VIRAL GENETIC DETERMINANTS OF NON PROGRESSIVE HIV-1 SUBTYPE C INFECTION IN ANTIRETROVIRAL DRUG NAÏVE CHILDREN

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Declaration

I, Demetrio Basil Tzitzivacos declare that this dissertation is my own, unaided work. It is being submitted for the degree of Masters of Medicine (Haematology) at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at any other University.

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Demetrio Basil Tzitzivacos

Publications

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Abstract

Objectives: Characterization of HIV-1 from slow progressors (SP) is important to facilitate vaccine and antiviral drug development. In order to identify virus attenuations that may contribute to slower rates of disease progression, the full viral genomes from primary isolates of six slow progressing HIV-positive children were sequenced.

Methods: Primary virus biological phenotypes were determined by growth in CCR5- and CXCR4-expressing U87.CD4 cell lines. Proviral DNA was isolated from co-cultured PBMCs, and the near full-length genomes and LTR regions were PCR amplified, sequenced and analysed. Predicted amino acid (aa) sequences for all the HIV-1 proteins were extensively analyzed.

Results: All primary HIV-1 isolates utilized CCR5, and were determined to be HIV-1 subtype C by phylogenetic analysis. Predicted aa sequence analysis revealed open reading frames for all HIV-1 genes which encoded for proteins of the expected length, with several exceptions. For example, isolate LT5 had a 2 aa insertion in the Vpr mitochondriotoxic domain. Isolate LT21 contained an additional 5aa in the C-terminus of tat exon 2, while the integrase enzyme in isolate LT39 had an additional 3aa at the C-terminus. Rev from isolates LT45 and LT46 did not have the characteristic subtype C 16aa truncation, and in addition, had a further 3aa. In addition, altered functional domains was noted in several isolates, such as the cAMP-dependent kinase PKA phosphorylation site in Nef (LT5), a Vpr mutation involved in decreasing pro-apoptotic activity (LT42), and the Nef ExxxLL motif involved in the interaction with AP-1 and AP-2 (LT46).

Conclusions: The slower HIV disease progression in these six children may be attributed to altered protein functions. For example, LT46 Nef is unable to bind AP-1 and AP-2 and therefore inactive on CD4 endocytosis. The biological relevance of these findings requires further investigation.

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List of Abbreviations

Aa:	amino acids
AIDS:	Acquired Immunodeficiency Syndrome
AP-1/AP-2:	Adapter protein 1 and 2
APOBEC3G:	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
ARV:	Antiretroviral
bp:	base pairs
cAMP:	Cyclic adenosine monophosphate
CCR5:	CC chemokine Receptor 5
CD4:	cluster of differentiation 4
CD8:	cluster of differentiation 8
cDNA:	complementary deoxyribose nucleic acid
CO_2:	Carbon dioxide
CTL:	cytotoxic T lymphocytes
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCR4:	CXC chemokine Receptor 4
DNA :	deoxyribonucleic acid
dNTPs:	deoxynucleotide triphosphates
EDTA:	ethylenediaminetetraacetic acid
ELISA:	Enzyme linked immunosorbent assay
Env:	envelope glycoproteins
FCS:	Fetal calf serum
Gag:	group antigen proteins
HAART:	Highly Active antiretroviral therapy
HIV-1:	Human Immunodeficiency virus type 1
HIV-2:	Human Immunodeficiency virus type 2
HLA:	human leukocyte antigen
IL-2:	interleukin 2
IN:	Integrase enzyme

kb:	kilobases
LTNP:	Long Term Non Progressor
LTR:	Long Terminal Repeats
MHC:	major histocompatibility complex
µl:	microlitre
µg:	microgram
Nef:	Negative factor protein
NSI:	Non syncytium inducing
ORFs:	open reading frames
P2:	passage 2
PBMCs:	peripheral blood mononuclear cells
PCR:	polymerase chain reaction
PHA:	phytohemagglutinin
PIC:	preintegration complex
PKA:	protein kinase A
Pmol:	picomole
Pol :	Polymerase
RNA:	Ribonucleic acid
RNF39:	ring finger protein 39
RRE:	Rev responsive element
RT:	Reverse transcriptase
SBBC:	Sydney Blood Bank Cohort
SDF-1:	Stromal derived factor 1
SI:	Syncytium Inducing
SP:	Slow Progressor
TAE:	Tris Acetate EDTA buffer
TAR:	trans-activation response
Tat:	Transcription Activator protein
Th1:	CD4 T helper 1 responses
UV:	ultraviolet
V:	voltage
Vif	Viral infectivity factor

VL:	Viral load
Vpr:	Viral Protein R
Vpu:	Viral Protein U
ZNRD1:	zinc ribbon domain-containing 1
°C:	degrees celsius
%:	percentage

1. INTRODUCTION

Globally there are currently over 34 million people infected with human immunodeficiency virus (HIV), the causative agents of acquired immune deficiency disease (AIDS) (http://www.unaids.org), and over 25 million people have died of AIDS since the start of the pandemic in the early 1980s. HIV type 1 (HIV-1) and HIV type 2 (HIV-2) are retroviruses which, upon infection causes the progressive depletion of $CD4^+$ T cells which are central in establishing and enhancing adaptive (cell-mediated and humoral) immune responses. This ultimately leads to vulnerability to common opportunistic infections, malignancies and death in the absence of antiretroviral therapy.

Rates of disease progression to AIDS amongst HIV-1 infected patients are highly variable and depend on a complex interplay between the host's genetic factors, immunological responses (reviewed by (Lama and Planelles, 2007) and the pathogenic potential of the virus (Pantaleo, Graziosi, and Fauci, 1993). These factors are interrelated and inextricably interdependent: the antigenicity and pathogenicity of the virus dependents on its own genetic complement, which in turn is altered by the host's specific immune responses which depend on the host's genetic constitution (especially human leukocyte antigen (HLA) loci).

1.1. Natural history of HIV infection

The natural history of HIV infection and disease progression has been well established in adults, particularly in those infected with HIV-1 subtype B. The median time from infection to development of AIDS is approximately 10 years for intermediate progressors (Langford, Ananworanich, and Cooper, 2007); Figure 1.1). The clinical course of disease progression can be divided into three phases; the acute phase or primary infection, the asymptomatic phase, and AIDS (Figure 1.1). Upon infection, HIV-1 replicates uncontrollably and HIV viral loads (RNA copies/ml) are extremely high (acute phase). This leads to a depletion of CD4+ T cells (cells/mm^3 or µl). Cellular immune responses (CD8+ cytotoxic T-lymphocyte (CTLs)) are mounted against HIV-1 to control the viral load at a set point, and the CD4+ cell counts recover (asymptomatic phase). The viral set point varies amongst patients, and it is well

established that the lower the set point, the slower the disease progression to AIDS. There is ongoing viral replication throughout the course of disease, and gradual loss of immune function. Once the CD4 counts drop to below 200 CD4 cells/µl, the individual is diagnosed with AIDS.

A small proportion of adult patients develop AIDS as early as 3 to 6 months post infection (rapid progressors)(Pantaleo and Fauci, 1996), or remain clinically asymptomatic (5% of infected patients) after 7-10 years in the absence of antiretroviral therapy (slow progressors (SP) or long term non-progressors, LTNP, (Learmont et al., 1992) (Figure 1.1). More recently, a subset of infected individuals (elite controllers) with spontaneous and sustained control of HIV infection, with viral loads <50 RNA copies/ml in the absence of therapy have been described (Pereyra et al., 2008; Walker, 2007). This spontaneous control occurs in approximately 1 in 300 HIV-infected persons, and the HIV Controller Consortium was recently established to study host genetics together with functional immunology studies in elite controllers (www.elitecontrollers.org).

By contrast, children with vertically acquired HIV-1 infection tend to have shorter clinical latent periods and survival times than HIV-1 infected adults (Saloojee and Violari, 2001). In the developed world approximately 20% of children progress rapidly to AIDS within the first 2 years after birth (reviewed in (Saloojee and Violari, 2001)) whereas in sub-Saharan Africa up to 50% of children are RP (rapid progressors) (Little et al., 2007)). Asymptomatic vertically infected children older than 7 years of age are unusual (Paul, 1997), and in a recent prospective study of the ANRS French Pediatric Cohort, only 2% of children infected showed no clinical progression at 10 years (Warszawski et al., 2007).

Interest in non-progressive HIV-1 infection has increased over time in the hope of understanding HIV pathogenesis and the mechanisms of protection involved, and providing insights relevant to viral attenuation, novel therapies and vaccine design (Walker, 2007). SP or LTNP cohorts provide a unique opportunity to study the complexity of the virus-host interaction with the aim of understanding the biological and molecular correlates of protection against HIV-1 infection and disease progression.

The duration of clinical latency following HIV-1 infection is regulated by the interplay of at least three virus-host factors: (i) genetic susceptibility of the host, (ii) the hosts ability to mount an effective immune response, and (iii) the genotypic properties of the infecting viral quasispecies (Alexander et al., 2000; Sheppard et al., 1993).

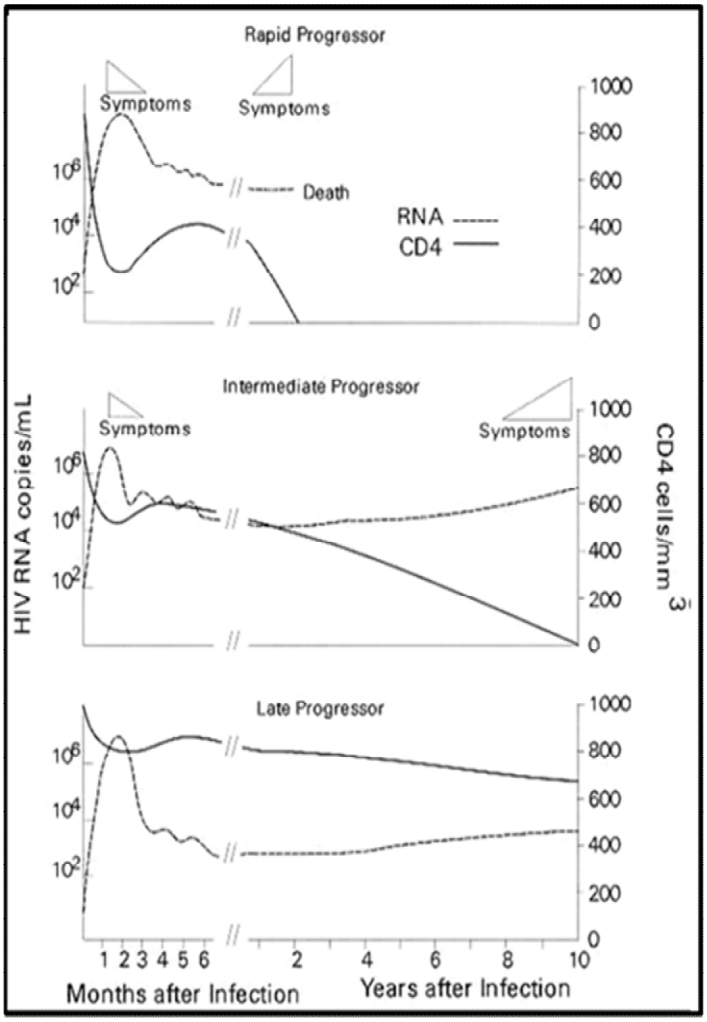


Figure 1.4: Typical course of the natural history of viral loads (HIV RNA levels) and CD4 counts at three rates of disease progression to AIDS (copied from (Langford, Ananworanich, and Cooper, 2007).

1.2. Host Factors impacting on HIV-1 disease progression

1.2.1. Immune factors.

Most research on LTNP patients has focused on the characterization of the specific antiviral host immune responses thought to be responsible for modulating disease progression. There are vigorous HIV-specific CD4+ T helper (Th1) cell responses in non-progressors, whereas these responses are absent or low in typical progressors (Rosenberg et al., 1997). LTNPs are more likely to develop strong neutralizing antibodies against HIV (Cecilia et al., 1999). Humoral immunity has, however, been shown to vary amongst HIV-1 subtype C vertically infected children but is not predictive of disease progression (Zhang et al., 2006). Antibodies against the CCR5 coreceptors have also been identified in a subset of adult LTNP's (Pastori et al., 2006) and have also been shown to block infection of macrophages and dendritic cells with R5 viruses (Bouhlal et al., 2005). In addition, LTNPS are more likely to develop strong and persistent cytotoxic T-lymphocyte (CTL) responses against several HIV-1 epitopes (Greenough et al., 1997; Harrer et al., 1996), albeit with viral attenuation (Cao et al., 1995).

1.2.2. Host genetics.

Susceptibility to HIV infection and disease progression can also be influenced by diverse host genetic factors such as the extent of heterozygosity at major histocompatibility complex class I (MHC-I) loci (Altfeld et al., 2006; Carrington et al., 1999; Carrington and O'Brien, 2003) and polymorphisms in the HIV-1 coreceptors CCR5 (Liu et al., 1996) and CXCR4 (Winkler et al., 1998).

