is accelerated and viral transcription increases. Research into viral kinetics demonstrated that the half-life of an active infected T cell or macrophage is significantly reduced[75], resulting in an increased cell turnover. This shortened life span is not matched by an equivalent rise in cell production rate, therefore, lymphocyte destruction must be directly induced as a result of HIV-1 infection, rather than loss of immune cell production[75]. The quiescent infected cell population maintains its longevity and is an important reservoir of virus, however it is still thought to contribute to viral pathogenesis. The underlying drive for high viral turnover is cited by many as the primary cause of HIV-1 pathogenesis [75,76]. Immune dysregulation related pathology has been demonstrated in a number of systems, but the relative impact on the immune cell population as a whole is still unclear. Apoptosis is the method by which a cell undergoes a pre-programmed suicide, this exists to regulate cell populations and also to promote the killing of cells infected by an intracellular pathogen. The latter is mediated by the binding of a cytotoxic ($CD8^+$) T lymphocyte or a natural killer cell to the infected cell. One apoptosis pathway is *via* the Fas protein cell surface ligand (FAS)[77]: once activated this signals the cell to shut down. In HIV-1 infection apoptosis is increased, with large numbers of infected and uninfected immune cells undergoing programmed cell death, contributing to population decline. Investigations into the expression of the FAS ligand have shown that as disease progresses, CD4 T cells have an increased number of FAS molecules on their surface[78,79], heightening the chances of cell:cell mediated cell death. A number of HIV-1 proteins have been implicated in augmenting apoptosis *via* the FAS ligand, these

include gp120[80], Tat[81,82] and Nef [83]. An increase in FAS expression can also be induced in stimulated T cells. The binding of CD4 or CD3 by their ligand results in activation and increased expression of FAS. Upon subsequent FAS stimulation, the cell undergoes activated induced cell death (AICD).

Cytokines play a vital role in the communication between cells of the immune system network. Certain cytokines drive a feedback system promoting two responses aimed at combating intracellular or extracellular pathogens. Cells and cytokines involved in a type 1 response target intracellular infections. The type 2 response is aimed at extracellular pathogens and is characterised by humoral involvement. This rationale can differentiate CD4 cells as either T helper 1 or T helper 2, dependent on which response they elicit. In HIV-1 infection a strong type 1 response characterised by type 1 cytokines IL-2, IL-12 and IFN-γ, has been suggested as being beneficial and immunoprotective, reducing the likelihood of progression to AIDS[84]. The same research has shown that HIV-1 drives the expression of type 2 cytokines; IL-4 and IL-10 inducing B cell activation and antibody response. This may be observed in some individuals as hypergammaglobulinaemia[85]. A type 1 to type 2 switch, if promoted by HIV-1, would downregulate type 1 cytokines *via* a positive feedback mechanism, resulting in the loss of an intracellular response. Furthermore, type 1 cytokines can be protective against apoptosis[86], which is increased during HIV-1 infection. Research into correcting this imbalance *via* the therapeutic administration of type 1 cytokines is underway and may give a better idea of how HIV affects this complex system, and whether it contributes to disease pathology.

In later stages of disease, T cell functional capacity is severely impaired, not only by a decrease in number, but by a failure to respond to antigenic stimuli. Several groups have demonstrated a loss of responsiveness even in early disease[87]. Initially, T cells fail to respond to recall antigens, later they lose their ability to recognise allo-

majorhistocompatability (allo-MHC) complexes. There follows a sequential and progressive loss of function, which occurs regardless of CD4 cell count[88]. However, some groups have found decreased responsiveness to selected pathogens only in patients with CD4 cell counts of < 300 cells/mm^3 [89]. Failure to respond to receptor stimuli or to secrete cytokines, is characterised as T cell anergy. An anergic T cell will not proliferate and hence T cell populations will decline, also reducing the memory T cell pool. Immunological memory is further impaired by the loss of antigen presenting cells early in infection[90,91]. Macrophages and dendritic cells, targeted and destroyed in early disease, cease to prime immune cells with antigenic stimuli. Only stimulated T cells can clonally expand and differentiate to acquire memory status. Infected cells have a significantly reduced capacity to divide as clones, so long-lived immunological memory to common pathogens decreases as the disease progresses, so increasing the possibility of infection with an opportunistic pathogen.

Viral phenotype can have a huge impact on the pathology of disease. In 1986 it was found that certain viral strains were able to induce syncytium formation of infected and uninfected cells *via* the gp120 and CD4 receptor binding[84,85]. An infected cell is induced to express gp120 on its surface, which in turn binds to CD4 on other cells. One cell can cause the aggregation of between 50-500 uninfected cells[92], allowing much more rapid transfer of infection from cell to cell. The cell cluster will frequently form a multinucleated giant cell that eventually dies. This phenotype is known as syncytium inducing (SI) virus and is associated with a more rapid decline of CD4 cells and progression to AIDS[93]. This phenotype is also linked to the use of co-receptor CXCR4[94], allowing it to infect a wider range of cells. Entry *via* CXCR4 will also negate any possible competitive inhibitory effects of β-chemokines for the CCR5 receptor. The SI phenotype can evolve during HIV-1infection and, therefore, a strong immune response or antiretroviral therapy, instrumental in slowing viral replication,

may prevent its appearance. Although it is possible to develop an SI phenotype over time, it is equally possible to receive SI HIV-1 at the point of infection[95,96]. Individuals with SI primary infection have been reported to advance more rapidly to symptomatic disease[97]. However, some studies have demonstrated SI virus but no correlation to an increased progression to AIDS[98]. Non-syncytium (non-SI) inducing virus can induce a rapid CD4 cell decline and aggravated progression similar to that seen with SI strains, and studies have shown that the risk of death, rather than disease progression, is similar for both phenotypes[99]. HIV-1 evolution usually results in more aggressive disease, sometimes characterised by an non-SI to SI switch. However, a direct link between SI formation and increased pathogenesis *in vivo* has yet to be determined.

1.5 Immune reconstitution during highly active antiretroviral therapy (HAART)

Treatment with highly active antiretroviral therapy (HAART) has been shown to bring about a rapid decline in circulating infectious virus. This can have a profound effect on the integrity of the immune system. Studies have shown that HAART can restore antigen specific CD4 T-cell responses to recall antigens, correlating with an increase in memory CD4 cells[100]. The same study showed that the decrease in viraemia that follows the introduction of HAART closely correlates with an improvement in the delayed type hypersensitivity response[100]. The expansion of CD4 memory cells, an improved inflammatory reaction and increased thymic output[101] provide strong evidence of good immune reconstitution and delayed disease progression following HAART. However, where antibody responses to opportunistic infectious agents improve, some studies have shown that even after a significant time on HAART, HIV-specific

lymphoproliferative responses remain suppressed[102]. Improved CD4 HIV-specific responses have been observed after treatment interruptions[103], implying that maintenance of response requires a low level or a repeated antigenic stimulus. However, other studies have shown that although CD4 and CD8 cell turnover increases after cessation of therapy, a high percentage of patients experience viral load relapse, implying that the immune response is not capable of controlling vireamia[104,105]. Some patients, termed long term non-progressors (LTNP) remain clinically asymptomatic although HIV-1 infected and sustain stable CD4 cell counts over a period of up to 10 years. Several studies have investigated why this should be so. As mentioned previously viral phenotype has been strongly associated with disease progression, or lack of progression. LTNP have lower levels of circulating virus leading to less immune pathology. Studies have suggested that the reason for this is the enhanced ability of CD8 cells in LTNP to suppress HIV-1 replication, leading to a reduction in the loss of CD4 cells[106]. If this is an accurate portrayal of virus versus host dynamics, then treatment aimed at preserving and maintaining the cytotoxic response and reducing viral load becomes of paramount importance.

1.6 Antiretroviral Therapy

The classification of HIV-1 as a retrovirus highlighted the replicative stages of its life cycle as potential targets for therapeutic intervention. Research into reverse transcription, in particular into reverse transcriptase (RT), the enzyme that catalyses this reaction, led to the rapid use of nucleoside analogue 3'-azido-3-deoxythymidine (AZT), previously synthesised as a possible anti-cancer treatment. This compound inhibits RT by preferentially binding to the enzyme in place of the regular nucleotide. The azido

group at the 3' position prevents the addition of further bases, effectively halting DNA synthesis.

Whilst the therapeutic benefits of AZT given by itself remain controversial[107,108] its use has sparked the discovery of other nucleoside reverse transcriptase inhibitors (NRTIs). The most widely used nucleoside analogues include didanosine (ddI)[109], dideoxycytidine (ddC)[110], stavudine (d4T)[111], and lamivudine (3TC)[112] and abacavir[113]. Advances in treatment include combining doses of two or more antiretroviral drugs which are commonly administered together. Examples of this are 'combivir' and 'trizivir'. These are tablets combining NRTIs AZT and 3TC[114] or AZT, 3TC and abacavir[115]. This improves the patient's adherence to the regimen by reducing the number of tablets taken. However, with all the NRTIs, including AZT, their usefulness has been limited by the development of viruses which are resistant to the drug and the associated clinical failure, often after a relatively short period of treatment, and by the toxicity frequently associated with these drugs. The similarity in action of the NRTIs has lead to the phenomenon of cross-resistance. A resistant virus evolves due to the selective pressure of one therapy but the mutation also confers resistance to a second similar antiretroviral agent. For example the M to V/I mutation at codon 184 of the RT gene confers resistance to 3TC, abacavir, ddC and in part to ddI.[116].

The NRTI class of antiretrovirals has been shown to be at their most effective when used in combinations of two or more. *In vitro* studies have shown that combination therapy achieves greater suppression of viral burden and viral replication[117], thereby inhibiting or delaying the development of drug resistance[118]. This was demonstrated *in vivo* in 1990 when AZT combined with ddC was given to patients with advanced disease. Those on the combined therapy showed greater and more persistent effects than those on a monotherapy regimen[119,120].

The second class of antiretroviral agent to act on RT is the non-nucleoside reverse transcriptase inhibitors (NNRTIs), which, although sharing a common target, are different in structure to the NRTIs. The NNRTIs do not bind to the nucleotide binding site: instead they compete with cellular nucleotides at the catalytic site and prevent completion of reverse transcription. Despite binding at the same site on the HIV-1 RT, NNRTIs are structurally different from each other and can have substantially different pharmacokinetic, protein binding, and metabolic profiles. Their toxicities and side-effects are relatively minor and do not overlap with other antiretroviral agents and the difference in target site to the NRTIs makes them an ideal combination option. *In vitro* studies have shown combinations of nucleosides and non-nucleoside RT inhibitors to have a synergistic or, at least additive activity. NNRTIs currently in clinical use include nevirapine (NVP), delavirdine (DLV), and efavirenz (EFV). Treatment with NNRTI and NRTI combinations has been shown to be very potent yet tolerable. However, as with the NRTIs, there is a risk of resistance evolution with incomplete suppression of viral replication.

The successful development of drugs inhibiting RT brought about significant interest in targeting other key enzymes in the HIV-1 replicative cycle. HIV-1 protease is an attractive target for therapeutic intervention since it is essential for the cleavage of Gag-Pol precursor polyproteins, required for the formation of mature virions. The crystallisation of the protease enzyme in 1989[121], and subsequent computer modelling, allowed the identification of compounds that would fit into the active site in the functional HIV-1 protease. Clinical evaluation of a number of protease inhibitors (PIs) has shown potent antiviral effects. Again, these are substantially augmented when used in combination with other antiviral compounds. The combination of a PI and two NRTIs produces marked reductions in plasma HIV-1 RNA levels, such that this strategy of triple combination therapy was defined as highly active antiretroviral therapy

(HAART). Now, the definition can include any triple or quadruple combination of mixed drug classes. Thus far, six protease inhibitors are approved for clinical use: saquinavir (SQV), indinavir (IDV), ritonavir (RTV), nelfinavir (NFV) kaletra and amprenavir (APV). Each of these drugs can have significant side-effects in the patient as well as having adverse interactions with other medications. As with NRTIs and NNRTIs, the protease inhibitors may also generate resistance through mutation evolution if viral suppression is incomplete. With PIs there is a much greater likelihood of cross-resistance, several of the PIs having almost identical resistance patterns. There is growing concern over the long-term side effects of all the conventional antiretroviral drugs, but particularly the protease inhibitors, which are known to be associated with hepatoxicity, diabetes, and a number of metabolic abnormalities e.g. lipodystrophy.

A better understanding of all types of current antiretroviral therapy has given rise to some caution, particularly with regard to the potential evolution of resistance, toxicity and adverse side-effects. However, the beneficial effects of HAART include a marked decline in mortality and incidence of opportunistic infections[122,123], and subsequently reduced rates of hospitalisation. As a result, the cost of HIV-1 clinical care has declined, despite an increase in pharmaceutical and outpatient spending, and it has been suggested that HAART is one of the most cost-effective medical interventions of the last decade.

The third viral enzyme integrase, is another potential target for therapeutic intervention. As yet no integrase inhibitor has shown a significant virological effect in clinical trials. Several other potential targets of antiviral intervention have been researched. The sites at which HIV-1 binds to the cell and the use of accessory molecules are prime candidates for a therapeutic strategy. Recombinant CD4[124] (an artificially created molecule that is similar enough to the CD4 receptor as to mimic action) and sulphated

polysaccharides[125] have both been directed at blocking virus attachment. More recently, molecules which block HIV-1 co-receptor binding have been developed. However, the ability of HIV-1 to use a variety of receptors and to switch receptor usage, means these drugs have some way to go before they will inhibit HIV-1 to the same degree as the current antiretroviral therapies.

Immunotherapy to restore immune function and delay HIV-1 pathology has been considered, but this strategy has been shown to be much more effective when used in combination with HAART. The administration of interleukin type 2 (IL-2) to patients receiving HAART greatly increased the numbers of CD4 cells, the numbers of naïve CD4 cells and reduced the numbers of infected CD4 cells[126]. Alternatively, therapeutic immunisation may also boost immune response, stimulating cytotoxic T cell activity and response to HIV-1antigens. Remune (manufactured by the Immune Response Corporation, 5935 Darwin Court, Carlsbad, California, USA) is a vaccine based therapy or immunogen (designed to stimulate a greater immune response to HIV-1), and has shown some promise in clinical trials, but it does not yet offer any lasting improvement to immune function[127].

1.6.1 Pharmacokinetics

Drug metabolism can have a profound effect on drug efficacy. Therapeutic drug monitoring has been used to determine how much active drug a cell is absorbing. The factors that govern drug transport and drug activation are various and can be specific to an individual. Gut absorption of antiretroviral compounds is affected by the presence of food, and most drugs have specific times, before and after eating, when drug absorption is optimal. Other compounding factors affecting absorption and uptake into cells are the enhancing or competitive effects of other drugs, antiretroviral or otherwise. Conditions which become contra-indications can cause little or no uptake, or alternatively cause so

much absorption that one drug causes toxicity. The pharmacokinetic enhancement activity of some antiretroviral drugs resulted in their prescription in pairs, for example ritonavir enhances the activity of other protease inhibitors by increasing the half-life or the peak concentration of the drug in the plasma[128-130].

Cellular drug metabolism has been highlighted as it was discovered that AZT and other NRTIs require cellular phosphorylation to be active. Some of the enzymes that catalyse this reaction show decreased activity with disease progression, thymidine kinase deficiency, for example, has been shown to correlate with disease progression[131].

Side-effects including nausea and vomiting are common with some antiretroviral therapies. This alone can reduce the incentive to take them as accurately as the treatment guidelines require. Taking a substantial number of tablets, most of which require specific gut conditions, requires considerable perseverance, so non-adherence to the regimen is a key factor in therapeutic failure. Therapeutic advances that allow combined use of drugs, reduction to a single dose per day, or even more manageable, use of drugs i.e. liquid form, can significantly improve patient compliance.

Incomplete viral suppression due to non-adherence or pharmacokinetics has been the subject of much research, in particular therapeutic drug monitoring. It becomes critical when a therapeutic regimen is seen to be failing, and the avoidance of resistance evolution is of paramount concern.

1.6.2 Treatment and disease status

Current opinion is still divided as to the merits of initiating antiretroviral therapy early in acute infection. Many clinicians and scientists believe that during primary HIV-1 infection (PHI) the virus is at its most vulnerable and so antiretroviral therapy can have the greatest effect. As mentioned in section 1.4.5 the immune response is most effective in controlling viraemia during PHI. Research suggests that early treatment may prevent

irreversible damage to the T-helper cell population[132], enabling the immune system to effect a cytotoxic response. It has also been suggested that early treatment might prevent viral replication destroying the lymphatic tissue, thereby slowing disease progression[133]. The preservation of the immune response may enable the immune system to control viraemia upon cessation of therapy. Some clinicians and scientists believe that initiating HAART as a long-term suppressive therapy in primary HIV-1 infection provides the best chance of eradicating the virus[134].

Treatment can also decrease an individual's infectious capacity, by reducing levels of functional virus present in the blood or genital secretions. This reduction in viraemia decreases viral 'seeding', helping to prevent the sequestration of virus in 'privileged' body compartments and known reservoirs, such as the CNS and lymph nodes.

A recent study described a PHI cohort where virus was isolated from plasma and cerebro-spinal fluid (CSF) and the different viral populations were analysed for resistance mutations[135]. This showed that compared to a group of patients with advanced stage HIV-1 disease, the patients with PHI had a single identical quasispecies in both plasma and CSF i.e. the same mutation was present in both viral populations or in neither[134]. This suggests that early HAART may affect both sites with greater efficiency than a later therapy would, when the viral populations show increasing divergence. Thus it may be that the potency and the potential long-term benefits of therapy are increased if that therapy is initiated early in PHI.

Others are cautious about the benefits of early treatment of during PHI. As drug treatment of HIV-1 moves into its third decade, there is a growing recognition of long-term toxicity associated with prolonged HIV-1 therapy. Starting therapy earlier may increase the likelihood of the development of side-effects such as lipodystrophy and liver and kidney damage. Perhaps more important is the increased risk of resistance to one or more classes of drugs. The longer a patient remains on a particular therapy, the

greater the chance of viral evolution of resistance mutations, leading to a viral population with significantly reduced sensitivity to that combination. With the potential of multi-drug resistance arising, a patient could lose several treatment arms relatively early in disease. This is of great importance should the patient be infected by a virus bearing one or more resistance-associated mutations. By unknowingly treating the patient with a drug to which the virus has reduced sensitivity, the selective pressure will cause rapid expansion of the resistant population, which might have otherwise reverted to wildtype in the new host.

Current British HIV-1 Association (BHIVA) guidelines suggest that if therapy is to be initiated during PHI, it should started as soon as possible, with a potent combination of at least two different drug classes, of three or more drugs, preferably as part of a clinical trial[136].

Patients who present with asymptomatic HIV-1 infection, but who acquired their virus at least one year previously, are of a slightly different clinical group than those undergoing acute infection. There are relatively little data suggesting an absolute time point for starting therapy in asymptomatic patients, based on surrogate markers. Arguments for and against treatment during this period are similar to those encountered when considering treatment during acute infection. However, treatment studies in this group have shown that the HIV-1 specific immune response is only partially improved upon initiation of HAART[137]. Furthermore, current guidelines suggest that those who start therapy at a CD4 cell count of 350 cells/mm^3 do no worse than those who start at 500 cells/mm^3 [136]. Given the risks of long-term side-effects, delaying treatment until necessary may prove the best choice for the patient's quality of life.

Treatment guidelines concur that patients with underlying immunosuppression characterised by symptomatic HIV-1 should be offered HAART. Initial therapies recommended by the BHIVA include at least three antiretroviral drugs of two classes.

Two drugs are typically NRTIs and make up the nucleoside analogue backbone. The additional drugs in combination should be either one or two PIs or an NNRTI.

Failure of therapy is demonstrated by the persistence of viral replication. In some patients this replication may still be suppressed below 400 copies/ml but may be detectable above 50 copies/ml. However, the persistence of viral load well into therapy presents a risk of the evolution of resistance and therapeutic change must be considered. Treatment after more than one previous therapeutic failure is defined as salvage therapy. The choice of salvage therapy is limited by the drug experience of the patient. It is more likely to succeed if the patient is naïve to one class of drugs. Improved outcome is also associated with concurrent use of drugs of which the patient has no experience, or to which resistance is unlikely. Also, salvage therapy is more successful in those who start treatment at a lower viral load.

Vertical transmission rates have been greatly reduced in the past 10 years by the use of antiretroviral treatment administered during pregnancy. Treatment has been aimed at suppressing viral replication to undetectable levels thereby reducing the likelihood of the transmission of virus. BHIVA guidelines recommend starting triple combination therapy during the first trimester[138]. In women with low viraemia and high CD4 cell counts, AZT monotherapy from the third trimester is suggested. The guidelines recommend an elected caesarean section and avoidance of breastfeeding. Alternative treatment includes the use of a single dose of Nevirapine (an NNRTI). In one study, Nevirapine administered during labour and to neonates shortly after birth, reduced the rate of perinatal HIV-1transmission by 47 % in women in Uganda[139].

Combination antiretroviral therapy has had considerable success in slowing disease progression in paediatric patients. Similar combinations to those administered to adults are currently in use, but with modified dosing strategies.

Antiretroviral drugs are also available to healthcare workers and in some instances, the general public, who have been exposed to HIV-1 infection following a potential transmission event. Occupational exposure most frequently involves percutaneous puncture with infected body fluids. In specific cases post-exposure prophylaxis (PEP) may be offered to individuals who have been exposed to potential infection *via* sexual contact. However, it has been suggested that this may discourage the "safe-sex" message[140]. The Department of Health recommends PEP treatment with AZT, 3TC and indinavir for 4 weeks following exposure[141]. As yet, only AZT has been studied as a PEP programme and no antiretroviral drug has been licensed for PEP. Therefore treatment strategies follow existing data for the treatment of patients of known HIV-1 status.

1.6.3 Alternative therapy in HIV-1 Infection

Complementary therapies for the treatment of HIV-1 infection have been described as the 'scientific and non-scientific interventions that diverge from the accepted traditional treatment of HIV-1 infection'[142]. This term encompasses various self-medications including vitamin and dietary supplementation and homeopathic remedies, but it can also mean alternative non-pharmacological therapies like acupuncture and massage. The use of complementary medicine for any illness has risen dramatically over the last ten years: recent estimates show that nearly half of Americans used a form of complementary medicine in 1997[143]. This proportion increases to 68% when investigating the HIV-1 infected population[144]. The percentage of patients using complementary therapy has not been altered by the expanded use of antiretroviral treatment: most patients see complementary medicine as an adjunct to, but in some cases, an alternative to conventional therapies.

1.6.4 Antiretroviral Drug Resistance

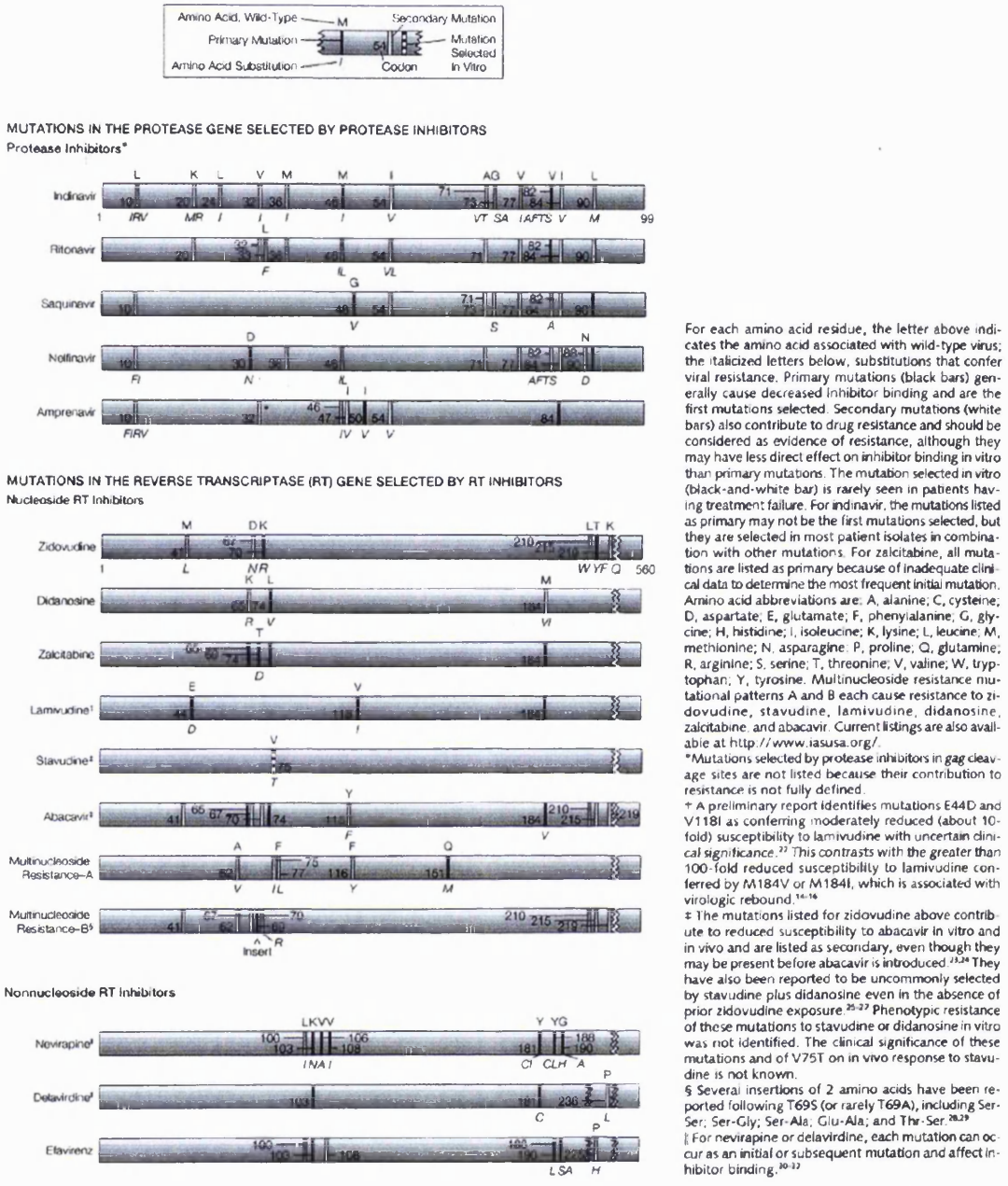
In the late 1980s, AZT was adopted as a first line strategy in combating HIV-1 infection. Subsequent clinical analysis demonstrated that AZT was effective in slowing viral replication *in vivo*, but the overall effect appeared to be short-lived. The long-term benefits to patients in terms of prolonging the asymptomatic phase or preventing disease progression were inconclusive. In 1989 it was demonstrated that HIV-1 sensitivity to AZT decreased over time[145]. HIV-1 sensitivity to a compound is measured as the amount of that drug needed to inhibit viral growth in infected cells by 50%: this is known as the IC_{50} for a given compound. The IC_{50} for AZT increased substantially over the course of treatment, implying the virus had evolved a degree of resistance to the drug. Sequencing of the resistant isolates demonstrated a number of mutations from the original sensitive virus (known as the wildtype virus). There are now known to be two primary and four secondary mutations associated with AZT resistance. Primary mutations are essential to allow the virus to replicate in the presence of a drug. However, primary mutations disrupt viral structure and can impair viral function making a resistant virus less viable. Secondary mutations appear to stabilise the mutated virus structure resulting in viral fitness equal to wildtype[146].

The presence of AZT suppresses sensitive strains of the virus. Therefore, a virus which bears a naturally occurring mutation, which allows replication in the presence of AZT will be selected for, so the population expands. Once a resistant mutation occurs the subsequent population expansion generates levels of the virus as high as those before treatment was started.

1.6.4.1 Mutations affecting a particular class of antiretroviral drug

Mutations conferring drug resistance tend to be clustered in the section of the genome that is being targeted for inhibition, either Pr or RT. Many drugs induce the same base change. For example the mutation at base 103 in reverse transcriptase causes the amino acid change from Lysine (K) to Asparagine (N), conferring a degree of resistance to all three currently used NNRTIs, Nevirapine, delavirdine and efavirenz. Hence, in this case, a virus with a K103N mutational change, may have resistance to three drugs, eliminating NNRTIs from a therapeutic strategy.

Figure 1-3 shows the most common mutations in HIV-1 genes conferring drug resistance, as demonstrated by gene and by drug class.



For each amino acid residue, the letter above indicates the amino acid associated with wild-type virus; the italicized letters below, substitutions that confer viral resistance. Primary mutations (black bars) generally cause decreased inhibitor binding and are the first mutations selected. Secondary mutations (white bars) also contribute to drug resistance and should be considered as evidence of resistance, although they may have less direct effect on inhibitor binding in vitro than primary mutations. The mutation selected in vitro (black-and-white bar) is rarely seen in patients having treatment failure. For indinavir, the mutations listed as primary may not be the first mutations selected, but they are selected in most patient isolates in combination with other mutations. For zalcitabine, all mutations are listed as primary because of inadequate clinical data to determine the most frequent initial mutation. Amino acid abbreviations are: A, alanine; C, cysteine; D, aspartate; 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. Multinucleoside resistance mutational patterns A and B each cause resistance to zidovudine, stavudine, lamivudine, didanosine, zalcitabine, and abacavir. Current listings are also available at http://www.iasusa.org/.

*Mutations selected by protease inhibitors in gag cleavage sites are not listed because their contribution to resistance is not fully defined.

† A preliminary report identifies mutations E44D and V118I as conferring moderately reduced (about 10-fold) susceptibility to lamivudine with uncertain clinical significance.[22] This contrasts with the greater than 100-fold reduced susceptibility to lamivudine conferred by M184V or M184I, which is associated with virologic rebound.[14-16]

‡ The mutations listed for zidovudine above contribute to reduced susceptibility to abacavir in vitro and in vivo and are listed as secondary, even though they may be present before abacavir is introduced.[23,24] They have also been reported to be uncommonly selected by stavudine plus didanosine even in the absence of prior zidovudine exposure.[25-27] Phenotypic resistance of these mutations to stavudine or didanosine in vitro was not identified. The clinical significance of these mutations and of V75T on in vivo response to stavudine is not known.

§ Several insertions of 2 amino acids have been reported following T69S (or rarely T69A), including Ser-Ser; Ser-Gly; Ser-Ala; Glu-Ala; and Thr-Ser.[28,29]

‖ For nevirapine or delavirdine, each mutation can occur as an initial or subsequent mutation and affect inhibitor binding.[30-32]

Figure 1-3 Most common mutations in HIV-1 genes conferring drug resistance.

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The presence of a resistance associated mutation does not necessarily allow the virus complete immunity from the drug's antiviral suppression. Sequential therapy of ddI

following AZT showed that although the virus maintained a primary mutation to AZT at codon 215, the later mutation at 74, associated with ddI resistance, suppressed the effect of the 215 mutation. This, in part, restored the virus's sensitivity to AZT[148]. This reduction in resistance, bought about by a resistance mutation to another drug, has been pivotal in the move towards combination therapy. In 1995 Brendan Larder and colleagues demonstrated that a mutation, bought about by 3TC therapy, from methionine (M) to valine (V) in codon 184 of reverse transcriptase, reversed phenotypic AZT resistance *in vitro*[149]. The addition of 3TC to AZT delays AZT resistance in previously untreated patients and can restore viral AZT susceptibility in patients who have already received AZT alone. In some AZT-experienced patients, the virological response to AZT-3TC therapy is not sustained and virus resistant to both drugs can be identified by a polymorphism at codon 333 of reverse transcriptase[150]. However, the AZT-3TC combination is still effective in the majority of patients and is a popular first line strategy, so much so that a dual drug pill containing doses of both AZT and 3TC marketed by Glaxo-Welcome has been available since 1998[151].

Once a patient has generated resistance to a drug regimen, they may decide to stop treatment all together. Following cessation of therapy there may be a genetic evolution of the virus from the drug resistant mutant back to the sensitive wildtype strain, usually because the wildtype virus is more viable than resistant mutants. This reversion to sensitivity can take anything between two weeks up to 12 months or more. If another drug is given before reversion, and resistance to the second therapy also evolves, the patient risks the evolution of multi-drug resistance. Repeated selection of drug resistant mutants from the viral population increases the likelihood of a mutation conferring resistance to more than one drug, especially if the virus already bears some resistance mutations.

Viral load is another factor which can have an effect upon the generation of resistance. The longer a patient maintains a detectable viral load during therapy, the more likely a resistant virus will emerge. A therapy which suppresses viral replication to undetectable levels therefore also limits the evolution of resistant virus. The persistence of a detectable viral load during therapy is termed virological failure, and has been highlighted as correlating with the evolution of resistance. The frequency of resistance evolution is dependent on viral kinetics and replication.

1.6.4.2 Transmission of drug-resistant variants

When decreased sensitivity to antiretrovirals was first observed, it became apparent that if a resistant virus was present in blood and genital secretions, then it could be transmitted to a non-infected individual. As the proportion of HIV-1 patients taking antiretrovirals increases, the chance of newly infected patients receiving a drug resistant mutant also increases. However, this likelihood is tied in with drug efficacy and viral load. An effective regimen will arrest viral replication, lowering circulating virus in blood and genital secretions to undetectable levels, thereby reducing the risk of compartmentalisation and of infecting others. A failing regimen, characterised by high levels of infectious virus, will increase the chances of transmission. If the regimen is failing due to the evolution of resistance, then the majority of the population of virus will be resistant, and this virus will be transmitted in blood or genital secretions. Infection with a drug resistant virus may severely reduce a patient's therapeutic options. Due to the homogeneous viral population immediately after infection, the first treatment strategy has been viewed by many as the most likely to have the greatest impact. If resistance transmission were not detected, drugs to which the virus was not sensitive may be included in the first strategy, negating its effect. The importance of detecting

the transmission of a resistant virus in choosing and preserving a first-line treatment strategy, has highlighted the potential role of testing for resistance at seroconversion. Characterisation of virus before a therapy regimen is selected, is a contentious issue as the current cost of identifying resistance through sequencing viral RNA is high. However, it can be argued that the cost may be balanced by the greater efficacy of a 'tailor-made' drug regime. When related to seroconverters this argument becomes more complicated. Early treatment prolongs the therapeutic period, thereby increasing drug expenditure and this may be added to the cost of resistance testing prior to selecting a drug regimen. In the argument for starting therapy early, as opposed to waiting for a clinical reason to treat, the financial burden must also be considered.

1.7 Viral load

1.7.1 Relevance in clinical practice

Whilst the main body of HIV-1 research in recent years has concentrated on developing antiretroviral therapies, it has highlighted the need to monitor disease progression and therapeutic efficacy. The impact of HIV-1 infection on the immune system is multi-factorial and not all areas of the disease pathology or immune dysregulation are understood. However, the observed outcome shows that the disease causes progressive immunosuppression, eventually resulting in chronic opportunistic infection leading to premature death. Consequently, the measurement of the number of circulating immune cells, in particular CD4 cells, has become a prominent marker of immune function and disease status. Before the advent of regular plasma viral load measurement, CD4 cell count was the predominant marker of disease status and was the background against which all clinical decisions were based. Today, where possible, both parameters are

measured. CD4 cell numbers ultimately demonstrate immune capability. However, viral load is more responsive to change, and a large variation in viraemia frequently predicts a later change in CD4 levels. Several other immunological and virological markers have been suggested as possible markers of disease status and progression; p24 antigenaemia, β2-microglobulin and proviral DNA levels have all been recognised as correlating with clinical outcome to some degree[152-155].

The quantification of HIV-1 RNA in plasma remains the most versatile measure of disease. This measurement can reflect the wider picture of viral dynamics within the body, the final product of an equilibrated system dependent on many other factors. Factors including immune control of viraemia, HIV-1 replicative ability or kinetics, and drug inhibition of replication, all, to some degree, affect a balanced system. The dynamics change as one factor predominates.

Viral load can be regarded as a marker for HIV-1 disease status, and if viral load starts to rise in an individual, it may give an indication of what factors might be responsible. For example, a patient on HAART with a persistently detectable viral load may have evidence of resistance associated mutations. Measurements of immune cell numbers and activation do not give as complete a picture, and are less responsive to system changes. A patient receiving a potent antiretroviral therapy will show a rapid decrease in viral load, in some cases within days, demonstrating therapeutic success. Measurement of immune cells takes much longer, by comparison, to show the benefits of a successful therapy. Antiretroviral drug efficacy has been most successfully investigated using plasma HIV-1 RNA quantification: the consequent fall in viral load upon commencing treatment can be easily compared for a range of single and combination therapies[156,157].

It has been possible to monitor patient status over time and compare this to viral load measurements, this has demonstrated that HIV-1 RNA levels can be linked to clinical

end points such as mortality or the development of AIDS, and that viral load is the most reliable predictor for clinical progression[67,158].

1.7.2 Viral load and its relationship to disease progression

Research has shown that a high level of virus in the plasma is a significant risk factor in disease progression[159,160] and that HIV-1 RNA levels are the most predictive marker of the progression to AIDS[161,162]. This assumption has lead to plasma HIV-1 RNA levels being used to distinguish between "progressors" and long-term asymptomatic patients. In studies a high baseline viral load at seroconversion has been linked significantly with the development of AIDS within a short time frame[66]. One such study followed a cohort of seroconverters over four years: those who developed AIDS or exhibited a rapid decline in T-cell activation and CD4 cell counts, were considered "rapid progressors". Those who consistently held CD4 cell counts over 400 cells/ mm^3 and who remained asymptomatic were considered "non-progressors". A year after seroconversion, there were no immunological or serological differences within the group: the only difference was in the level of HIV-1 RNA. Patients who were to become "rapid progressors" were found to have had initial viral load values which were significantly higher than the "non-progressors". The lack of immunological differentiation early in infection emphasises viral load as the most predictive marker of the onset of symptomatic disease.

1.7.3 Treatment Monitoring

The introduction of antiretroviral drugs has meant it has become increasing important to monitor the course of disease to maximise the clinical effect of treatment. Recently viral load has been the focus of research to find a marker that best demonstrates the

effect of treatment. Both the ACTG study 175[162,163] and the Delta 1 study[164] concluded that therapy-induced decreases in viral load, although transient, were the most significant prognostic indicators of positive therapeutic effects[165].

It is important to know as early as possible in the course of treatment of patient non-responsiveness or drug resistance in order to prevent clinical deterioration or an increase in aggressively resistant type virus. The emergence of HIV-1 resistance is a good reason to respond quickly to clinical changes. A beneficial antiretroviral therapy will suppress HIV-1 replication shown by a reduction in viral load[165]. Incomplete suppression means that viral replication, and therefore mutation is still possible. A regimen which fails to suppress virus in this way positively selects a resistant virus from the population. If a virus which survives in the presence of the drug evolves and which retains its function of replication and infection, it can cause HIV-1 RNA levels to rise with potentially detrimental clinical effects. Therefore, if a significant fall in viral load does not become apparent, indicating the patient is not responding to the therapy, that therapy can be altered before effects are manifested by clinical symptoms. Viral load measurement gives an estimation of the individual success of a therapeutic regime. This accounts for the increasing trend towards using viral load as the main prognostic tool for patients on therapy. In practice, a combination of markers, particularly HIV-1 RNA levels and CD4 cell counts, is seen as the best appraisal of the clinical status of the patient and their response to therapy. When a drug causes HIV-1 RNA levels to drop below the detectable limit, the length of time spent below detectability may be useful in predicting possible outcome of therapy.

1.7.4 Nucleic Acid Sequence-Based Amplification Technology

When methods for quantifying HIV-1 RNA were first developed in 1988, they centred around isolation of virus from plasma or serum which was then converted to template cDNA using reverse transcriptase. This cDNA could be amplified by polymerase chain reaction (PCR) to detectable levels[166]. Davis and colleagues were the first group to attempt a one step amplification of HIV-1 RNA known as a transcription-based amplification system (TAS)[167]. Like PCR, TAS used thermocycling, but did not require a separate reverse transcription step. They then used probes attached to dynabeads to detect the amplification products[167]. This work was taken further by Guatelli *et al* who modelled their system on an *in vitro* retroviral replication[168]. There are three enzymes essential for the retroviral replication and for the amplification of nucleic acids: reverse transcriptase to transcribe the target RNA sequence to DNA, a DNA-dependent RNA polymerase to create an RNA copy of the newly synthesised DNA, and RNase to degrade non-specific RNA. These enzymes were used cyclically to amplify cDNA and RNA templates of the original target sequence. The templates underwent a series of transcription and reverse transcription reactions which were self-sustained, under appropriate conditions. Like conventional PCR, the original target sequence could be amplified ten-million-fold. However, the reverse transcriptase reaction, traditionally the rate-limiting step of most RT-PCR methods, is the basis of the amplification cycle. Reverse transcription occurs cyclically, so if the primers fail to bind in one cycle to one RNA template, there is still the possibility of binding during another cycle. Kievits *et al* developed this technique further and applied this method to the quantification of HIV-1 RNA, it was described as nucleic acid sequence-based amplification (NASBA)[169]. The main differences between NASBA and RT-PCR based viral quantification are shown in Table 1-1 (adapted from Chan *et al*)[170].

Table 1-1 A comparison of RT-PCR technology with nucleic acid sequence-based amplification (NASBA).

Features	RT-PCR	NASBA
Reaction	Two sequential reactions, coupled together under non-isothermic conditions	One simultaneous reaction, isothermal at 41°C
Fragment size	May be optimised to amplify longer fragments	Optimal fragment length is <1000bp
Enzymes	RT and thermostable DNA polymerase. More recently a thermostable enzyme with dual ability to reverse transcribe RNA and polymerase DNA.	RT, RNase H, and T7 RNA polymerase.
Specificity	Variables of time, temperature and enzyme mix can be used to increase specificity	RNA polymerase is only functional at 41°C so conditions cannot be optimised for a specific target.
Amplification Product	dsDNA complementary to original RNA. Requires denaturation prior to detection with probe.	ss anti-sense RNA, may be probed or sequenced without further denaturation or strand separation.
Amplification Factor	Binary increase in product dependent on number of PCR cycles.	Exponential increase in product/transcription independent of number of cycles.
Fidelity	2×10^{-4} (for Taq polymerase)	2×10^{-4} (for both polymerases)
Additional features	Intron-flanking primers/DNases are necessary to prevent DNA amplification.	DNA background does not interfere with RNA amplification.

1.7.5 Development of an in-house viral load assay

It was proposed that an economic in-house viral load assay be developed and compared with NASBA in a clinical setting. The in-house assay was modified from an existing protocol describing HIV-1 immunocapture and lysis, reverse transcription, and subsequent radio-labelled nested PCR amplification and detection[156]. The existing assay protocol used anti-HIV monoclonal and polyclonal antibodies bound to latex microparticles (25μM), HIV-1 was captured via the antibodies to the particles. HIV-1 was then lysed and reverse-transcribed in one step before the resulting cDNA was added into a nested PCR incorporating a radiolabelled dNTP. The PCR amplicons from the second round could then be quantified, using a standard curve control included in every run, by measuring radiation. Proposed modifications included maximising the antibody resources (which were now limited) and by using a one-step reverse transcription (RT) and PCR. Alternative detection systems were investigated to try and quantify HIV-1 without using radioactivity. Proposed alternatives included using alkaline phosphatase (AP) labelled probes and substrate; incorporating fluorescein-labelled bases into the PCR and detecting cDNA using an anti-fluorescein antibody labelled with AP (NEN™, Life Science Products), or by a sandwich ELISA technique using an anti-fluorescein labelled antibody to which a large macromolecule studded with AP could be bound (AMDEX™, Amersham Biosciences). All the detection systems relied on the production of luminescence by enzyme AP and its substrate CDP-Star (NEN™, Life Science Products) and the detection and quantification of luminescence using a microplate scintillation counter (Topcount™). Details of the methods investigated are included in section 2.3 in Chapter 2.

The levels of antibody used were maximised by increasing the purification yield and by assessing the saturation point when binding the antibodies to the particles (this is

demonstrated in results section 3.7.1). This would allow less antibody to be used than was previously possible. Methods to optimise a one step RT-PCR were successful (as demonstrated in results section 3.7.2) and became routine for samples requiring RT-PCR such as routine DNA sequencing of HIV-1 for drug resistance genotyping.

Only the detection of DNA using a probe conjugated to alkaline phosphatase gave luminescence counts that were approximately proportional to the amount of DNA, but levels of background luminescence were variable and very high, generating a broad 'grey zone' where it was difficult to detect the levels of cDNA input. Methods were applied to produce a gradient of luminescence dependent on PCR cycles but these were largely unsuccessful due to the high background. Background luminescence was considerably reduced by using black plates and by blocking the plates with salmon sperm DNA to prevent non-specific binding when using labelled probes.