1.2.2.1. Human Leukocyte Antigen

The role of HLA is to clearly define the repertoire of the CD8+ epitope-restricted responses in HIV infection (Altfeld et al., 2006). The influence of HLA genotype on HIV response and disease progression has been reviewed by (Carrington and O'Brien, 2003). It has been demonstrated that the HLA background contributes to disease modification, where the presence of HLA-B27 (Goulder et al., 1997) and HLA-B57 (Migueles et al., 2000) appears to be linked to slower disease whereas with HLA-B35, the disease outcome is worse. Interestingly, Fellay et al. (2007) used a whole genome association strategy and identified a

polymorphism within an endogenous retroviral element which is associated with HLA-B*5701, and another near the HLA-C gene which impact on lowering viral load during the asymptomatic set-point period preceding disease progression to AIDS.

1.2.2.2. Gene polymorphisms

Approximately 25-30% of LTNP have polymorphisms in their HIV-1 coreceptors CCR5 and CCR2, and express mutant forms of these proteins. People that are homozygous for a 32-bp deletion in CCR5 are resistant to infection by CCR5-utilizing HIV-1 strains (Agrawal et al., 2004; Liu et al., 1996). This loss of function mutation prevents expression of the essential co-receptor (CCR5) for HIV attachment (Dean et al., 1996). Interestingly, heterozygosity for the 32-bp deletion in the CCR5 gene is associated with longer AIDS-free survival. Whether this is true for vertically infected children is debatable (de Angelis et al., 2007).

Chemokines have been shown to play a major role in HIV pathogenesis. Stromal Derived Factor -1 (SDF-1 or CXCL 12) the only known natural ligand for CXCR4, one of the two major coreceptors for cell viral entry for HIV-1 (reviewed by (Littman, 1998) is capable of blocking viral entry (Bleul et al., 1996). Patients who express the SDF 1-3'A variant are also associated with delayed disease progression (Fauci, 1996; Winkler et al., 1998). This polymorphism has however been shown not to be associated with LTNP disease of longer than 16 years in HIV-1 infected patients (Vidal et al., 2005).

The presence of polymorphisms such as H186R in the antiviral APOBEC3G (Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G) have been linked to accelerated progression to AIDS in African American populations (An et al., 2004). APOBEC3G is a cytidine deaminase which is responsible for hypermutation of the HIV-1 genome (G-to-A) and is inactivated by the viral encoded vif protein (Sheehy et al., 2002).Binding of Vif to APOBEC3G results in ubiquitination and subsequent proteosomal degradation of APOBEC3G .

The whole genome association study by Fellay et al (2007) also implicated polymorphisms in and near the ring finger protein 39 (RNF39) and zinc ribbon domain-containing 1 (ZNRD1) genes that were associated with time of disease progression to AIDS.

1.2.3. Co-infections.

Human co-infection of HIV-1 with other viruses such as Human T-cell lymphotropic Virus (Casoli, Pilotti, and Bertazzoni, 2007), Herpes simplex virus type 2 (Casoli, Pilotti, and Bertazzoni, 2007), Varicella zoster virus (Hung et al., 2005) and cytomegalovirus (Saillour et al., 1998) are associated with an increased rate of disease progression to AIDS. Interestingly, infections with helminths shift the immune response from Th1 response to a less protective Th0/2 type response (reviewed in (Bentwich et al., 2000). It appears that the aforementioned infections result in a more rapid progression to AIDS. Similarly, malaria has been associated with increases in HIV-1 viral load that may impact HIV progression in co-infected individuals (reviewed in (Slutsker and Marston, 2007).

By contrast, coinfection with GBV-C (hepatitis G virus) has been shown to result in a lower mean viral load and a higher mean CD4 cell count and subsequent retardation of disease progression (Tillmann et al., 2001). The latter findings have however, been disputed (Bjorkman et al., 2004) although these effects may be only noted in advanced HIV infection.

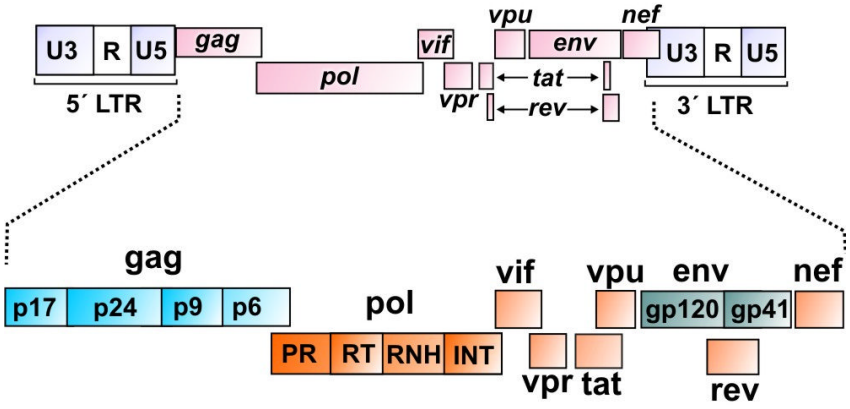
1.3. Viral Factors impacting on HIV-1 disease progression

Several viral factors that may impact on disease progression have been described. Controversy abounds in the literature with respect to their relevance. Infection with attenuated HIV viruses and/or mutations in functional sites of viral genes and proteins may impact on disease progression.

1.3.1. HIV-1 genome and gene functions

The HIV-1 full-length genome (~9.7kb) encodes 9 genes and is flanked by two identical Long Terminal Repeats (LTRs) (http://www.hiv.lanl.gov). The viral genes have been classified into 3 structural genes which include *gag, pol* and *env,* the 2 regulatory genes *tat* and *rev*, and four accessory genes which comprise of *vif, vpr, vpu* and *nef* genes which code for at least 16 distinct viral proteins. The gene sequences and the function of their products have been extensively studied (Table 1.1).

Table 1.1: Schematic representation of the HIV-1 genome organisation and HIV-1 derived proteins (top row), and description of the function and localization of each HIV-1 protein (adapted from http://www.hiv.lanl.gov and (Levy, 2007).



Name	Size	Function	Localization
Gag MA	p17	Matrix, Membrane anchor, env interaction, myristolated	Virion
CA	p24	Core capsid	
NC	p7	Nucleocapsid, helps in reverse transcription	
	p6	Binds Vpr, role in viral budding (L domain)	
Pol PR	p15	Gag/pol cleavage and maturation	Virion
RT	p51	Reverse transcription	
RNase H	p66	RNAse H activity	
IN	p31	cDNA provirus integration	
Env	gp120	External viral glycoproteins, bind to CD4 and coreceptor	Plasma membrane, Virion envelope
	gp41	Transmembrane subunit	
Tat	p16/p14	Viral transcriptional activator	Nucleolus/nucleus
Rev	p19	Regulation of viral mRNA expression, RNA transport, stability and utilization factor	Nucleolus/nucleus
Vif	p23	Promotes viral maturation and infectivity; inhibits APOBEC3G	Cytoplasm, virion
Vpr*	p10-15	Transactivation, promotes nuclear localization of preintegration complex, inhibits cell division, arrest cells in G2/M	Virion, nucleus (nuclear membrane?)
Vpu	p16	Promotes extracellular release of virion, degrades CD4 in the ER	Integral membrane protein
Nef	p27-p25	Pleiotropic, CD4 and MHC class I downregulation, can increase or decrease viral replication	Plasma membrane, cytoplasm

*Vpx in HIV-2 and SIV

1.3.2. HIV-1 life cycle

An overview of the viral life cycle is shown in Figure 1.2. HIV infection of a CD4+ T lymphocyte is initiated when the viral gp120 binds to CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984). This leads to conformational changes on the gp120 allowing co-receptor binding (CCR5 or CXCR4)(Deng et al., 1996; Dragic et al., 1996; Wyatt and Sodroski, 1998). The gp120 is shed, and the gp41 fusion peptide is formed which leads to fusion of viral and host membranes (Gallo et al., 2003; Weissenhorn et al., 1997). Once inside the cytoplasm, the viral p24 core is uncoated and releases the 2 copies of plus strand viral RNA and other viral proteins. The reverse transcriptase (RT) enzyme initiates reverse transcription of the viral RNA to cDNA (Fassati and Goff, 2001). The viral cDNA aggregates with the integrase enzyme, Vpr and other viral and host proteins to form the preintegration compex (PIC)(Marchand et al., 2006; Van Maele et al., 2006). The PIC localizes to the nucleus where the integrase enzyme initiates strand transfer leading to the irreversible integration of viral cDNA into the host chromosome (Mitchell et al., 2004). Following transcription and translation of the viral proteins, the viral protease enzyme cleaves specific viral polyproteins, and viral RNA and proteins assemble at the host membrane, leading to budding of an immature virion. Further maturation results in fully infectious viruses capable of initiating a new cycle of infection.

HIV is extremely efficient at reproduction, producing over ten billion particles daily in an average HIV-positive patient (one replication cycle takes about 2.5 days) (Ho et al., 1995; Wei et al., 1995). Because RT is error prone it introduces mistakes during each replication cycle, resulting in different viral quasispecies within the host (Roberts et al., 1988). Consequently HIV evolves rapidly, and can escape host immune surveillance, and rapidly establish drug resistant variants. Viral diversity presents the greatest challenge towards the development of effective antiretroviral drugs and HIV-1 vaccines (Papathanasopoulos et al., 2003).

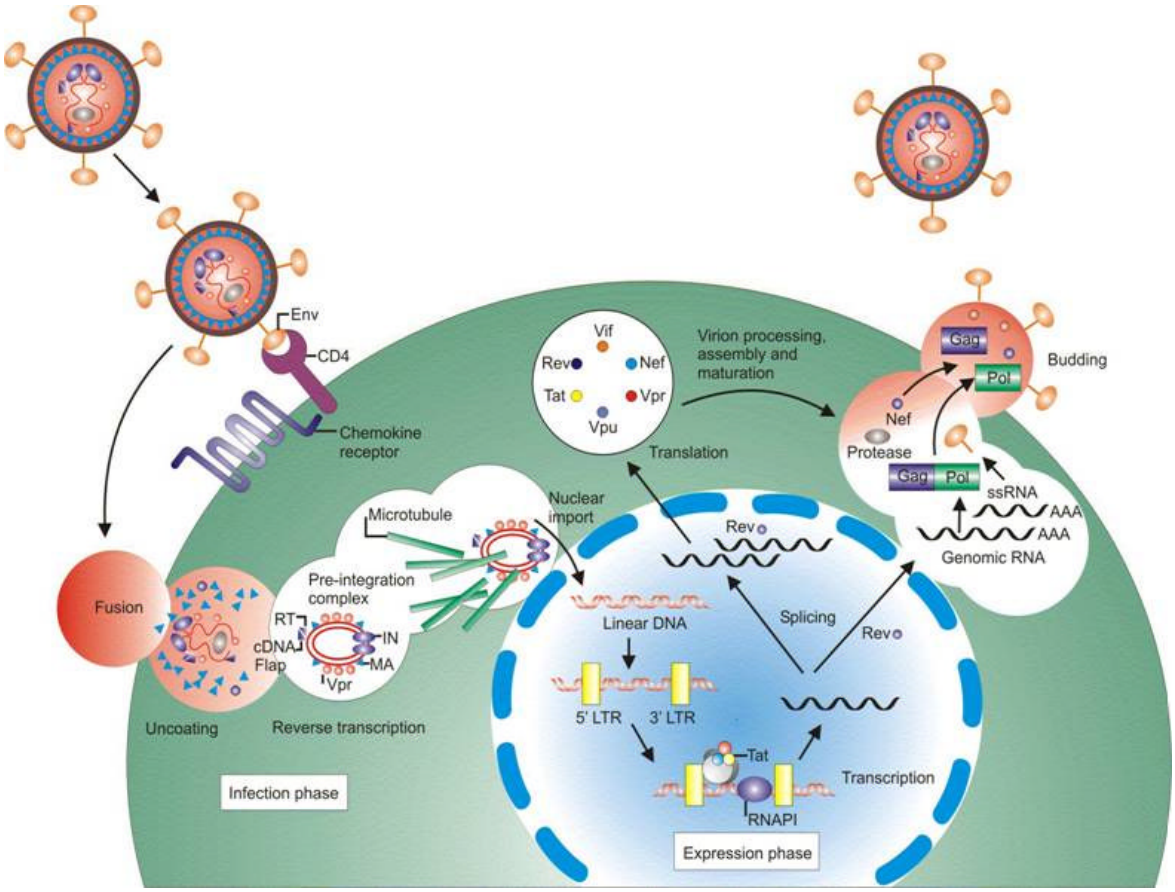


Figure 1.2: Schematic representation of the HIV-1 viral life cycle. Infection is initiated when free virions (top left) attach to CD4 + T lymphocytes via their CD4 receptor. (Copied from (Peterlin and Trono, 2003).

1.3.2.1. Viral subtype

The rapid ongoing evolution of HIV-1 results in extensive genetic diversity within a patient as well as between different isolates. There are three different groups of HIV-1 globally (M, N and O). HIV-1 group M is responsible for the pandemic worldwide, and is further subdivided into 9 distinct subtypes (A to K) and at least 43 circulating recombinant forms (http://www.hiv.lanl.gov). The subtype of the infecting virus may influence the rate of HIV disease progression to AIDS: in Senegal, patients infected with non A subtypes, such as C, D and G were shown to be 8 times more likely to progress to AIDS than those infected with subtype A (Kanki et al., 1999). Similarly, in Uganda subtype D was associated with faster disease progression compared to subtype A (Kaleebu et al., 2002). These findings were again

confirmed in a recent study in Rakai, Uganda, where the risk of progression to death was shown to be significantly higher for subtype D, recombinant subtypes, and multiple subtypes, as compared with subtype A (Kiwanuka et al., 2008).

1.3.2.2. Viral Biotype/Phenotype

Viral characteristics such as cell tropism, cytopathicity and replicative capacity have been associated with different stages of infection, suggesting an important role for HIV-1 phenotype in the clinical course of HIV infection. On sexual, vertical and parenteral transmission, infection seems to be initiated by CCR5-utilizing macrophage tropic (M-tropic) variants, while a shift to preferentially CXCR4-utilizing (T-tropic) HIV-1 populations is correlated with progression to disease (Connor et al., 1997; Koot et al., 1993; Koot et al., 1999; Koot et al., 1992). Patients infected with HIV-1 that exhibits the biological phenotype CCR5 (non-syncytium inducing (NSI) viral phenotype) appear to progress slower than the CXCR4 syncytium inducing (SI) phenotype. HIV-1 predominantly utilizes the CCR5 coreceptor for cell entry in early infection and during the asymptomatic phase of the disease. However, as the patient progresses to AIDS, a "switch"/emergence to CXCR4-utilizing viruses occur in approximately 50-90% of subtype B infected patients (Koot et al., 1993). It is arguable whether the emergence of the X4 viruses is a consequence or the cause for the rapid progression (reviewed in (Lusso, 2006)).

1.3.3. Viral attenuation

Limited information is available on the genotypic properties of infecting viral quasispecies that could contribute to the delay in, or absence of disease progression. However, persistence of defective and/or attenuated HIV genomes has been correlated with low replicative capacity and the lack of disease progression in at least some individuals.

1.3.3.1. Gene polymorphisms associated with attenuated viruses and delayed disease progression

The most studied defects associated with the absence of disease progression have been found in the *nef*/LTR region as documented in an asymptomatic patient from central Massachusetts (Kirchhoff et al., 1995) and eight patients in the Sydney Blood Bank Cohort (SBBC) (Deacon et al., 1995; Learmont et al., 1992). Full-length sequence analysis of the HIV-

1 genome from four Sydney cohort members confirmed that the deletions and rearrangements of the *nef*/LTR region were the most unusual features of these viruses (Oelrichs et al., 1998). Similar deletions have been found in a slow progressing Italian individual who acquired HIV-1 via sexual transmission (Salvi et al., 1998). Thus, infection with attenuated HIV-1 is responsible for slow or no progression in these patients. Interestingly, Geffin *et al*. (2000) found that one of five slow progressing HIV-1 vertically infected children harboured a virus with large deletions in *nef* and the remaining four contained discrete changes at a higher frequency compared to rapid progressors. Subsequently, numerous researchers have described "defective" *nef* alleles which impact on disease progression in various paediatric and adult cohorts (Brambilla et al., 1999; Catucci et al., 2000; Crotti et al., 2006; Kondo et al., 2005; Salvi et al., 1998; Tobiume et al., 2002; Verity et al., 2007; Walker et al., 2007).

The number of LTNP individuals exceeds by far the number of individuals infected with *nef*-deleted HIV strains, suggesting that host factors or defects in other viral genes may contribute significantly to their long-term survival. Several researchers have found LTNP with unusual or defective genes other than *nef*/LTR, including *gag, env*, *vpu, vpr, vif, rev* and *tat* genes (Alexander et al., 2002; Cho and Jung, 2008; Churchill et al., 2007; Huang, Zhang, and Ho, 1998; Iversen et al., 1995; Papathanasopoulos et al., 2003; Wang et al., 1996; Yamada and Iwamoto, 2000).

An LTNP with deficient activity in the viral *rev* gene has been described (Iversen et al., 1995). The authors demonstrated that a leucine-to-isoleucine substitution in the Rev activation domain contributed to virus attenuation *in vitro*. Slower disease progression in perinatally infected siblings may be partly attributed to an unusual 3 amino acid sequence extension (GCC) at the 3'end of Rev (Papathanasopoulos et al., 2003). Attenuated *rev* alleles in viruses from two of 4 studied SBBC members (in addition to *nef/*LTR) may contribute to viral attenuation and long-term survival of HIV-1 infection (Churchill et al., 2007).

Another LTNP with a consistently defective viral p17 sequence (Huang, Zhang, and Ho, 1998) and a non-progressing infant-mother pair infected with Vpr defective virus (Wang et al., 1996) have been identified. The R77Q mutation in Vpr has been associated with long term

control of HIV-1 infection in several studies (Mologni et al., 2006; Rajan et al., 2006; Rodes et al., 2004)). Yamada and Iwamoto (Yamada and Iwamoto, 2000) compared the proviral accessory genes of LTNP and progressors, and found that LTNP frequently harboured proviruses with mutated *nef, vpu, vpr, vif, rev* and *tat* genes, while almost all accessory genes in typical progressors were intact. Wang *et al*. (2000) found a V2 region extension of the gp160 unique to SP or LTNP.

Most of the abovementioned studies however, only looked at specific HIV-1 genes and their function and not the entire genome. Therefore, unlike the *nef* mutants described in the SBBC one cannot exclude the possibility that other defective genes may contribute or be responsible for viral attenuation. Alexander *et al*. (Alexander et al., 2000) however, examined the full genome sequences of eight individuals with slow or non-progressing disease. Seven of the eight patients were infected with virus containing one or more unusual polymorphisms, such as a one amino acid deletion in gp41, 4 amino acid insertions in Vpu, a 1-2 amino acid deletion in Gag, and deletions in Nef. Analysis of stored PBMC samples (ranging from 11 years prior sequencing to 4 years post sequencing for different samples) confirmed the viruses contained genes with unusual, difficult-to-revert polymorphisms, and these could be associated with slow or non-progression in these patients. The same group (Alexander et al., 2002) also described defective HIV-1 from a clinical nonprogressing mother and child pair with a two amino acid insertion in Vif responsible for their nonprogressor status. Functional studies with PBMC infections of recombinant HIV-1 containing the mutant or wild type Vif showed that the mutant's replication rate was approximately 20-fold lower than that of the wild type. Full genome sequencing and functional analysis of defective genes is thus an important way to establish an association between structural or functional alterations in various HIV-1 genes and the degree of disease.

1.4. Reasons for studying SP or LTNPs, and aims of study

Strong suppression of HIV-1 replication by highly active antiretroviral therapy (HAART) results in reconstitution of the immune system damaged by HIV-1 infection. As a

consequence, opportunistic infections and AIDS-related deaths have dramatically decreased since the introduction of HAART. However, immune reconstitution by highly active antiretroviral therapy (HAART) is only partial, and the immune responses against HIV-1 appear to be lost during the course of HAART (Rinaldo et al., 1999). On the other hand, LTNP have low viral loads and stable CD4 T cell counts, while maintaining strong anti-HIV humoral and cellular immunity (Cao et al., 1995). The presence of viral defects in LTNP tends to be associated with extremely low viral loads, and in several instances it has not been possible to recover HIV-1 isolates from plasma or other tissues (Cao et al., 1995). Despite this, immune responses to HIV-1 antigens are strong in LTNP, usually more so than in individuals with progressive infection (Cao et al., 1995; Montefiori et al., 1996). This raises the question of what drives the antigen-specific immune responses during such low levels of virus replication. The presence of quasispecies in viruses with mutated genes implies their replication, which suggests that they act as continuous immune stimulators for the patients. Although the mechanisms for long-term non-progression have not been elucidated, LTNP may harbour HIV-1 genomes that behave as live attenuated vaccines. The characterization of proviral genomes from LTNP can potentially help to gain insight into the design of potent therapeutic vaccines (Deeks and Walker, 2007).

Available evidence suggests that disease progression is a complex process, and justifies the need for studying different cohorts of patients infected with different HIV subtypes. South Africa has one of the fastest growing HIV-1 epidemics, with an estimated 6.2 million infected people (http://www.unaids.org). HIV-1 subtype C currently accounts for over 50% of all new infections by HIV-1 group M viruses worldwide, and is the major subtype responsible for the epidemic among South African heterosexuals (Van Harmelen et al., 1999). To date, the only report on the relationship between South African HIV-1 subtype C attenuated viruses and rates of disease progression describes two slow progressing vertically infected siblings with viruses comprising an unusual rev gene from a cohort of perinatally infected children at Chris Hani Baragwanath Hospital (CHBH) in Johannesburg (Papathanasopoulos et al., 2003).

Primary viral isolates are available from a further six vertically infected children in this cohort that have been identified as potential slow progressors. Since the date of HIV-1 infection in these children is known, it is an ideal opportunity to study disease progression in

this cohort. This study aims to investigate the virological features in a cohort of vertically infected children classified as SP to determine whether slow progression is associated with the presence of attenuated viruses harbouring defective HIV-1 genomes.

2. MATERIALS AND METHODS

2.1. Patient samples and primary virus isolates

Primary viral isolates obtained from six antiretroviral drug-naive vertically infected children attending a clinic at Chris Hani Baragwanath Hospital, Johannesburg, were selected for the purposes of this study. The children fit the following definition of slow progressors: documented infection lasting at least 6 years, asymptomatic or mild/moderate symptoms in the absence of antiretroviral drug use. The patient epidemiological data is shown in Table 2.1. Ethical approval was obtained by Professor Tiemessen, National Institute for Communicable Diseases, at the time of sample collection (M971010) from the University of the Witwatersrand Committee for Research on Human Subjects. No further human ethics was required for the purposes of this study (see Appendix A; Ethics waiver).

Infected peripheral blood mononuclear cells (PBMCs) from the six primary HIV-1 isolates (passage one) were obtained from Professor Tiemessen, and immediately co-cultured with 2 x 10^6 cells/ml phytohemagglutinin (PHA)-activated donor PBMCs in 10ml RPMI-1640 (Sigma, Steinheim, Germany), supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, USA), L-glutamine (Gibco), Penicillin/Streptomycin (Gibco) and 5% interleukin 2 (IL-2; Roche Diagnostics, Mannheim, Germany), according to standard PBMC co-culture techniques (Choge et al., 2006). Flasks containing the second passage (P2) of primary virus were incubated at 37°C with 5% CO_2 for 7 days. Viral isolates were maintained/fed by removing 50% of the total culture medium volume, and replacing it with fresh medium with IL-2 (Roche Diagnostics, Mannheim, Germany) on day 4, and monitored on day 7 for viral growth using the Murex p24 enzyme-linked immunosorbent assay (ELISA) kit (Abbott Murex, Dartford, UK). The expanded cultures were centrifuged at 1 400 rpm, and the infected PBMCs (resuspended in 90% FCS (Gibco) and 10% dimethylsulphoxide (Sigma) and supernatants were stored at -130°C and -70°C, respectively. The supernatant was stored at –70°C and used to extract viral RNA (High Pure RNA isolation kit; Roche) as required.

Proviral DNA was extracted from p24 antigen positive cultures from P2 PBMCs using the High Pure Template Preparation Kit (Roche), as per manufacturer's instructions. The virus

containing supernatant from P2 was used to establish primary virus phenotypes and biotypes by infection of MT-2 and CCR5 and CXCR4-expressing U87.CD4 cell lines, respectively, as described previously (Cilliers et al., 2003).

2.2. Polymerase Chain Reaction and sequencing

A nested polymerase chain reaction (PCR) was carried out to amplify the virtually full-length genomes, and LTR regions using the primers outlined in Table 2.2. The virtually full-length genomes were amplified using the outer primers MSF12 and OMFR1 and inner primers F2NST and OFM19 (when necessary), and cycle conditions as described previously (van Harmelen et al., 2001), using the Expand Long Template Taq Polymerase® kit (Roche) in the GeneAmp PCR systems 9700 thermal cycler (Applied Biosystems, Foster City, CA). A separate LTR amplicon was generated using the outer primers JL17 and LTR3, and inner primers VWLG-for and VWLG-rev, and cycle conditions as described previously (van Harmelen et al., 2001), using Amplitaq Gold®(Applied Biosystems) in the GeneAmp PCR systems 9700 thermal cycler (Applied Biosystems).