In conclusion using luminescence to detect HIV-1 was successful and implied that low copy numbers such as 10 – 100 copies would be detected if amplified in a PCR. Unfortunately, background luminescence was too high, precluding the use of this method for quantifying HIV-1. Concurrent with this work, a number studies substantiated conventional HIV-1 RNA quantification methods such as NASBA, RT-PCR and bDNA. These assays had achieved levels of specificity and sensitivity reliable for use in HIV-1 patient management. It was decided therefore to evaluate NASBA quantified viral load by comparing it against other conventional assays or HIV-1 disease markers, rather than by comparing it to a modified in-house system that was relatively untested.

1.7.6 Quality Assurance

Quality assurance is the process of testing the performance of an assay to ensure that all aspects of the assay satisfy the criteria demanded of it. Regular assay evaluation

identifies weaknesses in the assay design and performance. This feedback is invaluable to clinicians, scientists and assay designers alike, and ensures assays are continually updated to provide the highest possible standard. It is possible to look at results retrospectively generated over time to see if they reflect an accurate clinical picture. The integration of regular proficiency testing as part of the clinical application of viral load quantification, allows the evaluation of assay performance in a typical clinical setting. Proficiency testing is accomplished by designing a panel of samples, to be processed as ordinary clinical specimens, to evaluate all aspects of the assay. The panel is usually coded, or 'blind', so that the identity of the samples is not known to the operator. Performance is assessed through statistical analysis of assay results against either predetermined target values or against median values predicted by replication. Two factors are important in proficiency testing: accuracy and precision of results. Results are accurate if they are close to the true values for a sample. However, in some cases, the true values may not be known. Then, results can be compared to a reasonable estimate, such as the median given in several assays. If assays are replicated then the true estimate can be based on a measure of central tendency, such as the mean of the replicates.

Precision can be assessed by comparing results from coded replicates of the same sample. The intra-assay variability, or standard deviation is a measure of precision. The criteria for proficiency should reflect the goals of the clinical program, if the goal is diagnosis, then accuracy and precision may both be important. If the goal is to measure a change in response to therapy, then precision may be more important than accuracy. This is especially true in studies in which samples from a patient have been collected longitudinally and assayed in a series at the end of the study. In this case the background noise against which results are detected and change is gauged, as a measure of intra-assay variation.

This thesis will evaluate the NASBA method of quantifying HIV-RNA in a clinical setting. Many aspects of assay performance will be examined to give a comprehensive view of NASBA HIV-1 quantification in a clinical environment. The precision and accuracy of results will be assessed in a series of blind panels, where the expected values will be revealed upon analysis of results. The panels will be designed to test assay accuracy, reproducibility and sensitivity.

NASBA technology will also be assessed in its performance within selected patient study groups. Here, the target values will be unknown, and the assay will be assessed as a comparison against those values produced by alternative methods.

A further evaluation designed to assess viral quantification will use NASBA as a disease marker, and plot its correlation with alternative measurements of disease status.

1.8 Applications of viral load measurement.

An idea of the importance of viral load monitoring in the everyday care of HIV-1 infected patients can be gained by the frequency with which viral load monitoring is used by researchers and clinicians to answer questions about the disease.

Pharmacological studies use HIV-1 viral load as a marker of antiretroviral drug efficacy and pharmacokinetic effect. Immunological studies use viral replication as a measure of immune proficiency and can use it to estimate prospective immune cell pathology.

Clinicians may use viral load to assess whether a patient is likely to progress rapidly and therefore whether or not to initiate treatment. Viral load tests have been shown in some cases to diagnose HIV-1 prior to antibody positivity in those recently infected, particularly in babies born to infected mothers[58,171]

In Chapter 4 a study is shown in which viral load monitoring was used in addition to intracellular pharmacokinetics to assess the virological efficacy of antiretroviral therapy.

The versatility of HIV-1 RNA quantification was explored further in Chapter 5 where viral load monitoring played a pivotal role in the characterisation of a specific patient cohort.

This thesis evaluates the validity of viral load quantification by NASBA and looks at the expanding role of viral quantification in the field of HIV-1 research.

Chapter 2 Methods

2.1 Quantification of HIV-1 RNA by nucleic acid sequence-based amplification (NASBA)

2.1.1 NASBA HIV-1 QT (Organon Teknika ltd, Cambridge, UK)

The nucleic acid sequence-based amplification (NASBA) method uses the simultaneous activity of avian myeloblastosis virus (AMV) reverse transcriptase, T7 RNA polymerase and RNase H to amplify a specific target sequence of HIV-1 *gag* gene in HIV-1 RNA extracted from plasma. Once the target sequence has been amplified it is detected by induced electrochemiluminescence (ECL) and the quantity of RNA in the original plasma sample can be calculated.

2.1.1.1 HIV-1 RNA Extraction

HIV-1 RNA was extracted from the patient's plasma using the method described by Boom et al.[172]. This involves the lysis of HIV-1 virions in the plasma and adsorption of the released HIV-1 RNA to silica molecules. The silica with the attached RNA is washed to remove impurities, and finally the RNA is eluted into dH_2O. Briefly, the HIV-1 virus was lysed by adding 200µl of plasma to 900µl guanidinium thiocyanate (GuSCN) lysis buffer and the reaction tubes vortexed to ensure maximum viral lysis. The manufacturers provide three RNA calibrators from a non-HIV source, Qa, Qb, and Qc. Twenty µl of a mix of all three calibrators was added to each tube of plasma and lysis buffer. The silica suspension was vortexed vigorously before 50µl was added to

each reaction tube. The tubes were agitated for 10 minutes to maximise the binding of free RNA to the silica. The tubes were then centrifuged for 16 seconds at 13,000 rpm to pellet the silica with bound RNA. The pellet was washed to remove any bound impurities, twice with GuSCN, twice with 70% ethanol, and once with acetone, using 1ml volumes each time. After every wash, the silica was re-suspended and then pelleted by centrifugation at 13,000 rpm for 16 seconds. The supernatant acetone from the final wash was removed and the lids were left off and the samples dried for 10 minutes in a 56°C heating block. The silica pellet was resuspended in 50µl of dH_20 and incubated again at 56°C for 10 minutes, releasing any bound RNA into the water. After this incubation the silica was pelleted by centrifugation at 13,000 rpm for 2 minutes, leaving the extracted RNA in solution in the supernatant fluid.

2.1.1.2 Amplification

The amplification reaction as described by van Gemen et al[173] is initiated by the single addition of all reaction ingredients except the enzymes. Incubation at 65°C for 5 minutes to denature any secondary structure in the RNA, is followed by the addition of the enzyme mix and a 90 minute incubation at 41°C. Briefly, 5 µl of the extracted RNA was pipetted into a fresh 1.5ml conical tube into which 10µl of *gag* primer mix was added. The remaining extracted RNA was stored at -20°C. The reaction tubes were incubated in a 65°C heating block for 5 minutes to allow primer annealing and then transferred to a 41°C block for 5 minutes. Five µl of the enzyme mixture containing 0.1µg/µl BSA, 0.1 units RNase H, 40 units T7 RNA polymerase and 8 units AMV-reverse transcriptase, was added to each tube and the contents were pipetted gently to mix. The samples were incubated for a further 5 minutes to initiate the reverse transcription. The reaction tubes were transferred to a 41°C water-bath in the PCR

room to continue cyclic amplification of RNA for 85 minutes. The removal of the tubes from the water-bath and consequent cooling to room temperature stopped the reaction. If required, amplified RNA could be stored at -20°C for up to 6 months.

2.1.1.3 Detection of amplified product

The NASBA methodology utilises electrochemiluminescence (ECL) to detect the amplified RNA. The RNA is hybridised to a biotinylated capture probe that is in turn bound onto a streptavidin-coated magnetic bead. The whole complex is magnetically bound to an electrode where a ruthenium-labelled detection probe is hybridised to the target RNA. When voltage is applied to the electrode, the level of ECL (detected at 620nm) reflects amount of hybridised target RNA. The amplified RNA is firstly diluted by a factor specified by the manufacturer so that the detection values for Qa, Qb and Qc predict the values 10^6, 10^5 and 10^4 copies/ml respectively. For each sample a graph is plotted of the calibrator ECL against the RNA input. The ECL from the test sample is plotted on the same graph, and the HIV RNA copy number is determined. The lower limit of detectability for each sample is different, depending upon the amplification of the calibrators for that sample. This limit may be determined from each individual graph, but a limit of 2,000 copies/ml was suggested by the manufacturers to standardise all NASBA results. The curve does not give an accurate estimation of quantification below this limit, as the values plotted for the calibrators are approximate. For all NASBA results referred to in this text, the lower limit was 2,000 copies/ml unless otherwise stated.

Briefly, a specified volume of supplied detection diluent (Tris/HCl buffer) is added to 5µl of amplified RNA in a fresh 1.5ml conical tube. A biotinylated RNA probe for wildtype HIV-1 RNA and three biotinylated probes for detecting Qa, Qb and Qc RNA were supplied in each detection kit. One hundred and thirty µl of streptavidin-coated

magnetic beads was vortexed and added to four tubes labelled wildtype (wt), Qa, Qb and Qc. One hundred and thirty µl of the specific biotinylated probe, either wt, Qa, Qb or Qc was then added to the magnetic beads. Forty 5ml tubes (four for each reaction tube) were placed in a rack in four rows of ten, and 20µl of wildtype probe/bead mix was pipetted into first 10 tubes. Twenty µl of Qa mix was added to the second row of 10 tubes, then Qb and Qc to the third and fourth rows. Twenty µl wildtype probe/bead mix was added to an extra tube as a negative control, this did not have amplified material added to it. Five µl of diluted amplified RNA was pipetted into each one of four probe tubes. The tubes were covered with film, shaken by hand, and placed in a 41°C water bath for 30 minutes to hybridise the probe to the bead, and the sample RNA to the probe. The tubes were shaken every 10 minutes.

Whilst the hybridisation was taking place the computer was prepared to analyse the readings from the ECL detection machine. Patient identifiers for each reaction tube were entered and saved. Before every run the ECL detection machine was checked and re-calibrated if necessary using the assay buffer (100mM tripropylamine, pH 7.5) and cleaning solution (H_2O_2 solution) supplied.

Using a multi-dispenser, 300µl of assay buffer was added to each tube including the assay negative. To standardise the machine, 300µl of fresh magnetic bead mix was added to a clean tube and placed in position 1 of the carousel. The assay negative was placed in position 2. The sample tubes were then added in the order Wt, Qa, Qb, Qc, and so on until the carousel was full. The machine first analysed the ECL results of the reference solution and the assay negative. If these were valid the run continued. The printed result sheets were taken from the PCR room encased in a plastic folder preventing possible contamination with PCR products.

2.1.2 Nuclisens HIV-1 QT (Organon Teknika ltd, Cambridge, UK)

The Nuclisens assay differs from the original NASBA assay in that the calibration controls are specified exactly. In NASBA the calibration controls are taken to be approximately 10^6 (Qa), 10^5 (Qb), 10^4 (Qc) RNA copies/ml. In the Nuclisens version of NASBA, the calibrator copy input is specific to each product batch. Therefore, when the graph is plotted of ECL against copy number, each calibrator point has a smaller standard deviation. This allows the lower limit of detectability to go below 2,000 copies/ml. Again, the level of detectability is individual to each sample and can be read off the graph. In these studies the limit for all samples was taken to be 400 copies/ml unless otherwise stated.

2.1.3 Increased plasma input

In order to detect fewer than 400 copies/ml the assay requires a greater plasma input. NASBA and Nuclisens estimate viral load based on a plasma input of 100-200µl, the value per ml determined by the graph is therefore dependent on the input volume. Increasing the volume of plasma used in any PCR based assay, increases the sensitivity of detection, assuming that the extract is re-suspended in the same volume of diluent as before (50µl). The sensitivity of the assay depends on the number of copies/µl added to the amplification reaction. For example an input of 200µl of a sample with a concentration of 200 copies/ml would extract a maximum of 40 copies to go into the amplification reaction. This is likely to be undetectable by the Nuclisens and NASBA assays. Increasing the input to 1ml, increases the number of RNA copies going into the amplification to 200 copies, making it much more likely to be detected and quantified. Using 1ml of plasma allows a lower level of detection of 80copies/ml. Some of the

studies followed a protocol that extracted RNA from 2ml of plasma, lowering the detection limit to 40 copies/ml.

Similarly, diluting the plasma (or using a smaller volume) raised the upper level of detectability. In increasing the volume of plasma from 200 to 1000µl the potential HIV RNA present has multiplied by a factor of five. The volume of the NASBA lysis reagent, Guanidine thiocyanate (GuSCN) must likewise increase to maintain the proportion of HIV virus to lysis reagent. If the volume of lysis reagent is not increased there is a risk of incomplete lysis of virus, particularly if the plasma has a very high viral load. So for plasma inputs of more than 200µl the volume of lysis reagent was 9mls.

2.2 Alternative HIV-1 quantification assays

2.2.1 Amplicor HIV-1 Monitor (RT-PCR) Version 1 and 1.5 (Roche Diagnostics, East Sussex, UK)

The RT-PCR assay uses a single enzyme, rTth DNA polymerase, to reverse transcribe and amplify extracted RNA. A quantitation RNA standard (Qs) of known copy number is incorporated into the reaction to normalise the amplification. The amplified products are biotinylated and bound onto a microwell plate previously coated with HIV-1 or Qs - specific oligonucleotide probes. The bound amplicons are detected and quantified using avidin-horseradish peroxidase (HRP) conjugate and its colorimetric substrate. A series of five-fold dilutions of the amplicons allows comparison of the optical densities of the HIV-1 RNA and the Qs, from which it is possible to quantify the HIV-1 RNA copy number. Each set of reaction reagents allows the quantification of 21 plasma samples

and 3 controls supplied with the kit, a negative control and low positive and high positive control.

2.2.1.1 RNA extraction.

The extraction involves a single guanidinium thiocyanate (GuSCN) treatment of plasma and the addition of the Qs quantitation standard. The RNA is then precipitated with isopropanol and resuspended in dH_20 containing 400 copies of carrier RNA. Carrier RNA is a short strand of RNA that facilitates the initial binding of RNA extract to the enzyme rTth and also limits residual RNase degradation. Briefly, a set volume of Qs was added to the lysis buffer (GuSCN) dependent on each batch of reagents. 600µl of GuSCN and Qs was then aliquoted into 21 unskirted 2ml tubes labelled with a number and orientation mark. Into each tube 200µl of vortexed patient plasma was pipetted. The assay uses three controls which were prepared by adding 200µl of vortexed negative human plasma into tubes 22, 23 and 24. 50µl of the supplied negative control was added to tube 22, 50µl of the low positive control was added to 23 and 50µl of the high positive control was added to tube 24. After a 10 minute incubation at room temperature to ensure complete viral lysis, 800µl 100% isopropanol was added to precipitate the RNA, and the mixture vortexed. The RNA was pelleted by centrifugation at 13,000 rpm for 15 minutes with the orientation marks facing the centre of the centrifuge. The supernatant fluid was removed from the marked side of the tube (opposite side to the pellet) using a fine-tip pastette, and discarded. The pellet was washed with 1 ml of 70% ethanol and centrifuged at 13,000 rpm for 5 minutes before the supernatant fluid was removed as described. 400µl of dH_20 containing carrier RNA was added to each tube and vortexed to re-suspend the pellet. 50µl of the extracted RNA was transferred to a 0.5ml MicroAmp tube containing 100µl of RT-PCR mixture

made up of 20pmol of biotinylated primers Bio-SK431, Bio-SK462, 200μM dUTP, 150μM dNTPs and 10 units of rTth DNA polymerase. The Amplicor Version 1.5 brought out in September 1997 replaced the primers in Version 1.0 with an alternative pair of biotinylated *gag* primers, SK145 and SKCC1B, which have an improved affinity for diverse HIV-1 subtype viruses[174]. Extracted RNA remaining in the 2ml tube was stored at -20 °C.

2.2.1.2 Amplification.

The 0.5ml MicroAmp tubes were transferred to the PCR room and placed in a Perkin Elmer thermocycler 2400. Conditions were optimised for the different primers used in Version 1.0 and Version 1.5. The two different cycling profiles are detailed below:

Thermocycling Parameters	**HIV-1 Monitor Version 1.0**	**HIV-1 Monitor Version 1.5**
Hold Programme	2 min at 50°C	2 min at 50°C
Hold Programme	30 min at 60°C	30 min at 60°C
Cycle Programme	(4 cycles) 10 sec at 95°C 10 sec at 55°C 10 sec at 72°C	(8 cycles) 10 sec at 95°C 10 sec at 52°C 10 sec at 72°C
Cycle Programme	(26 cycles) 10 sec at 90°C 10 sec at 60°C 10 sec at 72°C	(23 cycles) 10 sec at 90°C 10 sec at 55°C 10 sec at 72°C
Hold Programme	15 min at 72°C	15 min at 72°C
Total Cycles	30	31

MicroAmp tubes were removed at any time during the final 15 minute hold at 72 °C. Once the final programme was completed the PCR reaction was quenched by the addition of 100μl of supplied denaturation solution.

2.2.1.3 Detection.

The HIV-1 Monitor system detected RNA on microwell plates of which rows A-F were pre-coated with an HIV-1 specific oligonucleotide probe, whilst rows G and H were coated with a Qs specific probe. 100μl of hybridisation buffer was added to all wells of the detection plates. 25μl of denatured sample was added to row A and pipetted to mix. 25μl was transferred to the next row and repeated to row F, producing a five-fold dilution series. 25μl of the same denatured sample was added to row G, mixed and 25μl transferred to row H for the detection of Qs. The plates were incubated at 37 °C for 60 minutes to hybridise the RNA to the probes on the plate. The plates were washed with a supplied phosphate buffer before the addition of 100μl of avidin-HRP conjugate to each well. The plates were incubated at 37 °C for 15 minutes to bind the conjugate to the biotinylated RNA . The plates were washed once more before adding 100μl of peroxidase substrate, the plates were left in the dark at room temperature for 10 minutes whilst the colormetric reaction developed. The reaction was stopped by adding 100μl of H_2SO_4 solution. The optical density (OD) of the fluid in each well was measured at 450nm and the results were calculated according to manufacturers instructions. Briefly, for each denatured sample the dilution giving the lowest OD was selected, background of 0.07 OD units was subtracted and then the total OD was calculated by multiplying the OD by the dilution factor of the sample.

The Qs value with the lowest OD in the range of 0.3 to 2.0 OD units was selected and the total Qs was determined by subtracting a background of 0.07 units and multiplying by the dilution factor. The actual copies of HIV-1 RNA can then be determined from the equation below:

HIV-1 RNA copies/ml = (Total HIV OD/Total Qs OD) x Input Qs x 40

The input Qs (copies per reaction) was different for each batch of reagents and was specified on the kit batch.

The upper limit of the assay was 750,000 copies/ml and the lower limit of detection was set at 400 copies/ml.

2.2.2 Amplicor HIV-1 Monitor with non-B subtype primers (RT-PCRnb) (Roche Diagnostics, East Sussex, UK)

This involved the addition of biotinylated primers SK145 and SK151 to the original primer pair to improve quantification of viral load in subtype non-B samples. The RT-PCR non-B followed the same protocol as a standard RT-PCR assay Version 1.0 with the addition of 20µl of a supplied primer mix to the PCR mix (section 2.2.1.1)[174].

2.2.3 Ultrasensitive Amplicor HIV-1 Monitor (RT-PCR) (Roche Diagnostics, East Sussex, UK)

The principle of the ultrasensitive version of the Amplicor HIV-1 Monitor was to lower the level of detection by increasing plasma input. The volume of plasma used was increased from 200µl to 500µl. When the maximum plasma input of 500µl was used the lower detection limit was 50 copies/ml.

2.2.3.1 RNA extraction.

Plasma samples from 21 patients were thawed and 500µl of each was transferred to a labelled 1.5ml conical tube. The negative, low positive and high positive controls were prepared from in-house standards and placed in tubes 22 –24. The samples were centrifuged for 1 hour at 24000 rpm to pellet the virus. The supernatant plasma was removed using a fine tip pastette. 600µl of GuSCN containing the quantitation standard (Qs) was added to each tube and vortexed to re-suspend the pellet. After a 10 minute

incubation at room temperature to ensure complete viral lysis, 600µl 100% isopropanol was added to precipitate the RNA, and the mixture vortexed. The tubes were centrifuged at 13,000 rpm for 15 minutes to pellet the RNA with the orientation marks on the tubes facing the centre of the centrifuge. The supernatant fluid was removed from the side opposite the pellet using a fine-tip pastette, and discarded. The pellet was washed with 1 ml of 70% ethanol and centrifuged at 13,000 rpm for 8 minutes. The supernatant fluid was removed as before. 100µl of dH_20 containing carrier RNA was added to each tube and vortexed to re-suspend the pelleted RNA. 50µl of extracted RNA was transferred to a 0.5ml MicroAmp tube containing 100µl of RT-PCR mixture which contained 20pmol of biotinylated primers Bio-SK431, Bio-SK462, 200µM dUTP, 150µM dNTPs and 10 units of rTth DNA polymerase. Extracted RNA remaining in the 2ml tube was stored at -20 °C.

The remainder of the assay proceeded as a standard Amplicor HIV-1 Monitor (RT-PCR) assay.

2.2.4 Cobas Amplicor HIV-1 Monitor (RT-PCR) (Roche Diagnostics, East Sussex, UK)

In 1999 the amplification and detection systems in the Amplicor HIV Monitor assay were automated using the COBAS machine. The COBAS machine incorporates a PCR thermocycler with an automated detection system using magnetic microparticles. The biotinylated PCR amplicons are denatured as before, then the strands are bound to magnetic microparticles *via* complementary oligonucleotide probes. The microparticles are immobilised by a magnet and any non-specific amplicons are washed away. The amplicons are detected as before using avidin-horseradish peroxidase conjugate and tetramethylbenzidine (TMB) to produce a colormetric product that can be quantified in

a photometer. Results were calculated by the machine and printed out as $\log_{10}$ copies/ml. Any Amplicor HIV-1 Monitor assays, standard or ultrasensitive, performed after January 1999, were automated using COBAS.

2.2.5 HIV-1 RNA 2.0 and 3.0 Assay (bDNA) (Bayer plc Diagnostics Division, Newbury, UK)

The principle of the HIV-1 RNA (bDNA) assay is the direct quantification of HIV-1 RNA in plasma using sandwich nucleic-acid hybridisation. The HIV-1 virus is extracted by ultracentrifugation and lysed using a proteinase K solution. RNA is then bound onto a microwell plate by a capture probe and a set of target probes (known as pre-amplifier probes) bind to different regions of the *pol* gene. Amplifier probes are then hybridised to the pre-amplifier probes forming a branched DNA (bDNA) complex to which multiple copies of an alkaline phosphatase (AP) labelled probe bind. AP reacts with a substrate to emit chemiluminescence proportional to the amount of HIV-1 RNA in the sample. A standard curve is plotted of the light emission from RNA standards of known concentration, enabling the HIV-1 RNA to be measured off the curve.

2.2.5.1 Sample preparation and HIV-1 lysis.

The assay manufacturers recommend quantifying 36 samples and 12 controls in each run. 1ml of patient plasma was added to a 1.5ml conical tube containing 50µl of polystyrene bead suspension to make it easier to see the centrifuged viral pellet. 1ml of appropriate standard was also added to 50µl beads in a 1.5ml tube for each of 12 standards, including negative control, and high and low positive controls. The tubes were then centrifuged at 4°C for 1 hour at 23,500 rpm. The supernatant fluid was

aspirated with a fine tip pastette avoiding the bead and virus pellet. Into each tube 120µl of supplied lysis reagent was added. This contained proteinase K, capture oligonucleotide probes and target oligonucleotide probes, both types of probe were designed to bind to HIV-1 *pol* or the internal standard RNA. The tubes were vortexed and incubated on a heating block at 63°C for 2 hours to ensure full viral lysis.

2.2.5.2 Hybridisation of the capture and target probes.

The tubes were allowed to cool to room temperature for 10 minutes then vortexed. 100µl from each tube was aliquoted into a capture well of a microwell plate pre-coated with oligonucleotide probes which bound to the capture probes in the suspension. The plate was then incubated for 16-18 hours at 52°C in the Bayer 340 bDNA analyser machine to hybridise the capture probes, bound to the RNA, to the plate, and the target probe to the RNA.

2.2.5.3 Hybridisation of the pre-amplifier and amplifier probes.

After incubation the plate was cooled to room temperature and washed automatically by the analyser machine with supplied wash buffer A before the addition of 100µl of pre-amplifier probe solution to all wells of the plate. The pre-amplifier probe was hybridised to the target probe during a 30 minute incubation at 45°C in the analyser machine. The plate was then washed as before prior to the addition to each well of 100µl of amplifier probe solution. The plate was again incubated at 45°C for 30 minutes to bind the amplifier probe to the pre-amplifier probe.

2.2.5.4 Hybridisation of the AP-labelled probe.

After the incubation the plate was washed as before prior to the addition of 100µl of alkaline phosphatase (AP) labelled probe solution. The plate was incubated at 45°C for 45 minutes to hybridise the AP-labelled probe to the amplifier probe. During this incubation sample identifiers and standard RNA concentrations were entered into the computer attached to the analyser. After incubation the plate was automatically cooled and washed with supplied wash buffer B before the addition of 80µl of the supplied dioxetane substrate solution. The plate was incubated at 37°C for 30 minutes in the analyser machine. After 30 minutes the analyser automatically measured the chemiluminescence emission at 630nm. The analyser plotted a curve from the light emitted by the standards of known RNA concentration, and, as long as the measurements for the negative control and high and low positive controls were within the batch range, values for HIV-1 RNA samples were read off the curve. Version 2.0 had an upper limit of 800,000 and a lower detection limit of 500 copies/ml. An updated version known as 3.0, bought out in 1999, has a improved detection of low copy number samples and can detect to 50 copies/ml. Version 3.0 has an upper detection limit of 500,000 copies/ml[175].

2.3 In-House viral quantification

2.3.1 Antibody purification

HIV-1 was captured from plasma using five antibodies coated onto latex microparticles. The five antibodies were chosen previously as having the best binding capacity for whole HIV-1 virus[156]. Supplies of antibody sera were obtained from the NIBSC AIDS Reagent Project (address), the supply of which was limited, so it became important to

optimise the amount of antibody required to coat the latex microparticles. The antibodies used are described below:

Antibody title	Description	Source type	Volume received	Specificity
ADP 360	Mouse monoclonal	Ascitic Fluid	100µl	gp120
ADP 401	Sheep polyclonal	Antiserum	1ml	gp120/gp160
ADP 403	Sheep polyclonal	Antiserum	1ml	gp120/gp160
ADP 408	Sheep polyclonal	Antiserum	1ml	gp160
ADP 412	Sheep polyclonal	Antiserum	1ml	gp120

Antibody was extracted from the source fluid using a protein G hi-trap column (Pharmacia, Biotech). Briefly, sera was put through microfilters to remove cellular debris, this filtrate was then diluted 1:2 with filtered 0.1M Tris-HCl (pH 7.6). The diluted antibody was loaded onto the column; previously washed 10 times with Tris-HCl. The column was then run through with 5mls 1M Acetic acid (pH 3) and the eluate was collected in 1ml fractions in tubes containing 0.1g 1M Tris-base to re-equilibrate the pH. The column was washed 10 times with Tris-HCl before loading a different antibody. Antibody fractions were measured in a spectrophotometer at 280nm. Fractions reading at > 0.2 OD were pooled for each antibody and pipetted into sealed dialysis tubing. The solution was dialysed overnight in a beaker containing 1L PBS to remove residual salts. Each separate antibody solution was analysed in a spectrophotometer to measure the antibody concentration using the following equation:

1mg/ml = 1.4 OD

Antibody title	Volume Obtained	OD reading	Concentration of antibody in mg/ml
ADP 360	3ml	0.883	0.63
ADP 401	11ml	1.244	0.89
ADP 403	11ml	1.397	1.00
ADP 408	10ml	1.687	1.21
ADP 412	9.5ml	2.043	1.46

2.3.2 Maximising the coating of antibody onto latex microparticles

An existing protocol used a solution of 80μg/ml of each antibody in 0.1M Tris HCl pH 7.6). For the evaluation latex microparticles were coated using solutions containing 20, 40 and 80μg/ml, then the solutions could be re-used to coat fresh particles in another 2 cycles to determine whether there was an excess of antibody at any of the concentrations. The approximate amount of bound antibody was determined using an anti-sheep antibody conjugated to horse radish peroxidase, HRP (DAKO Ltd). The amount of anti-sheep antibody bound to the HIV antibodies was then determined by adding the colormetric substrate of HRP, tetramethylbenzidine (TMB) to produce a colormetric product that could be quantified in a photometric plate reader.

Briefly, a conical bottom microtitre plate was blocked overnight prior to use with 1% BSA/PBS/Tween. 1ml of latex was also blocked with the same blocking solution. 100μl of latex was aliquoted into 9 tubes and centrifuged at 13,000rpm for 5 minutes, the supernatant was discarded and the pellet resuspended in 100μl Tris HCl, the latex was again pelleted by centrifugation as before and the supernatant discarded. The first 3 tubes (labelled first wash) had antibody coating solution added of either 20, 40 or

80µl/ml. These were incubated at 45°C for 20 minutes. The latex was pelleted as before, the supernatant removed and added to 3 tubes containing freshly washed latex, pelleted as described. The incubation step was repeated and the coating solutions removed as before to coat an additional set of fresh latex particles. Each antibody coating solution had been used to coat fresh beads three times. After each incubation and removal of the coating solution, the pellet was resuspended in Tris HCl and stored at 4°C.

One hundred µl of a solution containing 10% blocked latex microparticles in PBS/Tween was added to columns 1, 2 and 3 of row A on a microtitre plate(columns 1-12, rows A-H). 100µl of 10% latex coated with fresh 20µl/ml antibody wash was added to column 4 of row A, 100µl of 10% latex coated with the used 20µl/ml antibody (second wash) was added to column 5, and 100µl of 10% latex coated with the used 20µl/ml antibody (third wash) was added to column 6. In a similar fashion latex coated with 3 washes of 40µl/ml antibody solution was added to columns 7, 8 and 9, whilst latex coated with 80µl/ml antibody solution was added to columns 10, 11 and 12. A serial dilution of latex particles was applied down the plate ranging from 1:10 in row A (10% latex solution), to a dilution of $1:10^8$ in row 8. The plate was centrifuged at 2,000 rpm to pellet the latex and the supernatant was removed and the pellets were resuspended in 100µl of 200µg/ml HRP-conjugated antibody solution in 1 % BSA/PBS/Tween. The plate was incubated at 37°C for 60 minutes and then centrifuged at 2,000 rpm for 15 minutes. The supernatant was removed and the pellets washed with 1 % BSA /PBS/Tween and centrifuged three times. The final supernatant was removed and the pellet resuspended in 100µl TMB solution and kept in the dark at room temperature for 10 minutes. The colormetric reaction was stopped by the addition of 100µl 1M H_2SO_4. As the latex particles themselves absorb light, the plate was

centrifuged and the supernatant removed to a fresh flat-bottomed microtitre plate, before reading the OD at 450nm. The OD's reflected the amount of HRP-conjugated antibodies bound to HIV antibodies coating the latex.

2.3.3 Immunocapture of HIV

Latex microparticles coated with a 20μg/ml anti-HIV antibodies were used to capture whole HIV virions from patient plasma. The latex was pelleted by centrifugation, washed and re-pelleted before being suspended in a lysis/reverse transcription (RT) mix and incubated. This reaction used a single primer SPP1A (described further in section 2.4). After 90 minutes the mix was centrifuged and 10μl was removed and added into a nested PCR using primers SPP1A and SPP8, (described further in section 2.4). Target cDNA was visualised on a 1 % agarose gel.

Briefly, 100μl of plasma was aliquoted into a conical Sarstedt to which was added 20μl of latex - coated with 20μg/ml anti-HIV antibodies (described in section 2.3.1). Samples were placed on a shaking bed at room temperature for an hour. Samples were then centrifuged for 10 minutes at 10,000 rpm. The plasma was removed and discarded into a bleach pot, and the pellet was resuspended in 500μl PBS and then centrifuged as before.

2.3.3.1 Lysis and reverse transcription of HIV-1

Twenty μl of the following lysis/RT mix was added and the sample vortexed for 30 seconds to resuspend and lyse the virus. The samples were incubated for 90 minutes at 37°C.

RT Mix

Reagent	Vol. (µl)	Final conc.
MMLV-RT Buffer	3.0	1X
5mM dNTPs	3.0	0.5mM
10µM Primer SPP1A	0.3	0.1µM
RNAsin 40U/µl	0.55	22 Units
MMLV-RT 200U/µl	0.075	15 Units
0.1M Dithiothreitol (DTT)	3	10mM
Triton X-100 10 %	3	1 %
RNA Extract	10.00	
dH20	7.075	
Total	**30.00**	

After the lysis/RT reaction samples were centrifuged at 12,000 rpm for 5 minutes and 10µl of the supernatant was added into the following nested PCR reaction to amplify a 807bp region of RT gene.

PCR Mix

Reagent	Vol. (μl)	Final conc.
10X PCR Buffer	5.00	1X
5mM dNTPs	2.00	0.2mM
10μM Primer Spp1A	1.25	0.25μM
10μM Primer Spp8	1.25	0.25μM
Amplitaq G 5U/μl	0.34	1.7 Units
RNA Extract	10.00	
dH20	30.16	
Total	**50.00**	

The reaction tubes were then transferred to the PCR room to a 9700 Perkin Elmer thermocycler where reverse transcription and amplification took place under the following conditions: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 7 minutes and 4°C indefinitely until the tubes were removed.

2.3.3.2 Second round amplification

A second round 'nested' PCR amplification was performed on the first round product. However, one of the second round primers SPP2A was biotinylated, allowing the product to be bound to streptavidin coated plate. The following reagents were thawed and assembled on ice and a master mix was made up in the proportions shown overleaf, excluding the later input of the first round product. The master mix was pipetted into 0.5ml tubes according to the number of samples being run.

Nested PCR (second round)

Reagent	Vol. (µl)	Final conc.
10X PCR Buffer	5.00	1X
5mM dNTPs	2.00	0.2mM
10µM Primer Spp2A-bio	1.25	0.25µM
10µM Primer Spp6A	1.25	0.25µM
Amplitaq G 5U/µl	0.34	1.7 Units
1st Round sample	5.00	
dH20	35.16	
Total	**50.00**	

The tubes were transferred to the post-PCR room. After the first round product was thawed and vortexed, 5µl was added to the PCR mix. The samples were then amplified on a 9700 Perkin Elmer thermocycler for the following conditions: 95°C for 5 minutes, then 30 cycles of 95°C for 30 seconds, 55 °C for 30 seconds, 72°C for 30 seconds, followed by 72°C for 7 minutes and 4°C until the tubes were removed for storage or detection of the product.

The products of both first and second round amplification were stored at –20°C for up to 6 months.

2.3.4 Detection of biotinylated DNA using a probe conjugated to alkaline phosphatase.

The DNA from a biotinylated second round PCR was detected by binding it to the surface of a streptavidin coated microtitre plate. The antisense strand was removed and the DNA detected using probe LOPb conjugated to alkaline phosphatase. The amount of alkaline phosphatase present was detected by adding substrate CDP-star Star (NEN™, Life Science Products) which produced luminescence quantified by a microplate scintillation counter (Topcount™). Briefly, the streptavidin coated plate was washed once with PBS/ 0.05% Tween phosphate buffer. 90µl of the same phosphate buffer were added to all wells, 10µl of biotinylated DNA PCR product was added to column 1. A serial dilution of DNA was performed across the plate ranging from 1:10 in column 1 to $1:10^{11}$ in column 11, no DNA was added into column 12. The plate was incubated at 37°C for an hour before all wells were washed 5 times with phosphate buffer. 50µl of 0.15M NaOH was added to each well to denature the DNA and the antisense strand was removed by rinsing with phosphate buffer 5 times. 100µl of a 1:100 dilution of probe LOPb in hybridising SSC buffer was added to all wells of row A, whilst 90µl SSC buffer was added to all other wells. A serial dilution was performed down the plate so that the concentration of probe LOPb ranged from 1:100 ($1:10^2$) in row A to $1:10^8$ in row G, no probe was added to row H. The plate was incubated for an hour at 37°C. The plate was washed with phosphate buffer as before, then 100µl of CDP-Star was added to each well and the resulting luminescence was quantified by an automated microplate scintillation counter (Topcount™).

2.4 PCR Amplification of HIV-1 RNA

2.4.1 HIV-1 RNA Extraction for PCR

RNA was extracted from plasma using a QIAamp® viral RNA mini kit (QIAGEN Ltd, Crawley, West Sussex). Briefly, 140µl of patient plasma was treated with 560µl of supplied lysis reagent containing guanidinium thiocyanate to release viral RNA. 560µl of high-grade ethanol was also added to optimise the binding of the RNA to the column in the next step. The RNA in solution was then loaded onto a silica gel-based membrane of a QIAamp® spin column; the column rested in a standard 2ml microcentrifuge tube. The lysis reagent contained carrier RNA to improve the binding of the HIV-1 RNA to the membrane. The column and membrane were washed once with 500µl of guanidinium chloride buffer. This had a high salt content optimised to ensure maximum binding of RNA to the membrane. The membrane was washed once with 500µl of the supplied ethanol buffer to remove salts from the first wash. Finally, the RNA was eluted from the membrane by a single addition of 60µl of RNase-free water. Extracted RNA was stored for up to a year at -20°C.

2.4.2 Reverse transcription and PCR primers

Reverse transcription and PCR amplification of HIV-1 *pol* was achieved using four primer pairs amplifying a region of the reverse transcriptase (RT) and protease (Pr) gene in a nested PCR. Amplification of an 807bp region of RT used external primers SPP1A and SPP8, and internal primers SPP2A and SPP6A. Amplification of a 514bp region of

protease used external primers 5'Prot1 and 3'Prot1, and internal primers 5'Prot2 and 3'Prot2. Sequences for the primers used are shown below.

Primer	Type	Position in HXB2 genome	Sequence 5' to 3'
SPP1A	Sense	2481	GTAGGACCTACACCTGTCAACATAA
SPP8	Anti-sense	3354	GACTTGCCCAATTCAATTTTCCCAC
SPP2A	Sense	2518	TGTTGACTCAGATTGGTTGCACTTTA
SPP6A	Anti-sense	3325	TTCTGTATGTCTTAGACAGTCCAGCT
5'Prot 1	Sense	2078	AGGCTAATTTTTTAGGGAAGATCTGGCCTTCC
3'Prot 1	Anti-sense	2734	GCAAATACTGGAGTATTGTATGGATTTTCAGG
5'Prot 2	Sense	2136	TCAGAGCAGACCAGAGCCAACAGCCCCA
3'Prot 2	Anti-sense	2650	AATGCTTTTATTTTTTCTTCTGTCAATGGC

2.4.3 Reverse transcription and PCR amplification

2.4.3.1 One tube reverse transcription and PCR amplification.

Reverse transcription and PCR was performed in one tube consecutively using an in-house method which combined both sets of enzymes with first round primers. This method was developed as described below (and in results section 3.7.2), and further validated in the department to allow routine use. All the reagents were thawed to 4°C, kept on ice, and combined to make a reaction mixture. This was aliquoted in 40µl volumes into 0.5ml tubes. The RNA extract was heated to 80°C for 5 minutes before it was added to the prepared RT-PCR mix. Each reaction tube contained the following volumes in the concentrations shown:

Reagent	Vol. (µl)	Final conc.
10X PCR Buffer	5.00	1X
5mM dNTPs	2.00	0.2mM
10µM Primer Spp1A/5'Prot1	1.25	0.25µM
10µM Primer Spp8/3'Prot1	1.25	0.25µM
RNAsin 40U/µl	0.50	20 Units
AMV-RT 10U/µl	0.25	2.5 Units
Amplitaq G 5U/µl	0.34	1.7 Units
RNA Extract	10.00	
dH20	29.41	
Total	**50.00**	

The reaction tubes were then transferred to the PCR room to a 9700 Perkin Elmer thermocycler where reverse transcription and amplification took place under the following conditions: 42°C for 30 minutes, 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 7 minutes and 4°C indefinitely until the tubes were removed.

2.4.3.2 Second round amplification

A second round 'nested' PCR amplification was performed on the first round product. The following reagents were thawed and assembled on ice and a master mix was made up in the proportions shown overleaf, excluding the later input of the first round product. The master mix was pipetted into 0.5ml tubes according to the number of samples being run.

Reagent	Vol. (µl)	Final conc.
10X PCR Buffer	5.00	1X
5mM dNTPs	2.00	0.2mM
10µM Primer Spp2A/5'Prot2	1.25	0.25µM
10µM Primer Spp6A/3'Prot2	1.25	0.25µM
Amplitaq G 5U/µl	0.34	1.7 Units
1st Round sample	5.00	
dH20	35.16	
Total	**50.00**	

The tubes were transferred to the PCR room. After the first round product was thawed and vortexed, 5µl was added to the PCR mix. The samples were then amplified on a 9700 Perkin Elmer thermocycler for the following conditions: 95°C for 5 minutes, then 30 cycles of 95°C for 30 seconds, 55 °C for 30 seconds, 72°C for 30 seconds, followed by 72°C for 7 minutes and 4°C until the tubes were removed for storage or detection of the product.

The products of both first and second round amplification were stored at –20°C for up to 6 months.

2.4.4 Detection of PCR product

2.4.4.1 Agarose gel electrophoresis using a 1% agarose gel

Amplified PCR products were visualised on a 1% agarose gel. One gram of electrophoresis grade agarose was added to 100ml Tris-Borate EDTA (TBE) and heated in a microwave oven until dissolved. It was left to cool to 56°C. 10µl of ethidium

bromide was added and the mixture poured into a 11cm x 14cm gel tray. A loading comb with the appropriate number of wells was added, and the gel was left to set for 15 minutes.

The gel tank contained approximately 800mls of TBE. The gel tray was placed into the tank and the comb removed. 5µl of an HIV-1 molecular weight indicator was added to 1.5µl blue/orange loading dye and the mixture pipetted into the first well of each comb. 5 µl of each amplified product was mixed with 1.5µl loading dye and loaded into the appropriate well. The electrophoresis was run at 100 volts for 35 minutes. The gel was removed from the plate and the bands visualised on a transilluminator.

2.4.5 End point dilution series

This method allows the isolation and amplification of a single viral sequence from a heterogeneous sample. By the amplification of multiple replicates from a series of dilutions of sample RNA, it was possible to estimate the viral copy number of the original sample. A sample was diluted in a five-fold dilution series in replicates of 48 until the dilution ensures that only a proportion of the replicates gave a positive result on amplification. The dilution at which this proportion of positive signals is reached is termed the end point. The number of viral copies from which the positive signal was generated follows a Poisson distribution, so the signals which were amplified from single copies of virus can be determined.

In practise, an extracted RNA sample was diluted in dH_20 in a series of five fold dilutions from neat to create 1 in 5, 1 in 25, 1 in 125 and 1 in 625 dilutions. 48 replicates of each dilution were reverse transcribed and amplified in the single step reaction described previously. The number of positive signals out of 48 was determined by visualisation on an agarose gel. The Poisson formula was used to calculate the

likelihood of the positive tubes having originally contained one or more molecules of RNA[176]. Those determined to have originated from single copies were selected for single copy sequencing.

2.5 Sequencing DNA

A 1321 base pair region of HIV-1 *pol* gene was reverse transcribed and amplified by PCR (see section 2.3.2) to allow DNA sequencing of the genes coding for HIV-1 enzymes reverse transcriptase and protease. Reading of the DNA sequence and subsequent comparison to drug-naïve laboratory strain HXB2, determined polymorphisms and mutations associated with antiretroviral drug resistance.

2.5.1 Purification of PCR product

The amplified products were visualised on a 1% agarose gel before purification. PCR products were purified using a QIAquick® PCR purification kit (QIAGEN Ltd, Crawley, West Sussex). Briefly, 250µl of guanidinium chloride buffer was added to the 50µl PCR mix (see section 2.3.3.2) and the total mixture was then aliquoted onto the silica membrane of the provided spin column. The high salt content of the buffer was optimal for the adsorption of DNA to the membrane. The membrane was then washed with the supplied ethanol buffer to remove residual salts left from the first wash. DNA was eluted by the single addition of 30µl of 10mM TrisHCl. Purified DNA was stored at -20°C for up to 6 months.