All PCR products were resolved on a 0.8% Tris acetate EDTA (TAE) agarose gel, containing 0.5µg/ml Ethidium Bromide (Promega Corporation, Madison, WI, USA) at 100V for one hour. Gels were visualized on a UV transilluminator. All gels were run with the DNA molecular marker II and IX mixture (Roche; range 0.07 to 23.1kb), for amplicon size determination.

Pooled virtually full-length genome PCR products were purified on a Microcon-YM50 column (Millipore, Bedford, MA), and LTR PCR products were purified using a High Pure PCR Product Purification Kit (Roche) as per manufacturers' instructions. Primers spanning the entire HIV-1 genome (Table 2.2; (Rousseau et al., 2006) were used to sequence the PCR products in both directions. Amplicons were sequenced using the ABI Big Dye terminator system (version 3.1) according to manufacturer's instructions (Applied Biosystems) using 4µl of ready reaction mix, 2µl of 5x sequencing buffer and 1µl of each sequencing primer (3.2 pmol), PCR template, and ddH_2O to a final volume of 20µl. Reactions were placed in a MicroAmp 96-well optical reaction plate (Applied Biosystems). Terminator reactions were

then carried out with 25 cycles of 96^0C for 10 seconds, 50^0C for 5 seconds and 60^0C for 4 minutes in the GeneAmp PCR systems 9700 thermal cycler (Applied Biosystems). Sequencing reaction products were immediately purified of unincorporated dyes terminators, salts and dNTPs using isopropanol precipitation. Briefly, 80µl of 80% isopropanol (Merck,) was added to each well (containing 20 µl), mixed well and incubated in the dark for 15 minutes. The plate was then centrifuged at 2000Xg for 45 minutes, immediately inverted on a paper towel and centrifuged for a further 1 minute at 700xg. The plate was air-dried, and the purified sequencing products were resuspended in 10µl Hi-Di Formamide (Applied Biosystems). Sequencing plates were run on the ABI Prism 3100® Genetic Analyzer (Applied Biosystems), using a 50cm capillary filled with POP-6 polymer gel (Applied Biosystems).

2.3. Genome and Phylogenetic Analysis

All generated sequence data was edited using the Sequencing analysis 3.3 program (Applied Biosystems), and the near full-length and LTR sequences were assembled using Sequencher®4.5 (Genecodes, Ann Arbor, MI) to yield the complete full-length genome sequence in one contig. A multiple alignment of the near full-length genomes with reference sequences from HIV-1 subtypes A to K, and CRF01_AE and CRF02_AG (http://hiv-web.lanl.gov), were generated using Clustal X (version 1.83) (http://www.ebi.ac.uk/Tools/clustalx). The LTR regions were analysed in a separate clustal X alignment, since LTR data was not available for all the reference subtypes. Gaps were stripped prior to phylogenetic analysis. Neighbor-Joining phylogenetic trees were constructed in MEGA 4 (http://www.megasoftware.net) with the Kimura two-parameter model, and a transition/transversion ratio of 2.0. The stability of the nodes was assessed by bootstrap analysis (100 replicates). Bootstrap values greater than 70% were considered significant.

Similarity plots were performed against representative strains from each subtype using the RIP (recombinant Identification Program) 3.0 program in the Los Alamos database, with sliding windows of 300 nucleotides (http://hiv-web.lanl.gov). The nucleotide sequences and predicted amino acids for all the HIV-1 proteins of all isolates were analysed and known

functional motifs were identified. The sequences were annotated and submitted to Genbank using Sequin (http://www.ncbi.nlm.nih.gov/Sequin).

Table 2. 2: Epidemiological and clinical data of HIV-1 vertically infected children attending Chris Hani Baragwanath Hospital in Johannesburg, identified as potential slow progressors.

Sample ID	Age (yrs)*	Gender	Viral load (RNA copies/ml)	CD4 cell Count (cells/µl)
LT5	7	Female	1074	866
LT21	9	Female	6 588	546
LT39	7	Female	12180	760
LT42	8	Male	23564	416
LT45	7.2	Male	5524	410
LT46	10	Male	6024	467

*at time sample was taken

Table 2.2: Primers used for PCR amplification and sequencing of the near full-length genomes and LTR amplicons.

Primer name	Primer sequence 5'-3'	Used for:
MSF12 (outer)	AAATCTCTAGCAGTGGCGCCCGAACAG	Near full length PCR (van Harmelen et al., 2001)
OMFR1 (outer)	TGAGGGATCTCTAGTTACCAGAGTC	
F2NST (inner)	GCGGAGGCTAGAAGGAGAGAGATGG	
OFM19 (inner)	GCACTCAAGGCAAGCTTTATTGAGGCTTA	
JL17 (outer)	CATTCTGCAGCTTCCTCATTGAT	LTR PCR (F. McCutchan, pers. comm.)
LTR3 (outer)	ACTGTCTAGATGGATGGTGCTWCAAGYTAGT	
VWLG-for (inner)	AGATGCTGCATATAAGCAGCYGC	
VWLG-rev (inner)	TCTTTCCCCCTGGCCKTAAC	
U5Cc	CCTTGAGTGCTCTAAGTAGTGTGTGCCCGTCTGT	Sequencing (Rousseau et al., 2006)
U5Cd	AGTAGTGTGTGCCCGTCTGTTGTGTGACTC	
3_3_PlCb	ACTACTTAGAGCACTCAAGGCAAGCTTTATTG	
3_3_pICb	TAGAGCACTCAAGGCAAGCTTTATTGAGGCTTA	
2.U5Cd	AGTAGTGTGTGCCCGTCTGTTGTGTGACTC	
SQ2FC	CTTCAGACAGGAACAGAGGA	
SQ2FC(2)	GAGGAAGAACAAAACAAAAGTCAG	
SQ3FC(2)	GGGAGACATCTATAAAAGATGGATAA	
SQ4F	ACAGGCTAATTTTTTAGGGA	
SQ5F	AAACAATGGCCATTAACAGAAGAGA	
SQ6F	CTTTGGATGGGTTATGAACT	
SQ6.5FC	GCAGAGTTAGAATTAGCAGAGAACAG	
SQ7F(2)	AGGAGCAGAAACTTTCTATGTAGATGG	
SQ8FC	CCTGGTAGCAGTCCATGTAGC	
SQ9FC(3)	CAGGTAGACAGGATGAAGATCAGAAC	
SQ10FC	GGAGCCAGTAGATCCTAACCTAGAG	
SQ11F	ACTCTATTTTGTGCATCAGA	
SQ11FC	TCTATTCTGTGCATCAGGTGCTAAAGCATA	
SQ12F	CCTCAGCCATTACACAGGCCTGTCCAAAG	
DR7C	TCAACTCAACTACTGTTAAATGGTAGCCTAGC	
SQ13F(2)C	TATATAAATATAAAGTGGTAGAAATTAAGC	
SQ14FC	ACTCACGGTCTGGGGCATTA	
SQ15FC	GAGAGCGGTGGAACTTCTGG	
SQ16F	CCACACACAAGGCTACTTCC	
2.3_3_plCb	TAGAGCACTCAAGGCAAGCTTTATTGAGGCTTA	
SQ2R	CACCATCCAAAGGTCAGTGG	
SQ3R(2)C	GCTATGGTATCAAGCAGACTAATAGCACTC	
SQ3RC	TTTAGTTCCAGACCCCAATA	
SQ5.5RC	CTAGGAGCTGTTGATCCTTTAGGTAT	
4R(2)C	GTTGATCCCTTAGGTATCTTTCTA	
5R(3)C	CATTGCTCGTCCTACCCCCTGCCAC	
SQ6RC	ATAATGTATAGGAATCGGGT	
SQ6RC(2)	GAATTGGGTCAAAAGAGACCTTTGGA	
SQ7R(2)C	CTGTGGGTACACAGGCATGTGTAGC	
SQ8R	CTCCGCTTCTTCCTGCCATAGGAGAT	
SQ9'RC	ATATGAATTAGTTGGTCTGCCAGGCC	
SQ9RC(3)	TTGGATGTCTGCTTTCATAATGAT	
SQ8RC	TTCTACTACTCCCTGACTTTGGGGAT	
SQ10RC	GCCATTGTCTGTATGTATTACTTTGACTG	
RTC	CATTTGTCAGGATGGAGTTCATA	
SQ11RC(3)	TTTAGGAGTCTTTCCCCATATTACTAT	
SQ11RC(4)	GCTTGTAATTCAGTCTTCTGATTTGTTG	
SQ12R	TGTATGTCATTGACAGTCC	
HIP202	CTAATACTGTATCATCTGCTCCTGT	
13R(2)C(2)	GTGTTATATGGATTTTCAGGCCC	
14R(3)C	TCGAATCTGAAGCTCTCTGCTGGTG	
SQ15R	TTTGGTCCTTGTCTTATGTCCAGAATGC	
SQ16RC	CTTGTCTAGGGCTTCCTTGGT	
ED31	CCTCAGCCATTACACAGGCCTGTCCAAAG	
DR7	TCAACTCAACTGCTGTTAAATGGCAGTCTAGC	
DR8	CACTTCTCCAATTGTCCCTCATATCTCCTCC	
BH2	CCTTGGTGGGTGCTACTCCTAATGGTTCA	
1.5_5_PLC	GGGTCTCTCTAGGTAGACCAGATCTGAG	
1.3_3_ProC	AGGGTCTGAGGGATCTCTAGTTACCAGAG	

3. RESULTS

The HIV-1 primary virus isolates from six vertically infected children were successfully expanded using PHA-stimulated donor PBMCs. All six isolates were found to utilize the CCR5 coreceptor for cell entry, and exhibited the NSI phenotype (Table 3.1). Proviral DNA was successfully extracted and used to PCR amplify the near full-length genomes and LTR fragments (Figure 3.1). The near full-length genomes were amplified by nested PCR to yield an approximately 9.5 kb amplicon (Figure 3.1; Lanes 1 to 5), whereas for the LTR amplicons, the yield for all but one sample was adequate after the first round. The first round LTR amplicon was the expected size of approximately 1.2kb (Figure 3.1; Lanes 6 to 9), and second round amplicon was approximately 0.45kb (Figure 3.1; Lane 10). All amplicons were purified and successfully sequenced.

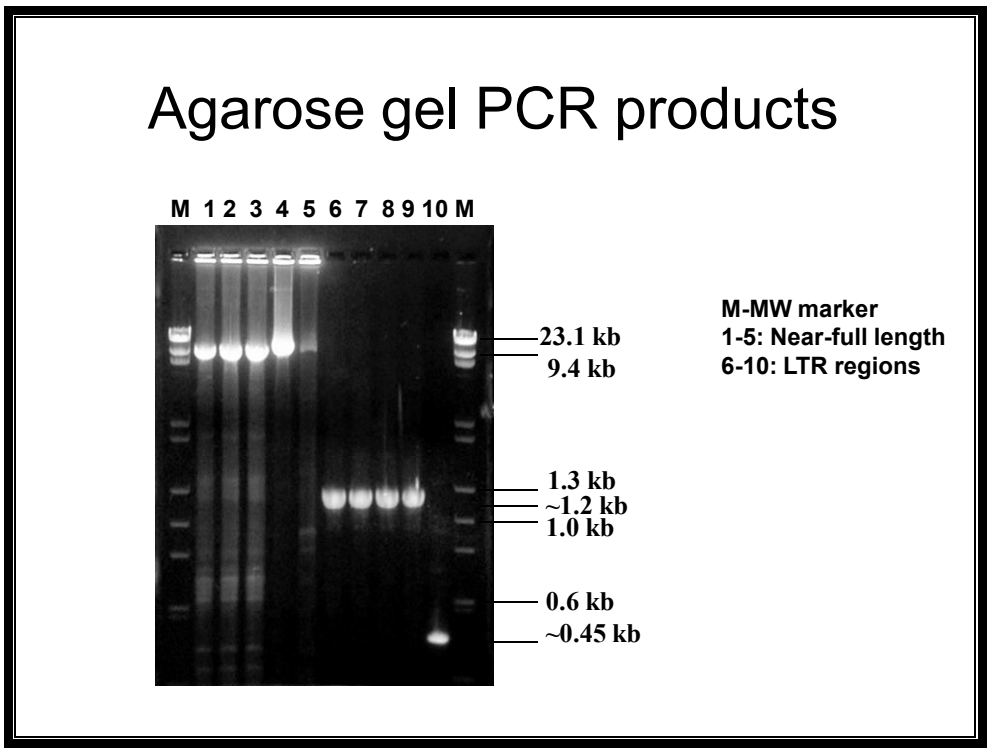


Figure 3.1: Representative agarose gel electrophoresis (1%) of the near full-length PCR (~9.5 kb, Lanes 1 to 5) and LTR PCR amplicons (~0.45-1.2 kb, Lanes 6 to 10) from the newly characterized HIV-1 subtype C isolates. The sizes of the molecular weight marker (M; Roche) are indicated in kilobases (kb) on the right.

Extensive sequence analysis revealed that all six full-length genomes encoded for the correct open reading frames (ORFs), and ranged from 9703 to 9767bp in length (Table 3.1). Nucleotide sequences for the full-length genomes were submitted to Genbank and are available under the accession numbers listed in Table 3.1. Phylogenetic analysis for the near full-length genomes and the LTR regions showed that all six isolates clustered within subtype C with a bootstrap value of 100% (Figures 3.2 and 3.3, respectively). In addition, all six isolates were subtype C throughout their genomes by similarity plots (Appendix B).

The nucleotide sequences and predicted amino acids for all the HIV-1 proteins of all six isolates were extensively analyzed and compared to each other to identify aberrant genes/proteins. All isolates were found to have unusual genes/proteins (listed in Table 3.1). An alignment of the predicted amino acid sequences for the unusual *integrase, vpr, tat ,rev* and *nef* genes are shown in Figures 3.4, 3.5, 3.6, 3.7 and 3.8 respectively. A description of the single letter amino acid (aa) sequence code is shown in Appendix C. The consensus C and ancestral C sequences were used for comparative purposes. Isolate LT39 had a four amino acid sequence extension at the C terminus of Integrase (IN) (Figure 3.4). Isolate **LT5** has a 2aa insertion in the mitochondriotoxic domain of Vpr, and isolates **LT21** and **LT42** contain the H77Q and LT45 has the A77Q polymorphism in Vpr (Figure 3.5). Isolate **LT21** contained an additional 5aa in the C-terminus of tat exon 2 (Figure 3.6). Rev in isolates **LT45** and **LT46** had a 3aa extension (Figure 3.7). Isolate LT5 contains the Nef N9S mutation and the Nef ExxxLM motif is disrupted in **LT46** (Figure 3.8). There were no obvious “defects” in the LTR regions, and remainder coding regions of the genome for all isolates (results not shown).

Analysis of the polymerase gene nucleotide sequences for the presence of antiretroviral (ARV) drug resistance mutations (Stanford database, http://hivdb.stanford.edu) revealed that no resistance mutations to RT or protease inhibitors were found, with the exception of V82A in the protease of LT46, which has been associated with phenotypic resistance to ritonavir, indinavir, nelfinavir and lopinavir *in vitro*.

The V3 loop region of the envelope glycoprotein (gp120) is involved in coreceptor usage, thus the predicted V3 amino acid sequences were extensively analyzed and compared

(Figure 3.9). The length, overall positive charge, amino acids at positions 11 and 25 and tetrapeptide motif at the tip of the V3 loop, are characteristic of CCR5 utilizing envelopes, and correctly correlated with the phenotype/biotype of each primary isolate (Table 3.1).

Table 3.1: Overview of biological and genotypic data obtained for the six newly characterized primary viral isolates from slow progressors.

Isolate name	Biotype/ Phenotype	Full length Genome (nucleotides)	Subtype	Genotypic Resistance profile[2]	Unusual genes	GenBank Accession number
99ZALT5	R5/NSI	9767	C	None	*vpr*	EU293445
99ZALT21	R5/NSI	9723	C	None	*vpr, tat*	EU293446
99ZALT39	R5/NSI	9744	C	None	*integrase*	EU293447
99ZALT42	R5/NSI	9729	C	None	*vpr*	EU293448
99ZALT45	R5/NSI	9703	C	None	*vpr, rev*	EU293449
99ZALT46	R5/NSI	9762	C	V82A in PR	*rev, nef*	EU293450

[1]R5/NSI: CCR5-utilizing, non-syncytium inducing.

[2]ARV drug naive, V82A reduces susceptibility to ritonavir, indinavir, nelfinavir and lopinavir *in vitro*.

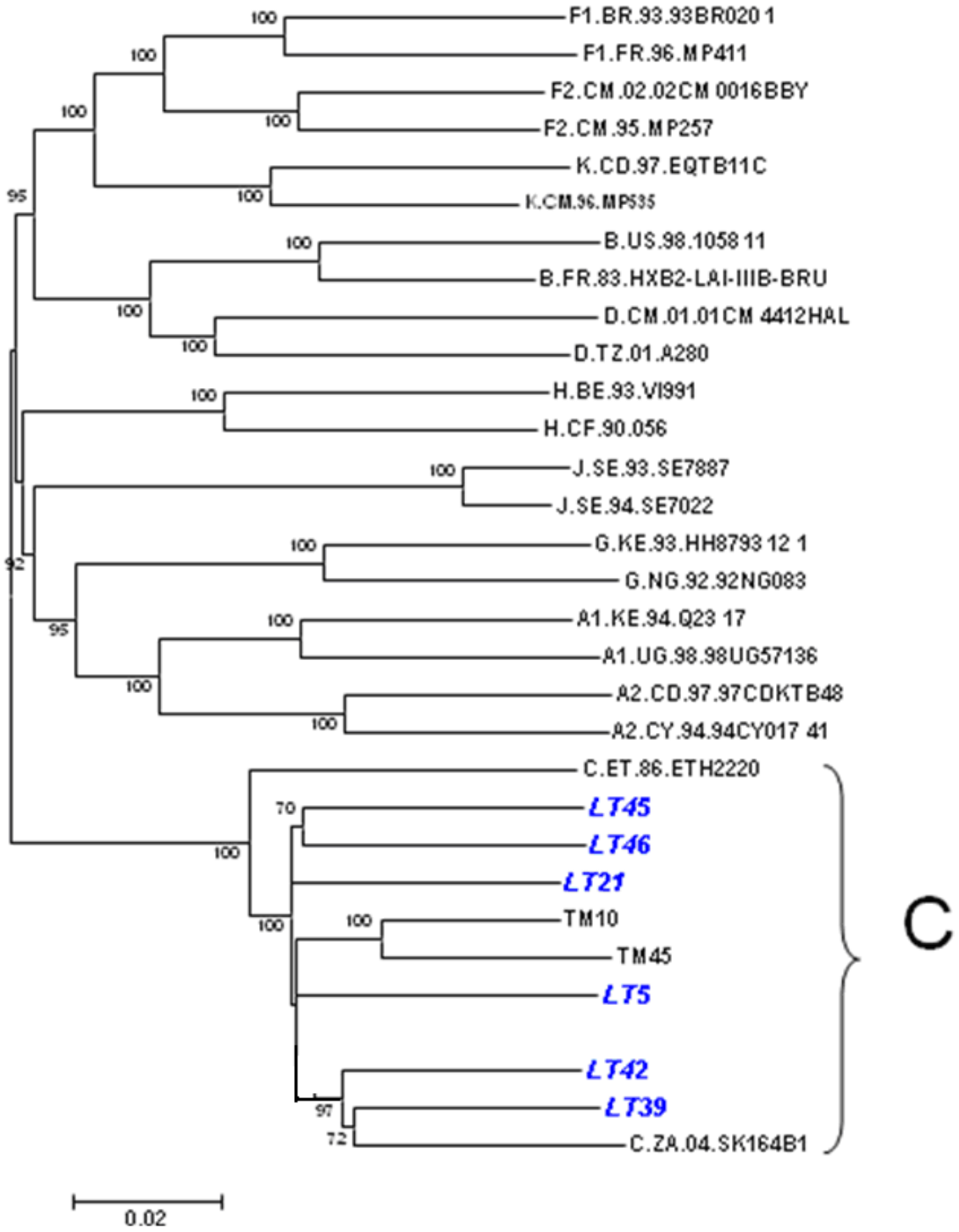


Figure 3.2: Phylogenetic relationships of the near full-length genomes of the six newly characterized subtype C viruses, with HIV-1 group M subtype reference sequences from the Los Alamos database. The newly sequenced genes are shown in blue italics. Phylogenetic trees were constructed from nucleotide alignments, using neighbour joining and the Kimura two-parameter distance matrix. The reliability of the branching order was estimated from 100 bootstrap replicates, and only bootstrap values of 70% or higher are shown.

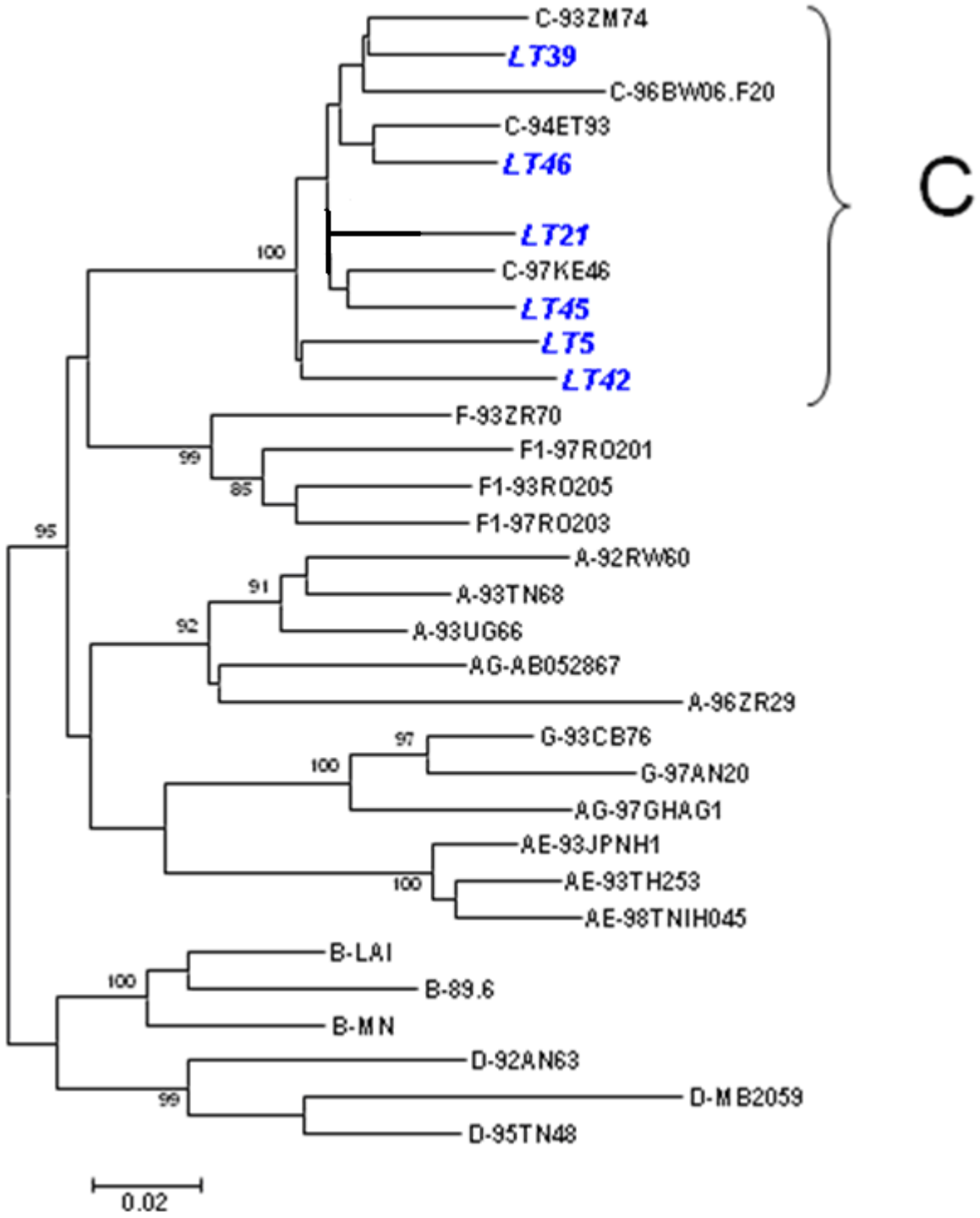


Figure 3.3: Phylogenetic relationships of the LTR regions of the six newly characterized subtype C viruses with HIV-1 subtype reference sequences from the Los Alamos database. The new sequences are shown in blue italics. Only bootstrap values of 70% or higher are shown. Phylogenetic trees were constructed from nucleotide alignments, using neighbour joining and the Kimura two-parameter distance matrix. The reliability of the branching order was estimated from 100 bootstrap replicates, and only bootstrap values of 70% or higher are shown.