2.5.2 Sequencing reaction

The following studies involved the sequencing of the reverse transcriptase (RT) and protease (Pr) genes for analysis of resistance associated mutations. After the RT-PCR amplification and purification of RT and Pr DNA, both products underwent a sequencing reaction using 3'dye labelled dideoxynucleotide triphosphates known as big dye terminators (BDT) (PE Applied Biosystems, Warrington, Cheshire, UK). These are fluorescently labelled dideoxy terminators for each of the four bases. A reaction mixture is made up, similar to that used for PCR, incorporating primers for both DNA strands, DNA polymerase, template DNA and BDT in place of dNTPs. Using primer annealing and thermocycling similar to PCR conditions, the primers bind and extend a complementary DNA strand. Instead of incorporating a dNTP and continuing DNA synthesis, a BDT is incorporated, terminating the DNA strand in a labelled ddNTP. The resulting reaction produces hundreds of DNA strands of different lengths, terminating in a labelled base. The DNA strands are loaded onto a polyacrylamide gel and subjected to electrophoresis so that fluorescence and therefore DNA length can be measured using a laser. The computer attached to the gel reader calculates which base emitted the fluorescence at which length of DNA.

The strength of DNA band seen on an agarose gel is proportional to the concentration of DNA, so if the band was weak subsequently the amount of DNA added into the sequencing reaction was increased from 2 to 4µl with appropriate reduction of volume of dH_20. The reagents were thawed and assembled on ice, the BDT were added last to prevent light degradation. Either 2 or 4µl of purified DNA plus 9 or 7µl of dH_20 with 1µl primer and 8µl of BDT were pipetted into 0.5ml tubes, 2 tubes per region amplified for sense and antisense sequences. The primers used were the second round PCR primers described in section 2.3.3.2. at a concentration of 3.2µM. A positive DNA

sample of known sequence supplied by the BDT manufacturer was also run, this included DNA from the M13 phage and M13 phage primers. 2µl of M13 DNA plus 6µl dH_20 was added to 4µl of M13 primer and 8µl BDT in a 0.5ml tube. The samples were amplified in a 9700 Perkin Elmer thermocycler under the following conditions; 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes and finally 4°C until the tubes were removed.

2.5.3 Purification of sequencing product

The sequencing reaction products were purified as follows to remove excess BDT which disrupt gel electrophoresis analysis. Sterile 0.5ml tubes were labelled for each reaction product. 2µl of sodium acetate was pipetted into each tube, to which 50µl of 100% ethanol was added. The entire sequencing reaction product was transferred into the appropriately labelled tube and vortexed to mix. The samples were held on ice for 10 minutes. The samples were then centrifuged for 15 mins at 13,000 before the supernatant fluid was removed from the side opposite the pellet. 250µl of 70% ethanol was added and vortexed to mix. The samples were centrifuged at 13,000 rpm for 5 mins. The supernatant fluid was removed as before. The pellets were incubated without a lid in a 37°C incubator for 20 minutes to remove excess ethanol and dH_20. The purified product was then loaded onto a polyacrylamide gel and sequenced as described in 2.4.4, or stored at -20°C.

2.5.4 Polyacrylamide gel electrophoresis using an ABI 377 (PE Applied Biosystems, Foster City, CA)

A polyacrylamide gel was made by adding 5mls of 10 x Tris Borate EDTA (TBE) to 45ml of sequagel automatrix, a ready made polyacrylamide mix (National Diagnostics, Hull, UK) and poured into a 115ml Nalgene (Nalge Company, New York, USA) filter. This was attached to a low pressure pump and left to de-gas for at least 20 minutes. 0.1g of ammonium persulphate was dissolved in 900µl of dH_20. Both the de-gassed gel and dissolved ammonium persulphate were removed to the PCR room. 250µl of ammonium persulphate and 30 µl of tetramethylethylenediamine (TEMED) was added to the gel to initiate the polymerisation reaction. The mixture was drawn up by syringe and gently poured between the prepared sequencing plates. Once the gel was poured the flat side of the comb was clamped between the plates at the top end and the gel left to set for 2 hours.

A loading comb was inserted into the gel and the gel rack was placed into the sequencer and clamped into place. Great care was taken to ensure that there was no smears or dust on the plates that might prevent the laser reading fluorescence from the gel. The upper and lower buffer chambers were filled with 1 x TBE. Sample identifiers were entered onto the computer attached to the sequencer. In order to equilibrate the temperature and electrical charge across the gel, the sequencer was run for 40 minutes. Product samples were prepared for loading as follows. Loading buffer was made up by adding formamide to blue dextran/EDTA loading dye in a ratio of 5:1, 6µl of the loading buffer was added to each product pellet and vortexed. The products were then heated to 90°C for two minutes, after which they were kept on ice until ready for loading. After the 40 minute pre-run the wells were rinsed through with TBE and 1.5µl of prepared product

was loaded in each well. The sequencing run was then started and run at 1300 – 1400 volts and 30 watts for approximately 9 hours until completed.

2.5.5 Analysis of Sequence

The DNA sequence electropherogram was analysed by computer using ABI PRISM® Sequence Navigator™ software (PE Applied Biosystems, Foster City, CA) by comparing the sense and anti-sense strands of each fragment with reference sequence HXB2.

2.6 HIV-1 p24 Antigen Quantification

HIV-1 p24 antigen was quantified using a Murex HIV p24 MAb kit (Abbott Laboratories Ltd, Berkshire, UK). Microtitre wells were pre-coated with human polyclonal antibodies to HIV. Patient plasma was incubated in the well together with a biotinylated anti-p24 monoclonal antibody. In a further step, peroxidase-conjugated streptavidin was added and bound to the biotin. Incubation with the peroxidase substrate produced a blue colour which turned yellow when the reaction was stopped with sulphuric acid. The optical density of the well was analysed and the concentration of p24 antigen in the plasma was calculated from the optical densities of series of dilution controls.

Briefly, 100µl of patient plasma or internal standard were added to a 100µl of biotinylated anti-p24 monoclonal antibody solution and incubated at 37°C for an hour in a microtitre plate pre-coated with human polyclonal antibodies to HIV. The plate was washed five times with phosphate buffer to remove unbound antibody and plasma proteins before 200µl of avidin-labelled horseradish peroxidase (HRP) was added. The

HRP bound to the biotinylated anit-p24 antibody during a 30 minute incubation at 37°C. The plate was washed again before the addition of 200µl of tetramethylbenzidine (TMB), the chromogenic enzyme substrate of HRP. The reaction was stopped with 50µl of 1M sulphuric acid producing a yellow colour proportional to the quantity of p24 antigen now bound to the plate. Optical density was read at 450nm and the concentration of p24 in each sample was determined by plotting a standard curve of the internal standards of known p24 concentration versus optical density. The lower level of detection was determined to be the mean optical density (OD) of the assay negatives + 0.5. Samples which gave a value below this were determined to be undetectable. The limit varied slightly for each assay, but all assays had a lower detection limit of 5pg/ml, so this figure was adopted as the standard detection lower limit. The lowest positive control was above 20pg/ml for all assays performed so this figure was taken as the lower limit of quantification. Samples showing a value above 5pg/ml but below the lowest positive control, were determined to be detectable but at a concentration less than 20 pg/ml. The upper limit was determined by the highest positive control, in all assays this was determined to be at least 300 pg/ml, so the standard upper limit was set at 300pg/ml. Samples which showed p24 antigen above this limit were re-tested diluted with negative human plasma to obtain a result.

2.7 HIV-1 Western Blot

HIV-1 antibodies were detected from patient plasma using a HIV-1 Western Blot kit (Cambridge Biotech Corporation, Rockville, MD, USA). The method involves the incubation of human plasma with a membrane embedded with HIV-1 proteins p17, p24, p31, gp 41, p51, p55, p66, gp120 and gp160. Any antibodies present bind to the

proteins and are visualised using an anti-IgG antibody conjugated to alkaline-phosphatase. Briefly, 2ml of supplied blotting buffer was added to each well of an incubation tray, the number of wells used was equal to the number of samples to be tested including three internal controls. A nitrocellulose membrane strip embedded with HIV proteins was added to each well and agitated for 5 minutes. 20µl of plasma sample was added to each well. Finally, 20µl each of supplied controls; negative, weak positive and strong positive were added to the last 3 wells. The tray was then incubated at room temperature for 2 hours to bind the any antibodies present to the HIV proteins on the membrane. The mixture was aspirated before 2ml of supplied wash buffer was added to each well and the tray agitated for 5 minutes. The wash was repeated before 2ml of working conjugate solution containing alkaline phosphatase-conjugated anti-human IgG was added to each well. The tray was incubated for 30 minutes at room temperature to allow the conjugated antibody to bind to any plasma antibodies bound to the membrane. The solution was aspirated before being washed twice with wash buffer as before. After the final aspiration, 2ml of supplied working substrate solution containing a precipitating alkaline phosphatase substrate was added to each well. The tray was incubated for 10 minutes at room temperature after which time the solution was aspirated and 3ml of reagent grade H_20 was added to each well and agitated for 5 minutes. The strips were then removed from the wells and left to dry for at least 2 hours before the analysis of antibody bands.

2.8 Statistical analysis.

The following statistical analyses were calculated using Microsoft ® Excel 97; standard deviation, paired and unpaired t-tests, correlation coefficient, Spearman's rank coefficient, and population correlation coefficient.

Statistical analysis of agreement, Mann-Whitney U test, Cohen's Kappa (κ) test and Standard error were calculated using equations cited in Medical Statistics at a Glance[177].

Chapter 3 Quality Assurance of Nucleic Acid Sequence-Based Amplification.

The following studies were aimed at investigating the quality of NASBA technology in the quantification of plasma HIV-1 RNA. The studies were designed to evaluate all aspects of this method including accuracy, upper and lower sensitivities, and reproducibility of results.

Two of these studies analysed proficiency panels which were quantified as part of wider studies investigating viral load quality assurance. One study was designed by the Virology Quality Assessment Program (VQA), an external quality assurance body. The second study was designed by the assay manufacturer, Organon Teknika Ltd, (Cambridge, UK). The proficiency panels incorporated plasma to which known quantities of viral RNA had been added, and clinical samples. In some instances the viral load was known before assay.

A further study investigated the sensitivity of NASBA HIV-1 QT (NASBA) in quantifying low copy numbers of virus, comparing it to that of an alternative plasma HIV-1 RNA quantification assay. Using the same panel and criteria, the study also evaluated Nuclisens HIV-1 QT (Nuclisens), a modified version of NASBA with improved sensitivity.

The range of viral load which could be quantified by NASBA was evaluated in two studies using selected panels of sequenced and subtyped viruses. The first of these investigated the ability of NASBA to quantify clinical subtype B virus, the most common type found in the developed world. The second of these studies investigated how well NASBA quantified a range of clinical non-B viruses. In both of these studies

NASBA performance was evaluated and compared to that of alternative quantification systems

3.1 Virology Quality Assessment Program

3.1.1 Introduction

The Virology Quality Assessment Program (VQA) is run as part of the Adult AIDS Clinical Trials Group (ACTG, USA). It aims to establish and implement quality assurance programs to ensure uniform performance in HIV-1 scientific analysis. An important aspect of quality assurance is proficiency testing. At present, the aim is to demonstrate that participating laboratories can reliably detect a five-fold change in HIV-1 RNA concentration in a patient. The VQA panel is a hypothetical representation of samples from a patient before and after therapeutic drug intervention. It was assumed that all samples would be assayed in the same batch, so that changes within a patient are then measured against a background consisting only of intra-assay variation. Under these criteria, the VQA generated a coded panel which consisted of replicates of five-fold dilutions over a broad range of concentrations, with which to evaluate assay proficiency.

3.1.2 Samples and Methods

The panel, PP07RA, consisted of three replicates of plasma to which HIV-1 RNA had been added at four concentrations (3.34, 4.04, 4.74 and 5.44 $\log_{10}$ copies/ml). In addition, there were three replicates of plasma from an HIV-1-infected patient at three five-fold dilutions, and eight plasma samples known to be negative, making a panel of 29 samples. Each test laboratory also received a series of four VQA standards, 3 of known HIV-1 concentration and one which was known to contain no HIV RNA. These were assayed with the coded panel, so the total number of samples was 33. In this laboratory, NASBA was used to quantify HIV-1 RNA concentrations for all samples.

The VQA protocol specified an input of 100µl of plasma, giving a lower level detection cut-off of 4,000 copies/ml or 3.6 $\log_{10}$ copies/ml. Any samples which gave an undetectable result were re-tested once. Plasma samples were received frozen on dry ice and stored until testing at -70°C.

Performance was based on the intra-assay standard deviation of $\log_{10}$ HIV-1 RNA concentration, rates of false differences detected (FDD), true differences undetected (TDU) and rates of false positives. An FDD error occurs when the difference in viral load between samples is so large that two samples with the same nominal concentration would be declared different. The number of true differences undetected (TDU) was calculated by comparing samples at adjacent dilutions. A TDU error occurs when the estimated concentrations for two samples with nominal concentrations that differ by a factor of five are given values so similar that they would be declared the same sample. Standard deviation was calculated from the four groups of replicated samples of known concentration.

3.1.3 Results

Twenty-nine unknown samples and the three VQA standards (containing known concentrations of HIV-1 RNA) were quantified for plasma HIV-1 RNA using NASBA. The eight negative samples and the VQA negative standard were tested, but the results were not processed by the VQA and not included in their analysis. Virus detected ranged from undetectable i.e. <3.6 $\log_{10}$ copies/ml to 5.71 $\log_{10}$ copies/ml. The viral load concentration was calculated from a curve plotted of the values of internal calibrators Qa, Qb and Qc. The VQA standards were quantified first and gave values ranging from undetectable i.e. <3.6 $\log_{10}$ copies/ml to 6.15 $\log_{10}$ copies/ml. The

nominal concentrations expected ranged from undetectable to 6.18 $\log_{10}$ copies/ml. The VQA standard values are given in Table 3-1.

Table 3-1 VQA standard controls quantified by NASBA.

	Viral Load $\log_{10}$ copies/ml			Absolute Difference
Sample	**Nominal**	**NASBA**	**VQA adjusted**	
VQA Std 1	<3.60	<3.60	<3.60	0.000
VQA Std 2	4.18	4.28	4.19	0.013
VQA Std 3	5.18	5.18	5.15	0.026
VQA Std 4	6.18	6.15	6.19	0.013

The VQA negative control, standard 1, gave an undetectable result and was repeated once as the protocol requested. However, the sample remained below the lower detection limit of 3.6 $\log_{10}$ copies/ml. A curve was plotted of the VQA standards and any given NASBA values were re-calculated by Organon Teknika (at the request of the VQA) to fit the curve, these values are shown additionally as 'VQA adjusted' in Table 3-1. The absolute difference between the nominal concentration and the VQA adjusted values was determined. The values for predicted (p) versus observed (o) were normalised and from these the total standard deviation for all four controls was calculated to be 0.004.

Figure 3-1 shows the known concentrations of the VQA standards plotted against values given by NASBA.

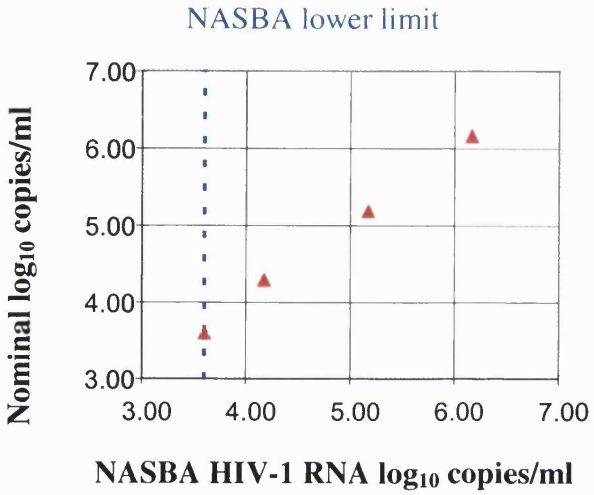


Figure 3-1 VQA Standard Controls quantified by NASBA. Three standards and one negative containing no HIV-1 RNA were quantified using NASBA, the NASBA values are plotted against the known HIV-1 RNA concentration.

The viral load values given for all the samples in panel PP07RA were then re-analysed using the VQA standard curve, these are shown as the VQA adjusted values. The nominal HIV-1 RNA concentration, the NASBA estimated values and the VQA adjusted values are shown in Table 3-2. The replicates of patient plasma (at 4.36, 5.00 and 5.59 $\log_{10}$ copies/ml) are included but were not used by the VQA to analyse assay performance. The standard deviation (St. dev.) for each value was calculated from the nominal concentration and the VQA adjusted value. Standard deviations greater than 0.15 were noted and from these a general assay standard deviation was calculated. This was calculated to be 0.42.

Table 3-2 Viral load and intra-assay standard deviation of Panel PP07RA.

	Viral Load log_{10} copies/ml			
Sample	**Nominal**	**NASBA estimate**	**VQA adjusted**	**VQA St. dev.**
VQA Std 1	3.60	3.60	3.60	0.00
VQA Std 2	4.18	4.28	4.19	0.01
VQA Std 3	5.18	5.18	5.15	0.02
VQA Std 4	6.18	6.15	6.19	0.01
PP07ra.14	3.34	3.58	3.44	0.07
PP07ra.24	3.34	3.61	3.48	0.09
PP07ra.27	3.34	4.85	4.80	**1.03**
PP07ra.04	4.04	3.92	3.80	**0.17**
PP07ra.11	4.04	4.04	3.93	0.08
PP07ra.15	4.04	4.40	4.32	**0.19**
PP07ra.09	4.36	4.00	4.37	0.01
PP07ra.16	4.36	4.36	4.28	0.06
PP07ra.28	4.36	4.45	3.89	**0.33**
PP07ra.03	4.74	4.68	4.83	0.06
PP07ra.22	4.74	4.88	4.62	0.09
PP07ra.26	4.74	4.91	4.86	0.09
PP07ra.01	5.00	4.93	4.89	0.08
PP07ra.19	5.00	5.00	5.12	0.08
PP07ra.21	5.00	5.15	4.96	0.03
PP07ra.05	5.44	5.40	5.39	0.04
PP07ra.08	5.44	5.43	5.46	0.01
PP07ra.10	5.44	5.46	5.22	**0.16**
PP07ra.06	5.59	5.58	5.58	0.01
PP07ra.17	5.59	5.59	5.72	0.09
PP07ra.29	5.59	5.71	5.59	0.00

Standard deviations greater than 0.15 are shown in bold type.

Eleven samples out of the 33 sample panel were undetectable and so were repeated. Eight out of the 11 remained undetectable. The number of undetectable panel samples and values after repeat testings is shown in Table 3-3.

Table 3-3 Repeat assay of those samples previously showing an undetectable viral load by NASBA.

	Log_{10} copies/ml		Repeated		Mean NASBA	Mean VQA Adjusted
Sample	Nominal	NASBA estimate	NASBA estimate	VQA adjusted		
PP07ra.02	0	<3.6	<3.6	<3.6	<3.6	<3.6
PP07ra.07	0	<3.6	<3.6	<3.6	<3.6	<3.6
PP07ra.12	0	<3.6	<3.6	<3.6	<3.6	<3.6
PP07ra.13	0	<3.6	<3.6	<3.6	<3.6	<3.6
PP07ra.14	3.34	<3.6	3.58	3.44	3.59	3.52
PP07ra.18	0	<3.6	<3.6	<3.6	<3.6	<3.6
PP07ra.20	0	<3.6	<3.6	<3.6	<3.6	<3.6
PP07ra.23	0	<3.6	<3.6	<3.6	<3.6	<3.6
PP07ra.24	3.34	<3.6	3.61	3.48	3.61	3.54
PP07ra.25	0	<3.6	<3.6	<3.6	<3.6	<3.6
PP07ra.27	3.34	<3.6	4.85	4.80	4.23	4.20

The three replicate samples at 3.34 log_{10} copies/ml were all undetectable by NASBA when first assayed. This was unsurprising as the nominal concentration of 3.34 log_{10} copies is below the NASBA lower detection limit of 3.6 log_{10} copies/ml. However, samples PP07ra.14, PP07ra. 24 and PP07ra.27 all gave detectable results when re-

tested. These repeated values were included in the VQA analysis and are shown in Table 3-2.

3.1.4 Statistical Analysis

The three replicate samples given a nominal concentration of 3.34 $\log_{10}$ copies/ml on re-testing by NASBA gave higher values (range 3.58 – 4.85 $\log_{10}$ copies/ml). Adjusted to fit the VQA standard curve, these values are still significantly different to those expected. Two of these NASBA estimates were higher than the third generating two false differences between values at the same concentration.

The three replicate samples with a nominal concentration of 4.04 $\log_{10}$ copies/ml also gave divergent results when estimated by NASBA (range 3.92 – 4.40 $\log_{10}$ copies/ml). One NASBA value was significantly different to the other two generating a false difference between values at the same concentration. Overall there were three false differences detected (FDD) and no false differences were detected at higher concentrations of viral load.

The higher NASBA estimation of the three samples at 3.34 $\log_{10}$ copies/ml resulted in the five-fold dilution difference between samples at 3.34 and 4.04 $\log_{10}$ copies/ml being undetectable in five out of six cases. A high estimation in a 4.04 $\log_{10}$ copies/ml sample also reduced the viral load difference between this and a sample at 4.74 $\log_{10}$ copies/ml. This gave a true difference undetected (TDU) value of six for the panel.

At higher concentrations there was significantly less variation between the expected concentration and the NASBA estimate. The total figures for FDD, TDU and intra-assay standard deviation, as given by the VQA, are shown in Table 3-4.

Table 3-4 VQA statistical results.

FDD	TDU	Intra-assay St. dev.
3	6	0.42

The NASBA estimated values and VQA adjusted values are shown graphically in Figure 3-2.

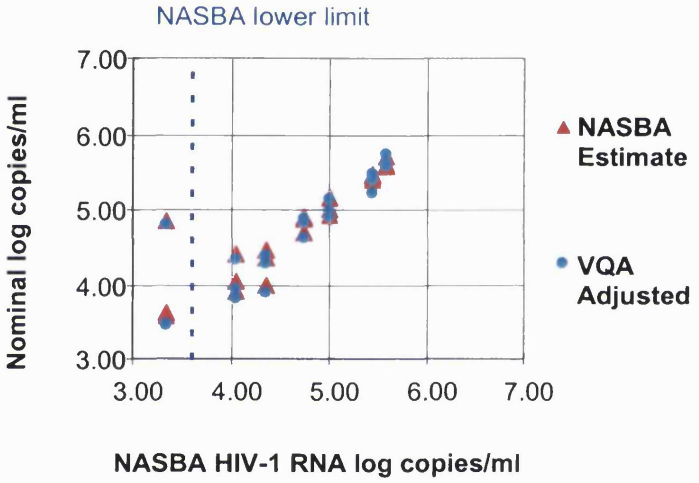


Figure 3-2 Proficiency panel PP07RA values quantified by NASBA (from Table 3-2) plotted against the nominal concentrations. Graph does not include VQA standards.

The VQA analysis was based on the repeated values for samples that were previously undetectable, and did not include the eight negative samples which contained no HIV-1 RNA. The intra-assay standard deviation was based only on the plasma with known concentrations added and not on the replicates of diluted patient plasma, and then only on standard deviations greater then 0.15.

Table 3-5 shows the results for all 29 samples in panel PP07RA including the negative samples. Where samples gave an undetectable result and were re-tested, a mean of the detection limit and second value was used. Standard deviations were calculated for each set of replicate samples, i.e. the VQA adjusted values for all samples at 3.34 $\log_{10}$ copies/ml was compared to the nominal concentration. The differences between the nominal concentration and the VQA adjusted value are shown as absolute numbers. The values for predicted versus observed, where the observed were taken as VQA adjusted values, were normalised and from these the intra-assay standard deviation was calculated to be 0.07.

Table 3-5 Plasma HIV-1 RNA in all samples of panel PP07RA.

Sample	Viral Load log_{10} copies/ml			Replicate sample st. dev.	Difference between Nominal and VQA adj.
	Nominal	NASBA estimate	VQA adjusted		
PP07ra.02	0.00	<3.60	<3.60		
PP07ra.07	0.00	<3.60	<3.60		
PP07ra.12	0.00	<3.60	<3.60		
PP07ra.13	0.00	<3.60	<3.60		
PP07ra.18	0.00	<3.60	<3.60		
PP07ra.20	0.00	<3.60	<3.60		
PP07ra.23	0.00	<3.60	<3.60		
PP07ra.25	0.00	<3.60	<3.60		
PP07ra.27*	3.34	4.23	4.20		0.86
PP07ra.24*	3.34	3.61	3.54		0.20
PP07ra.14*	3.34	3.59	3.52	0.33	0.18
PP07ra.15	4.04	4.40	4.32		0.28
PP07ra.11	4.04	4.04	3.93		0.11
PP07ra.04	4.04	3.92	3.80	0.17	0.24
PP07ra.09	4.36	4.45	4.37		0.01
PP07ra.16	4.36	4.36	4.28		0.08
PP07ra.28	4.36	4.00	3.89	0.19	0.47
PP07ra.26	4.74	4.91	4.86		0.12
PP07ra.03	4.74	4.88	4.83		0.09
PP07ra.22	4.74	4.68	4.62	0.08	0.12
PP07ra.19	5.00	5.15	5.12		0.12
PP07ra.21	5.00	5.00	4.96		0.04
PP07ra.01	5.00	4.93	4.89	0.08	0.11
PP07ra.08	5.44	5.46	5.46		0.02
PP07ra.05	5.44	5.40	5.39		0.05
PP07ra.10	5.44	5.43	5.22	0.09	0.22
PP07ra.17	5.59	5.71	5.72		0.13
PP07ra.29	5.59	5.59	5.59		0.00
PP07ra.06	5.59	5.58	5.58	0.05	0.01

* NASBA value is mean of first result and re-test value.

3.1.5 Discussion

The statistical analysis performed by the VQA evaluated assay performance based on four criteria: the number of false positives, intra-assay standard deviation, false differences detected (FDD) and true differences undetected (TDU). The VQA did not analyse the negative control results. However, the negative samples were all undetectable when tested twice.

The intra-assay standard deviation was based on the largest difference shown between samples from three of the eight panel groups (standard deviations of greater than 0.15). Basing the intra-assay standard deviation on the largest standard deviation cannot be a reflection of assay performance across the whole panel. It illustrates that when the observed value is different to the predicted value, the most it will be different by is 42 %. It does not, for example, show how much the observed value differs from the predicted value for all samples or how much greater or smaller the true viral load might be for any given sample. As such, it cannot be a measure of intra-assay standard deviation.

The analysis based on the normalised data for predicted and observed values gives a better idea of standard deviation within the assay for all samples. Essentially, a standard deviation of 0.07 shows that every observed value generated by NASBA (regardless of what level of HIV-1 RNA is predicted) can differ from the predicted value by 7 %. This is a useful figure for clinicians who need to know the accuracy of a viral load estimation. Small fluctuations in a patient's viral load can therefore be determined as significant, or within the intra-assay standard deviation.

The high results for panel samples of low HIV-1 RNA concentration produced an FDD value of three; this was a measure of how well the virus was quantified in samples of the same HIV-1 RNA concentration. The high estimation of one or two replicates, i.e. samples of the same nominal concentration, created a false difference; making replicate

samples appear different. All FDD were between samples of the lower HIV-1 RNA concentrations. This inaccurate measurement did not occur with samples of higher concentrations of HIV-1 RNA.

There were six TDU, also caused by inaccurate measurement of low HIV-1 RNA concentrations. The five-fold difference between the lowest HIV-1 RNA concentration and the second lowest was not detectable by this assay. In five cases the values obtained could have been measuring the same HIV-1 RNA concentration. At higher concentrations the assay showed all other replicate groups to be distinct from each other, and so the true difference in dilution was evident.

The sensitivity of the NASBA in detecting HIV-1 RNA at low levels was shown to be inaccurate in this study. The VQA analysis demonstrated high values for FDD and a TDU, and high values for standard deviations for individual groups. These were due to inaccurate measurement of low levels of HIV-1 RNA. By using 100µl plasma input, rather than 200µl, which is the recommended plasma input for NASBA, the protocol lessened the ability of NASBA to detect low viral load. A plasma input of 200µl would have given NASBA a lower detection limit of 3.3 $\log_{10}$ copies/ml, which would have been below that of the lowest samples tested (3.34 $\log_{10}$ copies/ml).

The accuracy of NASBA quantification values was also shown to be irregular in this panel. Low HIV-1 RNA samples, when they were detected, were quite significantly overestimated. This observation is corroborated by the standard deviations calculated for each panel group. The accuracy of measurement of the panel group with 3.34 $\log_{10}$ copies/ml is shown by a standard deviation of 0.33, the highest for any replicate group. The groups at 4.04 and 4.36 $\log_{10}$ copies/ml, have the second and third highest standard deviations at 0.17 and 0.19, respectively. This standard deviation decreases with increasing HIV-1 RNA concentration, so that for the highest HIV-1 RNA concentration at 5.59 $\log_{10}$ copies/ml the standard deviation is 0.05. All samples with an HIV-1 RNA

concentration of > 4.50 $\log_{10}$ copies/ml have a standard deviation < 0.10 showing a trend of increased accuracy with higher HIV-1 RNA concentrations. One reason for increased accuracy at higher virus concentrations might be that the NASBA protocol specified by the VQA had a lower detection limit of 3.6 $\log_{10}$ copies/ml, above that of the lowest panel samples. All the samples with HIV-1 RNA concentrations below this limit were undetectable in the first test and when repeated HIV-1 RNA was detected but at greater amounts than expected. A possible explanation might be that for very small amounts of HIV-1 RNA the NASBA has a variable effect. Either HIV-1 RNA is extracted and amplified very well producing higher than expected results, or it is not amplified at all.

In early 1997 when the panel was tested, the lower sensitivity of NASBA, at 3.6 $\log_{10}$ copies/ml, was known to be less than that exhibited by alternative viral load assays. The RT-PCR and bDNA assays at that time had lower detection limits of 400 and 500 copies/ml respectively. This prompted the development of Nuclisens, an ultra-sensitive version of NASBA which allowed a 2.6 $\log_{10}$ copies/ml detection limit. The Nuclisens was released with a modified protocol as the successor to NASBA. It is possible that quality assurance panels such as PP07RA indicated that the NASBA technique required greater accuracy and sensitivity at low HIV-1 RNA concentrations. The elimination of these weaknesses in assay performance enabled NASBA to compete with alternative assays, and be used with confidence in the clinical field.

3.2 Evaluation of Nuclisens using the alpha-trial proficiency panel

3.2.1 Introduction

A number of laboratories participated in this study, which aimed to assess the qualitative and quantitative performance characteristics of Nuclisens in comparison with NASBA. The Nuclisens was designed to extend the sensitivity of the NASBA protocol to give an approximate ten-fold increase in sensitivity. This was achieved by defining the quantities of internal calibrators, specific to each batch. By increasing the accuracy of the internal calibrators it was possible to extend the slope of the graph to detect virus at lower concentrations. As a consequence of combination antiretroviral therapy, viral load in patients post-treatment was frequently falling below the lower limit of the current NASBA protocol where detection was limited to 3.6 $\log_{10}$ copies/ml. The Nuclisens was designed to improve upon NASBA so that a wider range of HIV-1 RNA copy numbers could be detected. The range of the Nuclisens assay was individual to each sample, according to the NASBA technique of plotting a curve from the controls and reading off the sample RNA copies. However, for all samples tested the lower limit of the Nuclisens was at most 2.6 $\log_{10}$ copies/ml, the upper limit was not specified. Organon Teknika Ltd, created a panel consisting of terminal dilutions of a well-characterised HIV-1 RNA standard and of patient samples. The panel represented a range of reactivity that was of clinical interest. Variable sample input volumes were also to be assessed for improved sensitivity. The data produced by participating laboratories were to assess precision reproducibility, analytical sensitivity, effects of variable sample input, site-to-site variation of both NASBA based assays.

3.2.2 Samples and Methods

In proficiency panel 0297 there were 36 samples to be assayed using an input volume of 200µl of plasma in 0.9ml lysis buffer. Thirteen of these 36 were marked duplicates, the second of which was to be assayed independently from the first. A further 14 samples, seven marked duplicates were to be assayed using 2ml plasma in 9ml lysis buffer. The duplicates were to be assayed independently as before. The samples were assayed as shown in Table 3-6.

Table 3-6 Sample run order.

Run 1		Run 2		Run 3		Run 4		Run 5	
Code	ml*[1]	Code	ml	Code	ml	Code	ml	Code	ml
S1	0.2	S11	0.2	OT21	0.2	S1 dp*[2]	0.2	S11 dp	0.2
S2	0.2	S12	0.2	OT22	0.2	S2 dp	0.2	S12 dp	0.2
S3	0.2	S13	0.2	OT23	0.2	S3 dp	0.2	S13 dp	0.2
S4	0.2	S14	2.0	OT24	0.2	S4 dp	0.2	S14 dp	2.0
S5	0.2	S15	2.0	S25	0.2	S5 dp	0.2	S15 dp	2.0
S6	0.2	S16	2.0	OT26	0.2	S6 dp	0.2	S16 dp	2.0
S7	0.2	S17	2.0	OT27	0.2	S7 dp	0.2	S17 dp	2.0
S8	0.2	S18	2.0	OT28	0.2	S8 dp	0.2	S18 dp	2.0
S9	0.2	S19	2.0	OT29	0.2	S9 dp	0.2	S19 dp	2.0
S10	0.2	OT20	2.0	OT30	0.2	S10 dp	0.2	OT20	2.0

*[1] Sample input volume *[2] dp = duplicate sample.

All 30 samples were quantified first using NASBA and then repeated using Nuclisens, in the same run order. Duplicate samples were received as two tubes both identically

labelled. The panel was coded and the raw data was sent to Organon Teknika for analysis before the actual concentration in each sample was released. Samples of 0.2ml plasma input in 0.9ml lysis buffer were assayed in 1.5ml tubes, whilst those with a 2ml plasma input in 9ml of lysis buffer were in 15ml tubes. Samples with a plasma input of 0.2ml had a lower detection limit of 3.3 $\log_{10}$ copies/ml using NASBA and 2.6 $\log_{10}$ copies/ml using Nuclisens. When using a 2ml plasma input the lower detection limit for NASBA and Nuclisens were 2.3 and 1.6 $\log_{10}$ copies/ml, respectively. NASBA HIV-1 QT and Nuclisens HIV-1 QT were performed on all 30 samples according to the manufacturer's instructions. Samples were stored at -70°C until tested.

3.2.3 Results

Panel samples quantified by NASBA ranged from undetectable i.e. < 3.3 $\log_{10}$ copies/ml to 7.0 $\log_{10}$ copies/ml, whilst samples quantified by Nuclisens ranged from undetectable i.e. < 2.6 $\log_{10}$ copies/ml to 6.85 $\log_{10}$ copies/ml. There were three negative controls in the panel, S10, OT20 and OT30. All three were quantified with both assays and two samples, S10 and OT20, were quantified twice. All negative controls were below the detection limits of NASBA and Nuclisens on each occasion. The nominal concentrations for panel samples released by Organon Teknika and the values generated by this laboratory are shown in Table 3-7.

Table 3-7 Panel 0297 quantified by NASBA and Nuclisens.

Sample	Plasma Input ml	HIV-1 RNA log_{10} copies/ml				
		Nominal	NASBA	NASBA	Nuclisens	Nuclisens
S1	0.2	6.91	7.00	6.98	6.85	6.69
S2	0.2	6.21	6.37	6.29	5.97	6.15
S3	0.2	5.51	5.60	5.55	5.49	5.41
S4	0.2	4.81	5.00	5.02	4.75	5.08
S5	0.2	4.46	4.74	4.53	4.38	4.48
S6	0.2	4.11	4.34	4.81	3.93	3.60
S7	0.2	3.76	3.93	4.19	3.41	3.18
S8	0.2	3.41	3.95	<3.30	3.40	3.23
S9	0.2	3.06	<3.30	<3.30	3.18	2.76
S10	0.2	0	<3.30	<3.30	<2.60	<2.60
S11	0.2	2.71	<3.30	<3.30	2.93	<2.60
S12	0.2	2.37	<3.30	<3.30	<2.60	<2.60
S13	0.2	2.02	<3.30	<3.30	<2.60	<2.60
S14	2.0	3.41	3.51	3.37	O.o.T*	3.08
S15	2.0	2.71	<2.30	3.04	1.91	2.64
S16	2.0	2.02	<2.30	<2.30	<1.60	<1.60
S17	2.0	1.66	<2.30	<2.30	1.61	1.73
S18	2.0	1.32	<2.30	<2.30	<1.60	<1.60
S19	2.0	0.95	<2.30	<2.30	<1.60	<1.60
OT20	2.0	0	<2.30	<2.30	<1.60	<1.60
OT21	0.2	3.70	<3.30		3.86	
OT22	0.2	3.22	3.47		3.34	
OT23	0.2	3.70	3.78		3.68	
OT24	0.2	3.22	<3.30		3.36	
S25	0.2	3.70	3.93		3.45	
OT26	0.2	3.70	3.85		3.92	
OT27	0.2	3.22	<3.30		3.40	
OT28	0.2	3.70	<3.30		3.75	
OT29	0.2	3.22	<3.30		<2.60	
OT30	0.2	0	<3.30		<2.60	

* Upper calibrator signal out of tolerance, isolated to the specific batch reagents.

The nominal concentrations show that S1 – S9 and S11 – S19 were a dilution series. Samples S1 – S4 and S14 – S16 were separated by 0.7 $\log_{10}$ copies/ml, whilst samples S4 – S13 and S16 – S19 were separated by 0.35 $\log_{10}$ copies/ml. Samples OT21, OT23, S25, OT26 and OT28 were replicates of 3.7 $\log_{10}$ copies/ml and samples OT22, OT24, OT27 and OT29 were replicates of 3.22 $\log_{10}$ copies/ml.

The NASBA quantified virus from 17 of 18 dilution series samples (samples S1 – S9 and S11 – S19) at, or above, 3.41 $\log_{10}$ copies/ml. Two samples out of five from the diluted patient plasma duplicates at 3.7 $\log_{10}$ copies/ml were not detected using the NASBA. Using a 2ml plasma input did give the NASBA greater sensitivity, three out of four samples (from 2 duplicates) above the NASBA lower detection limit of 2.3 $\log_{10}$ copies/ml were detected and quantified.

The Nuclisens quantified virus from nine out of the 10 duplicate samples above the lower detection limit of 2.6 $\log_{10}$ copies/ml, using a 0.2ml plasma input. Increasing the plasma input, and thereby increasing the lower detection limit to 1.6 $\log_{10}$ copies/ml, did increase the sensitivity of Nuclisens, allowing quantification of virus at 1.61 copies/ml (S17), but sample S16, a higher concentration of 2.02 $\log_{10}$ copies/ml, was undetectable twice. Sample S14 quantified using a 2ml input was excluded due to excess calibrator control levels. This was due to excess amplification of the upper calibrator, Qa. This prevented the plot of the calibrator curve, and the subsequent estimation of virus in the test sample. Therefore, five out of eight samples above 1.6 $\log_{10}$ copies/ml were detectable above the Nuclisens lower detection limit, using a 2ml plasma input. In the diluted patient plasma panel OT21 – OT29 all nine samples were above the Nuclisens detection limit, 8 out of 9 samples were detected and quantified.

Samples S1-S7, S14, S15, OT21, OT23, S25, OT26 and OT28 were analysed by Organon Teknika Ltd and the results from all participating laboratories compared. The

average results for NASBA and Nuclisens from all laboratories and the average results obtained in this laboratory are shown in Table 3-8.

Table 3-8 Panel 0297: a comparison of selected results obtained from all laboratories.

Sample	Nominal conc.	HIV-1 RNA log_{10} copies/ml			
		All labs		This lab	
		NASBA	Nuclisens	NASBA	Nuclisens
S1	6.91	6.88	6.86	6.99	6.77
S2	6.21	6.26	6.10	6.33	6.06
S3	5.51	5.62	5.47	5.58	5.45
S4	4.81	5.01	4.77	5.01	4.91
S5	4.46	4.58	4.45	4.64	4.43
S6	4.11	4.25	4.10	4.58	3.77
S7	3.76	3.93	3.70	4.06	3.30
S14	3.41	3.44	3.42	3.44	3.08
S15	2.71	2.78	2.64	3.04	2.28
OT21	3.70	3.75	3.61	<3.30	3.86
OT23	3.70	4.04	3.87	3.78	3.68
S25	3.70	3.66	3.50	3.93	3.45
OT26	3.70	3.89	3.71	3.85	3.92
OT28	3.70	3.75	3.50	<3.30	3.75

Standard deviations for each sample were calculated for this laboratory and for all laboratories, the results can be seen graphically in Figure 3-3.

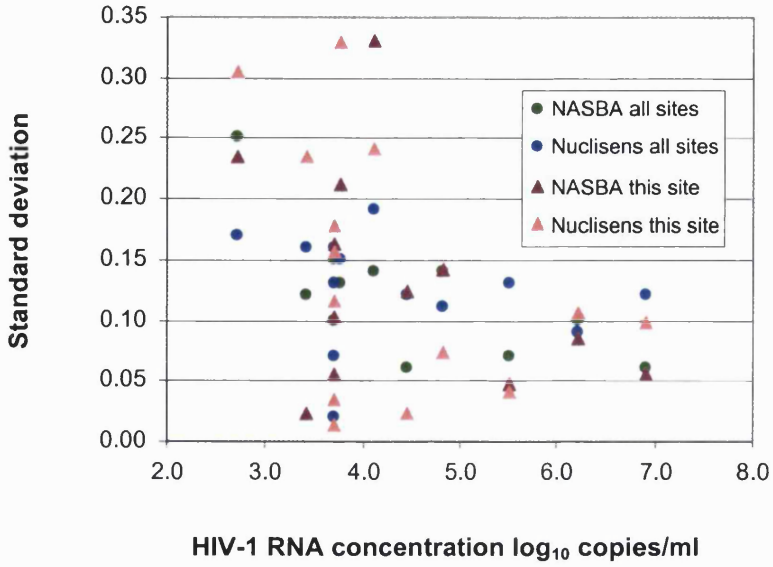


Figure 3-3 Standard deviations of results for samples from Panel 0297 selected by Organon Teknika Ltd for analysis.

When the data selected for analysis were normalised, standard deviations for viral loads quantified by NASBA and Nuclisens for all laboratories, and this laboratory, were calculated. These data are shown in Table 3-9.

Table 3-9 Standard deviations for samples selected for analysis from Panel 0297.

All sites		This site	
NASBA	**Nuclisens**	**NASBA**	**Nuclisens**
0.03	0.02	0.04	0.06

The figures for this laboratory change when all the samples in the panel are included in the analysis and are normalised. The standard deviation for NASBA for all samples

was 0.05, and for Nuclisens was 0.08. The standard deviations were calculated for all samples detectable in this laboratory and these are plotted graphically in Figure 3-4.

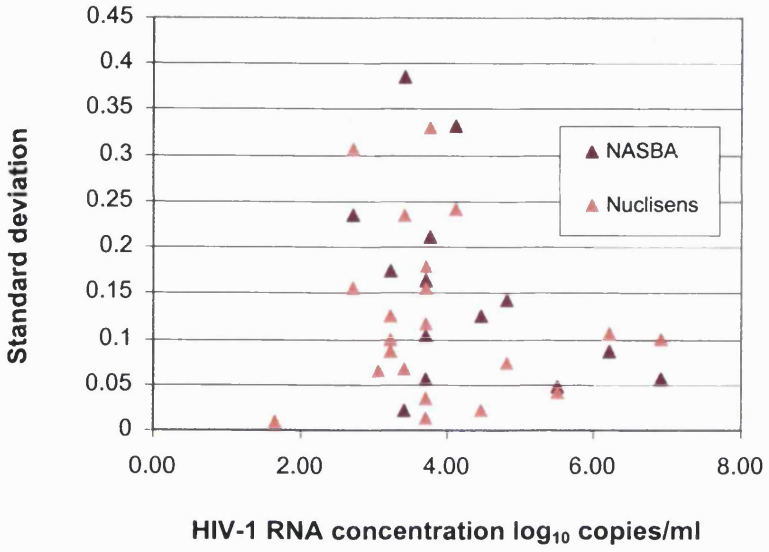


Figure 3-4 Standard deviations for all panel samples detectable in this laboratory.