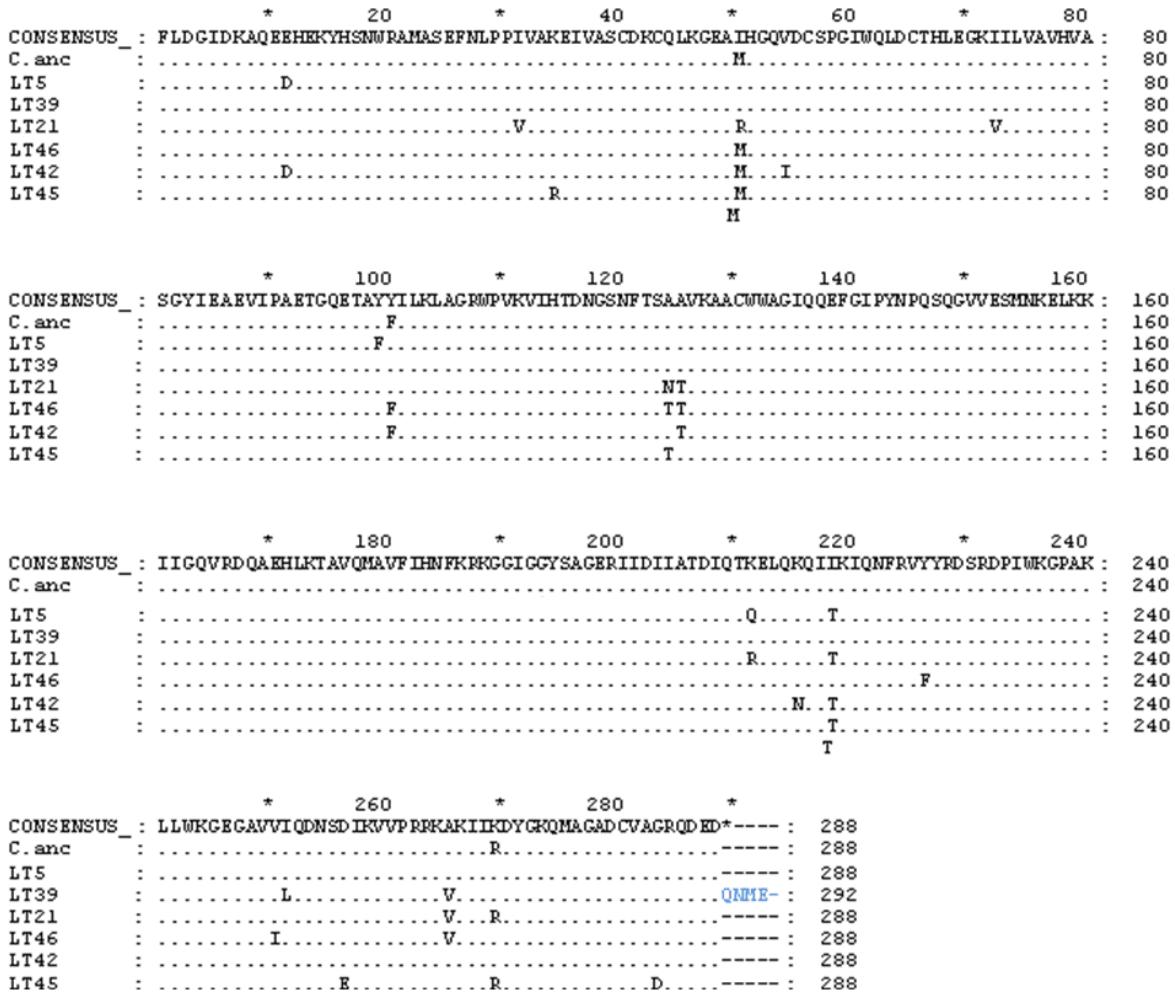


Figure 3.4: Alignment of the predicted amino acid sequences for the Integrase enzymes from the six newly characterized subtype C viruses and comparison to the consensus C and ancestral C sequences obtained from Los Alamos (www.hiv.lanl.gov). The unusual motif at the C-terminus of the LT39 integrase enzyme is shown in blue. The dots represent identical amino acids, and dashes indicate insertions/deletions.

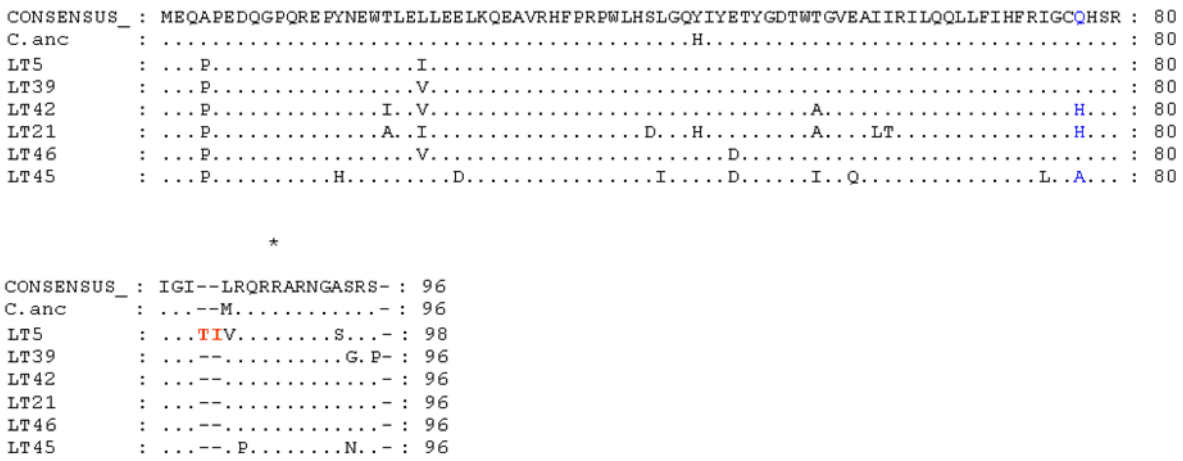


Figure 3.5: Alignment of the predicted amino acid sequences for the viral protein R (Vpr) from the six newly characterized subtype C viruses and comparison to the consensus C and ancestral C sequences obtained from Los Alamos (www.hiv.lanl.gov). The unusual 2 amino acid insertion at the C-terminus of LT5 Vpr is shown in red. Position 77 is highlighted in blue. The dots represent identical amino acids, and dashes indicate insertions/deletions.

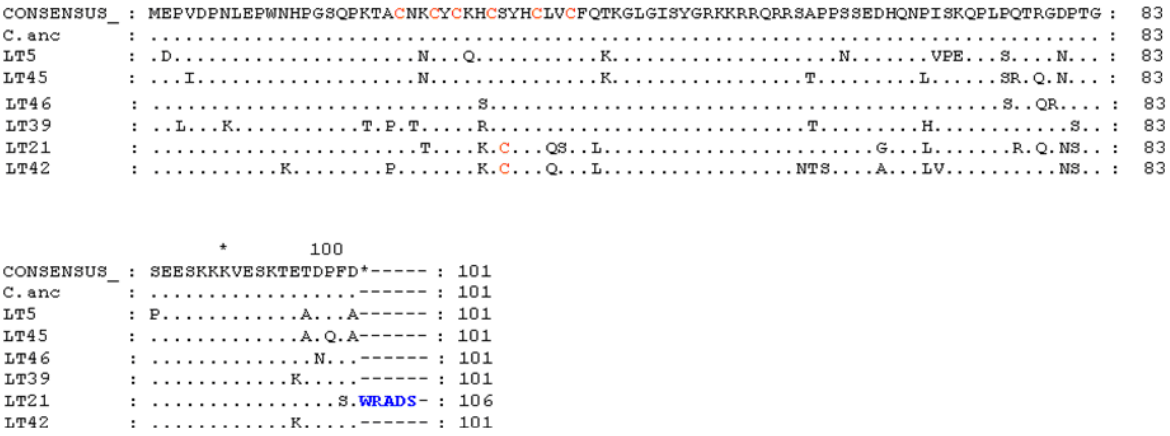


Figure 3.6: Alignment of the predicted amino acid sequences for Tat from the six newly characterized subtype C viruses and comparison to the consensus C and ancestral C sequences obtained from Los Alamos (www.hiv.lanl.gov). The unusual motif at the C-terminus of LT21 is shown in blue. The cysteine rich motif is highlighted in red. The dots represent identical amino acids, and dashes indicate insertions/deletions.

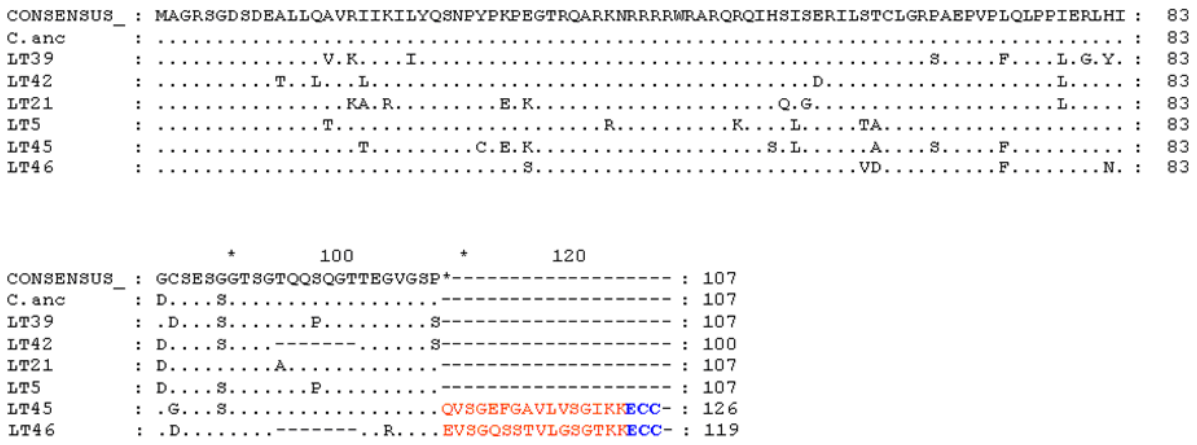


Figure 3.7: Alignment of the predicted amino acid sequences for Rev from the six newly characterized subtype C viruses and comparison to the consensus C and ancestral C sequences obtained from Los Alamos (www.hiv.lanl.gov). The unusual three amino acid insertion at the C-terminus of LT45 and LT46 is shown in blue. The dots represent identical amino acids, and dashes indicate insertions/deletions.

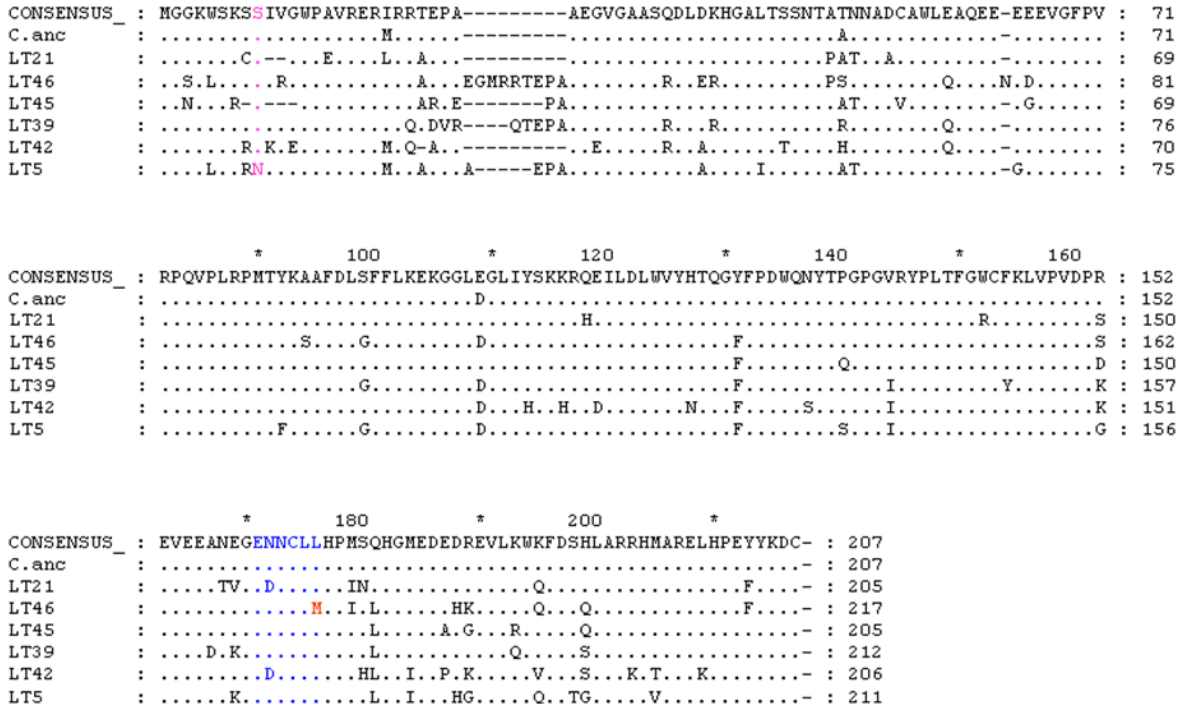


Figure 3.8: Alignment of the predicted amino acid sequences for Nef from the six newly characterized subtype C viruses and comparison to the consensus C and ancestral C sequences obtained from Los Alamos (www.hiv.lanl.gov). Position 9 is highlighted in pink,

and the ExxCLL motif is shown in blue. The dots represent identical amino acids, and dashes indicate insertions/deletions.