3.2.4 Discussion

The aim of this proficiency panel was to investigate the newly developed Nuclisens protocol to assess if it could succeed NASBA in accurate and sensitive quantification of plasma HIV-1 RNA. When data from all sites were analysed by Organon Teknika Ltd, the panel appears to have been quantified accurately by both assays. Standard deviations of the normalised values for NASBA and Nuclisens were 0.03 and 0.02 for all sites, demonstrating that quantification of virus was close to predicted values. All participating laboratories confirmed that Nuclisens was able to quantify virus in samples with lower HIV-1 RNA levels than could NASBA. This showed that the estimate for the Nuclisens detection limit was accurate, and that the revised assay could detect the majority of samples above this limit.

Interestingly, high standard deviations of one sample in this laboratory were matched by the average for all laboratories, implying that observed results, although different from predicted, were similar for all laboratories. Standard deviations for S15 were above 0.15 for all laboratories and for this laboratory, ranging from 0.17 to 0.31. Sample S6 and S7 also had standard deviations above 0.13, ranging from 0.13 to 0.33. This could imply a real difference between predicted and observed values, possibly as a result of dilution inaccuracy.

Nuclisens quantification at this laboratory was less accurate than the average for all laboratories. On four occasions virus was not quantifiable from samples which had predicted values above the Nuclisens detection limit. However, virus was quantified from other low HIV-1 RNA samples, demonstrating that it was possible to detect down to the lower limit, but not every time. Samples created by dilution could differ from their predicted nominal concentrations of HIV-1 RNA. Where the concentration level was close to the detection limit this could make a difference to detectability. The only other explanation would be that Nuclisens was unreliable at detecting samples of low HIV-1 RNA concentration close to the detection limit.

Quantification using increased plasma input in both protocols improved sensitivity, and both demonstrated the ability to detect virus at low levels, with Nuclisens detecting virus down to 41 copies/ml (1.61 $\log_{10}$ copies/ml).

The standard deviations for each sample as quantified by Nuclisens were slightly higher than those calculated for NASBA results. Seven Nuclisens results had standard deviations greater than 0.15, as opposed to six by NASBA, although one sample tested by NASBA showed the highest standard deviation of 0.38. The values for standard deviation appear similar for the two versions of the assay, demonstrating similar performance in the accuracy of quantification. The overall standard deviations for

NASBA and Nuclisens of 0.05 and 0.08, respectively, show that if anything, the Nuclisens results were slightly less accurate than those given by NASBA. Quantification of proficiency panel 0297 showed that 22 of 35 samples of spiked HIV-1 RNA and patient HIV-1 RNA were determined accurately, demonstrated by a standard deviation of less than 0.15. High sample standard deviations were sometimes matched at all laboratories, implying that sample plasma HIV-1 RNA concentration was significantly different to that predicted by the dilution series. Nuclisens did show improved sensitivity when compared to NASBA, and could detect down to the lower detection level of 2.6 or 1.6 $\log_{10}$ copies/ml depending on plasma input. However, in several cases the accuracy of Nuclisens detection was less than that of NASBA, giving Nuclisens a higher assay standard deviation overall. Feedback from the evaluation of panel 0297, by this laboratory and others, was invaluable to Organon in the development and consequent marketing of Nuclisens as the more sensitive successor to NASBA. The data produced from using an increased plasma input was important in that it demonstrated that higher sensitivity was possible using 2 ml of plasma. At the time of the study increasing the plasma input was not common practise and was only in the evaluation stage. Current Nuclisens protocol recommends a high volume plasma input and has replaced the original input volume; the assay information on the Nuclisens website (www.nuclisens.com/products/hiv/performance.html) cites a lower limit of detection of 1.6 $\log_{10}$ copies/ml as a result of the standard protocol. Advances in assay procedure rely on evaluation in the target setting and a constructed panel is often the first assessment of a new development in the clinical environment.

3.3 A comparative study of the sensitivities of RT-PCR and Nuclisens in HIV-1 patients on antiretroviral therapy.

3.3.1 Introduction

The objective of this study was to compare longitudinal changes in plasma HIV-1 RNA load in patients on antiretroviral therapy, using RT-PCR and Nuclisens. The study was also designed to evaluate whether the sensitivity of the Nuclisens assay could be increased by increasing the plasma input. The protocol for the study was designed collaboratively with Organon Teknika Ltd and all data produced were reported back to the company.

3.3.2 Patients and methods

Five patients receiving potent antiretroviral therapy of at least two NRTIs and one PI were selected. All the patients were receiving a new therapy, prior treatment with other antiretroviral drugs was not considered relevant at selection. For each patient there was a baseline plasma sample and four plasma samples taken at different intervals for each patient over the next four to 12 months. Viral load was quantified using RT-PCR version 1 described in section 2.2.1. Viral load for all time points was also quantified by Nuclisens using a 0.2ml plasma input. Both assays had the same lower level detection limit of 400 copies/ml or 2.6 $\log_{10}$ copies/ml. The protocol dictated that any samples giving an undetectable reading in the Nuclisens were re-tested using a 1ml plasma input to increase the level of detection to 80 copies/ml or 1.9 $\log_{10}$ copies/ml.

3.3.3 Results

The mean number of weeks of treatment at the fifth time point was 38 (range 33 – 47, median 36), with a maximum interval of 17 weeks between samples. The mean number of weeks between first and second time points was 8 weeks (range 2 – 15 weeks). The viral load results for the five samples of each patient are shown in Table 3-10.

Table 3-10 Plasma HIV-1 RNA in log_{10} copies/ml measured by RT-PCR and Nuclisens in patients receiving HAART.

Patient	Weeks of treatment	RT-PCR	Nuclisens	*Nuclisens Increased input
1	0	4.78	<2.60	4.54
	15	3.04	3.08	
	21	<2.60	<2.60	<1.90
	31	<2.60	<2.60	<1.90
	47	<2.60	<2.60	<1.90
2	0	4.94	4.85	
	6	3.15	<2.60	3.45
	9	2.87	<2.60	<1.90
	21	<2.60	<2.60	<1.90
	36	<2.60	<2.60	<1.90
3	0	5.11	4.98	
	8	3.28	3.08	
	14	<2.60	<2.60	<1.90
	21	<2.60	<2.60	<1.90
	36	<2.60	<2.60	<1.90
4	0	5.03	5.08	
	2	2.67	<2.60	2.18
	4	<2.60	<2.60	<1.90
	20	<2.60	<2.60	<1.90
	33	<2.60	<2.60	<1.90
5	0	5.27	5.26	
	8	2.90	<2.60	2.64
	15	<2.60	<2.60	<1.90
	30	<2.60	<2.60	<1.90
	38	<2.60	<2.60	<1.90

*Samples undetectable in Nuclisens were repeated using increased plasma input

Patients showed an initial baseline viral load of approximately 5.0 log_{10} copies/ml in both assays. Samples measured with the RT-PCR had a mean initial viral load of 5.03

$\log_{10}$ copies/ml (range 4.78 – 5.27 $\log_{10}$ copies/ml). Mean initial viral load measured by Nuclisens was 4.94 $\log_{10}$ copies/ml (range 4.54-5.26 $\log_{10}$ copies/ml). Patient 1 showed an initial viral load of 4.78 $\log_{10}$ copies/ml with the RT-PCR, but was undetectable (< 2.6 $\log_{10}$ copies/ml) with the Nuclisens. This sample was assayed again using the increased plasma input (1ml) and was shown to contain 4.54 $\log_{10}$ copies/ml. There was insufficient plasma to repeat the sample using 0.2ml input, so this figure was used as the baseline Nuclisens viral load for patient 1.

After baseline, the viral load for all patients declined to below detectability. At the second time point all samples were detectable using RT-PCR assay, but only two were detectable using the Nuclisens. However, the three not detected were quantified using the Nuclisens with increased plasma input, the values then were 3.45, 2.18 and 2.64 $\log_{10}$ copies/ml. At the third time point one sample, patient 2, was detectable with the RT-PCR assay but not by either Nuclisens or Nuclisens with increased plasma input. All samples from the fourth time point onwards were undetectable for all patients in both assays. The viral load for all samples at the last time point was < 2.6 $\log_{10}$ copies/ml for RT-PCR and Nuclisens, and < 1.90 $\log_{10}$ copies/ml for the Nuclisens with increased plasma input.

The largest fall in viral load was seen between first and second time points for RT-PCR and Nuclisens assays, with mean viral load drops of 2.02 and 2.15 $\log_{10}$ copies/ml, respectively, over a range of 2 – 15 weeks. If the values for viral load quantified by the Nuclisens with increased plasma input are included, the greatest drop becomes 2.9 $\log_{10}$ copies/ml. The mean viral loads for each assay at the different time points are shown in Table 3-11.

Table 3-11 Mean plasma HIV-1 RNA for all patients.

Time point	Viral load log_{10} copies/ml		
	Mean RT-PCR	Mean Nuclisens	Mean Nuclisens (increased input)
1	5.03	4.94	*
2	3.01	2.79	2.76
3	2.66	<2.60	<1.90
4	<2.60	<2.60	<1.90
5	<2.60	<2.60	<1.90

*Only one baseline sample was undetectable using a standard Nuclisens and was re-detected using increased plasma input, so the mean is not shown. 3 samples at time point 2 were undetectable using a standard Nuclisens and therefore were re-tested using increased plasma input.

The RT-PCR assay gave higher readings for most samples than the Nuclisens, on average values given for the same sample differed by 0.1 log_{10} copies/ml (range 0.01 - 0.50 log_{10} copies/ml). The virus was detectable by both the RT-PCR and Nuclisens in 10 samples. In seven samples, the RT-PCR gave a higher value than the Nuclisens, the mean difference was 0.20 log_{10} copies/ml higher (range 0.01 – 0.50 log_{10} copies/ml). In three out of 10 cases the Nuclisens gave higher results than the RT-PCR value, the mean difference was 0.12 log_{10} copies/ml (range 0.04 to 0.30 log_{10} copies/ml).

The RT-PCR also quantified virus in samples which the Nuclisens and the Nuclisens with increased plasma input could not. This is illustrated in Table 3-11 and graphically as Figure 3-5. The viral loads for samples at the third time point were undetectable

using either Nuclisens method, but the RT-PCR values gave a mean of 2.66 $\log_{10}$ copies/ml, higher than the Nuclisens detection limits of 2.6 and 1.9 $\log_{10}$ copies/ml.

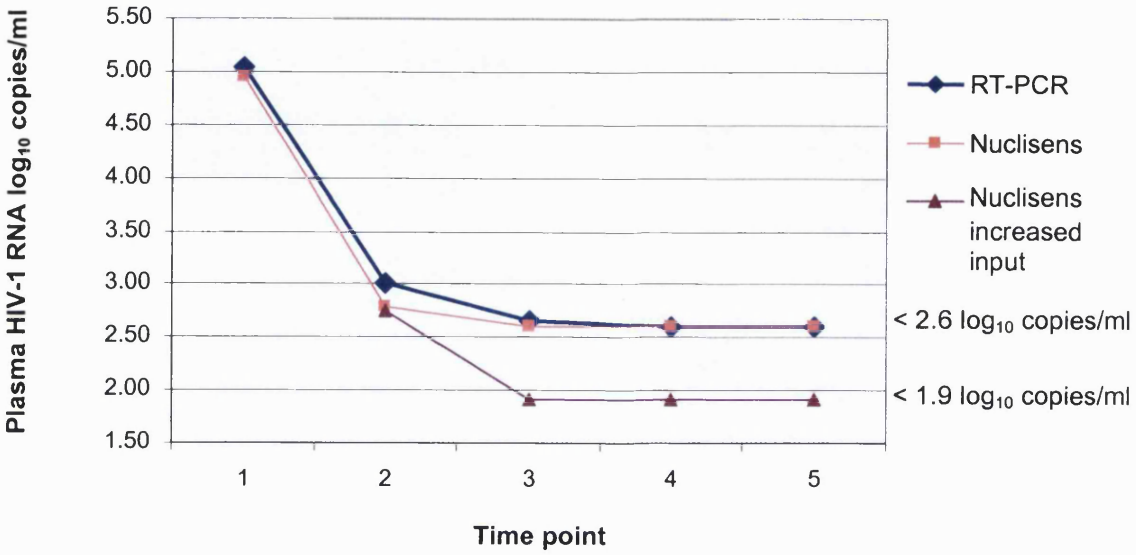


Figure 3-5 Mean plasma HIV-1 RNA quantified by RT-PCR, Nuclisens and Nuclisens with increased plasma input, for patients receiving antiretroviral therapy.

3.3.4 Discussion

The results for all assays followed a trend of decreasing viral load, aside from the anomaly of the baseline sample for patient 1. When quantified with RT-PCR, this sample had a value of 4.78 $\log_{10}$ copies/ml, whereas the Nuclisens was not able to detect virus. When quantified with the Nuclisens and an increased plasma input (1ml), the value given was 4.54 $\log_{10}$ copies/ml. There was no obvious fault in assay procedure and calibration levels for this sample were proportional when plotted graphically. The value given by the Nuclisens increased input was still lower than the RT-PCR value of 4.78 $\log_{10}$ copies/ml. No explanation is apparent other than technical error, so for analysis purposes the baseline Nuclisens value for this sample was assumed to be that given by the Nuclisens with increased input.

From Table 3-11 it is possible to see that the increased plasma input did not allow the Nuclisens assay to quantify HIV-1 RNA at lower levels than before. The viral loads for time point 3 onwards were undetectable using Nuclisens, and were still undetectable using the increased plasma input.

As shown in Figure 3-5 the main difference between the results estimated by RT-PCR and Nuclisens was that the RT-PCR results were slightly higher in the majority of samples measured (from Table 3-10 seven out of nine samples where both assays detected virus). It could be suggested that this was as a result of the difference in amplification step between the two assays, resulting in one method amplifying more HIV-1 RNA/DNA than the other. However, the use of HIV-1 RNA internal controls in both assays should make the actual degree of amplification irrelevant. Also there is a difference in the detection ability between the two assays. In one sample, patient 2 third time point, the RT-PCR detected virus whereas the Nuclisens, with increased plasma input, could not. This may be due to differences in HIV-1 RNA extraction. If RT-PCR extracted a greater proportion of HIV-1 RNA from the plasma than the Nuclisens, it would have a greater sensitivity to detect at low HIV-1 RNA levels, and possibly show as higher results for samples of the same HIV-1 level. The most likely explanation in this study is that technological differences resulted in more efficient low level plasma HIV-1 RNA determination by RT-PCR than by Nuclisens.

As a monitor of drug efficacy, both assays showed significant drops in viral load from initiation of treatment, and whilst the RT-PCR values remained higher than the Nuclisens, the drop in viral load was relatively similar for both assays. The difference becomes more apparent when the Nuclisens values show a greater decline in viral load due to a lack of detection. The detection lower limit is the same for both standard assays (< 2.6 $\log_{10}$ copies/ml). In this study, at the second time point (mean 8 weeks of treatment), the standard Nuclisens quantified two out of five patient samples, compared

to the RT-PCR which quantified all five. A clinician looking at viral load measured by Nuclisens as a guide of therapeutic potency, would assume a therapy was very effective in reducing viral load to undetectable levels by the second time point. If a similar therapeutic response was measured using RT-PCR, the detection of virus at the second and third time points by RT-PCR could imply that one therapy was less potent than another. This highlights the importance of using the same viral load assay when judging virological response to therapy.

The increased plasma input of 1ml did improve the sensitivity of the Nuclisens assay compared to using 0.2ml of plasma. However, Nuclisens, even with an increased plasma input, was not as sensitive at detecting low levels of virus as the RT-PCR assay. Nevertheless, the sensitivity comparison seen in this study may not be clinically relevant. For example a clinician reviewing viral load measured by either assay would assume that the therapy was achieving positive results. Only a comparison of values measured by different assays might prove inaccurate or misleading. Interchanging a patient's viral load measures from Nuclisens to RT-PCR or vice versa might generate quite a large alteration in viral load from one sample to the next. The maximum difference in viral load between RT-PCR and Nuclisens values in the same sample was 0.5 $\log_{10}$ copies/ml in this study. A rise in viral load of 0.5 $\log_{10}$ copies/ml could prompt a change in a therapy which did not appear to suppress viral replication, whilst a decline of 0.5 $\log_{10}$ copies/ml could misleadingly show a virological response to therapy. Either might be detrimental to the patient in the long-term.

The study did demonstrate that increasing the volume of plasma inputted could increase the level of detection down to the theoretical lower limit 1.9 $\log_{10}$ copies/ml (although the lowest value detected in this study was 2.18 $\log_{10}$ copies/ml), beyond that of the standard Nuclisens lower limit of 2.6 $\log_{10}$ copies/ml. As mentioned previously in section 3.2.4, the use of large volumes of plasma has superseded the previous protocol

and 1 to 2ml plasma input is now standard procedure for detecting RNA in samples containing low copy numbers of virus. Small scale collaborations between the manufacturer and the clinical user such as this provide clinical support for ongoing assay development.

The difference in HIV-1 RNA quantification values between the two assays emphasises the importance of consistently using the same assay to monitor a patient's viral load. The differences between values produced by different assays should be highlighted, thus, if viral quantification had to switch between assays a relative expected difference could be applied. From the results of this study, one might expect a steady viral load, previously quantified by RT-PCR, to drop by as much as 0.5 $\log_{10}$ copies/ml when measured by Nuclisens. This level of variability has been observed before in comparisons of the two methods[178] and it has been suggested that it may be due to differences in primer/probe binding sites. The difference in primer/probe binding sites between the two assays would mean that some viruses are optimally amplified and detected by Nuclisens and others by RT-PCR. This may account for the discordant values of ≤ 0.5 $\log_{10}$ copies/ml. This study agrees with previous findings that differences of ≤ 0.5 $\log_{10}$ copies/ml were seen for the same sample when measured by Nuclisens and RT-PCR[179-181], and were most likely due to inter-assay variability and did not reflect a disproportionate trend. Investigations into the maximum difference between values measured on alternative assays should be monitored periodically as an alternate measure of quality assurance of both assays.

3.4 Comparative quantification of subtype B population using NASBA and alternative commercial viral load assays

3.4.1 Introduction

Quantitative HIV-1 RNA testing is now an essential and standard part of the evaluation and clinical care of HIV-infected patients throughout the UK. Viral load testing is used to assess prognosis, to determine the need for antiretroviral therapy or to switch therapy, and to define a baseline laboratory value so that the response to therapy can be measured. Which of the three main methodologies of HIV-1 quantification, NASBA, RT-PCR or bDNA, is employed tends to be based primarily on the availability and cost to the specific healthcare body. However, assay performance issues such as sensitivity, accuracy and reproducibility have been highlighted by various studies and can have considerable impact on the choice of assay[179]. Each method of quantifying virus is subject to intra-assay variability and laboratory analysis of panels of plasma to which known levels of virus have been added, has demonstrated differences in detection levels between the various methods[182,183]. However, at the time of the study, little research had been done to compare how the different quantification methods performed in a routine clinical situation. Although viral load assays should not be interchanged in the monitoring of an individual patient, see section 3.3.4, this is sometimes unavoidable, for example, if a patient moves to a different treatment centre. It would be helpful to highlight any differences between the methodologies, so that clinicians are aware of how much inter-assay difference to expect.

In this study, a group of patients of known subtype B status were defined by *gag* sequence analysis. Each patient sample was evaluated by all three HIV-1 quantification methods, NASBA, RT-PCR and bDNA. The differences between values for any given sample were then plotted so that general trends could be observed.

3.4.2 Patients and samples

One hundred and fifty-two patients, selected from the Ian Charleson Day Centre (ICDC) at the Royal Free Hospital were identified by a clinician on the epidemiological evidence of having acquired their HIV-1 infection outside the UK (later described in 3.5.2), were analysed for non-B subtype diversity from 1996 - 1997. Sequence analysis of the *gag* gene (performed by Roche diagnostics, USA) identified 20 patients as being infected with subtype B virus. Plasma was separated within six hours of the blood being taken and was frozen at -70°C.

3.4.3 Viral load quantification

The viral load was measured using NASBA QT HIV-1 (Organon Teknika Ltd, Cambridge, UK), Amplicor HIV-1 Monitor 1.0 (RT-PCR, Roche Diagnostics, East Sussex, UK), and Quantiplex HIV-1 2.0 (bDNA, Bayer plc Diagnostics Division, Newbury, UK). Quantification was carried out according to the manufacturer's instructions. In each assay each sample was quantified once. The three assays used all have different lower limits of detection, ranging from 2.6 $\log_{10}$ copies/ml to 3.3 $\log_{10}$ copies/ml. To remove any bias in the analysis, a cut-off of 3.3 $\log_{10}$ copies/ml was used for all three assays, with any quantification below this level being taken as < 3.3 $\log_{10}$ copies/ml.

3.4.4 Results

The 20 samples gave viral load values in the range of < 3.3 log_{10} copies/ml to 5.81 log_{10} copies/ml when the values for all 3 assays were combined. Three samples out of the 20 were shown to contain < 3.3 log_{10} copies/ml in all three assays. Table 3-12 shows the values for all samples quantified by the three assays.

Table 3-12 Subtype B samples quantified by NASBA, RT-PCR and bDNA.

	HIV-1 RNA concentration in log_{10} copies/ml		
Sample	**RT-PCR**	**NASBA**	**bDNA**
1	3.84	3.85	< 3.30
2	5.81	5.74	5.30
3	4.79	4.81	4.14
4	3.86	4.06	3.38
5	3.84	< 3.30	< 3.30
6	4.13	4.28	3.41
7	3.32	< 3.30	< 3.30
8	4.49	4.35	4.18
9	5.07	4.98	4.51
10	3.34	< 3.30	< 3.30
11	3.85	3.74	3.36
12	5.28	< 3.30	4.88
13	4.79	4.62	4.04
14	< 3.30	< 3.30	< 3.30
15	< 3.30	< 3.30	< 3.30
16	3.86	4.13	3.64
17	3.45	3.81	< 3.30
18	< 3.30	< 3.30	< 3.30
19	5.51	5.41	< 3.30
20	3.41	3.35	NA*

*NA = Not available, insufficient plasma to perform the test.

Table 3-12 shows that the RT-PCR detected 17 out of 20 samples (85%). The NASBA assay detected 13 out of 20 samples (65%), whilst the bDNA assay detected 10 out of 19 samples (53%). The RT-PCR and bDNA assays did quantify samples below the 3.3 $\log_{10}$ copies/ml, however, because of the higher detection limit of the NASBA assay these samples were all categorised as below 3.3 $\log_{10}$ copies/ml.

The assays were compared in pairs, the mean difference and standard deviation between detectable values was calculated and expressed as $\log_{10}$ copies/ml. Previously, studies have used a difference of ≤ 0.5 to 0.3 $\log_{10}$ copies/ml to differentiate between concordant and discordant results[179,181,184]. In this study values that were different by ≤ 0.4 $\log_{10}$ copies/ml were categorised as concordant between the two assays. The percentage of concordant values, mean difference and standard deviations of the three assay pairs are shown in Table 3-13. The Table only includes differences calculated from actual values i.e. values that were undetectable in any assay were not included in concordance studies.

Table 3-13 Comparisons of performance of NASBA vs. RT-PCR, NASBA vs. bDNA and RT-PCR vs. bDNA in the quantification of subtype B HIV-1 virus.

	NASBA vs. RT-PCR n = 13	NASBA vs. bDNA n = 9	RT-PCR vs. bDNA n = 10
% detectable by both assays	65	45	50
Mean difference between values (log_{10} copies/ml)	0.02	0.53	0.51
St. dev. of absolute difference between values	0.10	0.20	0.17
% concordant values($\leq$ 0.4 log_{10} copies/ml difference)	100	22	20

Of the 13 samples quantified by both NASBA and RT-PCR all were within 0.4 log_{10} copies/ml of each other. Six samples were higher in the NASBA than in the RT-PCR, and seven were higher in the RT-PCR (mean difference 0.1 log_{10} copies/ml, range 0.01 – 0.37 log_{10} copies/ml). Of the nine samples quantifiable in both NASBA and bDNA, all nine were lower in the bDNA (mean difference 0.53 log_{10} copies/ml, range 0.18 – 0.86 log_{10} copies/ml). Of the 10 samples quantifiable by the RT-PCR and the bDNA, all were lower in the bDNA (mean difference 0.51 log_{10} copies/ml, range 0.21 – 0.75 log_{10} copies/ml).

The correlation between values plotted for each sample, between each assay pair is illustrated in Figure 3-6.

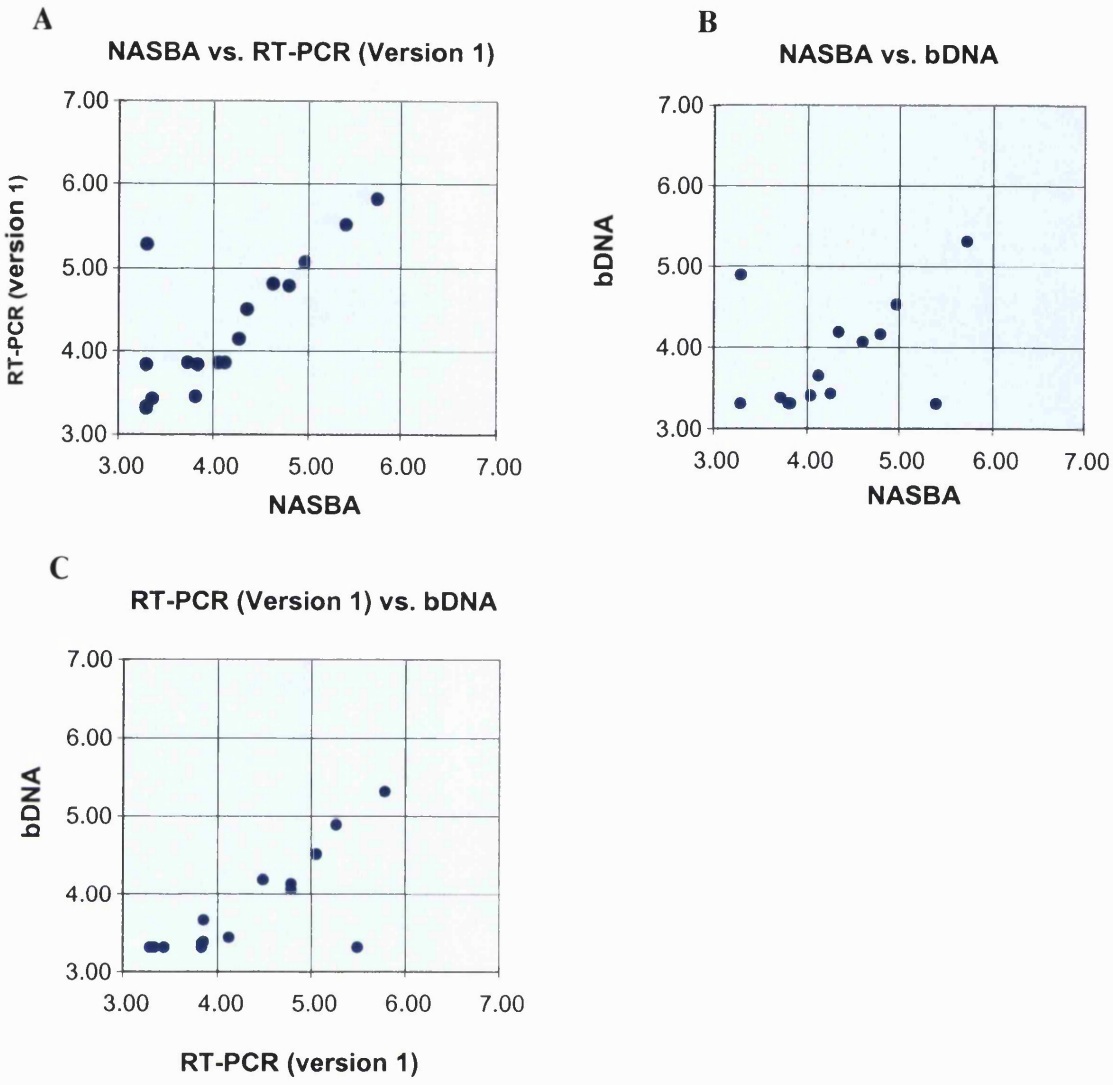


Figure 3-6 The correlation of viral load values in log_{10} copies/ml described by paired assays in plasma containing subtype B strains of HIV-1.

Statistical analysis (t-test) of the values given for each sample demonstrated the relationship of viral load values between assays. Correlation coefficients were also calculated for each assay pair. Both sets of results are shown in Table 3-14.

Table 3-14 Statistical analysis of the values given by the assay pairs.

	NASBA vs. RT-PCR	NASBA vs. bDNA	RT-PCR vs. bDNA
P value	0.295	0.045	0.0009
Correlation coefficient	0.822	0.548	0.796

Statistical significance was calculated using a paired t-test. P is significant at a value of < 0.05

Figure 3-6 A shows that viral load values produced by RT-PCR and NASBA show a fairly high correlation, demonstrated by a correlation coefficient of 0.822. Illustrating that the majority of values reported for the same sample differ only slightly between RT-PCR and NASBA. The Figure shows a few samples were recorded as higher values in the RT-PCR than the NASBA, but the t-test value between the two assays is $p = 0.295$, showing that the difference is not significant.

Figure 3-6 B shows that the majority of values produced by NASBA and bDNA were linearly proportional. However, the values frequently differed for the same sample, in some cases considerably. The bDNA gave consistently a lower value than the NASBA and the t-test comparison for these two assays has a value of $p = 0.045$. This demonstrates that the difference between the values is significant for this data set. The variability in values between both assays was also quite high, this is demonstrated by a poor correlation coefficient of 0.548 for NASBA versus bDNA (1 = positive correlation, 0 = no correlation). Therefore, there is only a minimal correlation between the results obtained by NASBA versus and bDNA.

Figure 3-6 C shows the correlation between the RT-PCR assay and the bDNA assay. As with the NASBA versus bDNA, there are differences between the values, but the correlation appears more linear. Again, the bDNA gave consistently a lower value for

the same sample than the RT-PCR. The t-test gives a value of $p = 0.0009$ for the comparison between the two assays, demonstrating that this difference between the values is significant. However, the correlation between the assays is relatively high, 0.796.

3.4.5 Discussion

The NASBA and RT-PCR assays produced values which correlated well for most subtype B viruses, whereas the correlation between the bDNA assay and either NASBA or RT-PCR was more unpredictable. The RT-PCR and NASBA methodologies show similarities which do not exist with the bDNA. Both the RT-PCR and the NASBA rely on PCR amplification of a target sequence, and the subsequent detection of that sequence. Both assays will favour an HIV-1 population which most closely resembles the primer sequences used in the amplification process. Although the population of viruses amplified optimally will differ between the two assays as a result of their different primer binding sites, similarity in procedure should mean that the range of values does not differ significantly. Some viruses gave a higher value in the RT-PCR than in the NASBA, whilst some were reported higher in the NASBA. Similar to study 3.3, the majority of the differences were in samples containing low HIV-1 RNA copy numbers. However overall, both assays showed relatively concordant results.

In this study, bDNA values could not be compared to those given by RT-PCR or NASBA, as the slope of values generated by the assay appeared to be significantly different, possibly as a result of the divergent methodology. This agreed with previously observed findings in a similar study from 1996. This study showed that whilst the mean difference between values produced by the NASBA and RT-PCR were not significant ($p > 0.9$), mean values between either NASBA or RT-PCR, and bDNA were significantly different ($p < 0.01$)[181]. In the current study, the bDNA assay gave

consistently lower values than NASBA and RT-PCR (mean difference 0.53 and 0.51 $\log_{10}$ copies/ml, respectively). Values produced by the bDNA were only occasionally within 0.4 $\log_{10}$ copies/ml when compared to either NASBA or RT-PCR. This difference between bDNA and NASBA or RT-PCR values has now been highlighted in further studies, Skidmore *et al*[185] observed bDNA values 0.50 $\log_{10}$ copies/ml lower than other HIV-1 quantification methods, including the RT-PCR and NASBA. This indicates that if the same sample is tested in bDNA and either NASBA or RT-PCR, it would appear to be significantly different. An earlier study did not observe a significant difference between the three assays[179], however, this study highlighted values where there was a difference of $\pm$ 0.5 - 1 $\log_{10}$ copies/ml, so a trend of a smaller difference may not have been observed. The variance within the bDNA assay may be minimal, so that repeated samples give very similar values. In four similar studies comparing bDNA, RT-PCR and NASBA performance, bDNA gave the most reproducible results between the three assays[181-183,185].

This study has demonstrated that, for this panel, values produced by NASBA and RT-PCR were all concordant to $\leq$ 0.4 $\log_{10}$ copies/ml. By comparison the values produced by bDNA and either NASBA or RT-PCR were relatively discordant and the bDNA values consistently lower. This study highlights that differences in methodology between assays may be responsible for the same sample showing a difference of > 0.5 $\log_{10}$ copies/ml. It is important that this relatively high level of inter-assay variability is recognised and accounted for in the comparison of viral load quantified by alternative assays. As previously recommended[185], wherever possible a patient's viral load should be measured by the same procedure. If a change in quantification method is necessary, it is essential that the variability observed in assay evaluations, be taken into account.

3.5 Comparative quantification of a non-subtype B population using NASBA and alternative commercial viral load assays

3.5.1 Introduction

At the beginning of the AIDS pandemic, Europe and the Americas saw an almost complete dominance of subtype B virus[186,187]. Viral load assays which were developed in the late 1980s and early 90s used laboratory strains of prevalent viruses circulating throughout Europe and North America. The commercial HIV-1 quantification assays were designed around sequence specific amplification and detection of sections of the HIV-1 genome[169,188]. Until recently, the target genome was specifically HIV-1 subtype B. NASBA uses amplification primers which bind in the *gag* gene, amplifying a nucleotide sequence *via* isothermal HIV-1 RNA amplification[169]. The RT-PCR and bDNA assays detect in HIV-1 the *gag* and *pol* regions respectively[189,190].

Over the last 20 years subtype distribution has shifted so that, except in Africa, subtype diversity has increased, particularly in Europe[39]. Subtype diversity was first associated with HIV-1 *env* gene in 1988[29]. Sequence analysis of the V3 hypervariable region in *env* demonstrated differences between geographical populations of HIV-1[29]. However, base changes in other parts of the genome have now been demonstrated[31]. Although none of the viral quantification systems detect in *env*, other genomic differences in non-B type viruses could affect the efficiency of subtype B sequence specific amplification and detection systems. Differences between the assay target sequence and the non-B sequence could mean inefficient primer or probe binding, reducing the amplification

and detection signal and so give rise to a false low result for the viral load, misrepresenting the clinical status of the patient.

This study was designed to investigate NASBA detection of diverse viruses, compared to alternative HIV-1 RNA quantification methods. A panel of non-B samples was selected and quantified using NASBA, RT-PCR and bDNA. Since the work was done, both RT-PCR and bDNA assays have been updated, the RT-PCR has been specifically altered to improve the detection of non-B subtypes[191].

3.5.2 Patients and samples

A cohort of patients was selected on epidemiological evidence of having acquired their HIV-1 infection outside the UK. Clinical notes specified either the infection was acquired outside Europe, or was transmitted by an individual from a population where non-B strains are highly prevalent. The criteria were such that those who had been infected in Africa or by an African individual were specifically selected for the study. Factors such as gender, transmission route, disease stage and therapy were not considered at selection. Primarily, the viruses were identified as B or non-B by a competitive peptide EIA technique[192]. Samples were then sequenced in the *gag* region (performed by Roche diagnostics, USA), with a variety of primers, to better define the viral subtype. Plasma was separated within 6 hours of blood being taken and was frozen at -70°C.

3.5.3 Viral load quantification

The viral load was measured using NASBA QT HIV-1, the Amplicor HIV-1 Monitor 1.0 (RT-PCR, Roche) and Quantiplex HIV-1 2.0 (bDNA, Chiron). The companies were consulted during the design of the study and it was suggested by Roche that we might

add new research primers SK145/SK151 (RT-PCRnb, Roche) to the existing amplification step of the Amplicor HIV-1 Monitor 1.0. At the time of this study, these primers were being evaluated internally by Roche as potentially improving the amplification and subsequent detection of non-B subtypes. This study allowed these additional primers to be tested against a characterised panel of non-B subtypes from this London clinic, evaluating the potential additional of the primers to the existing assay and the possibility of developing a new version of Amplicor HIV-1 Monitor. Quantification using all four assays was carried out according to the manufacturer's instructions. A single quantification was made with each assay of the viral load in each sample. All four assays used have different lower limits of detection, ranging from 2.6 $\log_{10}$ copies/ml to 3.3 $\log_{10}$ copies/ml. To remove any bias in the analysis a lower level cut-off of 3.3 $\log_{10}$ copies/ml was used for all four assays. Any quantification below this level was taken as < 3.3 $\log_{10}$ copies/ml. Where viral loads exceeded the specified upper limit for an assay, the sample was diluted in negative human plasma and re-tested to obtain a result.

3.5.4 Results

Of the 152 patients selected for the cohort, 119 were sequenced in *gag*. It was confirmed that 94 out of 119 (79%) were infected with non-B subtype virus, 20 out of 119 (17%) were infected with a B virus, and 5 viruses were could not be determined from the *gag* sequence. Six different non-B subtypes were characterised and the distribution of these amongst the 94 patients is shown in Table 3-15.

Table 3-15 Number of patients of each subtype (total = 94).

Subtype	No. of Patients	Percentage
A	45	48 %
C	30	32 %
D	14	15 %
F	1	1 %
G	3	3 %
H	1	1 %

Patient viral loads measured by all three assays ranged from < 3.3 copies/ml to 6.42 $\log_{10}$ copies/ml, with a wide spread of values. However, there was a number of samples in which viral load values differed from assay to assay. No one assay gave consistently higher values than all other assays.

Figure 3-7 shows the NASBA viral load values plotted against those for each alternative assay, to give a graph of one assay against another.

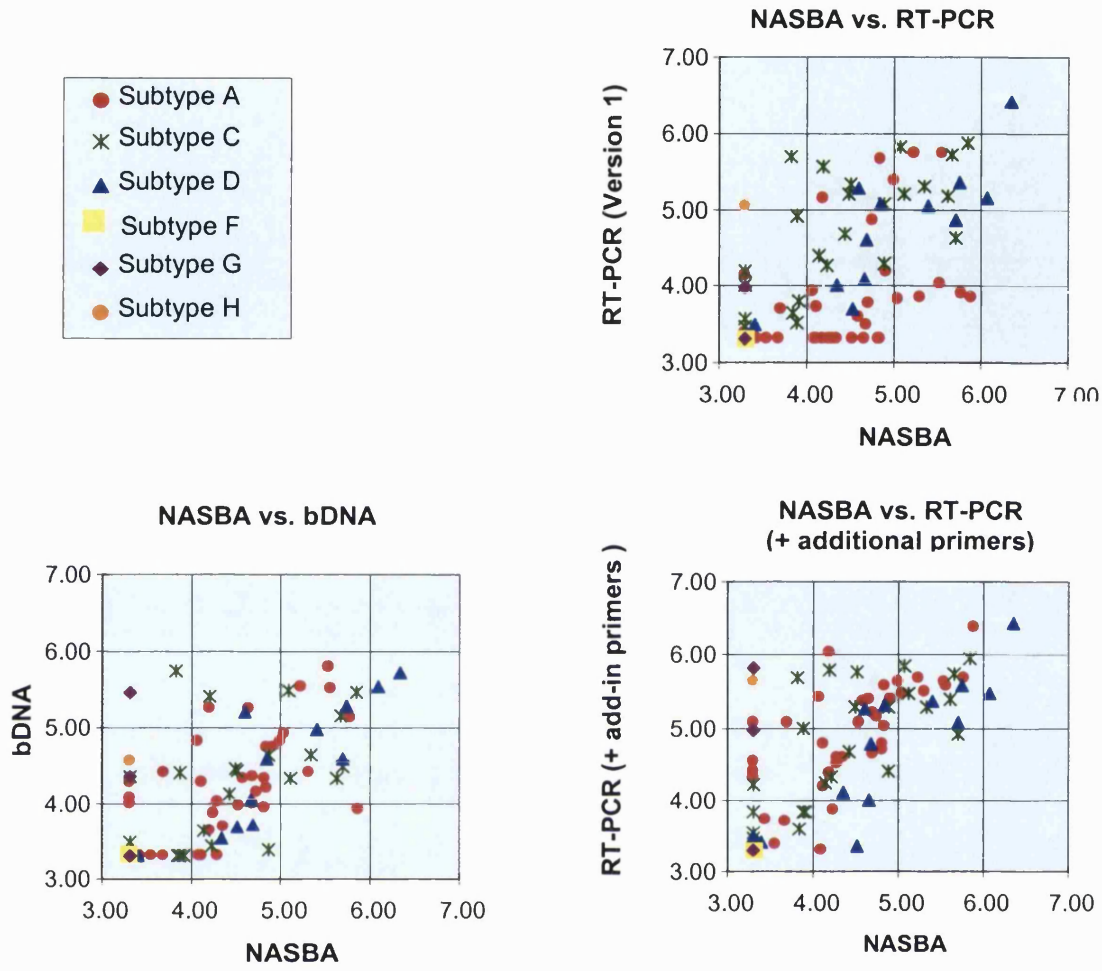


Figure 3-7 The correlation of viral load values in log_{10} copies/ml described by paired assays in plasma containing non-subtype B strains of HIV-1.

The t-test comparisons for the same assay pairs: NASBA versus RT-PCR, NASBA versus RT-PCR with additional non-subtype B primers, and NASBA versus bDNA for each different subtype group are shown in Table 3-16. Subtype groups F and H were omitted from t-test analysis, as there was only one sample of each subtype.

Table 3-16 T-Test comparisons of HIV-1 RNA assays quantifying different non-B subtypes.

Subtype (No. > 1)	NASBA vs. RT-PCR P value	NASBA vs. RT-PCR (+ additional primers) P value	NASBA vs. bDNA P value
A	0.00004	0.00001	0.06728
C	0.04955	0.00996	0.24433
D	0.07584	0.28065	0.00343
G	0.42443	0.20106	0.22802

P is significant at a value of < 0.05

Table 3-17 shows mean values calculated for each subtype as measured by all assays.

Table 3-17 Mean viral load in $\log_{10}$ copies/ml in each assay for each subtype.

Subtype	RT-PCR	RT-PCR (+ additional primers)	NASBA	bDNA	Standard deviation
A	3.74	4.67	4.25	4.09	0.38
C	4.43	4.51	4.21	4.06	0.21
D	4.54	4.63	4.78	4.34	0.18
F	< 3.30	< 3.30	< 3.30	3.34	0.02
G	3.52	4.69	< 3.30	4.38	0.67
H	5.05	5.62	<3.30	4.56	0.99

Table 3-18 shows the difference between NASBA and each alternative assay in predicting the mean values for each subtype.

Table 3-18 Difference in mean value between NASBA result and results of RT-PCR and bDNA assays.

Subtype	RT-PCR	RT-PCR (+ additional primers)	bDNA
A	0.51	-0.42	0.16
C	-0.22	-0.30	0.16
D	0.23	0.15	0.43
F	0.00	0.00	-0.04
G	-0.22	-1.39	-1.08
H	-1.75	-2.32	-1.26

Differences between NASBA and the RT-PCR assays in quantifying samples of subtype A and C were statistically significant. The NASBA mean for subtype A was higher than RT-PCR version 1 by 0.51 $\log_{10}$ copies/ml. The overall difference between subtype A samples quantified by NASBA and RT-PCR, had a significance of $p = 0.00004$. When NASBA was compared to the RT-PCR with additional primers, the NASBA mean for subtype A was lower by 0.42 $\log_{10}$ copies/ml, with a significance of $p = 0.00001$ for all samples.

The mean NASBA value for subtype C was lower than either RT-PCR assay, implying that the NASBA assay does not amplify subtype C virions as well as RT-PCR. T-test calculations showed that the difference between NASBA and RT-PCR had significance values of $p = 0.05$ for the RT-PCR version 1, and $p = 0.01$ for the RT-PCR with additional primers. However, Figure 3-7 shows a scattered distribution between NASBA and either RT-PCR for this subtype, suggesting that the difference may be sample dependent.

The NASBA values for subtypes G and H were also lower than either RT-PCR assay, but low sample numbers prevented statistical analysis. Unfortunately, it was not possible to repeat the quantification of these viruses due to a lack of sample.

NASBA gave values greater than the bDNA assay for subtypes A, C and D, but only subtype C showed a significant difference ($p = 0.0034$) between the assays. NASBA values were lower than the bDNA on samples of subtype F, G, and H, but again low sample numbers prevented analysis.

NASBA gave much lower values for subtypes G and H than all three other assays, suggesting that NASBA failed to amplify these subtype samples.

3.5.5 Discussion

Selecting patients on epidemiological evidence that they had acquired their infection outside the UK seemed to identify potential non-B subtype infections fairly well. Sequence analysis characterised 94 out of 119 (79%) patients were infected with a non-B virus. Of these 94 patients the distribution of non-B subtypes contained 48% subtype A and 32% subtype C. Subtypes A, C and D are the most common subtypes found in Africa[42,186,186]. This is comparable to a more recent study in Sweden where similar selection criteria were applied: Here they found out of 83 non-B viruses, 34% were subtype A, 31 % were subtype C and 24 % were subtype D[193]. In this study subtype D was less frequently observed; 15 % of patients showed infection with a subtype D virus. Subtype E (now known as recombinant A subtype[32]), which is the most common subtype found in Thailand[194], was not observed in this study. This may mean that this clinic has a very low prevalence of this recombinant virus. However, in highlighting patients potentially infected by an African source, the selection criteria may have missed those infected in Asia or Thailand. The discovery and reclassification of E as an

A/E recombinant was not known at the time this work was carried out, and thus the *gag* sequencing may not have detected a recombinant A/E virus.

The results show that there is considerable variation in viral load quantification between the different commercial assays for non-B viruses. An assay which failed to quantify virus from a non-B subtype virus would have lower viral load values for this population, compared to an assay that was able to quantify non-B virus. In this study, no assay consistently gave higher viral loads for all viruses, compared to any other assay.

The additional primers did improve the detection ability of the RT-PCR assay. The earlier version had, by comparison, lower mean values for subtypes A, C, D, G and H. Similar studies have highlighted the failure of the first generation RT-PCR to detect diverse subtypes, most notably the failure to detect subtype A[193,195]. In January 1997, an abstract from the 4th Conference on Retroviruses and Opportunistic Infections described a study of RT-PCR and RT-PCR with additional primers, confirming that there was a significant improvement in HIV-1 non-B subtype quantification by the incorporation of the new primers[174,193,196,197]. Later that year researchers observed that the RT-PCR version 1 could detect only a limited diversity of HIV-1 due to widespread primer mismatching associated with a number of subtypes [198]. Our study and others were instrumental in demonstrating the increased efficacy of the additional primers in quantifying clinical non-B subtype viruses. In December 1997, the new primers SK145/SKCC1B were incorporated into the RT-PCR and version 1 became version 1.5.

The NASBA appears to detect higher levels of subtype D viruses than any other assay. It gave higher values than the bDNA for A, C and D viruses, and higher values for A and D than the RT-PCR version 1. However, the RT-PCR with additional primers detected levels of A and D viruses similar to the NASBA. The ability of NASBA to detect more subtype A viruses than RT-PCR version 1, but not as many as RT-PCR with additional primers (now Amplicor HIV-1 Monitor version 1.5), has been

demonstrated subsequently, in a selected population infected with HIV-1 subtype A in Abidjan, Cote d'Ivoire[199]. The failure of NASBA to detect samples of subtype G and H has also been demonstrated elsewhere[181,200], but the limited numbers of subtype G and H samples available for testing has prevented conclusions from being drawn.

The bDNA assay showed a good detection of diverse HIV-1 subtypes in this study. Values produced by the bDNA were on average lower than either RT-PCR or NASBA, however, this is consistent with previous findings[185] (see section 3.4.4). This is most likely attributable to the different assay methodology of the bDNA rather than it being less sensitive in picking up viruses. The bDNA detected and quantified virus for all subtypes including F, G and H. Before the RT-PCR additional primers (or later version 1.5) were widely available, studies observed that the bDNA gave the best performance in quantifying diverse subtypes out of NASBA, RT-PCR and bDNA[181]and performed better against RT-PCR version 1 in detecting viruses of subtypes A-F[201,202].

The epidemiology of subtypes is now so widespread, that the country or place of infection can no longer be used as an indicator of subtype. For example, previously one might presume that a virus contracted in Asia had a high probability of being HIV-1 subtype A or recombinant A. Based on these results, an HIV-1 subtype A virus could be quantified comparably by NASBA, bDNA or RT-PCR with additional primers, but may fail to be detected by RT-PCR version 1. In the past it would have been prudent to test such a sample with bDNA or NASBA to ensure detection. However, the distribution of subtypes is now so widespread that the virus could be any of the known M subtypes. As it is no longer possible to predict from epidemiological evidence what HIV-1 subtype may have been transmitted, the best assay choice must be one that adequately detects all subtypes to some degree. Therefore, although NASBA and RT-PCR version 1.0 were proficient in detecting a number of subtype viruses, the failure to

detect certain subtypes cautions against the use of either assay to test a patient suspected of being infected with a non-B virus.

Current research using EIAs to detect the presence of non-B subtypes has so far only defined samples to be B or non-B subtype. As yet these techniques do not accurately differentiate between the other subtypes. If an EIA could detect the subtype of a virus, it would be possible to assess the ability of any given viral load assay to quantify the virus present. At present the only option for subtype classification is to sequence the virus. This is expensive and time consuming. Also, direct sequencing of *env* has a low success rate due to the variability of the region and consequent primer mismatches. However, it is possible to characterise subtype from *pol* sequences using BLAST analysis. This system uses a mathematical method to score the similarity of a DNA fragment to a reference sequence[203,204]. In this case, reference sequences from defined HIV-1 subtypes can be compared to *pol* sequences amplified from patient samples. Sequencing of this region is currently used to demonstrate the presence of any resistance-associated mutations. Therefore, it might be possible to use resistance testing to further investigate subtype diversity, without any additional test procedure. Alternatively every new patient could have a sample tested with all three currently available versions of each assay. From the three results it would be possible to see which gave the highest value. Whether this is the most accurate method is unclear, but it would be least likely to underestimate the viral burden. Future samples from that patient would be then tested with the chosen assay. This work implies that some assays are particularly suited to the testing of particular subtype, although this is very much a general rule and there will be anomalies. Therefore, the testing of each first sample using all three assays would choose the assay that is most efficient at quantifying that specific virus. However, on a financial basis, this procedure would cost almost the same as it would to sequence the sample.

Which plan is most cost effective by predicting which quantification assay best suits a patient, remains to be seen. Unfortunately, neither plan takes into account the possibility of a recombination event or evolutionary mutation producing a novel virus later on in disease. The possibility of such a situation is not unlikely, especially if different subtype quasispecies exist within a patient's viral population, either as a result of natural evolution or through superinfection. The Los Alamos database to date has 12 recognised recombinant strains and this number is increasing on a weekly basis. The prevalence of non-B and of recombinant subtypes up to October 2000 in this London clinic was 13 and 15% respectively, showing that the prevalence of recombinants is greater than the prevalence of parental subtypes. If a novel recombinant virus became the majority species, a viral load assay might be unable to detect it as accurately as the preceding virus, giving lower results and a false estimation of viral load.

If viral load quantification is to be relied upon as an aid to make complicated clinical decisions involving starting, continuing or changing therapy, it must be able to provide an accurate measure of viral status, throughout disease. The widening recognition of different genetic subtypes, and the problem this poses for any quantification method which relies on specific primer binding, must promote further research and development of all current viral load assays. This is necessary to maintain confidence in viral load as an accurate measure of HIV-1 status throughout disease.

3.6 Correlation between viral load quantified by NASBA and p24 antigen levels.

3.6.1 Introduction

Quantification of plasma HIV-1 RNA load has become one of the predominant methods, along with CD4 counts, used to monitor HIV-1 disease progression and antiretroviral drug efficacy. However, this is only the case in developed countries. As organisations such as The Joint United Nations Programme on HIV-1/AIDS (UNAIDS) endeavour to introduce antiretroviral therapy into countries previously denied this due to the prohibitive cost, the need to monitor HIV-1 therapy response in a cost-effective manner becomes a vital consideration. Many centres, even in the developed world, are unable to provide frequent regular viral load tests for each patient. However, if a quantitative p24 antigen (Ag) assay correlated well with viral load measurements and could detect rising viral load in an equivalent time frame, then this less expensive test could be substituted for certain patients or alternate with viral load monitoring.

The objective of this study was to evaluate the correlation between NASBA viral load measurements and p24 Ag levels in two different groups of patients with HIV-1 infection. The study selected samples from a cohort of patients with primary HIV-1 infection (PHI) and from patients with established infection. Patients with PHI have rapidly replicating virus and initial infection may be characterised by high viral load, and high p24 Ag. Alternatively, p24 Ag levels and circulating viral load may be lower in PHI compared with established infection, as the immune system is unimpaired and viraemia and antigenaemia are controlled by immune response. However, the immune response should control both factors, to the same degree, therefore p24 Ag should correlate with viral load in both scenarios. Patients with established infection are more

likely to have an impaired immune system and thus less control of viraemia and antigenaemia. Conversely, these patients are more likely to receive antiretroviral therapy, and this may be reflected in a lower viral load and low p24 Ag levels. Thus both groups may have the same or distinctly different profiles in terms of p24 Ag and HIV-1 RNA, but, if viral load correlates with p24 Ag then these two factors should still be linked, with high levels of one reflecting high values of the other. This study was designed to test the parameters of both assays and how well the values correlated.

3.6.2 Patients and samples

Patients were selected from either the Ian Charleson Day Centre cohort (ICDC, Royal Free & University College Medical School, London) or from a cohort selected by the Department of Pharmacology & Therapeutics, University of Liverpool. Those selected from the ICDC were taken from a seroconverter cohort during the first 4 months of infection. Patients from the Liverpool cohort had established HIV-1 infection. Therapy was not considered at selection. All plasma was separated within 6 hours of receiving the blood, and stored at -70°C. Samples from the University of Liverpool were shipped to this laboratory as frozen plasma.

3.6.3 Viral load and p24 Ag quantification

Viral load was quantified by NASBA or Nuclisens, depending on which version of NASBA was available at the time. Samples from patients with primary HIV-1 infection were tested from 1999 – 2000 with the Nuclisens assay (lower detection limit 2.6 $\log_{10}$ copies/ml). Samples from patients with established infection were first tested in 1997 with the NASBA assay (lower detection limit 3.3 $\log_{10}$ copies/ml) prior to the introduction of the Nuclisens. Any samples from the patients with established infection

falling below the lower level of detection using NASBA were re-tested using Nuclisens so that the lower level of detection was the same for all samples. All samples were then tested using the Murex HIV p24 MAb kit (Abbott Laboratories Ltd, Berkshire, UK) for p24 Ag quantification as described in section 2.5. The lower detection limit of the assay was < 5pg/ml whilst the lower limit of quantification 20 pg/ml. Samples showing a value above 5 pg/ml but below the lowest positive control, were determined to be detectable but at a concentration less than 20 pg/ml. The upper limit was determined by the highest positive control, in all assays this was determined to be at least 300 pg/ml, so the standard upper limit was set at 300 pg/ml. Samples that showed p24 antigen above this limit were diluted with negative human plasma to obtain a result.

3.6.4 Results

One hundred and nine samples from 43 patients with primary HIV-1 infection, and 100 samples from 17 patients with established infection, were tested for viral load and p24 Ag. Viral load values in patients with primary HIV-1 infection ranged from the lower limit of the Nuclisens assay, < 2.6 $\log_{10}$ copies/ml, to 7.15 $\log_{10}$ copies/ml (median 4.58 $\log_{10}$ copies/ml). The p24 Ag values for this group ranged from undetectable (< 5 pg/ml) to 1616.11 pg/ml (median of 39 quantifiable samples = 104.09 pg/ml).

Viral loads in established infection ranged from the Nuclisens lower limit < 2.6 $\log_{10}$ copies/ml to 6.48 $\log_{10}$ copies/ml (median 3.74 $\log_{10}$ copies/ml). The p24 Ag levels in this group ranged from undetectable (< 5 pg/ml) to above the upper detection limit 300 pg/ml (median of 33 quantifiable samples = 57.51 pg/ml). Unfortunately, two samples in this group that were above the upper p24 detection limit, did not have sufficient plasma to re-test.

Table 3-19 shows the number of samples tested, and the percentage of those detectable in both assays.

Table 3-19 Quantifiable p24 Ag and viral load in primary and established HIV-1 infection

	Primary HIV-1 infection		Established infection	
	p24 Ag	**Nuclisens**	**p24 Ag**	**NASBA**
Number of samples	109	109	100	100
% undetectable	23.9	8.3	58.0	35.0
% positive but < 20pg/ml	40.4	N.A*	9.0	N.A*
% quantifiable	35.8	91.7	33.0	65.0

* Not applicable

Only a third of samples (35.8 %) from patients with primary HIV-1 infection had quantifiable p24 Ag levels. But the number of samples with a detectable viral load by Nuclisens was 91.7 %, with the median viral load being 4.58 $\log_{10}$ copies/ml, almost 2 $\log_{10}$ above the Nuclisens lower limit of 2.6 $\log_{10}$ copies/ml. This demonstrates that in this group a significant number of samples with quantifiable viral load had undetectable levels of p24 Ag. Figure 3-8 shows the correlation between viral load and p24 Ag levels in patients undergoing seroconversion.

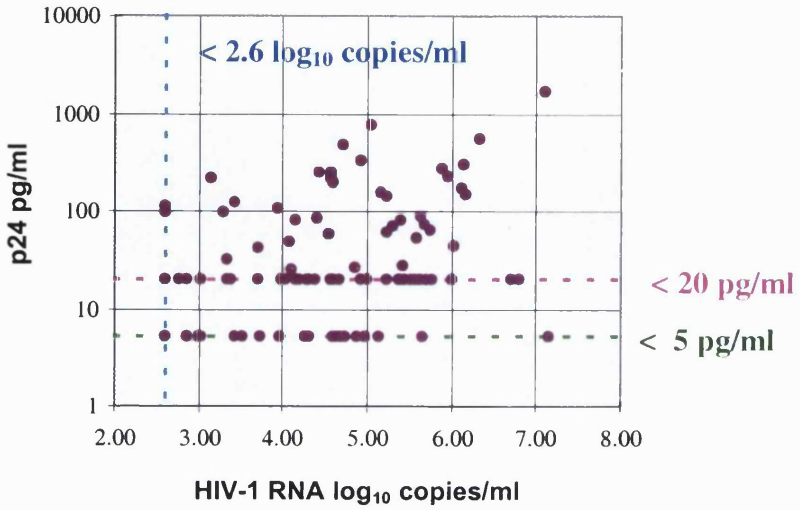


Figure 3-8 The correlation between viral load and p24 Ag levels in patients with primary HIV-1 infection.

The distribution of values is greatly affected by the number of samples in which p24 Ag is undetectable. The correlation coefficient for p24 Ag values and viral load values (not logarithmic) in primary HIV-1 infection is 0.50 (where 1 = complete correlation and 0 = no correlation), demonstrating that although values are not accurately matched, there is a distinct degree of relatedness.

If the values for viral load and p24 Ag are ranked and a Spearman's rank correlation coefficient calculated the value is lower at 0.28 (1 = complete correlation, 0 = none). This is because such a large proportion of the values are mismatched due to the lesser ability of the p24 Ag assay to generate a quantifiable value. If the data are selected so that only the values which are quantifiable in both assays are compared, the correlation of viral load and p24 Ag is much closer. The correlation coefficient for non-logarithmic viral load against p24 Ag is 0.86, demonstrating a much higher degree of relatedness.

If it is then assumed that p24 Ag shows good relatedness to viral load, when it is quantifiable, the issue is no longer matching high and low values, rather of matching detectability. It was possible to compare whether the quantification of viral load and p24 was achieved in the same sample, to distinguish whether both factors, neither or just one, was quantifiable. Table 3-20 shows the agreement between quantifiable p24 Ag and viral load, for the sample.

Table 3-20 Assessment of agreement between quantifiable viral load and quantifiable p24 Ag (> 20 pg/ml) in patients with primary HIV-1 infection.

		No. of patients with quantifiable p24 Ag	
		YES	**NO**
No. of patients with quantifiable viral load	**YES**	36	64
	NO	2	7

The frequencies with which the two values are quantifiable, and therefore agree, are shown along the diagonal.

The total observed agreement, (viruses undetectable in both assays + virus detectable in both assays) in this case is 43. If this is compared to the expected agreement (if the distribution was random) it is possible to calculate a value assessing agreement known as the Cohen's Kappa, κ. The κ judges agreement as poor if $\kappa \leq 0.2$; fair if $0.21 \leq \kappa \leq 0.4$; moderate if $0.41 \leq \kappa \leq 0.6$; substantial if $0.61 \leq \kappa \leq 0.80$ and good if $\kappa > 0.8$. In this case $\kappa = 0.03$ indicating poor agreement. Overall, patients undergoing

seroconversion show a poor relationship between the detection of viral load and p24 Ag, due to the high number of samples with undetectable p24 Ag.

In patients with established HIV-1 infection, like those with primary infection, the proportion of patients with a quantifiable p24 Ag was only a third of all analysed. Table 3-19 shows that 33 % of samples were quantifiable for p24 Ag as opposed to 65 % that were quantifiable for viral load. Compared to the primary infection patients, a greater proportion of in this group had undetectable viral load. In samples from patients with established infection 35 % viral load was undetectable as opposed to 8.3 % in patients with primary infection. This may have been because a higher proportion of patients with established infection were receiving therapy (100%), compared to those who had recently been diagnosed with HIV-1 infection (30%). The correlation between the non-logarithmic viral load and p24 Ag has a correlation coefficient of 0.33, showing a fair degree of relatedness. This is reflected in Figure 3-9, showing correlation between viral load and p24 Ag in patients with established infection.

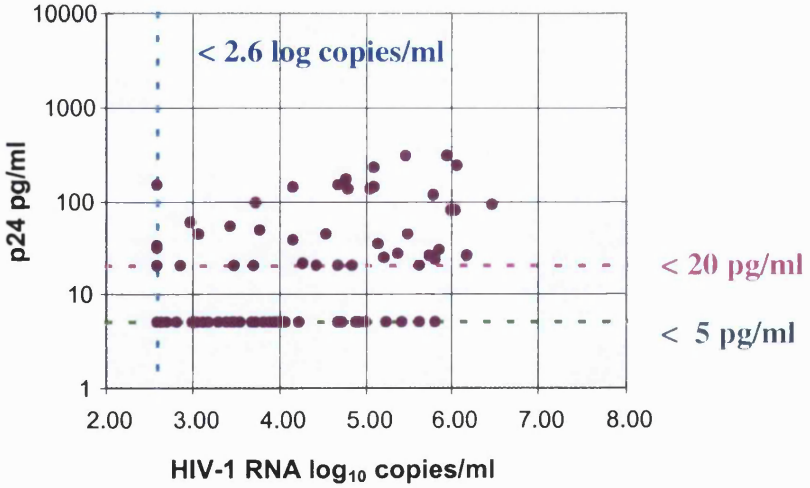


Figure 3-9 The correlation between viral load and p24 Ag levels in patients with established infection.

As before, in a large number of samples there is detectable viral load, but an undetectable or unquantifiable p24 Ag level. If the data are selected so that only the values which are quantifiable in both assays are compared, the correlation coefficient for non-logarithmic viral load against p24 Ag is 0.06, demonstrating a very low degree of relatedness. Agreement between the positive detection of p24 or viral load (not the values for either) shows a closer degree of relatedness between viral load and p24 Ag. Table 3-21 shows the observed number of samples with quantifiable virus in each assay, in patients with established HIV-1 infection.

Table 3-21 Assessment of agreement between quantifiable viral load and quantifiable p24 Ag (> 20 pg/ml) in patients with established infection.

		No. of patients with quantifiable p24 Ag	
		YES	NO
No. of patients with quantifiable viral load	YES	29	38
	NO	4	29

The frequencies with which the two value are quantifiable, and therefore agree, are shown along the diagonal.

In this case the Cohen's Kappa $\kappa = 0.23$ and shows fair agreement in quantification of viral load and p24 Ag. Compared with the primary infections, patients with established infection had a greater proportion of samples in which viral load was undetectable, possibly due to the increased use of highly active antiretroviral therapy (HAART). In samples where plasma HIV-1 RNA was undetectable, the majority also had

undetectable p24 Ag, demonstrating good agreement between the two parameters. However, the actual values produced by each assay did not reflect each other, indicated by the low correlation coefficient when analysing total data and quantifiable data.

3.6.5 Discussion

In a high number of samples from both groups p24 Ag was undetectable using the Murex p24 Ag assay. Whilst the number of samples with undetectable levels of viral load differed between the two groups, the number of samples with detectable p24 Ag did not, with a third of all samples tested in both groups having undetectable p24 Ag. This suggests that in this study, the Murex p24 Ag assay detected a more limited range than the viral load assay. This has been observed previously in other studies where the difference in detection ability has made it difficult to analyse any relationship between the two parameters[157].

This difference was more noticeable in the group of patients with primary infection. In a high number of samples from this group there was detectable viral load, and, on average, viral loads were higher in this group (median 4.58 $\log_{10}$ copies/ml) than in those with established infection (median 3.74 $\log_{10}$ copies/ml). Thus, when a third of samples had undetectable p24 Ag, the agreement between the two parameters was much lower in this group. However, when samples from seroconverters had detectable viral load and p24 Ag, the correlation between the values was good. This indicates that where detection was possible in the p24 Ag assay, the values given did reflect viral load.

In established infection, correlation between actual values (quantifiable values given in both assays) was poor. However, in the majority of samples in which viral load was undetectable p24 Ag was also undetectable, and those which had detectable p24 Ag also had detectable viral load, giving a fair agreement between the two assays.

This study shows that in some patients a p24 Ag value can reflect plasma viral load. This is illustrated more frequently in samples from patients undergoing seroconversion, for example the sample with the highest p24 Ag value of 1616 pg/ml, has the second highest viral load i.e. 7.11 $\log_{10}$ copies/ml. In cases where the correlation is good, a p24 Ag measurement would give an insight into plasma viral load i.e. changes in one would be reflected by changes in the other. However, correlation between the two measures was often poor. In a number of samples from both groups of patients, p24 Ag quantitative values did not reflect plasma viraemia. This is most apparent in the group of patients with established infection where there was very little correlation between values of p24 Ag and viral load. This difference in correlation between the two groups of patients may reflect their differing disease status.

The level of agreement between the number of positive values in both assays also differed with the stage of disease and possible treatment of the patients in the group. However, the lower ability of the p24 Ag assay to detect and quantify the antigen meant that the majority of samples in both groups had a value for viral load but not for p24 Ag. Thus, until p24 Ag detection is improved, the level of agreement can not be accurately assessed or related to disease stage.

It is recognised that specific neutralisation (by re-testing reactive samples using an anti-p24 antibody) in p24 Ag assays confers specificity of the test. However, the nature of the study meant that results were produced in accordance with protocol directions. Therefore, it was not possible to verify that reactive samples producing p24 Ag values were not influenced by non-specific binding.

A more sensitive version of the p24 Ag quantification assay was under development (but not available) at the time of this study. The new assay has a heat denaturation step to disassociate antibody-bound p24 Ag, raising the overall concentration of p24 Ag[205]. It gives a much greater sensitivity than previously possible[206]. Values for p24 antigen

as defined by the new assay were compared to viral load quantification for the same samples, and a Spearman rank correlation of 0.658 was observed, demonstrating a much improved correlation between HIV-1 RNA and p24 antigen[206]. If further studies endorse the measurement of p24 Ag as an indirect monitor of viral load, it may be possible to use p24 Ag measurement instead of viral load in certain circumstances. For example, where the detection of minor fluctuations in viraemia are not required i.e. in untreated patients, perhaps p24 measurement may be substituted for viral load such that standard of care could become monthly p24 Ag assessment and six monthly viral load measurement. This might be an attractive option for countries which are currently unable to afford regular viral load measurement.

3.7 Quantification of HIV-1 using an in-house assay.

3.7.1 Investigating the saturation point of antibody coating on latex microparticles.

3.7.1.1 Introduction

A previous protocol for the quantification of HIV-1 RNA employed an immunocapture step to extract HIV-1 from plasma, using antibody sera manufactured on site. This was no longer possible and supplies obtained from NIBSC AIDS reagent Project were limited. It became necessary to test the saturation point of the latex microparticles to see if a less concentrated antibody coating solution could be used to maximise antibody resources.

3.7.1.2 Methods

The antibodies and the concentrations of purified stocks are detailed in section 2.3.1. The previous protocol used an antibody coating solution of 80µg/ml, and this study tested three different antibody concentrations of 20µg/ml, 40µg/ml and 80µg/ml to coat three sets of latex microparticles. An additional three sets of fresh latex microparticles would then be coated in the same three antibody solutions, and a further three sets of fresh microparticles were coated once again in the three antibody solutions. The amount of bound antibody was measured using a ELISA technique incorporating anti-sheep antibodies conjugated to horse radish peroxidase. The addition of tetramethylbenzidine (TMB) would generate a colour change that could be used to quantify the amount of bound antibody from the coating solutions. If there was any

antibody bound to the second or third sets of microparticles (where the coating solution had already been used at least once) it would imply that the antibody concentration was in excess, and that the latex microparticles were saturated at that concentration. A full description of the method is shown in section 2.3.2.

3.7.1.3 Results

The absorbence of light at 450nm of all samples of the microtitre plate are shown in the table below. Where a well gave a value of >3.0 the absorbency was too high for the reader to measure. Higher values indicate that a large amount of antibody was bound to the latex, this was indirectly measured by anti-sheep antibody conjugated to horse radish peroxidase.

Table 3-22 Absorbency of light reflects the amount of anti-HIV antibody bound to latex particles after coating in three different antibody concentrations, three times over.

Latex dilution	Blocked latex microparticles			20µg/ml coating solution			40µg/ml coating solution			80µg/ml coating solution		
				X 1	X 2	X 3	X 1	X 2	X 3	X 1	X 2	X 3
1:10	0.803	0.396	0.649	>3.0	2.421	2.011	>3.0	>3.0	2.638	>3.0	>3.0	>3.0
$1:10^2$	0.144	0.687	0.839	>3.0	2.636	0.718	>3.0	>3.0	1.032	>3.0	>3.0	>3.0
$1:10^3$	0.216	0.338	0.319	>3.0	0.616	0.354	>3.0	2.764	0.472	>3.0	>3.0	>3.0
$1:10^4$	0.376	0.287	0.252	2.092	0.459	0.259	1.833	1.671	0.203	1.867	2.544	2.406
$1:10^5$	0.156	0.139	0.123	2.838	0.136	0.125	1.914	0.498	0.137	1.211	1.965	2.073
$1:10^6$	0.474	0.136	0.286	1.908	0.195	0.283	0.736	0.430	0.420	0.582	0.886	0.860
$1:10^7$	0.392	0.263	0.203	0.884	0.137	0.173	0.215	0.152	0.126	0.249	0.306	0.111
$1:10^8$	0.513	0.813	0.319	0.439	0.406	0.315	0.651	0.284	0.347	0.631	0.685	0.476

The first coating of antibody onto the latex gave very high values for all antibody concentrations down to a $1:10^3$ dilution of latex. The second set of latex microparticles to be coated in the same solutions also shows high values for antibody binding. However, only the 20μg/ml concentration gives actual values for the second coating, the results for antibody concentrations at 40μg/ml and 80μg/ml are still too high implying a very high level of antibody binding. Therefore at concentrations of 40μg/ml and 80μg/ml there is enough antibody left over after one coating of microparticles, to initiate a fresh coating.

It would appear that an antibody coating solution of 20μg/ml produces a high level of antibody binding and yet is still in excess as demonstrated by the second coating of a fresh set of latex microparticles. However, there is not enough excess antibody in the second coating to produce the same level of binding as seen in the first coating. From this it is possible to deduce that an antibody concentration of 20μg/ml is sufficient to produce good binding of antibody to the latex, and is economic of valuable antibody resources. Antibody concentrations above 20μg/ml can saturate the latex particles at least twice over, and are therefore at too great a concentration.

3.7.1.4 Discussion

This experiment determined two issues; firstly, that all the antibody solutions used were effective in coating the latex particles, and secondly that an antibody solution of 20μg/ml was sufficient to produce a good level of antibody binding without being too greatly in excess as to waste antibody resources. However, in coating the latex microparticles it was noticed that a discernible amount of latex was attaching to the side of the tube and this was not removed by centrifugation. Also, the latex microparticles

were difficult to handle using a pipette because of the attachment of the latex to the plastic of the pipette tip. This highlighted that latex microparticles may not be the best medium to use for HIV-1 immunocapture. Inconsistencies in pipetting or coating may have an effect on HIV-1 extraction. As the object of the protocol is to quantify HIV-1, a variable extraction method would jeopardise the validity of any results produced. Overall, considerable expertise was required in handling and manipulating latex microparticles which was regarded as a disadvantage.

3.7.2 Using a one step method to reverse transcribe and PCR amplify HIV-1 RNA.

3.7.2.1 Introduction

It was demonstrated in a previous protocol[156] that HIV-1 could be reverse transcribed and the cDNA produced in this reaction could then be added into a nested PCR. It was proposed to reduce this to a one step procedure incorporating reverse transcription and DNA amplification in one tube. This would save time and hopefully be more cost effective with regards to reagents. This study tests a panel of samples of known RNA concentration using a one step method. If the method produced cDNA proportional to the amount of RNA in the original sample, then the method could be used not only for routine amplification of HIV-1 RNA, but also provide a basis for an in-house viral quantification system.

3.7.2.2 Methods

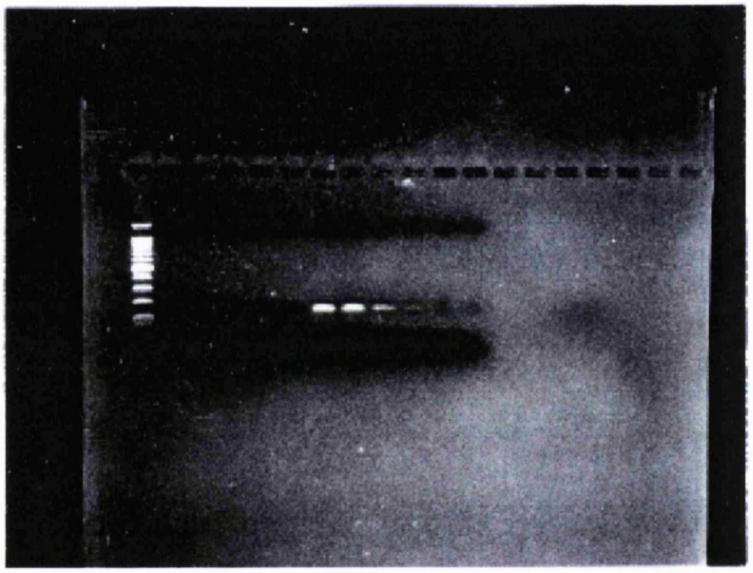
The original two step method used to reverse transcribe HIV-1 RNA and then amplify the cDNA is described in section 2.3.3 Briefly, extracted RNA was added into a reverse

transcription mix and incubated at 37°C for 90 minutes. The cDNA produced was then added into a nested PCR. The new method described here combined the two reactions in one tube. Extracted RNA was added into mix containing enzymes reverse transcriptase and DNA polymerase. The volumes and concentrations are shown in section 2.4. The DNA produced was visualised on a 1 % agarose gel. In this experiment both methods used primers 5' Prot1 and 3'Prot1 for the first round, and 5' Prot2 and 3' Prot2 in the second round so the any bands produced were 514bp. The strength of the DNA bands was regarded as proportional to the amount of DNA produced and therefore also proportional to the RNA in the original extract. The RNA extracts chosen were part of a panel produced by the NIBSC AIDS reagent project. The panel samples chosen contained 1000 copies/µl, 100 copies/µl, 10 copies/µl, 1 copy/µl and 0.1 copies/µl. The samples were run once using the previous method, and once using the new method. A 10µl input was used for all samples and one negative control of dH_20 was run making a total of 11 samples. Results of both methods were run on the same agarose gel and visualised with the aid of a DNA ladder. This experiment was repeated 3 times using the same RNA samples.

3.7.2.3 Results

The agarose gel pictures for the first of the three experiments is shown in figure 3-10. Lane 1 holds the DNA ladder, this demonstrates that all the bands seen are of the right order of magnitude for the expected size of product (514bp). Lanes 1 –5 were the PCR products from the two step method, while lanes 6-10 show PCR products from the one step method. Lane 11 is a negative control. Lanes 1 –5 show no visible bands whilst lanes 6-10 show four bands, the clarity and strength of the bands increases with increasing RNA input.

igure 3-10 Visualisation of DNA bands produced by two different methods run on a 1 % garose gel. Lanes 1-5 show DNA produced by a two step RT-PCR method, the RNA input in 0µl contained 10,000 copies, 1000 copies, 100 copies, 10 copy/µl and 1 copy respectively for anes 1-5. Lanes 6-10 show DNA produced by a one step RT-PCR method, the RNA input in 0µl contained 10,000 copies, 1000 copies, 100 copies, 10 copy/µl and 1 copy respectively for anes 6-10. Lane 11 was a negative PCR control.



The results of the first and subsequent experiments where both methods were repeated again twice are shown in table 3-23. The strength of the DNA bands is noted as either weak (w) or strong (s).

Table 3-23 PCR products produced from an RNA panel amplified by either a one step or a two step RT and PCR method. Strength of bands is denoted as either weak (w) or strong (s).

		DNA Band produced RNA copy input (in 10µl input)				
Method	Repeat	10,000	1,000	100	10	1
One step	1	0	0	0	0	0
	2	+ (s)	+ (s)	+ (s)	+ (w)	0
	3	+ (s)	+ (s)	+ (w)	+ (w)	0
Two step	1	+ (s)	+ (s)	+ (s)	+ (w)	0
	2	+ (s)	+ (s)	+ (w)	+ (w)	0
	3	+ (s)	+ (s)	+ (w)	+ (w)	0

It is possible to see from table 3-23 that after the results of the first experiment, the number of bands and the strength of the bands produced by both methods does not differ. The sample with a 1 RNA copy input and the negative control never gave a positive result in either method.

3.7.2.4 Discussion

The method employing two separate steps did not work on the first occasion. There was no obvious explanation of why this should be so, the method had subsequently

produced positive PCR results. The experiment is most likely to have failed during the reverse transcription step as no DNA was produced from any sample, yet the similar PCR in the alternative method and subsequent experiments with this method (using the same reagents) did work.

It would appear that a one step method for reverse transcription and PCR worked as well as the original one step method to produce detectable DNA down to an RNA input (in 10µl) of 10 copies/µl. This method was subsequently further validated in comparative studies conducted by the department (but not detailed here) and consequently replaced the existing two step method for routine reverse transcription and PCR amplification of HIV-1.

The strength of the DNA bands produced by the one step method appeared to be proportional to the RNA input implying that this method could be used for quantifying RNA.

3.7.3 Immunocapture of HIV-1 to latex microparticles

3.7.3.1 Introduction

An existing protocol for the capture of HIV-1 virions from plasma using anti-HIV antibodies bound to latex microparticles was modified to allow the coating of latex microparticles in a less concentrated antibody mix (see section 3.7.1). It was proposed that some fresh latex microparticles be coated in the new antibody solution. This latex could then be substituted into the previous protocol for extraction of HIV-1. Any captured HIV-1 could then be lysed and the free RNA could be first reverse transcribed and then amplified in a nested PCR. Any products visualised on an agarose gel would be a direct result of HIV-1 captured by the new antibody coating solution.

3.7.3.2 Method

The HIV-1 antibodies used in the immunocapture are described in section 2.3.1. Fresh latex microparticles were coated in the new antibody coating determined in section 3.7.1 according to the method detailed in section 2.3.2. It was decided to use a patient sample with a viral load that has previously been quantified by the RT-PCR giving a value of > 4 $\log_{10}$ copies/ml. A sample was chosen that had a viral load of 4.8 $\log_{10}$ copies/ml. One hundred µl of plasma was aliquoted into two tubes and from then on treated as two separate samples. Twenty µl of coated latex microparticles was added to the plasma and the remainder of the protocol followed that described in section 2.3.1. A positive RNA control (NIBSC Reagent Project) containing 10,000 copies/10µl input was added into the RT-PCR . The PCR products from all three samples and one negative control were then run on an 1 % agarose gel. The experiment was repeated three times.

3.7.3.3 Results

The number and strength of the DNA bands visualised on an agarose gel are detailed in table 3-24. The strength of the bands is denoted as either weak (w) or strong (s).

Table 3-24 The production and strength of DNA bands produced from HIV-RNA after immunocapture.

DNA Band produced after immunocapture and lysis/RT and PCR				
	Sample 1	Sample 2 (duplicate)	Positive Control	Negative Control
Experiment 1	0	+ (w)	+ (w)	0
Experiment 2	0	0	0	0
Experiment 3	0	0	+ (s)	0

The positive control gave a positive band two out of three times. Reverse transcription and amplification failed for all samples in the second experiment. There was DNA produced in sample 2 (a duplicate of sample 1) in the first experiment implying that virus had been captured by the antibodies bound to the latex, this virus had been subsequently lysed and reverse transcribed before being amplified by PCR. The negative control did not show a DNA band in any of the three experiments.

3.7.3.4 Discussion

This method worked on one of three occasions to capture HIV and subsequently reverse transcribe and PCR the RNA to a detectable amount. However, in two of the experiments no HIV RNA was detected. This may have been due to low levels of virus captured onto the latex-antibody complex due to poor avidity or binding stability Alternatively, and less likely, technical problems relating to RNA stability during manipulation steps, and lysis/RT degradation were considered.

Overall, the manipulation of the latex was found to be technically difficult in this and other experiments, with smears of latex attached to the side of the reaction tubes and to

the plastic of the pipette tips. The samples containing latex had to be pipetted very slowly and this difficulty may have contributed to the low success rate of the study.

3.7.4 Detection of biotinylated DNA using a probe conjugated to alkaline phosphatase.

3.7.4.1 Introduction

A previous in-house HIV-1 quantification method had used radioactivity to measure the amount of DNA produced by PCR. It was proposed to modify this system to enable the detection of PCR products without using radioactivity. One method for this was by using a DNA probe conjugated to alkaline phosphatase. The amount of DNA present could then be quantified by measuring the luminescence produced by the addition of the substrate to alkaline phosphatase.

3.7.4.2 Method

The experiment required DNA of known positivity. For this DNA samples were chosen that had previously produced positive results when run previously on an agarose gel. The first round product of one such DNA sample was then added in into a five sample biotinylated second round. The PCR mix and primers for producing biotinylated DNA are described in section 2.3.3.2. Ten µl of biotinylated DNA product was added to 90µl of hybridising buffer in the first column of a blocked microtitre plate, and then in 1:10 dilutions across the plate. The antisense strand was removed prior to the addition of a 1:100 ($1:10^2$) solution of probe LOPb (conjugated to alkaline phosphatase) in hybridising buffer, dilutions of probe were 1:10 down the plate. No probe was added to the last column and no DNA was added to the last row. The substrate to alkaline phosphatase, CDP-Star (NEN™, Life Science Products), was added and the resulting

luminescence was measured using a microplate scintillation counter (Topcount™). More detail of this method is shown in section 2.3.4.

3.7.4.3 Results

Luminescence counts as measured by the Topcount™ are shown in table 3-25.

Table 3-25 Luminescence counts produced by various dilutions of probe LOPb in response to dilutions of PCR product.

		Dilutions of PCR Product											
		1:10	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$	$1:10^7$	$1:10^8$	$1:10^9$	$1:10^{10}$	$1:10^{11}$	0
Dilutions of Probe LOPb	$1:10^2$	115747	138353	74863	16536	6116	4293	2710	3540	5827	4560	5022	4535
	$1:10^3$	52146	66472	23094	7153	2166	920	886	715	670	620	651	645
	$1:10^4$	7607	8034	3820	1393	633	400	380	360	366	303	316	290
	$1:10^5$	1980	1820	900	573	446	360	316	329	310	310	265	295
	$1:10^6$	1146	660	480	520	473	366	335	310	329	880	258	265
	$1:10^7$	653	633	573	473	466	473	354	322	316	316	276	271
	$1:10^8$	640	600	566	560	446	606	322	341	310	290	271	253
	0	466	453	420	460	506	393	322	297	303	297	268	289

The least diluted solution of probe LOPb gave the highest luminescence readings. The dilution of PCR product that gave the highest reading is actually the $1:10^2$ (1:100) not the least dilute (1:10). However, remaining dilutions of PCR product decrease in luminescence counts as the dilution increases. The proportionality of the decrease in luminescence compared to the increase in dilution factor for both the probe and PCR products are shown in figure 3-11.

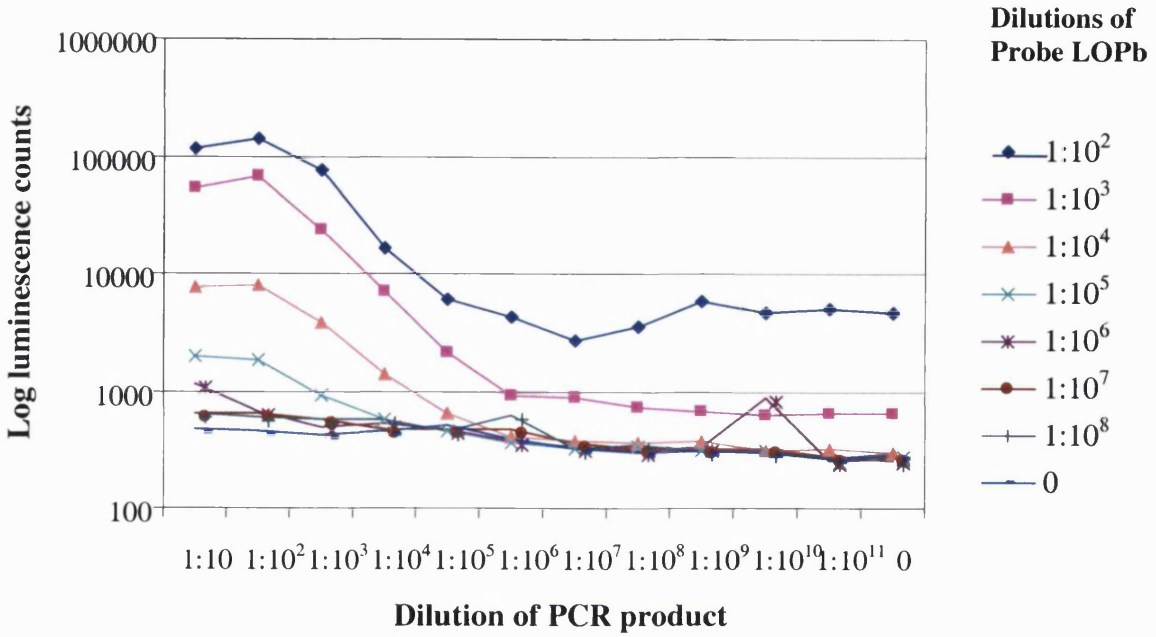


Figure 3-11 Log luminescence counts for various dilutions of biotinylated DNA PCR product and probe LOPb.

It is possible to see from figure 3-11 that a probe dilution of $1:10^3$ (1:1000) appears to give the best detection of the most dilutions of PCR products. This concentration of probe LOPb detects five dilutions of PCR products, down to a $1:10^5$ dilution, before the luminescence counts are the same as that for background (no PCR product). By comparison, at a less dilute probe mix of $1:10^2$ (1:100), the background is significantly higher and it is not possible to distinguish between background values (those the same as for no PCR products) and DNA more dilute than $1:10^4$.

All the wells in column 2 at a PCR product dilution of $1:10^2$ produce approximately the same luminescence as wells with PCR products diluted 1:10. This implies that the system is saturated at this level of DNA.

These results imply that the best dilution of probe LOPb would be $1:10^3$, whereas PCR products would be best detected at a dilution of $1:10^2$.

3.7.4.4 Discussion

The results of this experiment show that it was possible to detect biotinylated DNA using a conjugated probe. The results show that there is a difference between background luminescence counts and those containing PCR products. The level of luminescence detected decreases for increasing dilution of PCR product, implying that luminescence is proportional to the amount of DNA. However, at dilutions of 1:10 and $1:10^2$ for the PCR products, the luminescence counts are very similar, implying that at 1:10 the system is saturated.