Isolate	V3 loop sequence	# aa	Charge	Phenotype	Coreceptor usage
	11 25				
CONSENSUS C:	CTRPNNNTRKSIRIGPGQTFYATGDIIGDIRQAHC	35	+5	NSI	CCR5
C.anc	: ..........E.....G.....D..........	35	+5	NSI	CCR5
LT21	: .I.........E.....G....S.H...E...N.....	35	+6	NSI	CCR5
LT45	: .S.IG....T..G.....A.F...E...N..K...	35	+4	NSI	CCR5
LT39	: .I.......E.....G....A.....G..........	35	+3	NSI	CCR5
LT42	: ....G....Q.....G....A..-.NN..........	34	+4	NSI	CCR5
LT46	: ....S.....EV.....G....A.F...E..........	35	+4	NSI	CCR5
LT5	: .I.TG.....EV.....G.....DG......K.Y.	35	+3	NSI	CCR5

Figure 3.9: Alignment of the predicted V3 amino acid sequences of the 6 newly characterized HIV-1 subtype C isolates. The sequences are compared to the HIV-1 subtype C consensus and ancestral sequence (www.hiv.lanl.gov). The overall positive charge, number of amino acids, phenotype and biotype of each isolate is shown on the right. Dashes (-) indicate deletions/insertions, and positions 11 (green) and 25 (pink) and the V3 crown/tip (yellow) are highlighted.

4. DISCUSSION

In this study we have shown that the six primary HIV-1 subtype C viruses isolated from potential SP or LTNP all harboured potentially defective genomes. Analysis of the polymerase gene nucleotide sequences for the presence of ARV drug resistance mutations revealed that no resistance mutations to RT or protease inhibitors were found, with the exception of V82A in the protease of LT46, which reduces susceptibility to ritonavir, indinavir, nelfinavir and lopinavir *in vitro* (Larder et al., 2000; Shafer, Stevenson, and Chan, 1999). The presence of V82A in untreated individuals has been reported (http://hivdb.stanford.edu), and since protease inhibitors were not available in the public sector in South Africa at the time of sample collection, it is highly likely that this patient was ARV drug naïve. Thus, these patients were all considered to be ARV drug naïve, ruling out the possibility that antiretroviral therapy contributed to the delayed disease progression in these children.

Nucleotide and predicted amino acid sequence analysis revealed all viruses had open reading frames for all genes and encoded for proteins of the expected length, with several exceptions. Overall, patients **LT5, LT21** and **LT42** contain HIV-1 harboring Vpr with altered/decreased pro-apoptotic activity. Furthermore, HIV-1 from patient **LT21** exhibited an unusual tat exon 2 variant which could alter tat-mediated Reverse transcription. Patient **LT39** is infected with HIV-1 containing IN with potentially altered efficiency for full site integration. Finally, patients **LT45** and **LT46** harbored virus with an ECC extension in Rev which may alter Rev structure, impairing its ability to bind to RNA and other cellular factors or prevent ternary complex disassembly. In addition, the HIV-1 Nef from patient **LT46** contains a disrupted ExxxLM motif which may render LT46 Nef unable to bind adaper protein 1 (AP-1), and therefore inactive on CD4 endocytosis. It is possible that these alterations lead to altered viral replication which impacts on disease progression in these children.

The four amino acid (aa) QNME extension in the IN of **LT39** was unique to this isolate. IN mediates the integration of cDNA into the host chromosome, an essential step in HIV replication(reviewed in (Asante-Appiah and Skalka, 1999). Integration is mediated by a large nucleoprotein complex (PIC) containing viral DNA, Vpr , IN and several cellular proteins such as HMGa1 (Harris and Engelman, 2000), IN-1/SNF5 (Kalpana et al., 1994) and LEDGF/p75 (Rahman et al., 2007). The recognition sequence for the integrase enzyme was located at the ends of the viral LTRs (Roth, Schwartzberg, and Goff, 1989) binding to which requires at least a cross-linked tetrameric form of integrase to perform full-site integration (Faure et al., 2005). Integration of proviral DNA occurs preferentially into transcriptional units of active genes (Schroder et al., 2002). Centromeres and telomeres are not favoured targets (Carteau, Hoffmann, and Bushman, 1998). IN catalyzes the integration of viral cDNA into the host chromosome via 3' processing and strand transfer: the first is by processing the 3' ends of linear viral cDNA in which the GT nucleotide pairs of the viral DNA are removed leaving a CA dinucleotide at each 3'end (occurs in the cytoplasm), and the second is the strand transfer (or joining reaction) in which the processed viral DNA ends are inserted into host DNA (occurs in the nucleus). The C-terminal region of IN is involved in non-specific DNA binding. It is also important for oligomerization of the enzyme and is necessary since IN monomers/dimers can only integrate one side of the LTR. It is possible that the tetrapeptide QNME insertion identified at the C terminus of IN from isolate LT39 disrupts IN oligomerization, thereby altering the efficiency of the full site integration activity.

Viral protein R (Vpr) is responsible for multiple functions of HIV-1, including nuclear import of the pre-integration complex, apoptosis, fidelity of the RT process, G_2 cell cycle arrest and transactivation of the LTR and/or target genes thereby controlling HIV-1 gene expression (Cui et al., 2006). The 96 amino acid Vpr protein is packaged into the HIV-1 virion and released into the cytoplasm during initial cell infection where it can exert its actions. The *vpr* gene exhibits a wide range of naturally occurring polymorphisms (reviewed in (Tungaturthi et al., 2004), and the association between these and disease progression warrants further investigation. The Vpr R77Q mutation has been associated with long term non progression and affects the mitochondriotoxic domain of the wild type gene which has been shown to induce T cell apoptosis (Lum et al., 2003), and has been argued to be necessary for virulence (Brenner and Kroemer, 2003). Isolate **LT5** has a unique 2aa insertion in the

mitochondriotoxic domain of Vpr. Previous research showed that R77Q was associated with slower disease progression, by decreasing the pro-apoptotic activity of Vpr (Lum et al., 2003). Isolates **LT21** and **LT42** contain H77Q, which represents a conservative amino acid change vs R77Q. Thus, these three isolates likely contain Vpr with altered/decreased pro-apoptotic activity. Interestingly, Vpr has neurotoxic effects (Jones et al., 2007) and a defective *vpr* gene has recently been implicated in HIV-1 dementia (Thomas et al., 2007).

The tat viral gene is mainly responsible for transactivation of HIV-1 gene expression in conjunction with the TAR element, (Bannwarth and Gatignol, 2005) and other diverse Tat functions have been reviewed in (Harrich et al., 2006). Tat is encoded by two exons, and in natural infection both Exon 1 and Exon 2 are transcribed by cells, so different forms have different molecular functions. Tat exon 1 is highly conserved and its main function is to mediate transactivation of the viral LTR promoter. It can also induce monocyte chemotaxis and protein-protein interactions as well as uptake of soluble tat by cells. Exon 2 conveys improved viral replication and has been implicated in chemoattraction of monocytes and dendritic cells (Izmailova et al., 2003), in cell adhesion and binding to cell surface integrins, modulation of MHC class I gene expression, ability to inhibit reverse transcription, tat mediated apoptosis (Peruzzi, 2006), neurotoxicity (Sabatier et al., 1991) and can bind human translation elongation factor 1 delta (Xiao et al., 1998). Isolate **LT21** contained an additional 5aa in the C-terminus of tat exon 2. There are no previous reports of a 5 aa extension in the databases (rarely see 4 aa). Tat exon 1 has been shown to facilitate the first strand transfer reaction of RT to cDNA, while Tat exon 2 (regions 86 to 101) inhibits cDNA synthesis when tat concentrations reach a critical level. Future work needs to elucidate whether the LT21 tat exon 2 (86 to 106) variant is an enhancer or suppressor of tat-mediated reverse transcription.

Rev initiates the late phase of virus gene expression by interacting with the RRE on the full length 9 kb and singly spliced 4 kb viral RNA transcripts, and their nuclear import and export is mediated by binding of the Rev nuclear localization signal to importin β, or the Rev nuclear export signal to Crm1/Exportin-1, respectively. Rev thus plays a critical role for the production of HIV-1 structural proteins (reviewed in (Cochrane, 2004). Rev in isolates **LT45** and **LT46** had a 3aa extension. There are only two other reports of this Rev polymorphism:

the SBBC LTNP viruses (Churchill et al., 2007), and SP from vertically infected siblings (TM10 and TM45) from the same cohort in South Africa (Papathanasopoulos et al., 2003). Rev activity is defined by domains or motifs for nuclear export of RNA, RNA binding, and nuclear import signals, which have been shown to be conserved during vertical transmission (Ramakrishnan et al., 2005). Thus the presence of the ECC extension may alter Rev structure, impairing its ability to bind to RNA and other cellular factors or prevent ternary complex disassembly.

The functions of nef have been reviewed in (Das and Jameel, 2005). Nef causes endocytosis and lysosomal degradation of CD4 and downregulation of MHC class I and II. The leucine residues in the Nef ExxxLL motif are required for CD4 downregulation and are involved in the interaction with adapter protein 1 (AP-1). This motif is disrupted in **LT46** (ExxxLM). This may render LT46 Nef unable to bind AP-1, and therefore inactive on CD4 endocytosis. Thus, no/low degradation of CD4 in an infected cell will most likely impact on the production of infectious virions. Nef gene deletions are probably the most extensively studied, since the initial identification of this deletion in adult LTNPs in the SBBC (Deacon et al., 1995). In addition, the cAMP-dependent kinase PKA phosphorylation site in Nef of **LT5** is altered. Nef aberrations have been well described (Brambilla et al., 1999) and frequently involve the LTR regions of the viral genome, the inability of the virus to cause downregulation of MHC and CD4 molecule may contribute to immune recognition of the infected cells, subsequent cytotoxicity, and lead to host resistance. In contrast, variations in nef dependant epitopes have been shown to facilitate immune escape and an attenuated response of the cytotoxic T-lymphocytes (Ueno et al., 2007). Nef mutations have recently been linked to different rates of disease progression in children (Walker et al., 2007). The latter studies were in a cohort of 34 children and sequencing was notably done only on the *nef* gene.

Overall, this study describes the identification of six potential HIV-1 subtype C attenuated viruses and unusual genes coding for possibly defective Integrase, Vpr, Tat, Rev and Nef proteins that may affect viral replicative capacity which may be partly attributed to the long term survival of these children. However, as mentioned previously there are many confounding variables which contribute to long-term non progression of HIV-1 infected individuals. This study concentrates on the genotypic characteristics of the infecting viral

isolates but that is not to say that this is the sole determinant which contributes to the extended survival of these patients. Importantly, this study provides clues on future function analysis to establish an association between structural or functional alterations in various HIV-1 proteins and their impact on disease progression.

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Appendix A

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

Ref: W-CJ-070514-1
14/05/2007

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **D Tzitzivacos**

Project title: Non-progressive HIV-1 subtype C infection-viral determinants.

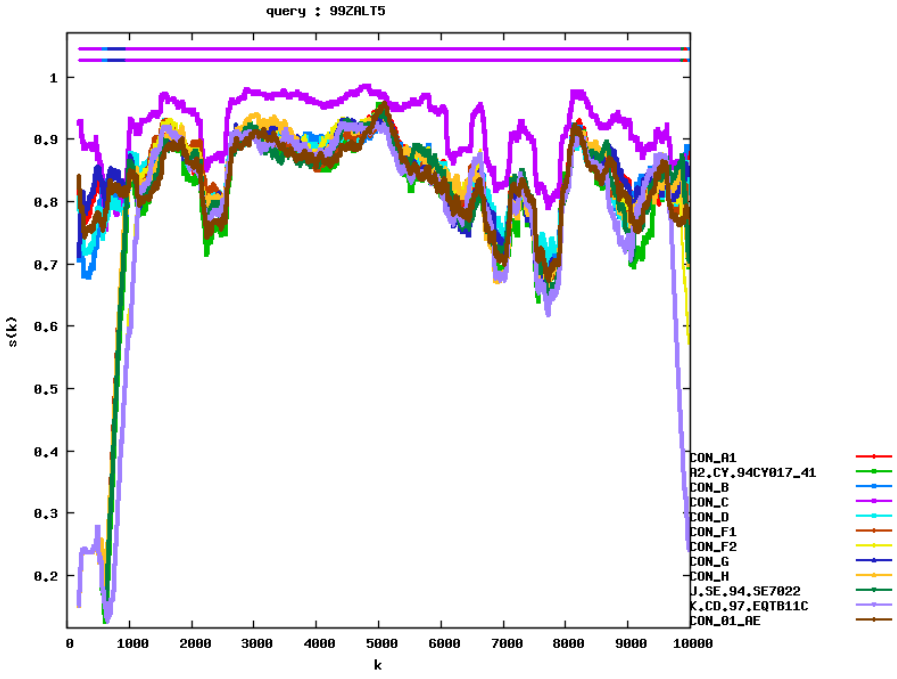
Reason: This MMed project uses primary virus isolates collected by Prof C Tiemessen (NICD) under ethics clearance M01–05-59. There are no human participants.