Repeat runs of these studies showed variability in the production of luminescence, the background was much higher in subsequent trials and the results of this first experiment could not be repeated. This level of variability could be due to inter-assay differences. However, frequently it was not possible to distinguish low levels of DNA from high levels of background luminescence. Also the high background prevented the calculation of a standard curve of luminescence versus DNA input as the background luminescence was not the same for every sample.

Chapter 4 Applications of HIV-1 viral load quantification by NASBA.

Applications of viral load quantification

Since its inception, viral load quantification has had a prominent role in HIV-1 disease clinical management, and new applications are frequently proposed. HIV-1 RNA levels have been used as a predictor of disease progression [67,207,208] and of non-progression[209]. Levels of circulating virus have been used to monitor therapeutic success and have been said to be predictive of therapeutic failure[210-213]. HIV-1 RNA has been shown to correlate inversely with competency of immune system and some have hypothesised that it can be predictive of immunological decline[214]. Recently, HIV-1 RNA was used to diagnose HIV-1 status prior to the immunological development of antibody response in infants born to infected mothers[58,171].

The following studies looked at the current applications of HIV-1 RNA quantification and assess the value of viral load measurement in the current clinical setting. Drug efficacy as measured by viral load was related to intracellular pharmacokinetics, to determine if viral load can accurately represent therapeutic success. Viral dynamics in primary HIV-1 infection were monitored and compared to traditional markers of acute infection to evaluate the use of viral load measurement in this distinct group.

4.1 Virological monitoring of intracellular antiretroviral drug metabolism

4.1.1 Introduction

Viral replication has been shown to be greatly reduced by effective antiretroviral therapy. The Delta trial of 1995[164] and ACTG 175[215] both demonstrated the improved efficacy of combination therapy in reducing viral replication. Current British HIV Association (BHIVA) therapeutic guidelines[136] suggest treatment with two nucleoside analogue reverse transcriptase inhibitors (NRTIs) plus either a protease inhibitor (PI) or a non-nucleoside analogue reverse transcriptase inhibitor (NNRTI).

NRTIs such as zidovudine (AZT) require intracellular phosphorylation to the active triphosphate form before being able to inhibit HIV-1 replication. Although dual therapy shows improved efficacy compared to monotherapy[164,215], recent studies have shown that NRTIs which require the same intracellular kinase compete for phosphorylation, resulting in lower concentrations of the active forms of both drugs[216]. In particular, *in vitro* studies have shown that AZT has a greater affinity than d4T for thymidine kinase, resulting in the preferential phosphorylation of AZT over d4T[217]. This was demonstrated *in vivo* when patients receiving both drugs showed less virological response than those receiving d4T alone[218]. Data from the ALTIPHAR study (pharmacological follow-up of the ALTIS 1 and 2 trials of d4T/3TC in patients treatment naïve or experienced) demonstrated decreased active phosphates of d4T and 3TC in patients who had previously taken AZT, as opposed to those who had no record of treatment[216].

The aim of this study was to determine whether plasma HIV-1 RNA load could reflect intracellular active drug concentrations. The study was initiated in 1995 when typical

anti-HIV drug treatment involved dual NRTI therapy, prior to the introduction of PIs or NNRTIs. Therefore, viral load and intracellular phosphorylation were measured in patients starting antiretroviral therapy with AZT and another NRTI. A correlation between viral load and intracellular levels of AZT would link drug metabolism and virological failure. As mentioned in section 1.6.4, the persistence of detectable viral load throughout therapy has been highlighted as correlating with the evolution of resistance[212,219,220]. The effect of previous antiretroviral therapy was also studied, categorising patients as NRTI-naïve or NRTI-experienced to see if a link between virological failure due to decreased phosphate levels and AZT experience and could be established.

4.1.2 Patients and Methods

The study was undertaken between 1995 and 1997. HIV-1 positive patients who wished to start antiretroviral therapy with a dual nucleoside combination were selected by the Department of Pharmacology & Therapeutics, University of Liverpool from those attending the Liverpool Royal Infirmary. Twenty-three patients were enrolled on the study, 21 male and two females, whose ages ranged between 29-58 years (median 38 years). All patients had acquired HIV-1 through sexual transmission, and were not taking any medication known to interfere with AZT metabolism. Hepatic and renal functions in each patient were shown to be normal. Each participant provided written informed consent. Of the 23 patients, 11 had received AZT prior to commencing the trial. Of these 11, the median length of AZT treatment was 12 months (range 2-68 months). Twelve patients were antiretroviral-naive. At the start of the study, one patient (patient H, AZT-experienced) had already commenced therapy, all other patients commenced on day 1. Patients were given a dual nucleoside combination of either AZT and ddI (n = 17), or AZT and 3TC (n = 6).

Patients were subdivided into therapy-naïve and therapy-experienced groups, depending on prior experience of AZT. Of the 23 patients who started the trial, 17 patients were followed for 12 weeks or more. Nine patients continued to 12 months of follow-up. The median follow-up time was 28 weeks (range 4 – 57 weeks). The major reason for leaving the study was a change in therapy due to an adverse clinical event or disease progression. Three patients withdrew due to intolerance of side-effects or from personal choice. One patient (patient L, AZT-experienced) could not be included in the study because his HIV-1 RNA remained at undetectable levels as measured by NASBA throughout the study period. This subtype was ascertained using an in-house serotyping method and was found to be a non-subtype B virus. The viral load in Patient L was subsequently quantified using RT-PCR with non-B primers. This gave results ranging from 4.82 – 5.08 $\log_{10}$ copies/ml.

Patients were monitored at baseline, 2 weeks and 1, 2, 3, 6 and 12 months. Blood was taken at each time point following the supervised ingestion of 300mg AZT. The plasma was quantified for HIV-1 RNA with NASBA. Samples which were below the detection limit were re-tested with Nuclisens. This had a lower detection limit of 2.6 $\log_{10}$ copies/ml. AZT concentration and AZT metabolites were measured by the Department of Pharmacology & Therapeutics, University of Liverpool. AZT concentration in the plasma was measured at 0, 1 and 2 hours (Radioassay; RIA; Sigma, Poole, UK). Peripheral blood monocytes (PBMCs) were isolated and AZT and AZT metabolites were extracted and analysed by HPLC[221]. Plasma was stored at -70°C until analysis.

4.1.3 Results

Virological Efficacy

The median baseline viral load for all 22 included patients was 5.64 $\log_{10}$ copies/ml (range undetectable, < 2.6 $\log_{10}$ copies/ml, to 6.61 $\log_{10}$ copies/ml). Twenty patients showed a decline in viral load from start of dual NRTI therapy to the second sample taken, whilst two patients showed a slight increase, both had prior experience of AZT. Of these 20 patients, 15 demonstrated a viral load decrease between baseline and second sample of > 0.5 $\log_{10}$ copies/ml.

All patients were categorised as AZT-naïve n = 12, or AZT-experienced n = 10. All 12 of the AZT-naïve patients showed a > 0.5 $\log_{10}$ copy drop in viral load between baseline and second sample whilst only three out of 10 AZT-experienced patients showed a similar drop in viral load.

The mean values for viral load for each time point at median weeks are shown in Table 4–1.

Table 4-1 Mean HIV-1 RNA for median time points in patients taking dual NRTI antiretroviral therapy.

AZT-experienced			AZT-naïve		
Weeks	**HIV-1 RNA log_{10} copies/ml**	**n**	**Weeks**	**HIV-1 RNA log_{10} copies/ml**	**n**
0	5.21	10	0	5.69	12
2	4.71	9	2	3.58	11
5	4.99	10	4	3.37	11
9	4.72	8	9	3.62	10
13	5.19	6	13	3.65	9
27	4.71	5	26	4.34	8
39	4.54	4	41	4.39	6
53	4.92	4	52	3.82	5

n = Number of patients. Data is shown according to an on-treatment analysis which only includes patients actively taking part in the trial at each time point.

These results are shown graphically in Figure 4-1.

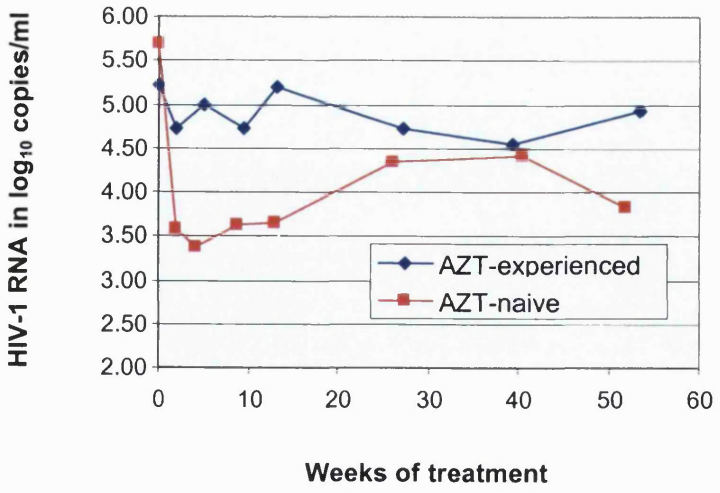


Figure 4-1 Mean plasma HIV-1 RNA in AZT-naïve and AZT-experienced patients, taking a dual NRTI regimen containing either AZT and ddI or AZT and 3TC.

Figure 4-1 shows a reduction (mean 2.07 $\log_{10}$ copies/ml) in viral load in patients with no AZT experience between baseline and 10 weeks. However, this reduction is not sustained and viral load rises to 4.34 $\log_{10}$ copies/ml at week 26 (1.34 $\log_{10}$ copies/ml difference from baseline) and remains around 4 $\log_{10}$ copies/ml for the remainder of the study. Patients with prior AZT experience show very little virological response to the therapy. Although viral load does decline in some patients, most in this group remain at baseline levels. Viral load seems to decline over weeks 15-40, but, by the end of the study, mean viral load had declined from baseline by only 0.29 $\log_{10}$ copies/ml.

A comparison of efficacy between the two groups can also be viewed by assessing the number of patients achieving an undetectable viral load (< 2.60 $\log_{10}$ copies/ml) for any mean time point. Patients who had no prior experience of AZT achieved an undetectable viral load more often then patients who had previously taken AZT. This is demonstrated in Table 4-2. The Table indicates that, initially, therapeutic efficacy in reducing viral load was greatly influenced by prior experience of AZT. However, at

later time points the number of patients achieving undetectable levels of virus declines in the therapy-naïve group, making the difference in virological efficacy less apparent. Data are shown according to an on-treatment analysis.

Table 4-2 Number of patients achieving undetectable viral load whilst on dual NRTI therapy.

Mean weeks of therapy	AZT-experienced patients		AZT-naïve patients	
	Undetectable viral load	n	Undetectable viral load	n
0	0	10	0	12
2	2	9	3	11
5	0	10	5	11
9	1	8	5	10
13	0	6	4	9
27	0	5	1	8
40	1	4	0	6
53	0	4	1	5

The significance of AZT experience was calculated by assessing viral load change over time, and comparing mean differences between the two groups in an unpaired t-test. Viral load change at 2 weeks, 3 months and 12 months for both groups is shown in Table 4-3.

Table 4-3 Viral load change from baseline in AZT-naïve and AZT-experienced patients taking dual NRTI therapy.

	Viral load change in log_{10} copies/ml from baseline		
	2 weeks	**3 months**	**12 months**
AZT-naïve (n = 12)	-2.14 ± 0.81 (n= 11)	-2.08 ± 1.05 (n = 10)	-1.78 ± 1.43 (n = 5)
AZT-experienced (n = 10)	-0.61 ± 0.83 (n = 9)	-0.60 ± 0.67 (n = 6)	-0.68 ± 0.47 (n = 4)
p value from unpaired t-test	0.0006	0.008	0.188

Values are mean values ± standard deviation. p denotes a significant difference where $p < 0.05$.

Table 4-3 illustrates that although AZT-naïve patients have a much greater virological response to therapy than the AZT-experienced patients, this is not sustained to 12 months. The mean log_{10} viral load decrease in therapy-naïve patients at 12 months is 1.78 log_{10} copies/ml, still larger than that shown in experienced patients, but the standard deviation between the samples is also larger. This demonstrates a greater variation between the samples of naïve patients at 12 months, indicating a significant log_{10} decrease in viral load from baseline in some patients, and virtually no decrease in others. The p values show significant differences in virological efficacy between the two groups at 2 weeks and 3 months. At 12 months the difference between the two groups is no longer significant.

Pharmacology

There was no significant decrease in total intracellular AZT phosphate over the twelve months of the study in either group. Levels of total phosphates remained comparable in both groups to 12 months of therapy, demonstrating that patient withdrawal did not select out a population with different phosphorylation metabolism. There was no change in the mean intracellular AZT diphosphate (DP) or triphosphate (TP) metabolite during the course of the study. In both groups of patients mean AZT monophosphate (MP) rose to above 3 pmol/10^6 cells after 12 months of treatment. However, the standard deviation within both groups was high and the small number of samples available after 12 months reduces the likelihood of this marking a trend in increasing AZT MP.

In all patients, levels of AZT MP were higher than DP or TP metabolites. This is because the phosphorylation of AZT MP by thymidine kinase takes longer than the subsequent phosphorylation of DP or TP, making the initial phosphorylation of AZT MP the rate-limiting step of the entire reaction. Thus, levels of AZT MP build up over time in comparison to DP or TP levels. Table 4-4 demonstrates mean levels of AZT phosphate metabolites over time.

Table 4-4 Comparison of mean AZT metabolites in AZT-experienced and AZT-naïve patients at three time points.

Concentration in pmol/10^6cells	AZT-experienced	AZT-naive	p value
AZT (total) Plasma			
0 months	3.74 ± 2.66 (n = 11)	4.70 ± 3.68 (n = 10)	0.654
3 months	4.55 ± 0.87 (n = 7)	5.59 ± 3.24 (n = 10)	0.807
12 months	4.21 ±1.52 (n = 4)	4.55 ± 2.38 (n = 5)	0.730
AZT MP			
0 months	1.93 ± 2.00 (n = 11)	2.52 ± 2.27 (n = 9)	0.656
3 months	1.95 ± 2.12 (n = 7)	1.77 ± 0.93 (n = 10)	0.601
12 months	3.01 ± 2.30 (n = 4)	3.08 ± 1.65 (n = 4)	0.886
AZT DP			
0 months	0.27 ± 0.17 (n = 11)	0.27 ± 0.14 (n = 9)	0.897
3 months	0.24 ± 0.14 (n = 7)	0.25 ± 0.13 (n = 10)	0.759
12 months	0.22 ± 0.12 (n = 4)	0.31 ± 0.09 (n = 4)	0.286
AZT TP			
0 months	0.11 ± 0.08 (n = 11)	0.09 ± 0.06 (n = 9)	0.752
3 months	0.13 ± 0.11 (n = 7)	0.12 ± 0.08 (n = 10)	0.792
12 months	0.13 ± 0.12 (n = 4)	0.14 ± 0.08 (n = 4)	0.543

Differences between AZT-experienced patients and AZT-naïve patients were analysed by Mann-Whitney U test.

The Table indicates that AZT intracellular phosphates were variable from patient to patient. Large standard deviations for total phosphate and MP levels were seen for both groups of patients, e.g. mean baseline total phosphate levels for therapy-naïve patients were 4.70 pmol/10^6 cells ± 3.68, indicating a wide variation in levels between patients. Diphosphate and TP levels were much lower, but in some cases the standard deviation equals the mean value, e.g. triphosphate levels in AZT-experienced patients at 12 months were 0.13 pmol/10^6 cells ± 0.12.

Overall, there was no apparent difference in any AZT metabolite from baseline values in either AZT-experienced or AZT-naïve patients.

4.1.4 Discussion

The study demonstrated differences in virological response to therapy between the two groups of patients. Viraemia in therapy-naïve patients declined much more steeply upon initiation of AZT-containing dual therapy, than in those who had previous experience of AZT. However, this difference was shown to be transient, with no significant difference in viral load seen in those who continued to 12 months (n = 9). This contrast in virological response to therapeutic intervention has been demonstrated before. Data from the ALTIS trial[222] (a pilot open study of d4T/3TC in antiretroviral naïve and experienced patients) demonstrated that therapy-naïve patients starting d4T/3TC showed a 1.66 $\log_{10}$ copy drop in viral load compared to AZT-experienced patients who showed a 0.55 $\log_{10}$ drop on starting the same combination. Prior exposure to AZT had reduced the potency of the new regimen, even though neither group had received d4T or 3TC before. In another study by Gomez-Cano *et al* looking at patients treated with AZT and ddI, only those who were treatment naïve showed a reduction in viral load, and this was transient before returning to baseline levels[223]. Research into the pharmacokinetics of d4T and 3TC by Sommadossi *et al*[216] indicated that prior AZT treatment hampered the phosphorylation of d4T, and to a lesser extent, of 3TC. In agreement with both of these studies, the results of this study show a significant difference in virological response to initiation of therapy, between AZT-experienced and AZT-naïve patients. However, the pharmacokinetic monitoring did not connect the difference in virological response to intracellular phosphate levels. Total AZT metabolites and individual phosphates maintained a steady state throughout the study in both groups of patients. Although individual patients varied substantially, there

was no significant difference in AZT phosphorylation between the groups of AZT-naïve and AZT-experienced patients. Therefore, the virological failure seen in AZT-experienced patients, and later at 12 months, in the AZT-naïve patients, cannot be attributed to intracellular AZT phosphate levels. Published studies have demonstrated that the phosphorylation of AZT to the active triphosphate by thymidine kinase is the rate-limiting step in the inhibition of HIV-1 by AZT[224]. This study did show reduced levels of triphosphates in comparison to AZT monophosphates or total phosphates. However, the levels of triphosphate were reduced in both groups of patients and did not correlate with levels of viral replication, as measured by plasma viral load. Therefore, in this study, virological failure (no discernible decline in viral load) was not due to inefficient metabolism of AZT to the active triphosphate. A more recent study found that prior treatment with any NRTI did not affect later virological inhibition with a different NRTI[225]. This study also concluded that the ability of a cell to phosphorylate AZT or d4T was not affected by the use of either in a prior regimen[225].

This study did not measure levels of ddI or 3TC active phosphates, therefore it was not possible to see if prior AZT-experience was important. If prior AZT-experience caused a decrease in the phosphorylation of either ddI or 3TC, the reduced levels of active drug may have been in part responsible for the virological failure.

Another factor which may have an impact upon the study is the potential presence of AZT-associated resistance mutations in patients previously treated with AZT. It was unfortunate that more plasma was not available, or had been set aside, for genotypic testing. Prior resistance would explain why such a small number of AZT-experienced patients responded to the treatment. If a significant number of AZT-experienced patients had started the study with a quasispecies of mixed AZT sensitivity, evolution of a majority of AZT resistant virus would be inevitable. This could also explain the difference in response in AZT-naïve patients, most of whom showed a good primary

response to therapy, achieving undetectable viraemia, significantly different to that of AZT-experience patients. However, the response in therapy-naive patients was not sustained, and at 12 months virus had become detectable with a mean of 3.82 $\log_{10}$ copies/ml. This virological profile could demonstrate the evolution of resistance in both groups of patients. NRTI resistance-associated mutations are known to emerge fairly frequently in dual combination therapy[226]. This profile also agrees with the findings of the Delta trial[165] which showed that dual NRTI therapy had the greatest efficacy, characterised by the largest viral load decrease, in patients who were AZT-naïve.

Since 1995 when this study was initiated, several studies have observed that dual NRTI therapy frequently fails to suppress viral replication below the level of detection[227], and any reduction in viral load achieved is often transient. Frequently, viral load returns to baseline levels in the majority of patients[227], particularly in those who are NRTI-experienced[223]. This study verified previous findings that dual NRTI therapy demonstrates limited and transient viral inhibition. Virological failure was apparent in AZT-experienced patients at an early stage in the trial. At first, AZT-naïve patients showed good viral inhibition as a result of therapy. However, at the close of the trial both AZT-experienced and AZT-naïve patients demonstrated drug failure characterised by the persistence of viral load throughout treatment. Because there was no overall benefit of therapy to those patients who were AZT-naïve at the start of the trial, the failure of therapy in those who were AZT-experienced cannot be distinguished from those who were AZT-naïve. This observation precludes any conclusions being drawn as to why those who were AZT-experienced failed therapy, ultimately those who were AZT-naïve also showed persistence of viral load whilst on treatment and thus it is not possible to distinguish between the two groups.

The dual nucleoside therapy used in this trial was not effective in inhibiting HIV-1 replication in the long-term. This incomplete suppression may have been due to prior

resistance mutations (in those pre-treated with AZT) or may have contributed to the evolution of resistance. Whether this lack of efficacy was due to prior resistance or the evolution of resistance is unconfirmed. However, the lack of potency of the dual NRTI combination shown in this study requires further investigation to ascertain why this regimen failed even in those who were AZT-naive. Although dual nucleoside therapy has been surpassed in the developing world by the introduction of HAART, there are still many countries where antiretroviral treatment is constrained by financial costs. In this situation a dual nucleoside therapy may be the only affordable regimen. In this study the combination of AZT and one other NRTI was not greatly beneficial to either group studied and the pattern of un-sustained viral decline was similar to that seen by AZT alone[228].

This study was designed before the advent of multi-drug combination therapy or HAART as it is now defined. The observation that dual NRTI therapy has a reduced potency in AZT-experienced patients still has relevance to current treatment guidelines. At present, the BHIVA recommends a starting regimen containing three antiretroviral drugs of at least 2 classes, typically two drugs are NRTIs and make up a nucleoside analogue backbone[136]. If prior drug experience lessens the efficacy of one or more components of HAART, it will have an effect on the regimen as a whole.

Perhaps most importantly the results show that reduced drug potency was not due to intracellular levels of phosphates. Therapeutic drug monitoring was not able to predict or demonstrate virological failure or a lack of inhibition in any of the patients who fitted the criteria of therapeutic failure. Although intracellular drug levels can demonstrate potency and activity in various regimens currently used today, here the observations demonstrate that therapeutic drug monitoring cannot always determine the reasons behind virological success or failure.

Chapter 5 HIV-1 Disease Monitoring in Primary HIV-1 Infection

5.1 Viral detection versus Western Blot during primary HIV-1 infection.

5.1.1 Introduction

As previously described in section 1.4.5, upon HIV-1 infection the body mounts a humoral response, but it can be weeks or even months before the levels of antibody are high enough to be detectable by commonly used antibody tests such as the ELISA. In the new-born infant, at a risk of vertical transmission, the immature immune system and the possibility of transfer of maternal HIV-1 antibodies compound this situation, hence an HIV-1 diagnosis can take months to confirm. If a patient is aware of a possible transmission event, the time to confirmation of diagnosis (usually regarded as a three month window period) can be extremely stressful. Polymerase chain reaction (PCR) using *gag* and *pol* primers has been used to diagnose some cases where it is too early for an immune response[57,58]. However, this method may be dependent on cellular HIV-1 DNA or plasma viral HIV-1 RNA, levels of which can also take a number of days to weeks to accumulate. Also there are problems concerning false positives and the use of PCR where such low copy numbers are involved[229]. Measurement of p24 antigen has been used in some cases. However, current assay limitations, such as sensitivity, highlight the disadvantages of diagnosing HIV-1 by p24 antigen ELISA (see section 3.6 for sensitivity of p24 ELISA). In this study viral load was retrospectively analysed

from the first available plasma sample of patients undergoing seroconversion. Viral load measurement and Western Blot analysis of antibody profiles were compared, to determine if viral load could be detected where antibody responses were undetectable or weak.

5.1.2 Methods

Patients were selected for analysis from those attending the Ian Charleson Day Centre (ICDC), Royal Free Hospital, London, for diagnostic HIV-1 testing. Blood was taken and the separated plasma stored at -70°C. Plasma samples were analysed for antibody responses to HIV-1 using a Western Blot kit (Cambridge Biotech, Rockville MD, USA) by the Department of Virology, Royal Free Campus, Royal Free and University College School of Medicine (see section 2.6). Bands corresponding to antibodies to HIV-1 proteins p17, p24, p31, gp41, p51, p55, p66, gp120 and gp160 were measured. Western Blots showing no bands or very weak bands unlikely to be detected using a conventional gp41, p24 antigen diagnostic ELISA were highlighted and if plasma was available, it was retrospectively tested for HIV-1 RNA using Nuclisens. Where no plasma was available the plasma nearest in time from the same patient was tested.

5.1.3 Results

From a cohort of 47 patients who had been proven to be seroconverting to HIV-1 plasma samples from 40 were analysed by Western Blot for antibodies to HIV-1. Eleven out of 40 patients showed a developing immune response characterised by no visible antibody bands or weak bands of only one or two antibodies. Where no antibody band was visible from the first available plasma, Western Blot was repeated at later time points until a firm HIV-1 diagnosis could be confirmed. Three patients had no visible

antibody bands from the first available plasma, two out of the three were analysed at later time points by Western Blot for the appearance of bands. Plasma samples at equivalent time points were analysed for viral load for all 11 patients. Results of Western Blot and viral load quantification are shown in Table 5-1.

Table 5-1 Western Blot (WB) detects HIV-1 antibodies compared to viral load.

Patient	Date WB	Bands detected in Western Blot			Viral load		
		None	Weak	Strong	log_{10} copies/ml	Date	Days after WB
4	10/09/96	none			N/A		
	17/09/96	none			N/A		
	25/09/96		p24		3.94	25/09/96	0
	03/11/96		p24	gp120, gp160	<2.6 *	25/10/96	-9
6	15/06/99	none			N/A		
	29/06/99		gp160, p24		6.18	06/07/99	7
14	08/11/96		p24		6.14	08/11/96	0
	13/11/96		p24, gp160		6.04	13/11/96	0
17	12/09/97		gp160		N/A		
	15/09/97		gp160		5.30	22/09/97	7
	23/09/97		gp41, gp160, gp120	p24	N/A		
	01/10/97		gp41, gp160, gp120, p31, p17	p24	4.54	01/10/97	0
18	02/07/99	none			6.15	07/07/99	5
22	04/12/98		gp120		5.23	04/12/98	0
29	30/01/98		p24		3.72	30/01/98	0
32	19/03/98		p24, gp41, gp160, gp120, p17		5.18	18/03/98	-1
35	17/04/98		p24, gp160		5.88	21/04/98	4
40	19/10/98		p24		7.34	19/10/98	0
46	02/07/99		p24		4.92	06/07/99	4

WB = Western Blot * Patient receiving HAART N/A = no plasma available

The Table shows that individual patients develop detectable antibodies at different time points. Eight out of 11 patients developed p24 antibody response first. In two patients this was concurrent with anti-gp160. One patient developed a response to gp120 first and another patient responded to gp160 first. Patient 18 showed no bands on a Western

Blot. This patient was not tested by Western Blot at a later time point, but was confirmed to have HIV-1 in later samples using a diagnostic HIV-1 antibody ELISA. Where a quantitative viral load test was performed, HIV-1 RNA was always detected. Mean HIV-1 RNA was 5.43 $\log_{10}$ copies/ml (range 3.72 – 7.34 $\log_{10}$ copies/ml). Unfortunately six of the earliest samples were unavailable for retrospective testing for HIV-1 RNA. Five out of 11 patients had either no visible antibodies or a response which was unlikely to be detected by the conventional diagnostic method i.e. ELISA for detecting p24 and gp41. Three out of these five patients had samples available for HIV-1 RNA quantification. All three had high levels of HIV-1 RNA (range 5.23 – 6.15 $\log_{10}$ copies/ml) in the samples tested.

5.1.4 Discussion

This small retrospective study shows the levels of antibody response are variable in patients undergoing seroconversion. The majority of patients in this study generated a response to p24 antigen before any other. However, a proportion did not produce anti-p24 antibodies first. Patients who showed no Western Blot bands or who produced antibodies other than to p24 and gp41 would be unlikely to give a concurrent positive result in a diagnostic antibody ELISA.

Five patients had either no detectable antibody response or weak antibodies not including those to p24 or gp41. Three of these patients were tested for viral load and all three had significant levels of circulating virus. This implies that in some cases viral load can be detected before anti-p24 and gp41. Therefore a viral load test might identify a new infection earlier than the currently used diagnostic antibody test. Current use of antibody testing to diagnose new HIV-1 infections emphasises the time lag where neither clinician nor patient is aware of the HIV-1 status. Recent research has highlighted the importance of early diagnosis of infection for two reasons. Many

patients are aware of the limitations of HIV-1 testing and of the "window period" before seroconversion. This may deter patients from having an early test, preferring to wait for 3 months to get a definitive answer. Epidemiological evidence has suggested that a significant proportion of the new HIV-1 epidemic in developed countries is driven by recently infected individuals[62]. Earlier testing might be encouraged if patients could be assured of a more immediate answer. It would increase awareness in this potentially infectious recently infected population.

This early period of infection may also be critical in terms of host immune pathology, and sequestration of the virus into sanctuary sites. Early treatment of seroconverters has been shown to be effective in preventing irreversible immune cell damage[230]. Early treatment would also reduce viral load in this potentially infectious patient population[231].

This study also encourages the use of viral load testing in new-born babies at risk of maternal transmission. The immature immune system takes a longer time to mount a measurable antibody response to HIV-1 infection. A viral load test could confirm infection at a much earlier stage. A number of studies in Europe and North America detecting viral RNA have shown high specificity and sensitivity in the early determination of HIV-1 infection in HIV-1-exposed children[171,232,233].

In the USA the Department of Health and Human Services recommends the use of HIV-1 viral load assays for at-risk patients suspected of recent infection as the preferred method of diagnosis[234]. However, a recent report put the rate of false positives in the quantification of HIV-1 RNA at between 1.9%-3.0%[235]. Quality assurance panels previously described in sections 4.1.1 and 4.1.2 showed NASBA and Nuclisens to have 100% accuracy in detecting negative control samples. However, another study in 1998 showed the range of false positives for all three viral load assays (bDNA, RT-PCR and Nuclisens) to be 5% – 20%[229]. Thus the use of viral load testing as a diagnostic assay

should be recommended with caution. A more recent study in Brazil looking at the detection of HIV-1 in babies less than 12 months old, at risk of vertical transmission, showed that the NASBA assay detected two infected babies less than 3 weeks old, and 23 infected infants over 3 weeks, with no false positives in any of the 251 children studied[58].

This current study, which looked at recently infected adults in a London clinic, assessed the value of diagnostic viral load testing in cases where recent transmission was suspected. The greater cost of viral load testing compared to that of antibody ELISAs, compounds the issue. It is necessary to investigate further in a larger population whether HIV-1 can be detected earlier by quantitative viral load assays than by antibody ELISA. If viral load can detect HIV-1 earlier, analysis is needed to assess whether there would be a significant gain from early diagnosis to balance the greater financial cost.

5.2 Virological monitoring in primary HIV-1 infection

5.2.1 Introduction

The events during the period of time immediately following transmission of HIV-1 to a new host have been theorised as some of the key determinants in the disease process. Severity of the acute infection illness and the levels of disease markers during PHI have been linked with speed of progression[71,236,237], and immune proficiency[238]. Several studies have shown viral load during primary HIV-1 infection (PHI) to correlate with disease progression[65,66,239,240]. It is thought that the evolution of quasispecies takes place in the initial period of viral population expansion, immediately after infection[241]. The infiltration of the virus into sanctuary sites such as lymphatic tissue and central nervous system also occurs during this time[72,242]. Both events could have a significant impact on the course of disease[243-246]. Initial viral load levels indicate the replicative capacity of the virus[241] and the subsequent rate of decline may provide insights into the immune response and control of viraemia[247]. Successful HAART initiated early in PHI would constrain viral expansion and possibly be more effective in controlling viraemia than therapy started later in the disease process[248-250].

In this study a cohort of patients undergoing primary HIV-1 infection was monitored virologically. The natural history of primary HIV-1 infection was monitored in untreated patients. In those starting HAART, plasma HIV-1 RNA was analysed against time to initiation of treatment in order to assess whether receiving HAART early in PHI achieved a better virological response, than in those who started treatment later.

5.2.2 Patients and Methods

Patients were selected from those attending the ICDC clinic, Royal Free Hospital, London. Patients were diagnosed as undergoing PHI by Western Blot analysis or presentation with seroconversion illness. Western Blots were performed using a Western Blot kit (Cambridge Biotech, Rockville MD, USA) by the Department of Virology, Royal Free Campus, Royal Free and University College School of Medicine (see section 2.6). Antibodies to p17, p24, p31, gp41, p51, p55, p66, gp120 and gp160 HIV-1 antigens, if present, were observed as either weak or strong bands. Plasma HIV-1 RNA was analysed at regular intervals using NASBA from 1996 – 1997 which had a lower limit of detection at 3.30 $\log_{10}$ copies/ml, and Nuclisens 1997 –2000 which had a lower limit of detection at 2.6 $\log_{10}$ copies/ml. Where adequate plasma was reserved, early samples from 1996 –1997 that gave a NASBA value below 3.30 $\log_{10}$ copies/ml were retrospectively re-evaluated with Nuclisens. Where patients initiated antiretroviral treatment virological response was monitored and compared against those who remained untreated using an on-treatment analysis. This analysis included only those who continued on therapy. To include patients who later stopped therapy viral loads for all patients were re-analysed using an 'intent to treat' analysis.

5.2.3 Results

Forty-seven patients were enrolled onto the study, many of whom were also participating in the 'QUEST' study investigating the early initiation of antiretroviral therapy during PHI. All of the patients were infected between 1996 and 2000. Patients were selected if a plasma sample was available within five months of probable infection. Table 5-2 shows the total number of patients and in which year they are believed to have been infected.

Table 5-2 The number of PHI patients and the year in which they were infected.

Year	Number of patients
1996	6
1997	20
1998	12
1999	7
2000	2
Total	**47**

The probable date of infection was estimated from a recalled significant risk event, such as unprotected sex with a high-risk group partner, or severe seroconversion illness.

Forty out of 47 patients had plasma analysed for anti-HIV-1 antibodies by Western Blot. The multinational scientific committee of immunologists and virologists in the QUEST study defined PHI specifically: criteria for study entry was the appearance of bands by Western Blot. Those patients who exhibited less than or equal to this number of bands were admitted onto the trial. The selection criteria for the study detailed here necessarily incorporated these criteria as a basis for differentiating between patients early, middle or late in PHI.

The seven patients who were not tested by the Western Blot method were categorised according to clinical notes describing the risk event or seroconversion illness. The groups were defined as follows:

Patients in the early PHI period: 0 – 4 antibody (Ab) bands of any strength, or notes indicating infection or PHI illness 0 –1 month prior to first sample, n = 15.

Patients in the mid-PHI period: > 4 Ab bands that are a mixture of weak and strong bands, or where notes indicate infection 1 – 2 months prior to first sample, n = 13.

Patients in the late PHI period: All Ab bands present (weak or strong) or notes indicating infection > 2 months but < 5 months prior to first sample, n = 19.

Full Western Blot analysis results can be seen in appendix I. Of the seven patients for whom there was no Western Blot data, two were categorised as early, two as middle and three as late in primary HIV-1 infection. More description from their clinical notes on these seven patients is shown in appendix II.

The mean viral load in the first clinical samples was 5.07 $\log_{10}$ copies/ml (range < 2.6 – 7.45 $\log_{10}$ copies/ml). Three patients (patients 10, 25 and 47) did not have a detectable viral load at first presentation. Patient 47 had an undetectable viral load using NASBA (< 3.3 $\log_{10}$ copies/ml) and patients 25 and 47 had an undetectable viral load using Nuclisens (< 2.6 $\log_{10}$ copies/ml). Samples from patients 25 and 47 were re-tested using RT-PCR with non-B primers (RT-PCRnb) to see if virus could be detected using alternative primers designed to amplify a greater range of non-B subtypes. This assay quantified the virus to be < 2.6 $\log_{10}$ copies for patient 25 but > 5.9 $\log_{10}$ copies/ml for patient 47, implying that virus from patient 47 was of a subtype not quantified by NASBA/Nuclisens primers. Unfortunately, there was insufficient plasma to re-test patient 10. However, the patient acquired the infection in Zimbabwe and may well have been infected by a non-B virus. All three patients were more than a month into their infection. These three patients were excluded from baseline viral load analysis.

Patients defined as early in PHI (n = 15) had a mean viral load of 5.76 $\log_{10}$ copies/ml (range 3.94 – 7.45 $\log_{10}$ copies/ml). Patients who were categorised as mid-PHI had a mean viral load of 4.71 $\log_{10}$ copies/ml (range 2.77 – 5.77 $\log_{10}$ copies/ml), late seroconverters had a mean viral load of 4.70 $\log_{10}$ copies/ml (range 2.79 – 7.15 $\log_{10}$ copies/ml).

During the assumed first 120 days of infection 19 patients (40%) who were not receiving therapy were analysed for virological response to investigate the natural history of the virus. Viral load was determined and the percentage change from the sample of first presentation (baseline) was calculated over time in days. This was expressed as percentage change per week. Patient 21 was excluded from this analysis as no sample was taken during the first 120 days. Calculated results for all 18 patients are shown in Table 5-3.

Table 5-3 Percentage change from baseline viral load to second sample during the assumed first 120 days of infection in untreated patients.

PHI Status	Patient no.	Viral load in log_{10} copies/ml		Days after baseline	% change in log_{10} copies/ml per week	Mean % change in log_{10} copies/ml	St. dev.
		Baseline	Second sample				
Early	6	6.18	5.00	22	↓6.10		
(n = 4)	27	6.00	5.23	37	↓2.43		
	17	5.30	4.70	28	↓2.84		
	18	6.15	5.92	35	↓0.74	↓3.03	2.24
Middle	1	4.59	4.20	5	↓11.87		
(n = 4)	15	5.69	4.58	38	↓3.58		
	43	5.45	5.23	13	↓2.17		
	23	4.15	3.11	26	↓6.72	↓6.09	4.30
Late	9	3.34	4.08	5	↑-31.02		
(n = 10)	11	4.28	4.08	18	↓1.82		
	12	4.88	4.66	34	↓0.94		
	13	3.04	<2.6	10			
	16	7.15	5.85	35	↓3.64		
	42	4.58	4.43	14	↓1.62		
	41	4.98	4.90	23	↓0.46		
	28	5.40	5.62	19	↑-1.54		
	8	5.38	5.61	31	↑-0.98		
	26	4.32	4.41	28	↑-0.56	↑-2.85	10.68
mean		**5.05**	**4.80**	**23.39**	**0.64**		
median		**5.14**	**4.70**	**24.50**	**1.82**		
St. dev.		**1.03**	**0.74**	**24.69**	**8.78**		

Difference calculated as second sample minus baseline, therefore a viral load increase from baseline is denoted as negative decline.

The table shows that in most cases the second sample measured was 3 – 4 weeks after baseline (mean 23 days, range 5 – 38 days). Second samples were selected as close to 3 weeks as possible to ensure measurements were within 120 days of infection, even for patients late in PHI. Patients in the early period of PHI all show a reduction in viral load from baseline to second sample, with a mean decrease of 3.03 %, range 0.74 – 6.10 % (standard deviation for % reduction was 2.24). All patients in mid-PHI also show a reduction of viral load from baseline, but the decline is greater with a mean percentage reduction of 6.09 % (range 2.17 – 11.87 %). The standard deviation is higher at 4.30, but this reflects the higher level of reduction seen in this group. Six out of 10 of the patients in the late period of PHI also show a decrease in viral load but greatly reduced in magnitude. In this group 4/10 patients show an increase in viral load from baseline. The mean percentage reflects this and shows an increase from baseline values of 2.85 % (the difference from baseline value to second sample ranges from 31.02 % increase – 3.64 % reduction). This may reflect the natural history of the virus in terms of replication rate versus immune response, i.e. the second sample from patients early in PHI was within 2 months of probable infection, therefore the immune response, although effective in reducing viral load, was probably still developing. The second sample from mid-PHI patients was within 3 months of infection. Here, the immune response should have been fully developed and at its most effective in reducing viral load. The second sample of the late PHI patients was taken 3 – 4 months after infection. By this time the virus should have infiltrated lymph nodes and tissues, infecting a wider variety of cell types and this could have caused viral load to level out or even to rise.

The mean for the group as a whole is 0.64 % reduction from baseline. However, the standard deviation is high at 8.78. The maximum reduction in viral load achieved in any one patient was 11.87 %.

Antiretroviral therapy during PHI

The entire group of 47 patients was then re-analysed according to whether therapy was received at any point during follow-up (not just during the first 120 days). Fourteen out of 47 patients started antiretroviral therapy less than 5 weeks after Western Blot or the clinical notes indicated probable infection. Two patients started therapy between 5 – 18 weeks after infection, three patients started therapy between 19 – 35 weeks and four patients started therapy after 1 year of infection. Five patients initiated therapy but stopped. Sixteen patients elected not to take therapy throughout follow-up. The number of patients and time intervals between probable infection and start of treatment are shown in Figure 5-1. Three patients who did not receive any therapy, failed to attend the clinic after a two or less samples had been taken, and therefore were characterised as lost to follow-up.

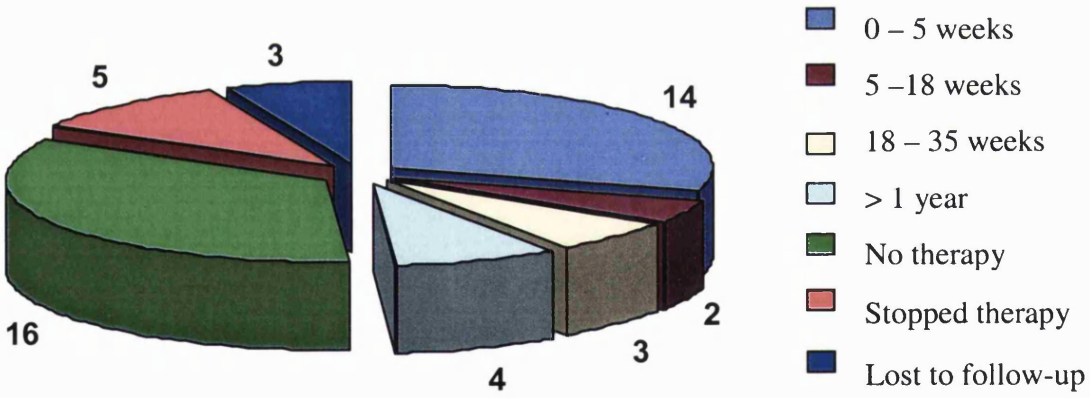


Figure 5-1 Number of patients and time between probable infection and start of treatment (if any) in patients with primary HIV-1 infection. n = 47.

The number of patients in each group who achieved an undetectable viral load (by Nuclisens i.e. < 2.6 $\log_{10}$ copies/ml) is shown in Table 5-4.

Table 5-4 Patients achieving an undetectable viral load (< 2.6 log_{10} copies/ml) according to when therapy was initiated during primary HIV-1 infection (total follow-up, n= 47).

Treatment Initiated weeks post probable infection	Undetectable at 3 weeks of treatment	n	%	Undetectable at 6 months of treatment	n	%	Undetectable at 1 year of treatment	n	%
0 – 5 weeks (n = 14)	3	14	23	10	14	71	8	11	73
5 –18 weeks (n = 2)	0	2	0	1	2	50	1	2	50
18 – 35 weeks (n = 3)	0	2	0	2	3	67	2	3	67
> 1 year (n = 4)	0	2	0	3	4	75	2	4	50
No therapy (n = 19)	0	8	0	0	11	0	0	9	0
Stopped therapy (n = 5)	1	3	33	0	4	0	1	5	20

Three patients failed to attend after two visits, none of the three received any antiretroviral treatment and so are included in the 'no therapy' group. n denotes the number of patients with a sample available for testing at each time point. The number of patients in each group with an undetectable viral load was calculated as a percentage of n.

Table 5-4 shows that no untreated patient was undetectable for HIV-1 RNA at either 3 weeks, 6 months or a year. However, in three patients of this group HIV-1 RNA did drop transiently below the level of detection during follow-up. In all three cases, the sample in which the virus was undetectable was the second sample available for testing.

One patient (included in those who stopped therapy) received therapy for more than a year, before stopping.

The patients who stopped therapy can be included in the results on an intent-to-treat (ITT) basis i.e. patient viral loads from those who started therapy within 5 weeks (regardless of when therapy was stopped) were included with the results of those treated within 5 weeks. Revised results for the percentage of patients achieving an undetectable viral load are shown in Table 5-5 and graphically as Figure 5-2.

Table 5-5 Percentage of PHI patients who had an undetectable viral load at 3 weeks, 6 months and 1 year after the start of treatment which was initiated at varying times, based on an intent-to-treat (ITT) analysis.

Treatment initiation (weeks)	No. of patients	% patients with an undetectable viral load		
		3 weeks post treatment	6 months post treatment	1 year post treatment
0 – 5	15	29	67	67
5 –18	4	0	25	25
19 – 35	5	0	50	60
> 1 year	4	0	75	50
No Rx	19	0	0	0

The three patients who did not attend after two clinical visits were included in the group who did not receive any therapy.

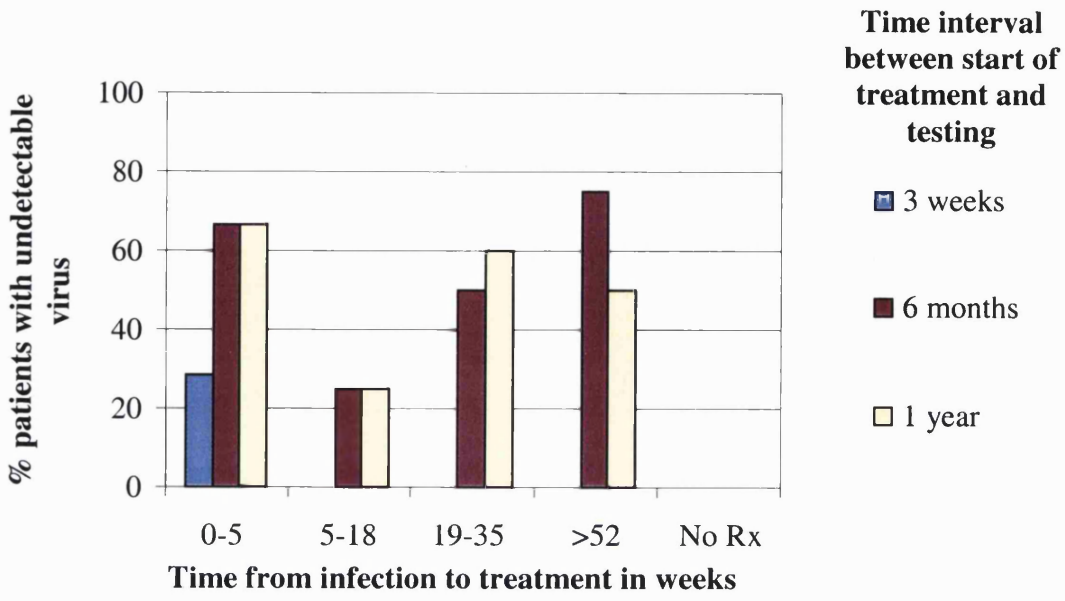


Figure 5-2 Percentage of PHI patients achieving an undetectable viral load at 3 weeks, 6 months and 1 year, according to when treatment was initiated in weeks after probable infection.

Patients who elected not to start treatment during PHI did not achieve undetectable levels at either 3 weeks, 6 months or 1 year. Three out of 19 untreated patients did have at least one undetectable viral load (< 2.6 $\log_{10}$ copies/ml) during the year follow-up, but in all cases this was a transient fluctuation in viral load. All three patients displayed relatively low viral loads throughout the study in comparison with the rest of the group. In all three patients the sample in which virus was undetectable was within the first 4 months of infection.

Figure 5-2 shows that within the first 3 weeks of treatment, only patients who initiated their treatment within 5 weeks of probable infection achieved an undetectable viral load. This could be because therapy is more effective when initiated early in infection, possibly before the virus has a chance to evolve into quasispecies. Alternatively, during

the early months of PHI, the virus is also being suppressed by the immune system, and this, combined with HAART could be responsible for the increased level of suppression in patients treated early in PHI.

The percentage of patients achieving an undetectable viral load at 1 year was always equal to, if not higher than, the percentage who achieved an undetectable viral load at 6 months, irrespective of time of initiation of therapy. This implies that the majority of patients achieving an undetectable viral load after 1 year of treatment, would have had undetectable virus at 6 months. Three out of four patients who started therapy after 1 year of infection achieved undetectable viral loads after 6 months of therapy, a higher percentage than in any other group, implying this was the most successful therapy group. However, at 1 year only two patients in this group had sustained undetectable levels of virus. All three of the groups treated after 5 weeks have low numbers of patients and this precludes any objective comparison between the groups of which time point it is most beneficial to start therapy.

The debate surrounding the initiation of therapy during PHI generally concerns the period immediately after infection, during which time therapeutic intervention may avert irreversible damage to the immune system and prevent the virus from entering sanctuary sites. To investigate the success of early intervention, viral load decline during the first month of therapy in patients who started treatment within the first 5 weeks after probable infection was compared to those who started therapy later. This analysis allows all patients treated after 5 weeks to be grouped together, increasing the number of patients in the group treated late in PHI. The percentage change over time was measured for each patient and adjusted accordingly to the number of days between samples in order to calculate a percentage change in viral load over 1 week. Tables 5-6 and 5-7 show percentage change in viral load per week for patients treated within 5

weeks of infection and for those treated who started therapy more than 5 weeks post infection.

Patients shown in Table 5-6 and 5-7 were analysed on an intent-to-treat (ITT) basis so patients who later stopped therapy were included. Five patients stopped therapy. However, all five stopped after at least 8 weeks of therapy, so for the analysis based on the first month sample all were still receiving therapy.

Table 5-6 Percentage decrease in viral load in patients with PHI who started treatment within 5 weeks of infection. n = 15.

Patient no.	Viral load log_{10} copies/ml		Days interval between samples	% reduction in viral load (log_{10} copies/ml) per week
	Baseline	Second sample		
4	3.43	< 2.6	16	
5	5.83	3.38	29	10.14
14	6.04	2.96	21	17.00
19	5.74	3.00	30	11.14
22	4.86	2.88	27	10.56
29	3.72	< 2.6	54*	
32	5.17	3.08	27	10.48
34	7.45	3.97	17	19.23
35	5.88	3.52	15	18.74
38	4.04	N/A	*	
39	4.08	2.86	27	7.75
40	7.34	4.89	28	8.34
45	6.71	4.08	28	9.80
46	5.00	< 2.6	28	
47	5.13	3.58	27	7.83
Mean	**5.36**	**3.47**	**26.71**	**11.91**
St. dev.	**1.24**	**0.63**	**9.38**	**4.30**

*Although patients 29 and 38 were treated within 5 weeks of probable infection, neither had a second sample available for testing within the first month of starting therapy. The second sample for patients 29 and 38 were taken at 54 and 89 weeks respectively.

Patient 29 was included in the analysis as the second sample was determined to be approximate to 1 month of receiving therapy, patient 38 was disqualified from the group calculations.

Table 5-7 Percentage decrease in viral load in patients with PHI who started treatment after 5 weeks post infection. n = 13.

Patient no.	Viral load log_{10} copies/ml		Days interval between samples	% reduction in viral load (log_{10} copies/ml) per week
	Baseline	Second sample		
8	4.88	3.40	21	10.11
10	3.18	2.65	36	3.24
17	5.08	3.08	25	11.02
18	5.72	5.05	27	3.04
23	4.11	3.11	9	18.92
24	5.53	4.68	21	5.12
25	N/A	N/A		
26	5.40	N/A		
28	5.62	N/A		
30	5.04	3.00	28	10.12
33	4.71	3.15	21	11.04
37	N/A	N/A		
41	5.00	4.41	35	2.36
Mean	**4.93**	**3.61**	**24.78**	**8.33**
St. dev.	**0.74**	**0.86**	**8.20**	**5.40**

Patients 25 and 37 did not have a baseline sample taken at the start of therapy. Table 5-6 and 5-7 were compared to the values from Table 5-8 for untreated patients (including those that were lost to follow-up)

Table 5-8 Percentage decrease in viral load for patients who remained untreated throughout follow-up. n = 19

Patient no.	Viral load log_{10} copies/ml		Days interval between samples	% reduction in viral load (log_{10} copies/ml) per week
	Baseline	Second sample		
1	4.59	4.20	5	11.87
2	4.59	< 2.6	76	
3	2.77	< 2.6	7	
6	6.18	5.00	22	6.10
7	4.98	5.57	61	-1.36
9	3.34	4.08	5	-31.02
11	4.28	4.08	18	1.82
12	4.88	4.66	34	0.94
13	3.04	<2.6	10	
15	5.69	4.58	38	3.58
16	7.15	5.85	35	3.64
20	5.13	N/A		
21	4.66	5.04	11	-5.21
27	6.00	5.23	37	2.43
31	3.97	4.05	66	-0.21
36	2.79	N/A		
42	4.58	4.43	14	1.62
43	5.45	5.23	13	2.17
44	5.62	N/A		
mean	**4.72**	**4.77**	**25.07**	**-0.62**
St. dev.	**1.19**	**0.60**	**19.53**	**9.74**

Patients 20, 36 and 44 did not have a second sample available for testing. Table 5-9 shows the percentage change in viral load per week for all patients, treated and untreated.

Table 5-9 Percentage decrease in viral load per week for all patients.

Therapy Initiation	Baseline viral load log_{10} copies/ml	n	Mean time point between baseline and second sample in weeks	n	% change in viral load per week	No. of patients with undetectable virus
< 5 weeks after probable infection	5.36	15	3.6	14	↓ 11.91	3
St. dev.	1.24		0.7		4.30	
> 5 weeks after probable infection	4.93	13	3.77	9	↓ 8.33	0
St. dev.	0.74		1.63		5.40	
None	4.72	19	4.0	16	↑ -0.62	3
St. dev.	1.19		3.3		9.74	

The mean baseline viral load for each group of patients was comparable, mean 4.99 log_{10} copies/ml (range 2.77 – 7.45 log_{10} copies/ml). For the majority of patients the second time point analysed was 3 – 4 weeks after treatment started or 3 – 4 weeks after presentation in those not receiving treatment. The percentage change in viral load per week was variable for all patients, demonstrated by a high standard deviation. Those receiving treatment showed a distinct trend of a reduction in viral load. Patients treated early (< 5 weeks into PHI) showed a mean reduction in viral load of 11.91 %, range 7.75 – 19.23 %. Patients treated later showed a mean reduction of 8.33 %, range 2.36 – 18.92 %. In the group of untreated patients the viral load went up as well as down, thus

the mean percentage change in this group was –0.62 %, ranging from a 31.02 % viral load increase to a viral load decrease of 11.87 %.

Statistical analysis of the significance of the percentage change was calculated using an unpaired t-test. The percentage change per week of patients treated early (within 5 weeks) versus untreated patients has a significance value of p =0.001, late treated patients (> 5 weeks after infection) versus untreated has a p value of 0.03. This demonstrates that the decrease in viral load during first 3 weeks is significantly greater in those on therapy. A comparison of the percentage change observed in patients treated early versus late, was analysed using an unpaired t-test to give a p value of 0.115. This indicates that there is no significant difference in viral load change between the two groups.

If Figure 5-2 is revised so that the groups receiving treatment after 5 weeks are combined to create a larger group, the percentage of undetectable patients at 6 months is quite similar to that of the group receiving early treatment. The combined groups of those receiving therapy later than 5 weeks against those receiving therapy within 5 weeks is shown in Figure 5-3.

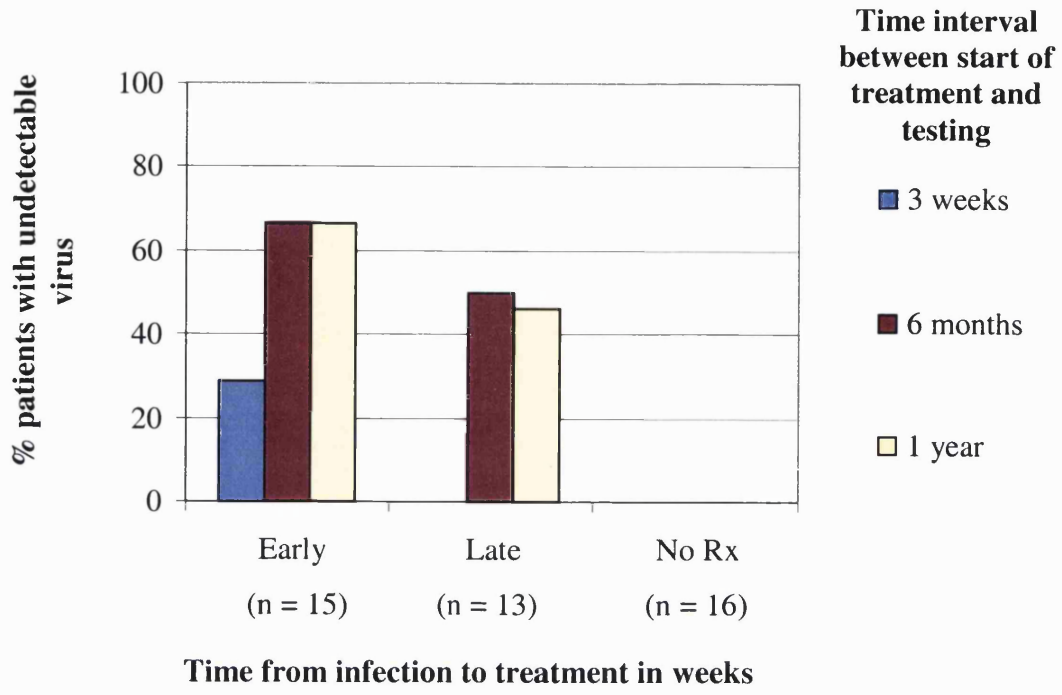


Figure 5-3 Percentage of PHI patients achieving an undetectable viral load at 3 weeks, 6 months and 1 year, according to when treatment was initiated. Early = treatment started 0 – 5 weeks from probable infection. Late = treatment started more than 5 weeks from probable infection. No Rx = no therapy initiated throughout follow-up.

More patients achieve an undetectable viral load within 3 weeks in those starting therapy early than in those starting therapy later. As mentioned previously, this is probably as a result of suppression resulting from the combination of HAART and the early immune response. At 6 months, both groups show therapy is successful in suppressing virus to undetectable levels in 50 – 65 % of patients.

67% of patients who started therapy early still had undetectable levels of virus after 1 year of treatment, versus 46% of patients who started therapy later. This indicates that therapy has a greater efficacy in suppressing and sustaining undetectable virus levels if initiated early in PHI. The proportion of patients achieving an undetectable viral load at

1 year in both groups is very similar to the proportion at 6 months. This implies that the majority of patients who start therapy and suppress virus to undetectable levels, do so by 6 months or not at all, regardless of when therapy is started. This shows that therapy is as effective as possible within 6 months in both groups of patients. In patients treated late, the proportion achieving undetectable viral load declines after 1 year of treatment indicating that therapy was less effective over the long term in this group of patients. In addition, the study highlights that a number of patients in both groups do not achieve full viral suppression. Combining both groups of patients, early treated and late treated, the percentage who did not achieve an undetectable viral load was approximately 40 %, including those who stopped therapy.

One reason for the success of early treatment in reducing virus to undetectable levels may be that therapy was aided by the additional early immune response. In the natural history of infection the reduction in viral load seen during early or mid-PHI (see Table 5-3) must be due to the early immune response. Patients early or mid-PHI showed a mean reduction of 4.56 % per week, compared to late PHI patients who had a mean increase in viral load of 2.85 % (see Table 5-3). The reduction in viral load potentially attributable to the immune system can be subtracted from those patients who started therapy within 5 weeks of seroconversion to give a value for the reduction achieved by the therapy alone. A comparison of early treatment percentage change (minus immune response 4.56 %) and the percentage change shown by late treated patients using an unpaired t-test gave a p value of 0.85 and demonstrates that there was no significant difference between the two groups.

5.2.4 Discussion

This study of viral load in a cohort of patients undergoing PHI shows that while there is a degree of variability from patient to patient, some trends can be established or

confirmed. Viral load during PHI, particularly the first month of infection, is high. In this study viral loads exceeding 6.5 $\log_{10}$ copies were observed. The high level of HIV-1 RNA observed was consistent with other studies of PHI[68,241,251], but this level of viraemia is very rarely seen, even in AIDS and chronic immune suppression. The immunological profile and measurement of viral load in the untreated patients follows accepted theories about viral replication upon transmission, and the natural history of disease[68,241,251]. Early in the first few months following transmission, the immune response was evolving with Western Blots of patients during this time showed limited or even absent antibody responses to HIV-1 proteins.

However, it has been observed that Western Blot results are highly variable and differ from patient to patient in time and strength. One patient may produce a broad clonal response with a wide range of antibodies, of high concentration relatively very quickly, for example a month after infection, but this would be unusual with full responses in Western Blot taking 6 –9 months. The definition of PHI used by QUEST and by this study would have defined this patient as late in PHI regardless of how recently the patient had been infected. The ideal method for defining how recently a patient was infected is to measure specific antibody concentrations in the sera using normalised optical densities. Unfortunately, this was not part of the QUEST protocol and was not performed on the original samples.

A previous study in 1996 observed the rate of viral replication increasing immediately after infection, characterised by a high viral load, which was later suppressed by immune response and viral load was reduced to well below baseline levels[241]. This correlates with the results of the untreated patients in this study who had indeterminate or weak antibody profiles on a Western Blot, characterised as early or mid-PHI. The viral load in these untreated patients was high at baseline, but was rapidly reduced by the time of the second sample. This contrasted to those in late PHI, who showed a mean

reduction in viral load by the second sample but at a much lower rate of reduction. This indicated that the immune response was still suppressing viraemia in some late-PHI patients. However, others in this group showed slight rise in viral load, indicating that HIV-1 was now evading the immune response, or was replicating faster than the immune system could control it. This was possibly as a result of the infection of lymphoid tissue and sanctuary sites during the period of increased viral replication[72]. Previously high levels of viraemia had infected multiple tissues and sites, increasing the number of HIV-1 infected cells. These cells, primed by ongoing infection, would then go on to produce more virions.

Antiretroviral treatment appeared to suppress HIV-1 replication and was greatly effective in reducing viraemia to undetectable levels. The time of initiation of treatment did not appear to affect the reduction in viral load once the early immune response was taken into account. Previous studies in untreated patients have estimated the immune response during the first 120 days to suppress viraemia by 6.5 % per week[68]. This is similar to that seen in the early and mid- PHI patients in this study (mean viral load reduction 4.56 % per week). If this percentage reduction is taken into account, the difference in efficacy between early and late treatment indicates that much of the effectiveness of early treatment is due to the immune response. However, the increased suppression resulting from combined affects of treatment and the immune response, reduced the viral replication to undetectable levels in three out of 14 patients (21 %) of the early treated group within 3 – 4 weeks, as opposed to none of eight patients in the late treated group.

In this study all patients diagnosed as HIV-1 positive during PHI were given the option of antiretroviral therapy. Figure 5-1 showed how many elected to take therapy and when it was initiated. The two largest groups were those who started therapy early (within 5 weeks of infection) and those who elected not to take therapy. This implies

that those who want to take therapy will take it immediately upon diagnosis, whereas those who have reservations about antiretroviral therapy will not take therapy until they feel it is necessary. It may also indicate that clinicians believe that starting therapy early in PHI will be more effective than saving it until later. In this study, therapy alone did not reduce the level of viral load more effectively when started early. During the first 3 weeks of treatment, patients who initiated therapy early (< 5 weeks into PHI) showed a higher number of patients with an undetectable viral load than those who started therapy later. This disparity is maintained at 6 months and 1 year of therapy, the percentage of patients achieving undetectable levels of virus was greater in those treated early than those treated late. However, there was no significant difference in the reduction in viral load as result of treatment, particularly when the early immune suppression was also taken into account.

This study shows that during PHI a large percentage of patients are recruited to receive antiretroviral therapy. Based on an ITT analysis, 40 % of patients did not achieve successful viral suppression, regardless of when therapy was started. The patients for whom therapy did not suppress viral replication after 6 months to 1 year, risk the evolution of resistant viral strains and immune suppression. If therapy was started early, patients risk a treatment failure very early in their disease course, potentially eliminating the use of a number of drugs in their first year of infection. Unfortunately, this study did not include immunological data comparing the immune system in those receiving therapy and those not taking treatment. A number of studies have indicated that early treatment preserves vital anti-HIV-1 responses and cellular differentiation and function[230,252,253].

Early treatment of PHI gave an increased likelihood of full suppression of viraemia to 6 months and 1 year, a greater likelihood than if started late in PHI. However, a small but significant percentage never achieved full suppression, regardless of how early therapy

was started. The potential loss of treatment options early in disease due to the evolution of resistance must be weighed up against the irreversible immunological damage that may occur if viral replication is left unchecked. Therefore, both virological and immunological benefits need to be ascertained before intensive HAART is unequivocally recommended to patients recently diagnosed with PHI.

5.3 Frequency of mutations associated with resistance to antiretroviral drugs, in patients undergoing Primary HIV-1 Infection.

5.3.1 Introduction

The advent of antiretroviral therapy has brought about a marked decline in mortality and in the incidence of opportunistic infections in patients with HIV-1[122,123]. Therapy can considerably prolong the asymptomatic period before onset of AIDS, and has lessened patient anxiety over impending ill health[254]. However, therapeutic failure resulting from low drug levels, drug resistance and patient non-compliance can give rise to a growing number of patients who are left without effective treatment options[255,256].

There is growing evidence that resistant strains are as capable of infecting new individuals as the original sensitive virus (known as the wildtype virus)[257]. The widespread use of antiretroviral drugs and the growing number of patients whose virus has one or more mutations associated with drug resistance, may mean an increase in the transmission of resistant strains[258]. This would have important implications concerning treatment during acute infection; a resistant virus may compromise the response to initial therapy[259].

It may be appropriate to perform resistance testing prior to starting therapy, so that the most effective treatment strategy can be chosen. Before assessing the benefits of such a scheme, it is necessary to assess the extent of transmission of resistant viruses. A number of international studies have investigated resistance-associated mutations in cohorts of patients recently infected with HIV-1[258,260,260,261,261]. All the studies agree that there is now transmission of virus resistant to all three classes of drugs[261] and some

report that transmission of NRTI resistant strains, in particular those resistant to AZT, is most prevalent[262,263,263]. Other reports suggest that transmission of PI resistant strains has an equivalent frequency[264,265]. However, the distinction between primary and secondary mutations, and therefore by how much a mutation will decrease sensitivity to the drug, is less clear for PIs than for NRTIs or NNRTIs. There is a wide variety in reported frequencies, cohort studies show transmission of resistant strains of around 3 – 16% for NRTIs, 0 – 4% NNRTIs and 1 – 18% for PIs[262-266]. Some studies report a high frequency of the transmission of strains with mutation M184V, the rapidly emerging mutation associated with high level resistance to 3TC and low level resistance to abacavir, ddC and ddI[266,267].

The study reported here was designed to assess the level of genoptypic resistance in a well described cohort of recently infected individuals and to estimate the likelihood of transmission of a drug resistant strain. This is also a pilot study for resistance testing during seroconversion, and will demonstrate how viable this strategy would be.

5.3.2 Patients and Methods

Clinical assessment of patients attending the ICDC clinic, Royal Free Hospital, London, selected a cohort of 47 as having recently been infected with HIV-1 (patients described in section 5.1 and 5.2). Disease status was further characterised by Western Blot analysis of anti-HIV-1 antibody profiles. Blood was taken at the first clinic visit and separated within 6 hours. The plasma was frozen and stored at -70°C.

HIV-1 RNA was extracted using a QIAmp® viral RNA mini kit (QIAGEN Ltd, Crawley, West Sussex). The HIV-1 RNA was then reverse transcribed and the RT and Pr genes amplified in a nested PCR according to in-house methods (see section 2.3.3). Once a positive DNA band was identified by agarose gel electrophoresis, both regions

were purified using a QIAquick® PCR purification kit (QIAGEN Ltd, Crawley, West Sussex). The DNA was then put into a sequencing reaction, purified and finally loaded onto a polyacrylamide gel and sequenced using an ABI PRISM® 377 DNA Sequencer (see section 2.4.4).

DNA sequences were analysed for resistance mutations using ABI PRISM® Sequence Navigator™ software. RT and Pr gene sequences were compared to wildtype reference sequence HXB2 and analysed for mutations or polymorphisms associated with resistance cited in the Los Alamos Resistance Database[268]. Definitions of primary or secondary resistance were defined according to the recommendations of the International AIDS Society-USA[269].

5.3.3 Results

Forty-five out of the 47 patients were further characterised as being between 0 – 5 months of PHI by Western Blot analysis or by a recalled risk event. Forty out of the 45 had incomplete antibody profiles characteristic of PHI.

Forty-one out of the 47 patients had samples available from the first clinic visit, all samples were extracted and amplified by in-house RT- PCR. Three samples failed to amplify in either RT or Pr regions after three separate attempts. Two of these patients had low viral loads, patient 25 was undetectable (<2.3 $\log_{10}$ copies/ml) whilst patient 29 had a viral load of 3.72 $\log_{10}$ copies/ml, both values are unusually low for patients undergoing PHI. The low values may be accurate or they may be falsely low due to diverse viruses (see section 3.5). However, PCR is able to amplify low amounts of DNA. A failure of reverse transcription may have occurred, the fidelity and reliability of reverse transcription is much less than that of PCR . Patient 10 had a substantial viral load of 4.98 $\log_{10}$ copies/ml which should have been possible to reverse transcribe

and PCR amplify. The sequence of this viral genome may be incompatible with the PCR and/or sequencing primers. The viral load was quantified by Nuclisens and if this was a diverse subtype the expected Nuclisens value would be much lower (see section 3.5). However, the Nuclisens primers bind in *gag* and the PCR and sequencing primers bind in *pol* so the possibility of a good binding of the Nuclisens primers and the virus, whilst having a mismatch with the PCR primers remains a distinct possibility.

The remaining 38 samples were amplified and sequenced for one or both regions. Five samples failed to amplify in RT or Pr on the first attempt but a positive PCR result was obtained after repeated amplifications. Thirty-four samples were sequenced in both RT and Pr regions. Four samples failed to amplify in the RT region and so were sequenced in the Pr region only.

Number of mutations associated with all three drug classes (NRTI, NNRTI and PI) are shown in Table 5-10.

Table 5-10 Frequency of mutations associated with all three drug classes in patients with PHI.

Drug Class	PI	%	NRTI	%	NNRTI	%
No. of patients with primary mutations :	0	0	5	15	2	6
No. of patients with secondary mutations :	21	55	3	9	2	6

Appendix III shows the resistance mutations in the RT gene selected by NRTIs. Out of 34 patients sequenced in RT, five patients (15%) had one or more primary mutations associated with NRTIs. Three patients (9%) had secondary NRTI associated mutations. One patient (3%) had two primary NRTI associated mutations. There was an average of 0.32 NRTI mutations per person. The percentage of patients and the number of NRTI mutations are shown in Figure 5-4.

Observed primary mutations associated with NRTI resistance were T69N, K70R, M184V and T215Y. Secondary mutations included M41L, L210F and T215D or T215E.

Appendix IV shows the resistance mutations in the reverse transcriptase (RT) gene associated with NNRTIs. Figure 5-5 shows the percentage of patients with the different NNRTI mutations.

Two patients (6%) had one or more primary mutations, a further two patients (6%) had one secondary mutation. There was an average of 0.24 NNRTI mutations per person. Primary mutations associated with NNRTI resistance observed were K103N, V108I and G190A. The only secondary mutation observed was A98S.

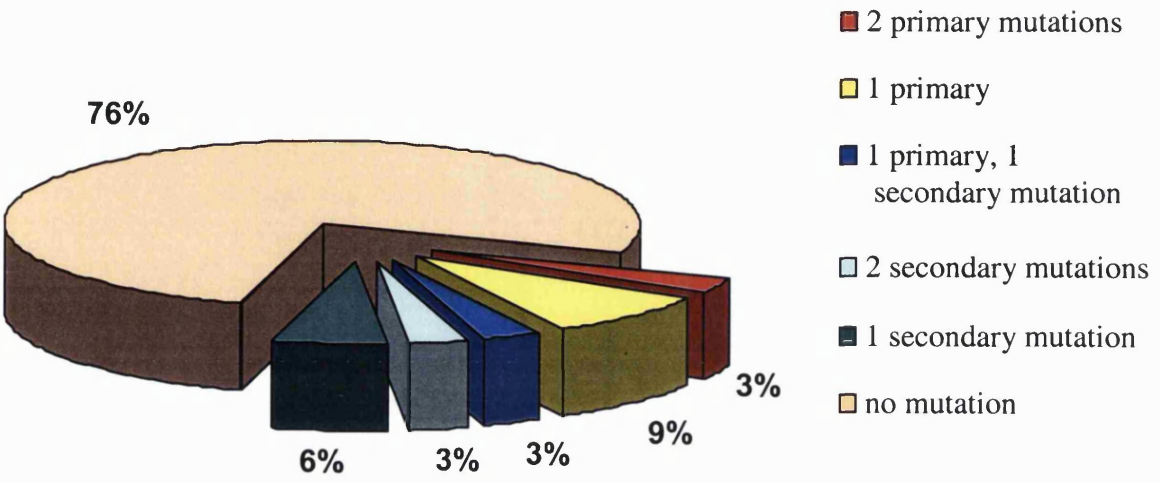


Figure 5-4 Breakdown of mutations associated with NRTI resistance in PHI patients.

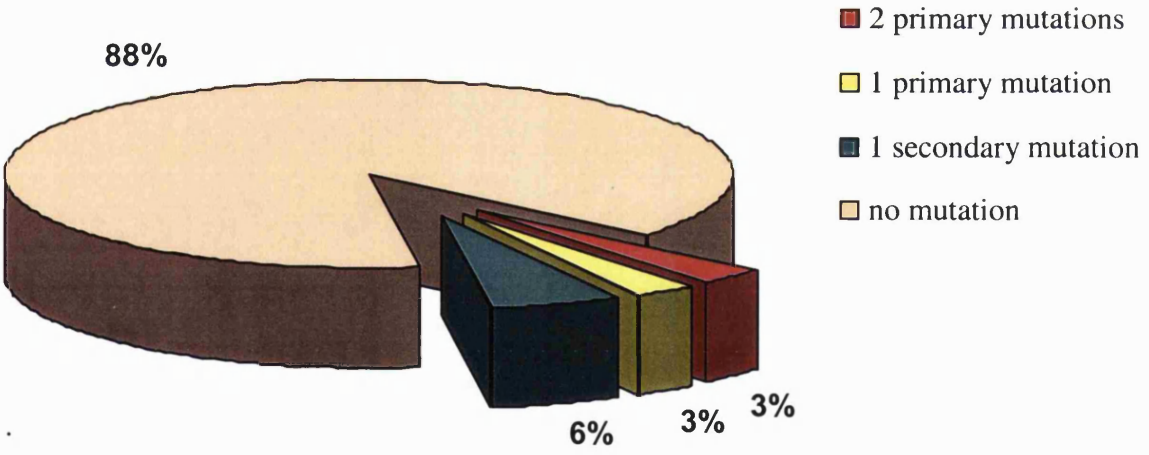


Figure 5-5 Breakdown of mutations associated with NNRTI resistance in PHI patients.

The distribution of observed mutations in RT, associated with both drug classes, is shown in Figure 5-6.

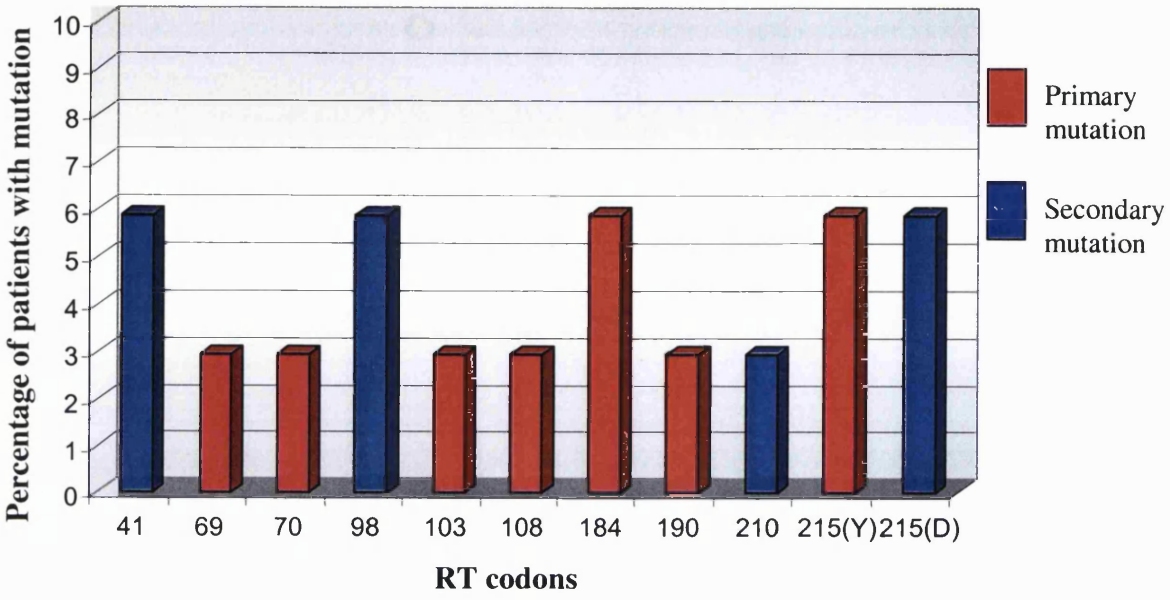


Figure 5-6 Distribution of mutations associated with RT resistance in 34 PHI patients.

Only two (6 %) patients had the prevalent M184V mutation. Three patients (9 %) had AZT-associated primary mutations. Two (6 %) patients had mutations that conferred cross-resistance to more than one NNRTI.

Appendix V shows the number of resistance associated mutations in the Pr region for the 38 samples sequenced. Twenty-one (55%) patients had one or more secondary protease mutations, but no primary mutations associated with PI resistance were observed in this study. Seventeen (45%) patients had wildtype virus with no known resistance mutations. Figure 5-7 shows the percentage of patients with one or more secondary mutations associated with PI resistance.

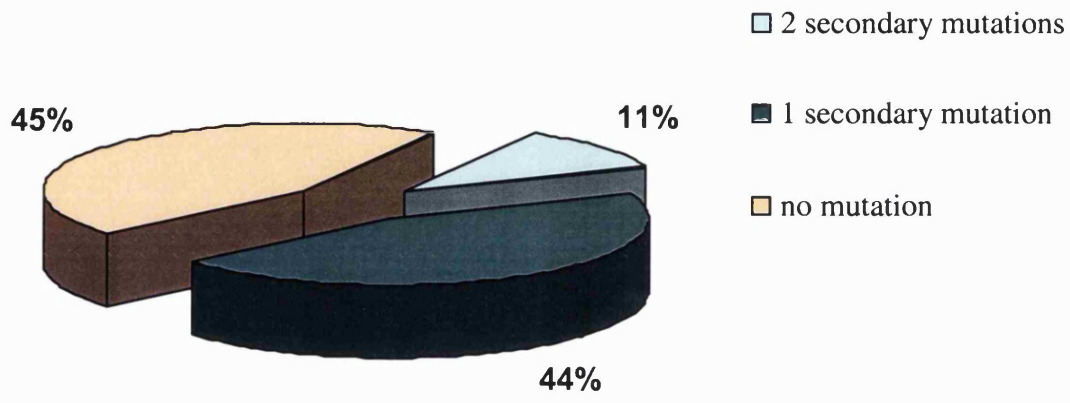


Figure 5-7 Breakdown of mutations associated with PI resistance in PHI patients.

Eleven patients (32%) had the V77I mutation associated with secondary resistance to indinavir, ritonavir, nelfinivir and saquinavir. Other secondary mutations observed in Pr included L10V/I, K20M, L33V, M36I and A71T/V. Overall, there was an average of 0.66 mutations and a median of 1 mutation per person. Figure 5-8 shows the number and type of mutations observed in Protease.

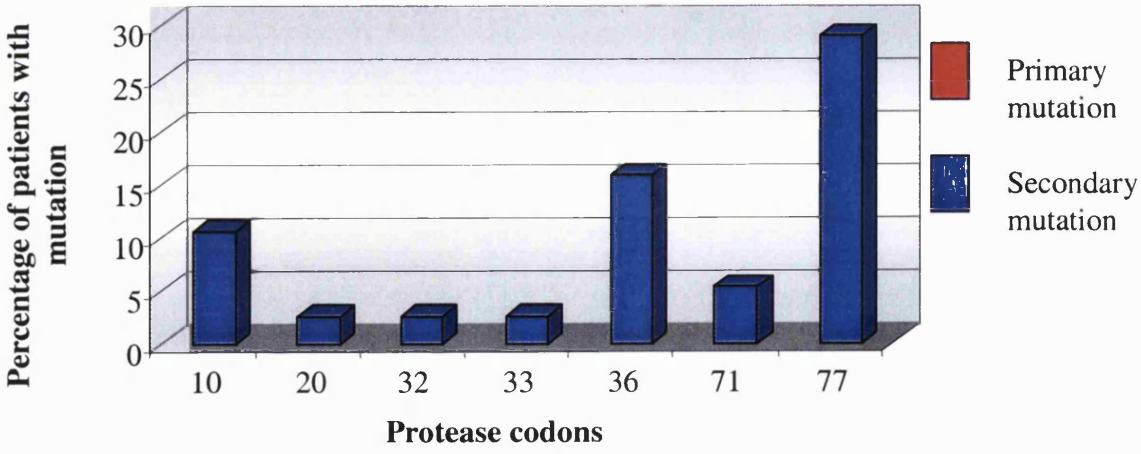


Figure 5-8 Distribution of mutations associated with PI resistance in 38 PHI patients.

The Pr gene was amplified in four samples (Patients 10, 32, 45 and 47, appendix V) where it was not possible to amplify RT. BLAST analysis was performed on these samples using an internet database of current HIV-1 subtypes[270]. Three out of four of these samples were defined as subtype C from analysis of the Pr gene. However, due to the short length of the gene (297 base pairs) a definition of non-B subtype could not be confirmed. Three out of four patients were infected by a partner from either Ethiopia or Zimbabwe.

Overall, there was an average of 1.07 mutations per person, with a median mutation frequency of 1 per person.

A number of mutations were observed which are not currently associated with resistance. Table 5-11 shows the most prevalent polymorphisms observed in this cohort.

Table 5-11 Mutations not currently described to be associated with resistance observed in PHI patients

Codon	Gene	Number of patients	%
63	Pr	28	73.7
122	RT	22	64.7
179	RT	3	8.8
214	RT	27	79.4

5.3.4 Discussion

The overall prevalence of key resistance mutations in this population was 16%, comparatively low when compared with the prevalence described in the USA of rates as high as 21 %[271] and 28%[272]. However, this study appears to correlate with the lower frequencies of transmission of resistant virus seen in Europe and the UK, where the prevalence of transmission ranges from 5%[260] to 10%[261].

The frequency of transmission of virus resistant to NRTIs showed 15% to have primary resistance-associated mutations while 24% had primary and secondary or secondary only. The greatest number of NRTI mutations were associated with AZT resistance, 9 % of patients had a primary AZT mutation associated with resistance. This reflects the widespread use of AZT in the infecting population. In a similar study of 82 Swiss seroconverters seven patients (9 %) were observed to have AZT resistance-associated mutations[261]. A previous study of UK seroconverters showed AZT resistance associated transmission to be in only one patient out of 20 (5 %)[260].

Of 34 patients sequenced in RT, 6 % had primary NNRTI resistance mutations, with 12% having primary and secondary or secondary only associated with NNRTIs. One patient (3 %) had two primary resistance mutations associated with NNRTI use. Previous studies have reported NNRTI resistance transmission to be low, the Swiss study saw NNRTI resistance-associated mutations in only 2 out of 82 PHI patients (2 %)[261] whilst a UK PHI study did not observe any NNRTI resistance-associated mutations[260]. One patient had primary resistance mutations associated with two classes of drugs, NRTI and NNRTI.

Of 38 patients sequenced in Pr, 55 % showed secondary mutations associated with PI resistance. No primary mutations to PIs were observed in this study. This is comparable with previous studies showing low prevalence of PI resistance in PHI populations[260,261]. The Swiss study observed PI primary resistance-associated mutations

in 3 out of 82 patients (4 %)[261] whilst no primary resistance mutations associated with PI use were observed in a previous UK study[260]. The high level of secondary mutations in Pr observed in this study was also observed in previous studies[260,261], the natural polymorphism of Pr has been reported and it is unlikely that the presence of a PI secondary mutation alone could confer reduced susceptibility to PI use.

A number of mutations not directly associated with resistance was observed across the population. Polymorphism L63P was very prevalent in this population, indicating that this may be a wildtype polymorphism evolving away from the reference HXB2 strain. However, L63P is emerging with second generation PIs, suggesting that it may play a role in the evolution of PI resistance[273].

There was a high incidence of polymorphisms in codons adjacent to resistance sites. Mutation L214F in RT was observed in 79.4% of the population. This mutation and others (RT 98 and 179) could have implications for viral fitness. Two possible explanations suggest themselves. Possibly the mutations surrounding important resistance sites reflect a structural change facilitating the increase of replicative capacity in the presence of drug and drug-associated resistance mutations, or, these mutations are a result of the genetic evolution of HIV-1 away from a previous 'wildtype' species, and are not associated with drug resistance, posing no direct threat to HAART.

This study was completed retrospectively after some of the patients were recruited into antiretroviral therapy studies. Successful treatment during PHI relies on choosing a highly potent regimen. The presence of resistance mutations in the newly infected patient could jeopardise the potency of the regimen. Six patients (16%) had one or more primary mutations associated with antiretroviral resistance in their baseline sample. As described previously in section 5.2.3, a sizeable percentage of PHI patients are electing to start antiretroviral therapy within 5 weeks of infection. In this instance resistance testing would have been relevant for six patients (16 %) who had genotypic

evidence of reduced susceptibility to antiretrovirals. Currently the cost of resistance testing does not permit routine genotypic testing of all PHI patients. However, this study has shown that genotypic testing may offer some guidance to clinicians on selecting the most potent therapy available.

5.4 Viral genetic diversification during HAART in two patients infected by a common source.

5.4.1 Introduction

During primary HIV-1 infection (PHI), the virus is present as a relatively homogenous population, closely related to the virus transmitted during the infecting event[59]. Once the infection becomes established, a wide variety of virus genotypes (quasispecies) evolves[59]. Sequence variation is a result of the inaccuracy of reverse transcriptase in transcribing HIV-1 RNA to DNA[274,275]. The error rate is typically one nucleotide per 5900 in the DNA template[275], at least one nucleotide change per replicative cycle. The extent of this evolution is exacerbated by the rapid turnover of HIV-1 during PHI[59]. If a successful HAART regimen is administered during PHI it should suppress viral replication and thereby viral evolution. However, in creating a drug intensive environment to prevent viral replication, the regimen is also creating selective pressure for drug resistant mutants to arise as long as residual replication is still able to occur. The generation of resistance reflects the extent to which HAART has inhibited viral replication. If no resistance mutations occur, viral evolution has been suppressed by effective inhibition of viral replication.

This study investigated viral genetic evolution in two patients who had acquired their infection from the same source at the same time. Sequence analysis of their viral populations compared the degree of genetic relatedness between the virus in the baseline samples from each patient. Then patients were monitored virologically over an 18 month period and at 16 months their virus was sequenced for comparison of evolution. At both time points, baseline and 16 months, sequence analysis included

consensus and single copy sequences to compare the divergence in minority species of the viral population.

5.4.2 Patients and Methods

Patient 1 and patient 2 were established homosexual partners who presented with PHI syndrome following a simultaneous sexual exposure to a casual partner in the Canary Islands. Both patients had a detectable HIV-1 RNA load less than a week after infectious exposure. Both patients were also tested using Western Blot demonstrating that both had an evolving immune response characteristic of early PHI (results shown in PHI cohort appendix I, patient 1 is referred to as patient 14, patient 2 is referred to as patient 19). They received AZT, 3TC and indinavir at approximately 2 weeks after exposure. Blood samples were taken at presentation approximately 7 days after exposure, and at regular intervals over the next 18 months. Plasma was separated and stored at -70°C. Plasma viral load was quantified by NASBA and repeated using Nuclisens which had a lower detection limit of 2.6 $\log_{10}$ copies/ml. A mean of both quantification results was used. Where viral load fell below detectability the virus was quantified using RT-PCR, which had a lower detection limit of 1.7 $\log_{10}$ copies/ml. HIV-1 RNA was extracted using a QIAmp® viral RNA mini kit (QIAGEN Ltd, Crawley, West Sussex). The HIV-1 RNA was then reverse transcribed and the RT and Pr genes amplified in a nested PCR according to in-house methods (see section 2.3.3). Once a positive DNA band was identified by agarose gel electrophoresis, both regions were purified using a QIAquick® PCR purification kit (QIAGEN Ltd, Crawley, West Sussex). The DNA was then put into a sequencing reaction, purified and finally loaded onto a polyacrylamide gel and sequenced using an ABI PRISM® 377 DNA Sequencer (see section 2.4.4).

Single copies were produced using a previously described end point dilution series (see section 2.3.5). A single heterogeneous RNA extract was diluted to the point at which only single copies could be amplified in a first round RT-PCR, these single copy samples were then amplified in a second round of PCR and sequenced. RT and Protease sequences were compared to the wildtype HIV-1 reference sequence HXB2 for analysis of mutations.

5.4.3 Results

Both patients had baseline viral loads in excess of 6 $\log_{10}$ copies/ml, patient 1 (P1) had 6.14 $\log_{10}$ copies/ml and patient 2 (P2) had 7.11 $\log_{10}$ copies/ml. Both patients maintained a high viral load until initiation of HAART. Patient 1 started HAART five days after first presentation, whilst patient 2 started HAART 7 days after first presentation. For both patients initiation of HAART was within 14 days of exposure. Viral loads for both patients, before and after initiation of HAART are shown in Table 5-12 and Table 5-13 and in Figure 5-9.

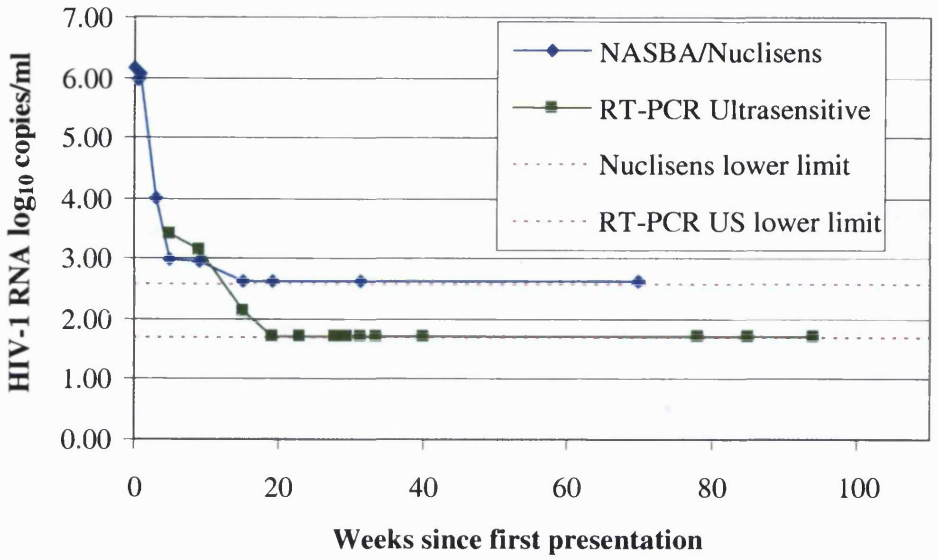
Table 5-12 Plasma HIV-1 RNA in patient 1 following infection and onset of HAART.

	HIV-1 RNA log_{10} copies/ml	
Weeks from presentation	**Mean NASBA/Nuclisens**	**RT-PCR ultrasensitive**
0	6.14	
3 days	5.96	
Onset of HAART → 1	6.04	
3	3.99	
5	2.99	3.40
9	2.96	3.15
15	<2.60	2.12
19	<2.60	<1.70
23		<1.70
28		<1.70
29		<1.70
31	<2.60	<1.70
34		<1.70
40		<1.70
70	<2.60	
78		<1.70
85		<1.70
94		<1.70

Table 5-13 Plasma HIV-1 RNA in patient 2 following infection and onset of HAART.

	HIV-1 RNA log_{10} copies/ml	
Weeks from presentation	**Mean NASBA/Nuclisens**	**RT-PCR ultrasensitive**
0	7.11	
3 days	6.35	
Onset of HAART → 1	5.74	
3	3.70	
5	3.00	3.66
9	3.04	3.23
15	<2.60	2.50
23	<2.60	<1.70
25	<2.60	<1.70
28		<1.70
30	<2.60	1.74
32	<2.60	<1.70
34		<1.70
40		<1.70
46		<1.70
58	<2.60	
70	<2.60	
78		<1.70
85		<1.70
94		<1.70
105		<1.70

Patient 1



Patient 2

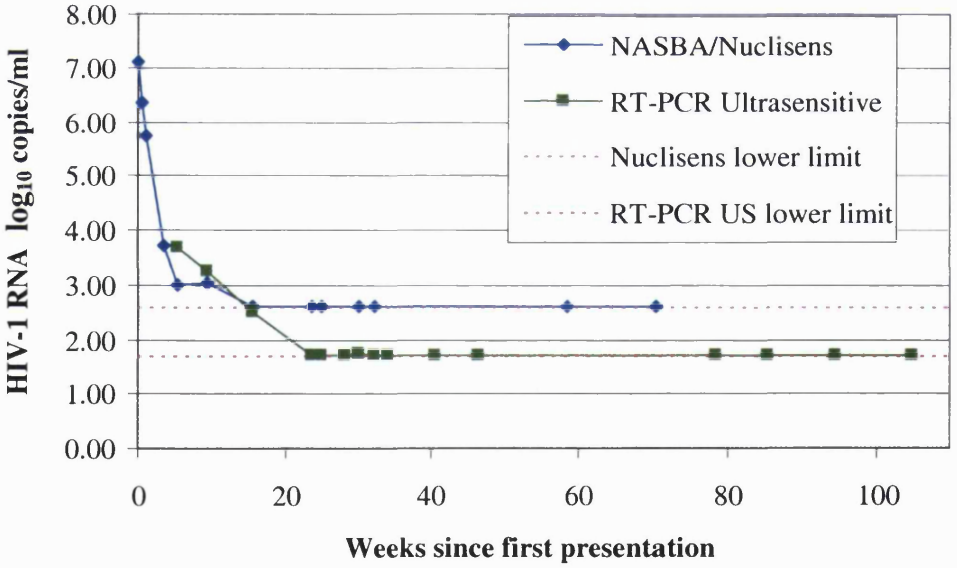


Figure 5-9 Virological profile of patients 1 and 2 following infection and onset of HAART.

Both patients had a rapid decline in viral load on initiation of HAART. Patient 1 had a decline of 2.05 $\log_{10}$ in just over two weeks from starting HAART. Although patient 2 had a higher baseline viral load, he too showed a similar decline of 2.04 $\log_{10}$ copies/ml in the first two weeks of treatment.

Consensus sequence analysis of baseline samples revealed that neither patient carried any primary mutations associated with drug resistance. However, both patients had mutation L214F. There was a single base difference in RT at codon 122; P1 had the GAA wildtype giving amino acid E, whilst P2 had AAA, giving the amino acid change E122K. In the Pr gene both patients had differences from wildtype (CTC) at codon 63, although neither showed the common polymorphism L63P. Patient 1 showed L63S (CTC to TCC) whilst patient 2 had L63H (CTC to CAC).

Single copy sequences at baseline revealed no other mutations differing from the consensus sequences in either patient. Overall, there was less than 1% divergence between sequences of P1 and P2 in RT andPr. Thus, at this early disease stage, the evolution due to a different host pressure was not apparent.

Consensus sequencing at 16 months showed no new mutations associated with drug resistance. The L214F mutation was still present in all sequences analysed in both patients. The only difference from baseline sequence was seen in the Pr of P2. Consensus sequencing showed a mixed base population in P2 at codon 63, previously at baseline this had the base sequence CAC. Figure 5-10 shows the mixed population observed in the electropherogram. Single copy sequences isolated two populations differing at base 63. The codon had a mixed population of bases CAC or TCC, giving amino acids H and S. The isolated populations are shown in Figure 5-11 (a) and 5-11 (b).

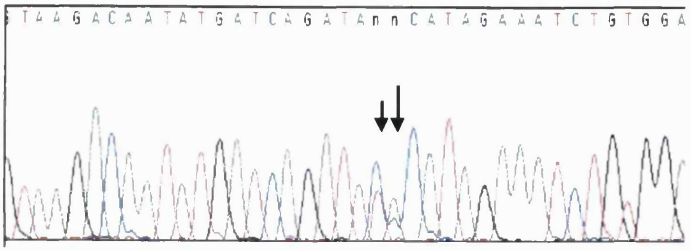


Figure 5-10 The mixed base population observed in the consensus sequence electropherogram for Patient 2 at 16 months

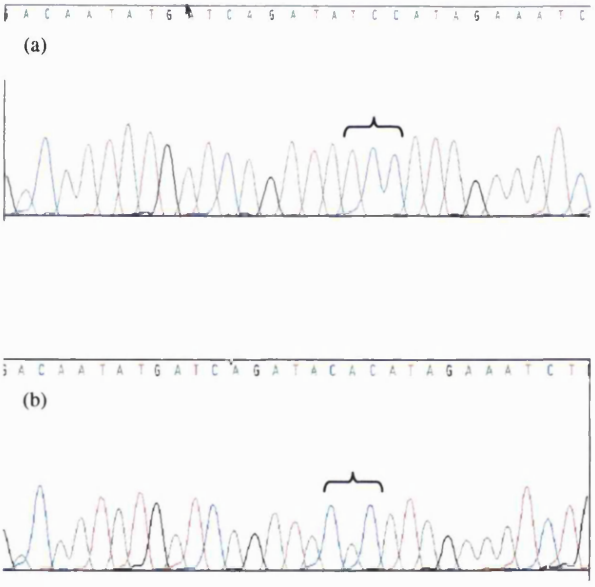


Figure 5-11 Single copy sequences demonstrated two different viral species in patient 2 at 16 months, shown as (a) and (b).

Patient 2 has a mixture of two viral populations (a) and (b), (a) has base sequence TCC (serine), the second population (b) has base sequence CAC (histidine), at codon 63. This base sequence gave a population mixture of 63S and 63H in the single viral copies. Previously at baseline all single copies had the base sequence CAC, 63H.

There were no other new mutations in either patient at 16 months. An example of sequence alignment with the reference sequence of HXB2 can be seen in Figure 5-12.

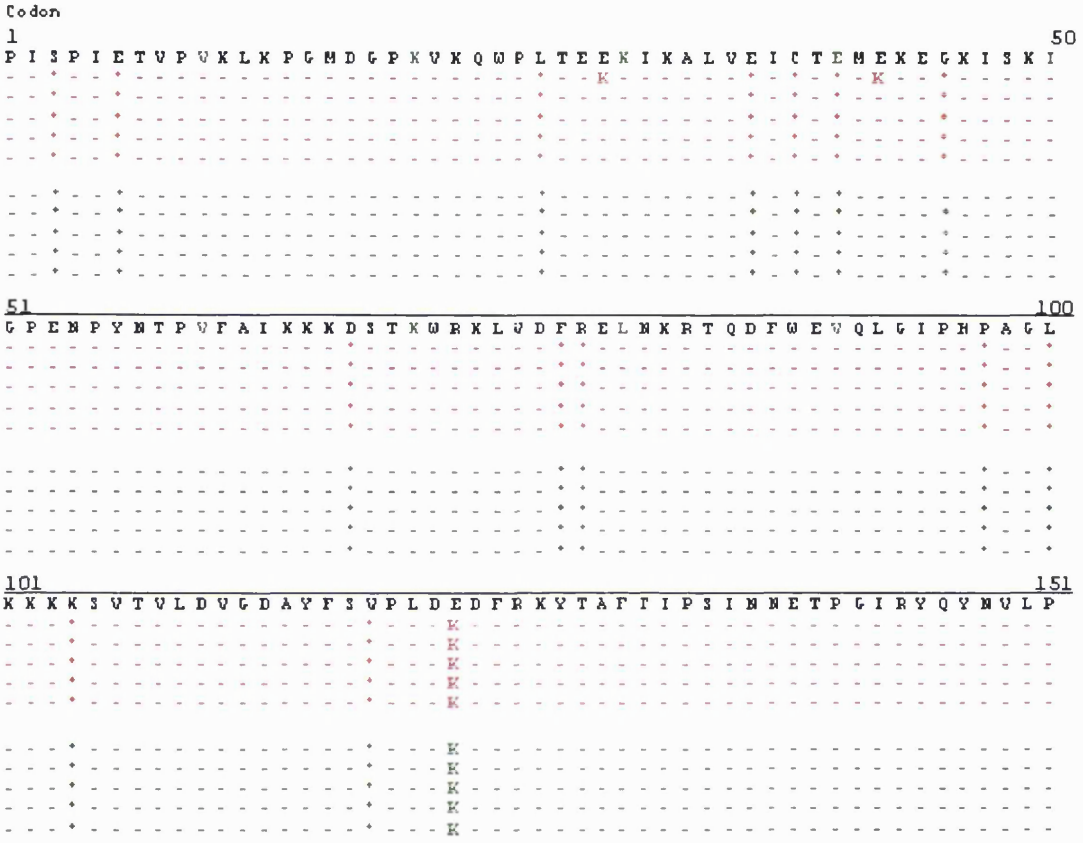


Figure 5-12 Protein sequence alignments of the first 150 codons of RT from 5 single copy sequences of P2 at baseline (red) and 16 months (green) against the reference wildtype sequence HXB2.

Asterisk * indicates a base change within the codon (silent mutation not affecting the amino acid). Dashes indicate homology with reference sequence HXB2. P1 sequences from 5 single copies (at baseline and at 16 months) were wildtype at codon 122 but otherwise were identical and are not shown here.

5.4.4 Discussion

There was less than 1 % sequence difference between viral populations of P1 and P2 at baseline and after 16 months. Consensus sequencing demonstrated that both patients had very close sequence similarity at baseline. The lack of viral evolution in either patient over 16 months following the onset of HAART meant that this sequence similarity was maintained.

In this study consensus sequencing gave a realistic and accurate representation of all viral populations present. Single copy sequencing showed no more minority species than had been present in consensus sequencing. Where a mixed population was present (in P2 codon 63 of Pr at 16 months) consensus sequencing demonstrated the different viral species as mixed bases at codon 63. Single copy sequencing then identified the individual populations. Single copy sequencing detects individual virions and may have a better chance of detecting a minority species than consensus sequencing. However, single copy sequencing is more costly and takes more time in comparison to consensus sequencing.

Occasionally the minority species may carry resistance and here identification becomes critical. However, in this study, consensus sequencing adequately detected both populations present, the electropherogram showed a mixed base population, although it was not possible to read the different codon sequences. If this had been a primary resistance site it would have been possible to repeat single copy sequencing to isolate the different populations and give a better description of potential resistance. This study showed that consensus sequencing is capable of identifying mixed populations, and is much quicker and more cost effective than single copy sequencing.

Neither patient showed any primary mutations associated with resistance at baseline or at 16 months. The rapidly emerging lamivudine-associated mutation M184V in RT was not seen in either patient implying that viral replication was significantly attenuated so

that evolution was not able to occur. If evolution had been possible the M184V mutation may have been selected for. However, it is equally possible that three drugs in combination, regardless of potency, create a difficult selective pressure, requiring HIV-1 to produce more mutations balancing fitness and drug resistance. Therefore any resistance would take longer to occur.

Aside from the lack of resistance-associated mutations, very few non-resistance mutations arose during therapy. This indicates a lack of evolution as a result of almost complete suppression of viral replication. This clarifies the information given by viral load quantification. It also implies that whilst viral load is undetectable (< 1.7 $\log_{10}$ copies/ml) little or no replication is occurring in these patients, characterised by a lack of viral evolution.

This study and others[248,276], looking at HAART during PHI, have shown that early intervention with antiretroviral therapy reduces viral load to undetectable levels in a significant proportion of patients[277,278]. Whilst viral load remains undetectable the likelihood of resistance evolving is low. Therefore, it is possible to use viral load as an indicator of the probability of resistance emergence. However, it remains to be seen whether viral load can predict resistance, or whether resistance occurs first, characterised by the resulting rise in viral load. For ease of monitoring, viral load is more accessible and can be performed more frequently than resistance testing.

Chapter 6 Discussion

In November 1997 the Department of Health and Human Services of the USA issued guidelines regarding the clinical healthcare of HIV infected patients. In these guidelines a panel of experts stated that viral load testing was "the essential parameter" in decisions to initiate or change antiretroviral therapy[234]. The panel also agreed that viral load had a much stronger correlation with disease progression than CD4 cell counts, and later that year the Food and Drug Administration recommended that plasma HIV-1 RNA levels be used as primary endpoints in clinical antiretroviral studies[279]. In the intervening years viral load quantification has been improved and expanded to support clinical decisions. However, there is still a need to continually evaluate viral load and its inter-relationships to understand how combined measures may best assist in HIV patient management.

This thesis has investigated and validated the ongoing development and applications of viral load assays, in particular the NASBA method of quantifying HIV-1 RNA. However, RT-PCR and bDNA systems were also appraised with reference to subtype B and non B subtypes: both assays detecting more non B subtype viruses when compared to NASBA. New versions of all three assays were tested and reviewed at this site. The panel of non-B subtype viruses has been a valuable tool for investigating the ability of all the assays in quantifying diverse viruses, particularly as the frequency of these viruses is increasing in Europe and North America. The ongoing development of viral load assays must take epidemiological factors such as this into account.

For HIV-1 subtype B well above the assay detection limit, quantification of virus was comparable for all three assays, NASBA/Nuclisens, RT-PCR and bDNA. When non-B subtypes were excluded viral RNA values for the same sample quantified by two or

more methods differed by a maximum of 0.86 $\log_{10}$ copies/ml. This demonstrates that continuity in the choice of assay is very important in virological monitoring of HIV infection. If virological quantification methods are interchanged, a disparity of up to 1 $\log_{10}$ copies/ml may be attributable to inter-method variation. Excluding non-B subtypes, differences between NASBA/Nuclisens and RT-PCR values for the same sample rarely exceeded a difference of 0.5 $\log_{10}$ copies/ml. However, differences were apparent between NASBA and bDNA and, to a lesser extent, between RT-PCR and bDNA. In all of the studies comparing all three types of quantification technique, regardless of subtype, the bDNA produced values slightly lower than either RT-PCR and NASBA. This was most probably due to the similarity between the RT-PCR and NASBA in methodology, as opposed to that of bDNA, and is confirmed in similar studies[181,185].

The evaluation of virological quantification of non-B subtypes demonstrated distinct variation in values dependent on subtype and method. NASBA gave low estimates for samples of subtype C, F, G and H compared to the other three assays RT-PCR, RT-PCRnb and bDNA. Whereas NASBA quantified subtype A samples gave comparable values. Subtype D samples gave higher values in the NASBA than in any other assay. This study highlighted differences between the quantification methods in the sites selected within more or less conserved regions on the HIV genome for primer/probe binding. The target region of the HIV-1 genome specific for each assay has a significant impact on which subtypes are preferentially quantified. The first generation of RT-PCR and NASBA HIV-1 quantification assays preferentially amplified and quantified HIV-1 variants of subtype B and D, with variable success in quantifying more diverse subtypes A and C, and limited success with F, G and H. The first generation bDNA assay (version 2.0) by comparison, in this study and others, performed better with a larger range of diverse subtypes[181,201]. The additional primers

used in this investigation did show improved quantification of diverse subtypes and this has been confirmed in other studies[174,193,193,197]. The level of improved detection seen justified the development of a new version of Amplicor HIV-1 Monitor, version 1.5, incorporating the new primers. The clinical verification of the success of the new primers was vital in their overall evaluation. This study was an example of how collaborations between manufacturers and clinical users continue to drive the development of HIV-1 quantification assays and justify their increased use in the clinical field.

The assays have been investigated for reproducibility of results and for their ability to quantify low copy numbers of viruses. A number of studies evaluated the performance of NASBA, comparing it to alternative quantification systems, like the RT-PCR, or by comparing results against those of other disease markers. NASBA was not as sensitive as either the RT-PCR or bDNA in quantifying HIV-1 RNA in samples with low copy numbers. These studies show that the development of the Nuclisens assay improved assay sensitivity, detecting virus to a lower level of 2.6 $\log_{10}$ copies/ml. This sensitivity was further increased by using a larger volume of plasma, with virus in one sample being detected at 1.61 $\log_{10}$ copies/ml, demonstrating that the assay was capable of detecting virus at the calculated theoretical lower level of 40 copies/ml. This is comparable to the current versions of RT-PCR Ultrasensitive and bDNA which detect to a lower level of 1.7 $\log_{10}$ copies/ml. However, the most sensitive Nuclisens assay with increased plasma input was found to be technically difficult to process due to the required larger volumes of plasma (2mls) and lysis buffer (9mls). This protocol is now the standard plasma input for samples containing low copy numbers of virus. The studies described in this thesis verified that Nuclisens was capable of detecting down to the theoretical lower limit of 1.7 $\log_{10}$ copies/ml and this information was reported to Organon as feedback of our collaborations. Evaluations of assay proficiency and of

new assay developments allow manufacturers to improve upon existing procedures and performance, and to demonstrate this in a clinical rather than company setting.

A number of studies presented here showed that the accuracy of quantification of low copy number virus was less using NASBA and Nuclisens than with RT-PCR and bDNA. The ENVA panel analysis demonstrated that in NASBA the standard deviation of quantified viral load increased significantly with decreased viral load. Evaluation of the Nuclisens assay, demonstrated improved sensitivity, but also identified problems quantifying at low RNA copy numbers. In the study comparing Nuclisens and RT-PCR quantified virus in samples from patients starting HAART, the Nuclisens detected virus in two out of five patients who had started treatment, compared to detection in five out of five patients using the RT-PCR. Virus which was detected using the RT-PCR (with a theoretical sensitivity of 2.6 $\log_{10}$ copies/ml) remained undetectable using the most sensitive Nuclisens which had a theoretical sensitivity of 1.6 $\log_{10}$ copies/ml. Low level viraemia, particularly in patients on HAART, is of clinical relevance because of the implications for the evolution of resistance. The detection and possible quantification of virus at very low copy numbers will highlight this disparity between assay sensitivities and any method which allows reliable quantification of virus at levels < 1.7 $\log_{10}$ copies/ml should be preferentially selected for clinical trials and patient care.

An existing protocol for in-house viral quantification was modified and investigated as a viable alternative. The use of antibodies bound to latex microparticles to extract HIV-1 from plasma was evaluated and although the antibody binding itself was successful the method, and in particular the use of latex microparticles as the medium was found to be technically demanding and gave variable results. Variable and often high background in the detection system precluded further study using a probe conjugated to alkaline phosphatase. However, the production of luminescence was very sensitive, producing

very high counts when DNA was present, so that when background remained low there was a large range over which DNA was detectable. Although the modified in-house assay was not endorsed by this evaluation, there is clearly still a need to develop cost-effective viral load quantification, particularly for use in the developing world. It is hoped that this centre will continue to research possible projects for use in areas where viral load quantification would aid research, but is not a financial possibility. However, any assay for use in the developing world must be optimised for a wide range of diverse subtype viruses. This challenge has impeded assay development by both commercial and research groups.

Viral load was evaluated compared to the surrogate disease marker p24 antigen (Ag). In this study the p24 antigen assay appeared to detect only a third of the population and this reduced the possibility of statistically correlating viral load with p24 antigen levels. In patients with primary HIV-1 infection (PHI) there was a positive correlation between p24 antigen levels and viral load where both measures were quantifiable ($r = 0.86$), and this was less apparent in patients with chronic infection ($r = 0.06$). In PHI the level of p24 antigen is the product of a dynamic process dependent on the evolving immune response and the generation of antibodies. New versions of the p24 antigen assay cite improved sensitivities, but there is a limit to the amount of p24 antigen that these can detect. Secondly, our experience suggests that neutralisation shows specificity to be limited in all versions of this assay. If p24 antigen measures are to be utilised as an indirect measure of viral load in countries where viral load quantification is too costly, the p24 assay sensitivity must be improved such as has been demonstrated with newer versions of the assay[206]. More research is needed into the correlation of viral load and p24 antigen and the relationship of both to disease progression and therapeutic efficacy. Alternative disease markers such as p24 antigen, should not be recommended for use in developing countries purely on the basis of cost alone. It may be better to wait until the

cost of viral load assays themselves is reduced, rather than install a system that may not be as accurate or is only a poorly correlated indirect measure of viral RNA. This research suggests that it would be very difficult to use p24 antigen measures to indicate viral load changes in order to monitor HIV-1 disease or antiretroviral therapy. Nuclisens, the new version of NASBA, improved the sensitivity of detection of HIV-1 RNA but did not include any changes to the primer binding sites and so preferentially detected the same population of viruses as NASBA. The failure of NASBA/Nuclisens to detect diverse viruses has been demonstrated previously[181,185,200], and yet this issue has still to be addressed. This is of particular importance in the light of growing evidence of increased subtype diversity in the UK and USA[39] and the increased use of viral quantification in the third world, where subtype diversity is greatest.

The study evaluating NRTI therapy in AZT-experienced versus AZT-naïve patients, found that the decrease in viral load upon NRTI initiation was significantly more pronounced in patients with no prior experience of AZT than in those previously treated with AZT. When pharmacological data of the AZT metabolites was evaluated and compared to viral load, it became apparent that the difference in virological response was not due to incomplete metabolism of AZT to the active form. Therefore, this study confirmed previous findings that patients starting a NRTI regimen showed a much more vigorous initial response if they were therapy naïve[165]. However, the cause of the reduced impact of therapy in the AZT-experienced patients studied here was not determined. The lack of efficacy may have been due to the presence of resistance-associated mutations in the AZT-experienced patients. If resistance-associated mutations were evolving in the AZT-naïve patients from the start of therapy, the mutations may have taken some time to reduce the sensitivity of the virus to the drug, explaining why these patients show good initial virological response that diminishes after 30 weeks. If this were the case, genotypic testing of patients of both groups before

and after would have demonstrated resistance as the differential factor predicting initial response, unless resistance mutations were only present in minority species. Unfortunately, due to the copious amount of plasma required for pharmacological monitoring and viral load testing, there was insufficient plasma available for genotypic testing. Ultimately however, pharmacological analysis and virological response demonstrated there was a significant difference between the groups, but incomplete AZT metabolism, inhibited by prior AZT exposure, was not the differential factor in this trial.

Virological response to therapy was the major determination of therapeutic success in the investigation of pharmacological metabolism. In the characterisation of the seroconverter cohort, viral load was used to investigate diagnosis during the window period, viral evolution and viral replication rates (with and without antiretroviral therapy) throughout the course of primary HIV-1 infection. The first of these studies looked at the detection and quantification of viral load in patients with an incomplete antibody profile as characterised by Western Blot. This study confirmed that there are cases of seroconverters who demonstrate a quantifiable viral load prior to diagnosis by conventional serology. This study also demonstrated that the majority of patients develop p24 antibodies as the early phase of immune response. In 1996/1997 when this study was done, the majority of serological tests were designed to detect antibodies to gp41. However, a proportion of patients developed other antibodies first, such as to gp160 or to gp120. Viral load was detected prior to p24 and gp41 antibody development in certain patients, demonstrating that viral load could be a valuable diagnostic tool during the seroconversion window period. Viral load testing has already demonstrated good specificity and sensitivity in determining perinatal HIV-1 infection of new-born babies born to infected mothers[58,171,232,233]. However, the cost of viral load testing adults at risk of HIV-1 infection, is significantly greater than that of an HIV-1

diagnostic ELISA, and so conventional viral load testing would not be cost effective for mass screening. However, recent advances such as plasma pooling and multiple probing have meant that modified mass screening technologies are becoming available[280]. Our understanding of the dynamics of virus immediately following transmission has improved greatly over recent years. Unfortunately the nature of first presentation in the case of HIV-1 infection, is that generally a patient presents whilst experiencing seroconversion symptoms associated with the primary immune response. Therefore, it is relatively unusual for a patient to present prior to the seroconversion illness at a time when they might have a high virus load but no measurable immune response. This has limited our ability to research widely virological and immunological response immediately after infection. To understand better the systemic effect of HIV-1 transmission, all possible seroconverters should be tested for HIV-1 RNA and for HIV-1 antibodies using conventional ELISA and Western Blot technologies. Complementary to this, recently infected individuals should be encouraged to seek testing and take part in further characterisation studies.

No other viral load assay was evaluated in this laboratory, but false positives have been described with all three HIV-1 quantification assays[281-283] with one study demonstrating rates ranging from 1.9 – 20%[229]. Although the accuracy of NASBA/Nuclisens was shown to be variable at low copy numbers of virus, there were no false positives detected in any of the studies which included negative samples. This is particularly important for the detection of HIV-1 RNA in seroconverting patients. If NASBA/Nuclisens is reliable in detecting positive seroconverters with a negligible rate of false positives, as demonstrated here, it should be reliable to use as an HIV-1 diagnostic tool in cases where HIV-1 antibodies may be absent. The only drawback with this might be the inaccurate quantification of virus at low copy numbers. However, all seroconverters studied showed significantly higher viral loads than those

with chronic infection due to the lack of immune modulation, making it unlikely that virus was replicating in the new host at levels below 2.6 log_{10}copies/ml, therefore Nuclisens should detect virus adequately in the seroconverting population. To ensure that all virus was detected in the newly infected such as seroconverters or neonates it might be best to use the assay with the highest sensitivity. Ultrasensitive RT-PCR and bDNA both detect down to 1.7 log_{10} copies/ml and although Nuclisens is capable of detecting at a similar level, in these studies virus at low copy numbers was more frequently undetectable using Nuclisens than by RT-PCR. The choice of which quantification assay to use to diagnose HIV infection in these unique populations would have to take account of the differing assay sensitivities and the diversity of viruses in the population. The latter may be facilitated by combined testing to ensure that the assay detects virus from the donor or mother as well as from the infected recipient. Virological monitoring of the PHI cohort confirmed existing concepts relating to virological expansion and immune control in the new host. Although it was not possible to verify immune function in these patients, there was a clear distinction between the viral load in patients early in PHI compared to those who were several months into their infection. Viral load measurement during the first 120 days of infection enabled distinction to be made between patients infected within 1 month (early in PHI) and patients infected over a month previously (mid-PHI). Viral load declined more sharply in patients mid-PHI compared to those early in PHI, either this was as a result of decreasing viral replication, or it was evidence of developing immune control. This study also demonstrated that in the first 120 days of PHI, a nadir of viral suppression was reached after which viral load started to rise again, rather than staying static. PHI patients who elected not to take therapy in the later months following infection showed a decline in the reduction of their viral load such that some patients showed viral levels beginning to rise once more. This reflects what we currently

believe about the natural history of virus in the new host. During the first period of PHI high viral load reflects massive population expansion and subsequent general distribution and sequestration of virus into secondary infected sanctuary sites. The developed immune response then suppresses circulatory virus and reduces viral load. However, the infection of densely populated immune cells at sanctuary sites, would eventually allow viral replication to overtake immune system control, resulting in a slow rise in viraemia. In this case viral load is a measure of a dynamic system, the two main factors being viral replication and immune control, the viral load changing to reflect which factor is prevailing. Conversely, patients treated early in PHI may have restricted this first step of viral distribution and sequestration, reducing the infection of cells in sanctuary sites and reducing the potential of immunopathology of lymphoid tissue. In this study patients treated early did show improved reduction in viral load compared to patients treated later in PHI, but this was not statistically significant. Here, virological monitoring alone was not able to differentiate whether or not the time of initiation of HAART was an important factor in virological response.

The evaluation of genetic evolution of virus in two simultaneously infected PHI patients taking early HAART confirmed the findings of the virological monitoring of the PHI cohort. The study showed that the early initiation of HAART in these two patients had rapidly suppressed viral load to undetectable levels and thereby reduced the potential for viral evolution. However, the absence of a control made it impossible to determine whether this was more effective because of the timing of HAART initiation or whether similar data would have been produced if therapy had been initiated later in PHI. The absence of viral evolution was the same in both patients. This implies that the natural pathology of the virus may have been affected by the early onset of treatment since both patients had very similar clinical profiles (and yet each patient was genetically distinct) and therefore there were no differences that could be attributed to host factors.

Both studies investigating therapy in PHI appeared to suggest real benefits to patients taking HAART during PHI. Early therapy appears to restrict both viral evolution and the ability of the virus to undergo a secondary population expansion, subsequent to distribution and infection of sanctuary sites. Although the importance of the time to initiation was not determined, it was highlighted as a key issue and worthy of further investigation, particularly given the high proportion of patients taking HAART early in PHI compared to those who wait longer to start therapy.

To further characterise the PHI cohort a study was undertaken to investigate the prevalence of resistance-associated mutations in newly transmitted HIV-1. Up until 2000 transmission of drug resistant HIV was described as occurring at rates of prevalence ranging from 5% to 28%[260,261,271,272,284] in newly infected patients. The UK appears to have slightly lower rates of resistance transmission, from 1994 until June 2000 the frequency was believed to be around 13%[258] although this appeared to be rising. The study of seroconverters at the Royal Free Hospital confirms this frequency, estimating a transmission of resistant viruses in 16% of the current PHI population[285], slightly higher than before. However, this retrospective study did not differentiate between patients on basis of the year of probable infection, the majority of patients were infected from 1997 to 1999. If resistance transmission is increasing steadily year by year, a study focused around 1997 to 1999 would be more likely to show higher rates of resistance than a study that included data from pre-1997, and we shall review this issue again in two years.

Recent estimates put the transmission of resistance in the UK for the year 2000 at 27%, much higher than in previous years[286], with an adjusted relative risk per year of 1.74. It is not yet clear if this increase in the level of resistance transmission can be differentiated into a rise in protease inhibitor (PI) resistance, and non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance, associated with their increased use in

clinical practise, or whether it is a rise in resistance across all drug classes. Previously, it was possible to assume a viral quasispecies was drug naïve unless the patient had already been treated. This allowed the clinician open choice of all possible therapeutic options, enabling a switch in regimens if resistance emerged or the regimen was intolerable. Current data imply that for up to a quarter of new infections, the virus will not be drug naïve and therefore the patient may already have lost treatment options in all three drug classes before starting their first regimen. This has a significant impact on prognosis and could limit regimen choice for patients relatively early in infection and could therefore influence progression to AIDS in a similar time frame as in the pre-HAART era. It is clearly essential to detect patients who may have been infected with a resistant virus. Unfortunately at present this is confounded by the limitations of genotyping in detecting minority resistant viral populations. The nature of viral replication during PHI argues for genotypic testing to be performed, as early in PHI as possible. It is particularly important to genotype within the early period of PHI when the quasispecies are still relatively similar, the viral population at this time may also determine which virus infects the long-lived cells in lymphoid tissue. It is quite likely that late in untreated PHI the virus which replicates and infects most effectively will become the majority. If the most effective viral cycle is achieved by a drug-naïve virus, the circulating population will shift to portray quasispecies of naïve viruses. At this late stage of PHI, resistant viruses in the minority may well go undetected by consensus sequencing.

For the small proportion of patients with resistance-associated mutations, this study highlighted the importance of resistance testing during PHI. The high level of viral replication and expansion, and possible early sequestration of virus, coupled with the high number of patients electing to take HAART early in PHI demonstrated in these studies, increases the clinical relevance of using genotyping technology in the

management of PHI patients. Genotypic analysis of the PHI cohort determined 6 patients who had primary resistance-associated mutations. This represented a low proportion of PHI patients and may argue on cost grounds against genotyping all PHI patients. However, the population of resistant viruses demonstrated in these patients could impact upon clinical decisions throughout disease management.

This thesis has demonstrated not only the versatility of viral load measurement but also its limitations. Though NASBA/Nuclisens quantified viral load it has yet to inform all the issues surrounding low copy number sensitivity and subtype diversity. Furthermore, no one measure can ever be used confidently in HIV-1 disease to monitor patients. Management must take into account a host of other variables that impact upon clinical status. However, good quality assurance and continual evaluation, and judicious use of viral load as a diagnostic and research tool, particularly in molecular epidemiology studies, will maximise the value of viral load estimates in patient clinical care.

Appendices

Appendix I - Western Blot Analysis of Primary HIV Infection (PHI) Cohort

Of 47 patients, 40 had plasma analysed for antibody to HIV-1 at first presentation. Antibodies to the following antigens were detected by Western Blot analysis, p17, p24, p31, gp41, p51, p55, p66, gp120 and gp160.

Patient	Sample Date	Positive bands	Weak bands	Strong bands
3	08/05/98	all	p17, p31, gp41, gp120	p24, gp160, p66, p51
4	10/09/96	none		
	17/09/96	none		
	25/09/96	only p24	p24	
	03/11/96		p24	gp120, gp160
5	10/12/98	all	all but p24 and gp 160	p24, gp160
6	15/06/99	none		
	29/06/99		p24, gp160	
7	17/12/97	all	all	
8	28/04/98	all	all but p24	p24
9	25/02/98	all	all	
10	24/06/97	all	all	
11	17/06/97	all	all	
12	16/04/97	all	all	
14	08/11/96	only p24	p24	
	13/11/96		p24, gp160	
15	09/08/99		all but no p31	
16	10/12/97	all	all	
17	12/09/97	only gp160	gp160	
	15/09/97	only gp160	gp160	
	23/09/97		gp41, gp160, gp120	p24
	01/10/97		gp41, gp160, gp120, p31, p17	p24

Patient	Sample Date	Positive bands	Weak bands	Strong bands
18	02/07/99	none		
19	05/11/96		p12, gp41	p24, gp160
20	06/03/98	all		all very strong
22	04/12/98		gp120	p24, gp160
23	08/01/98		p17, no p24	all but p17 or p24
	14/01/98	all	p17, p24	all but p17 or p24
24	08/08/97	all	all	
25	?/7/96	all		all very strong
26	27/06/97	all	all	
28	17/12/97	all	p24	all but p24
29	30/01/98	only p24	p24	
30	20/12/98	all	p17	all but p17
31	17/09/97	all	all	
32	19/03/98	all	all	
33	13/08/97	all	p17	all but p17
35	17/04/98		p24, gp160	
36	09/09/97	all		all very strong
37	04/04/97	all		all very strong
38	04/02/98		gp160, gp120, p51, p66	p24
39	06/03/97		p66, p55	p24, gp120, gp160
40	19/10/98	only p24	p24	
41	12/05/97	all	all	
43	02/04/97		gp120, gp41, p17	p24, gp160
44	08/09/99		all but p55	
45	04/03/99	all	all but p24	p24
46	02/07/99	only p24	p24	
47	10/10/97		gp41, p51, p66	p24, gp120, gp160

Appendix II – Categorisation of PHI patients

Categorisation of primary HIV-1 infection patients (PHI) as either early, middle or late in PHI, according to either Western Blot (WB) analysis (see appendix I) or clinical notes (see page 221 for criteria).

Patient	Description in clinical notes	Category
1	Infected 1 month previously, WB at previous hospital diagnoses seroconversion.	Middle
2	Risk 0-2 months previously, WB (not present in notes) diagnoses seroconversion.	Middle
3	WB	Middle
4	WB	Early
5	WB	Early
6	WB	Early
7	WB	Late
8	WB	Late
9	WB	Late
10	WB	Late
11	WB	Late
12	WB	Late
13	Results of WB (not present in notes) diagnose late seroconversion	Late
14	WB	Early
15	WB	Middle
16	WB	Late
17	WB	Early
18	WB	Early
19	WB	Early
20	WB	Late
21	Flu 1 week ago, flu also 4 months ago, HIV-1 negative 7 months ago.	Late
22	WB	Early
23	WB	Middle
24	WB	Late
25	WB	Late
26	WB	Late

Patient	Description in clinical notes	Category
27	Seroconversion illness one week previous, risk < 1 month ago.	Early
28	WB	Late
29	WB	Early
30	WB	Late
31	WB	Middle
32	WB	Early
33	WB	Middle
34	Negative test 2 weeks prior to presentation, then re-tested after flu, a month previous to first available sample.	Early
35	WB	Early
36	WB	Late
37	WB	Late
38	WB	Middle
39	WB	Middle
40	WB	Early
41	WB	Late
42	Risk 4 months ago, fever 0 - 2 months ago.	Late
43	WB	Middle
44	WB	Middle
45	WB	Middle
46	WB	Early
47	WB	Middle

WB = Western Blot. Where only WB is indicated full antibody profiles were available, shown in appendix I.

Time in PHI	n
Early	15
Middle	13
Late	19
Total :	**47**

Appendix III

Resistance Associated Mutations : Reverse Transcriptase (NRTIs)																			Total
Patient No.	Comment	41	44	62	65	67	69	70	74	75	77	115	116	118	151	184	210	215	219	
1																				0
2																				0
3																				0
4																				0
5																				0
6																				0
7	Neg PCR																			
8																				0
9																				0
10	Pr only																			
13																				0
14																				0
15																				0
16		1																1*		2
17																				0
18																				0
19																				0
22																				0
23																				0

Resistance Associated Mutations : Reverse Transcriptase (NRTIs)																				Total
Patient No.	**Comment**	**41**	**44**	**62**	**65**	**67**	**69**	**70**	**74**	**75**	**77**	**115**	**116**	**118**	**151**	**184**	**210**	**215**	**219**	
24																				0
25	Neg PCR																			
26																				0
27																				0
28																				0
29	Neg PCR																			
30																				0
31																				0
32	Pr only																			
33																	1			1
35																				0
37																1				1
38																1				1
39							1	1												2
40																				0
41																				0
42																		1*		1
43																				0
44																		1		1
45	Pr only																			
46		1																1		2
47	Pr only																			
41		2		0	0	0	1	1	0	0	0	0	0	0	0	2	1	4	0	

Appendix IV

Resistance Associated Mutations : Reverse Transcriptase NNRTIs													Total
Patient No.	Comment	98	100	103	106	108	179	181	188	190	225	236	
1													0
2													0
3													0
4							1						1
5													0
6													0
7	Neg PCR												
8													0
9													0
10	Pr only												
13							1						1
14													0
15													0
16													0
17		1											1
18													0
19													0
22													0
23													0

Resistance Associated Mutations : Reverse Transcriptase NNRTIs													Total
Patient No.	**Comment**	**98**	**100**	**103**	**106**	**108**	**179**	**181**	**188**	**190**	**225**	**236**	
24						1							1
25	Neg PCR												
26													0
27													0
28													0
29	Neg PCR												
30													0
31		1											1
32	Pr only												
33													0
35													0
37													0
38							1						1
39													0
40													0
41													0
42													0
43													0
44													0
45	Pr only												
46				1						1			2
47	Pr only												
41		2	0	1	0	1	3	0	0	1	0	0	

Appendix V

Resistance Associated Mutations : Protease Inhibitors																					Total
Patient No.	Comment	10	20	24	30	32	33	36	46	47	48	50	54	63	71	73	77	82	84	88	90	
1																						0
2														1								1
3																	1					1
4														1								1
5														1			1					2
6														1			1					2
7	Neg PCR																					
8														1								1
9														1								1
10	Pr only																					0
13														1			1					2
14														1								1
15														1			1					2
16		1												1								2
17								1						1								2
18		1					1							1								3
19														1								1
22														1								1
23														1								1

: Protease Inhibitors		Resistance Associated Mutations																				Total
Patient No.	Comment	10	20	24	30	32	33	36	46	47	48	50	54	63	71	73	77	82	84	88	90	
24			1					1														2
25	Neg PCR																					
26														1			1					2
27																	1					1
28														1								1
29	Neg PCR																					
30								1						1								2
31																						0
32	Pr only													1	1		1					3
33		1												1								2
35																						0
37														1								1
38														1	1							2
39														1			1					2
40		1																				1
41														1			1					2
42														1								1
43																						0
44														1								1
45	Pr only							1														1
46								1						1			1					3
47	Pr only							1						1								2
41		4	1	0	0	0	1	6	0	0	0	0	0	28	2	0	11	0	0	0	0	

Key to Appendixes III, IV and V

Primary Mutations[269] shown in red

Secondary Mutations[269] shown in black

* T215D/E mutation from threonine to asparagine/glutamic acid (not T 215F/Y threonine to phenylalanine/tyrosine) has an unconfirmed resistance effect, therefore patients with T215D/E are included as having a secondary rather than primary mutation.

Pr only indicates where a virus failed to amplify in RT but was amplified in Pr.

Neg PCR indicates that a virus failed to amplify in both RT and Pr.

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