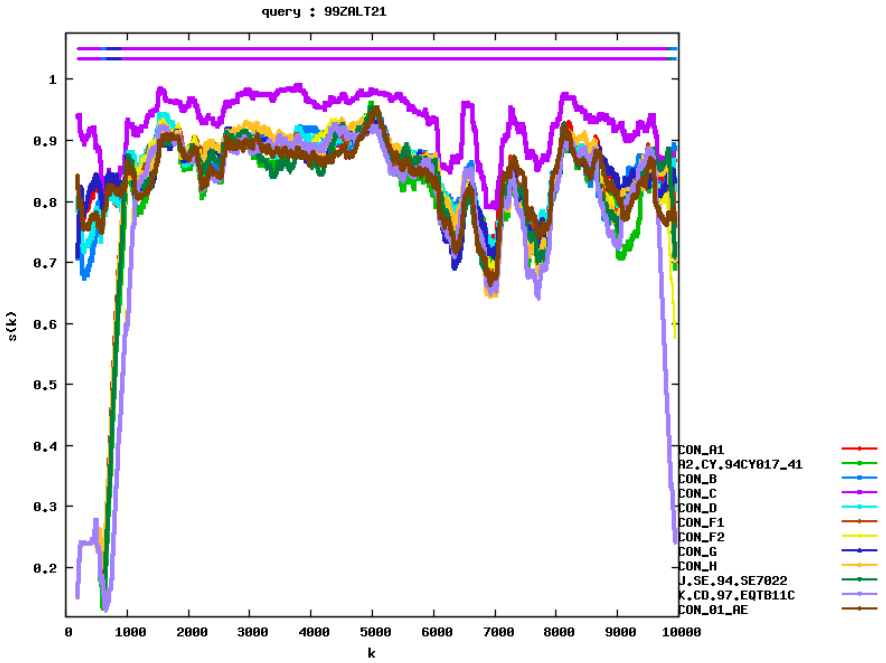
UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON - JONES
HREC (MEDICAL)
2007 -05- 14
JOHANNESBURG

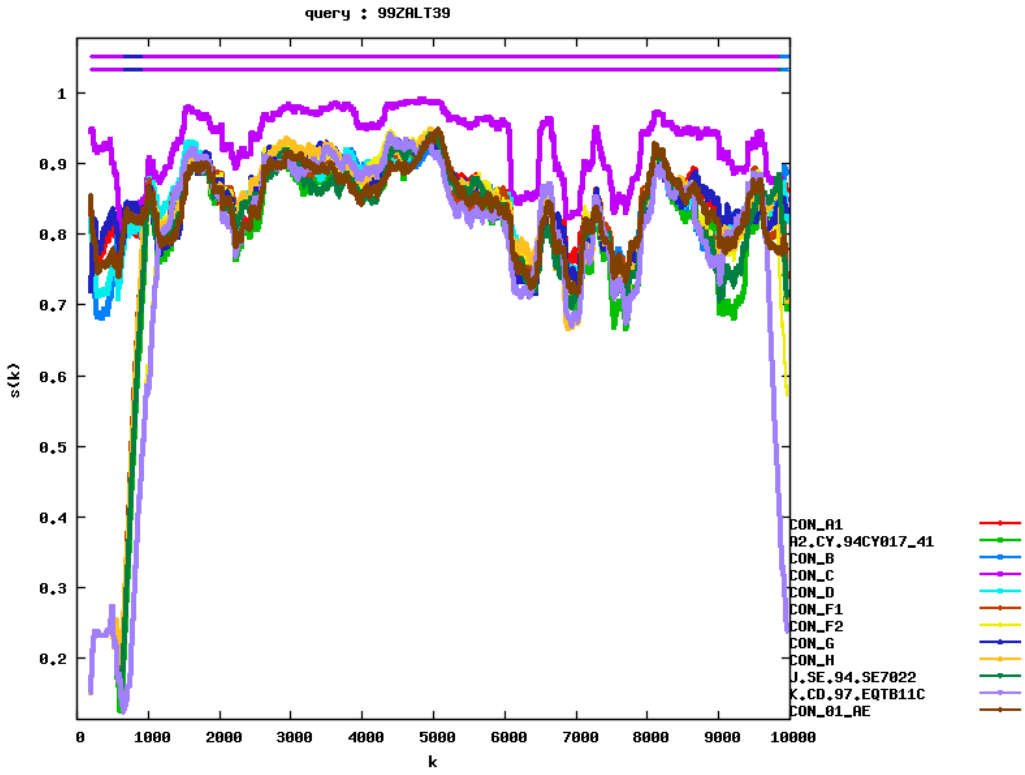
Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

Appendix B: Similarity plots of the full length genome sequences of the 6 newly characterized sequences, confirming they were HIV-1 subtype C. The test sequence is compared to consensus sequences of all subtypes, and the sequence it shares the most homology with is shown closest to s(k) values of 1.



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0.5
0.6
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0.9
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CON_B
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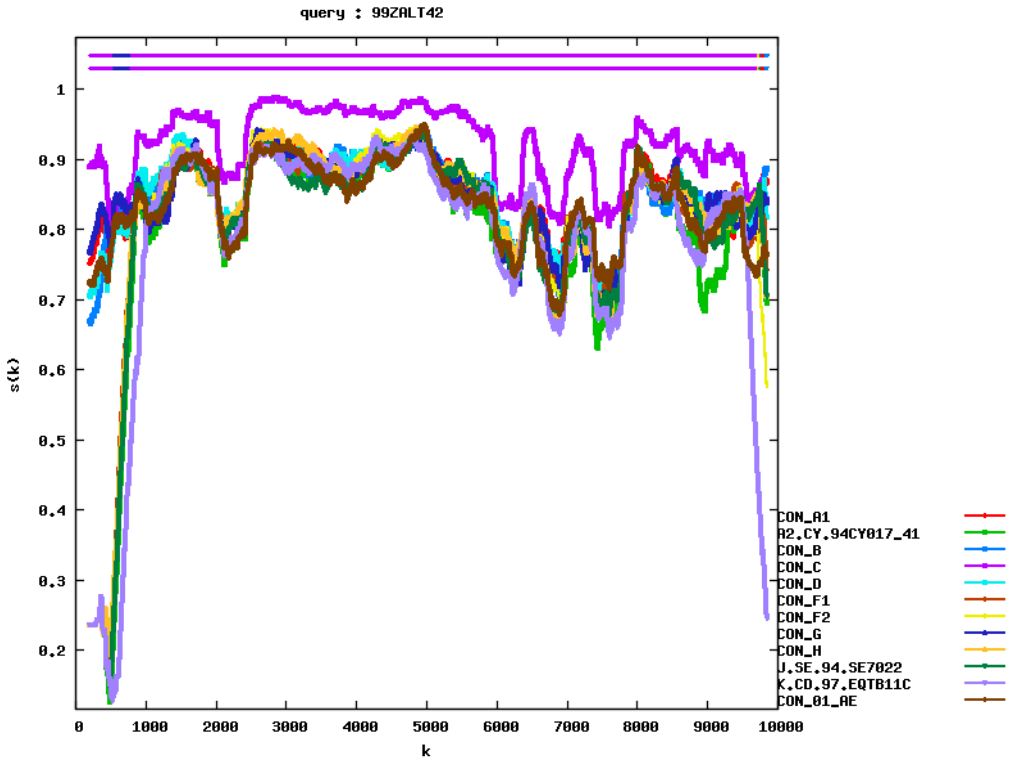
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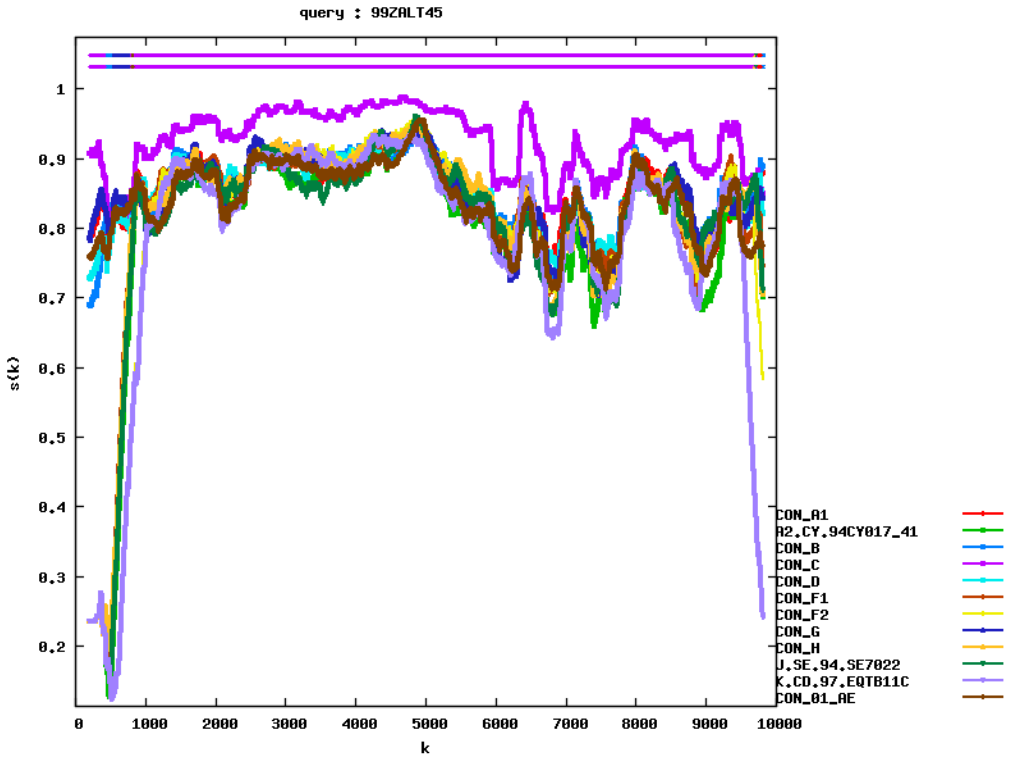
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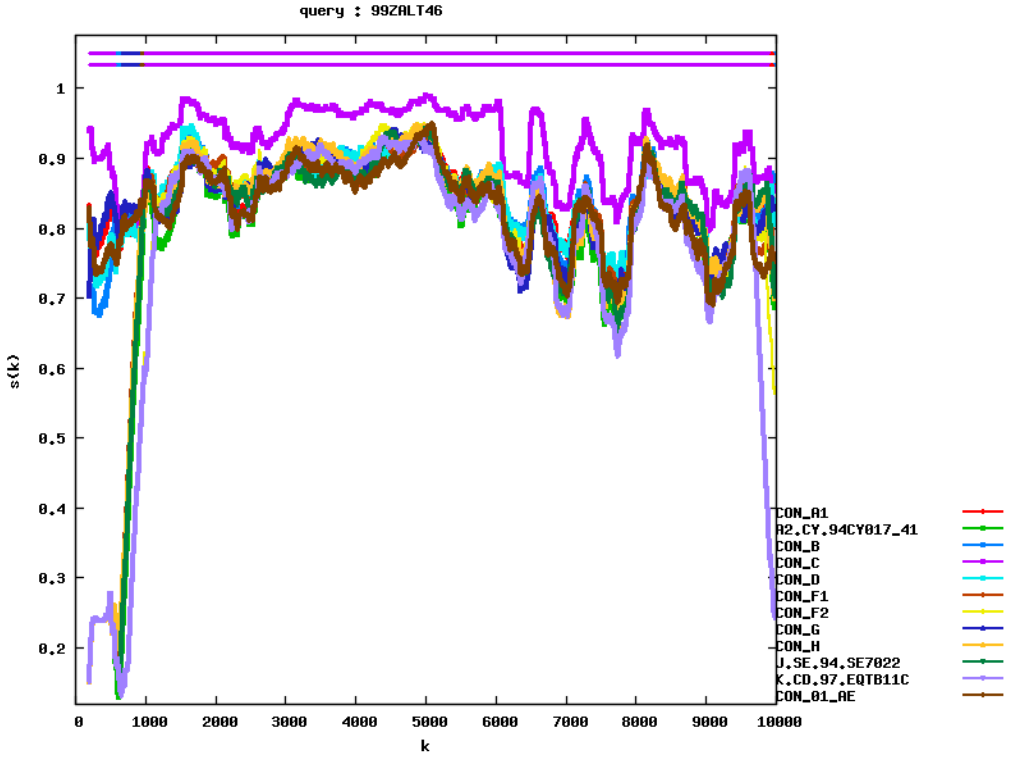
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CON_F2
CON_G
CON_H
J.SE.94.SE7022
K.CD.97.EQTB11C
CON_01_AE

Appendix C: Amino acid codes

single-letter code	*abbreviation*	*full name*
A	Ala	Alanine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	Aspartic acid
C	Cys	Cysteine
Q	Gln	Glutamine
E	Glu	Glutamic acid
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Leu	Leucine
K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine