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Genetic variation in gp120 V1/V2 and neutralization sensitivity of HIV-1 subtype C isolates from children with rapid and slow disease progression

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Isaac Ang'Ang'A Choge

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, for the Degree of Master of Science in Medicine (Virology).

Johannesburg, 2003

ABSTRACT

This project explores genetic variation and antibody neutralization sensitivity in gp120 V1/V2 and C2-V5 regions amongst HIV-1 subtype C pediatric isolates. Isolates from 25 slow progressing children and 16 rapid progressing infants were used in subtyping and quasispecies analysis. The V1/V2 and C2-V5 regions were amplified by nested PCR and subtyping analysis performed with a novel V1/V2 heteroduplex mobility assay (HMA). This was compared to the well-established C2-V5 HMA. Population-based sequencing and phylogenetic analysis was done on all samples for both regions. The C2-V5 HMA indicated that forty of the isolates were HIV-1 subtype C, with one subtype B, which was confirmed with phylogenetic analysis. However, the V1/V2 HMA subtyped only 66% of the isolates correctly. Further genetic analysis showed that this region was too small and contained extensive hyper-variability making it unreliable for subtyping. The ratio of non- synonymous to synonymous base substitutions per site in V1/V2 was higher in children with slow disease progression. This suggested that the V1/V2 region in slow progressing children was under stronger diversifying pressure than children with rapid disease progression. However, in the C2-V5 region there was stronger diversifying pressure in the rapid progressing children.

Three isolates were selected and analysed further to identify neutralization sensitive and resistant quasispecies. A peripheral blood mononuclear cell (PBMC) based neutralization assay was used together with a more sensitive V1/V2 heteroduplex tracking assay (HTA). This new approach demonstrated the selection of viral variants in increasing concentrations of autologous serum. Two of these isolates showed both neutralization sensitive and resistant bands at different serum concentrations, whilst the third isolate did not show any differences despite showing a good neutralization profile. Isolation and sequencing of individual bands from the two isolates may identify envelope genes that are

highly sensitive or resistant to antibody neutralization. These data could contribute towards the design of an *env* immunogen that includes neutralization-sensitive epitopes.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Medicine (Virology) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.



Isaac Ang'Ang'A Choge

~~30th~~ 6th day of ~~August~~ May 2004 ~~2003~~

I dedicate this work to my personal Lord and saviour Jesus Christ, for his everlasting strength and courage throughout my life and this work.
To My late Mother, who was always there in prayer to her last day. She was a Hero of a mother
To the rest of my family, and to my loving girlfriend, Lydiah Waki Wambugu. God Bless you all, always.

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LIST OF ABBREVIATIONS

ng	nanogram
µg	microgram
µl	microlitre
°C	degrees celcius
ag	antigen
AIDS	acquired immunodeficiency syndrome
ATP	adenosine triphosphate
AZT	3'-azido-3 deoxythymidine
bp	base pair
C1-C5	constant regions 1-5
CDC	Centre for Disease Contol
CD4	cluster of differentiation 4
CD4bs	cell determinant 4 binding site
cDNA	complementary deoxyribonucleic acid
CHBH	Chris Hani Baragwanath hospital
CNS	central nervous system
CO_2	carbon dioxide
CRF	circulating recombinant form
CTL	cytotoxic T lymphocytes
DC	dentritic cells
DC-SIGN	specific intercellular adhesion molecule grabbing nonintegrin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
$d_n d_s$	ratio of non-synonymous to synonymous base substitutions
dsDNA	double stranded DNA
DTT	dithiothreitol
EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunoabsorbent assay
env	envelope glycoprotein gene

Env	envelope glycoprotein
Env gp120	Envelope glycoprotein 120
Env gp160	Envelope glycoprotein 160
EthBr	ethidium bromide
FCS	fetal calf serum
gag	group associated antigens gene
Gag	group associated antigens
Gly	glycosylation site(s)
GTR	general time reversible
H_2O	water
HAART	highly active antiretroviral therapy
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HMA	heteroduplex mobility assay
hr	hour
HTA	heteroduplex tracking assay
HTLA III	Human T-cell lymphocyte virus type 3
IL-2	interleukin-2
IPTG	isopropyl-β-D-thiogalactoside
IVDU	intravenous drug users
LAV	lymphocyte associated virus
LB	Luria Bertani
LTNP	long-term non-progressor
LTR	long terminal repeat
m	molar
MA	milliampere
MAbs	monoclonal antibodies
mg	milligram
$MgCl_2$	magnesium chloride
M group	major group
min	minute
ml	millilitre
ML	maximum likelihood
mM	millimolar
MTB	*Mycobacterium tuberculosis*

MTCT	mother to child transmission
mRNA	messenger RNA
N group	novel variant
NAbs	neutralizing antibodies
NaCl	sodium chloride
nd	not done
NSI	non-syncytium-inducing
O group	outlier group
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
PNS	peripheral nervous system
R5	CCR5 using viruses
RANTES	regulated on activation normal T-cell expressed and activated
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonucleases
RP	rapid progressor
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
SABTS	South African blood transfusion services
sec	second
SI	syncytium inducing
SIV	simian immunodeficiency virus
SP	slow progressor
ssDNA	single stranded Deoxyrobonucleic acid
STD	sexually transmitted disease
STI	sexually transmitted infection
TBE	tris-borate ethylenediaminetetraacetic acid
$TCID_{50}$	50% tissue culture infectious dose
TEMED	N,N,N',N'- tetramethylethylenediamine
U	unit

UV	ultraviolet
V	volts
V1/V2	variable regions 1-5
VC	virus control
VL	viral load
WHO	world health organisation
X4	CXCR4 using viruses
X-gal	5-bromo4-chloro-3-idolyl-β-D-galactosidase

PRESENTATIONS ARISING FROM THIS DISSERTATION

Isaac Choge, Cilliers T, Taylor N, Gray G, Meyers T, Papathanasopoulos MA, Morris L. Genetic variation in V1/V2 and neutralization sensitivity of HIV-1 subtype C isolates from infants with slow disease progression. *Poster* presentation (pg054) at the 16th conference of the South African Society of Biochemistry and Molecular Biology (SASBMB) and the International Union of Biochemistry and Molecular Biology (IUBMB). Cape Town, November 2001.

Isaac Choge, Cilliers T, Taylor N, Gray G, Meyers T, Papathanasopoulos MA, Morris L. The use of a V1/V2 heteroduplex tracking assay for identifying antibody neutralization sensitive viral quasispecies. *Poster* presentation (T1-P22) at the first South African AIDS conference. Durban, August 2003.

Isaac Choge, Gray G, Meyers T, Papathanasopoulos MA, Morris L. Novel use of the gp120 V1/V2 heteroduplex mobility assay for viral subtyping. *Oral* presentation at the 13th International Conference on AIDS and STI's in Africa (ICASA). Nairobi, Kenya. September 2003.

Isaac Choge, Gray G, Meyers T, Papathanasopoulos MA, Morris L Novel use of the gp120 V1/V2 heteroduplex mobility assay as a viral subtyping tool. In preparation.

Polly Walker, **Isaac Choge**, Melissa K. Cilliers T, Myers T, Holmes E.C, Morris L Positive selection and infectivity of subtype C primary isolates from children with differing rates of disease progression. In preparation.

CHAPTER ONE

INTRODUCTION

1.1 The Global AIDS pandemic

Infection with human immunodeficiency virus type 1 (HIV-1) leads to severe immunodeficiency in the majority of individuals, a condition known as the acquired immunodeficiency syndrome (AIDS). The most recent United Nations Program for AIDS (UNAIDS) statistics of December 2002 reveals that globally, a total of 42 million adults and children are living with HIV/AIDS (http://www.unaids.org). In sub-Saharan Africa alone, the epicenter of the HIV pandemic, an estimated 29.4 million people (>70% of global infections) currently live with the virus. In 2002, there were an estimated 3.5 million new infections worldwide with at least 14,000 infections occurring daily. The highest prevalence has been recorded in Botswana, where 39% of the population is reported to be HIV infected.

In South Africa, the first two documented cases of AIDS occurred in 1982 among homosexual flight stewards who had previously visited America (Ras *et al.*, 1983). The epidemic thereafter extended to the heterosexual population with the index case of AIDS in a black heterosexual subject being reported in December 1987 (Schoub, 1990). Since the epidemic began, an estimated 4.7 million HIV-1 infections have occurred in South Africa, accounting for more than 10% of the total number of global infections (UNAIDS, 2002). The South African Medical Research Council reports that about 40% of adult deaths in the year 2000 were among persons aged between 15-49, largely due to the HIV/AIDS epidemic (http://www.mrc.ac.za/bod). By 2010, it is forecast that 5 to 7 million cumulative

AIDS deaths will occur, unless treatment to prevent AIDS is effected immediately (Birmingham, 2000; Dorrington R, 2001).

1.2 Discovery of HIV-1 and HIV-2

AIDS was first described in North America in 1981, after a number of homosexual men developed either of two hitherto uncommon diseases, Kaposi's sarcoma (a rare skin cancer) and pneumonia caused by the parasite *Pneumocystis carinii,* a yeast-like organism (CDC, 1981). These men were all previously healthy and between the ages of 20 and 45. In 1983, the virus HIV-1 (then known as human T-cell lymphotropic virus type 3 (HTLV-III) or lymphocyte-associated virus (LAV)) was isolated and used to infect an Epstein-Barr virus (EBV) transformed B cell line (Barre-Sinoussi *et al.*, 1983). The virus produced from this line was also able to grow in other lymphoblastoid cell lines and subsequent experiments showed that the virus was cytopathic for primary $CD4^+$ T-cells (Montagnier *et al.*, 1984).

HIV-1 is a retrovirus along with several other well-characterized lentiviruses including equine infectious anemia (EIA), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV) and human immunodeficiency virus type 2 (HIV-2) (Luciw, 1996). Common to all lentiviruses is infection of bone-marrow–derived cells, persistent viremia, life long infection, prolonged sub-clinical infection, weak neutralizing antibody (Nab) responses, continuous virus mutation, antigenic drift and neuropathy (Kahn and Walker, 1998). Amongst these, HIV-1 is not only the most globally dispersed, but it is also highly pathogenic with the most devastating effects (Kanki *et al.*, 1994).

1.3 Transmission of HIV

HIV is found in large quantities in the body plasma fluids, including genital secretions (semen and vaginal), breast milk, lymph and blood of people with an established HIV infection. Transmission occurs mainly via sexual transmission, intravenous injections or the use of contaminated blood products and vertically from mother to child.

Sexual contact is the major route of HIV infection. Transmission is via infected $CD4^+$ T lymphocytes or free virus present in genital fluids (Lehner *et al.*, 1999). Infected men transmit the virus by up to eight times more effectively than infected women (Padian *et al.*, 1997). In North America and Europe, male homosexual activity is responsible for most infections but rates of heterosexual transmission are reported to be on the increase. In contrast, HIV is spread predominately by heterosexual contact in Africa whilst bisexual and homosexual activities are less common (UNAIDS, 2002). Sexual transmissions occur at the mucosal lining of the gut, vagina, or penis where the virus attaches to dendritic cells (DCs) via DC-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN) prior to anchoring onto $CD4^+$ immune cells (Turville *et al.*, 2002). The risk of transmission is further increased by bleeding during sexual intercourse caused by abrasive sexual contact, as well as the presence of other sexually transmitted infections (STIs) (Royce *et al.*, 1997).

Injection using hypodermic needles contaminated with HIV provides a high-risk conduit for infection. The virus spreads quickly between injection drug users who share contaminated needles (Liitsola *et al.*, 1998; Naganawa *et al.*, 2002). Similarly, there is a risk with nosocomial infections especially in clinics and hospitals that do not have proper and effective sterilization. Before 1995 regular blood transfusion was cited as a major route of transmission (> 90%), especially for people with hemophilia where blood contaminated

with HIV-1 was used to prepare clotting factor VIII (Dyer *et al.*, 1997; Greenough *et al.*, 1999; Schoub, 1994). Heat sterilization and screening of blood donors have now almost entirely eliminated this risk in most countries.

Mother to child transmission (MTCT) has become one of the main target areas for prevention of HIV infection. Compared to developed countries where the implementation of antiretroviral regimens have reduced MTCT rates to less than 2% (McGowan and Shah, 2000), surveillance reports reveal that currently in developing countries MTCT rates are between 21 and 43% (McIntyre and Gray, 2002; UNAIDS, 2002). The exact mechanism of transmission is not known, but three pathways have been proposed. Prepartum transmission occurs across the placenta, causing *in utero* infections and is thought to confer rapid disease progression in infants (Courgnaud *et al.*, 1991). Intrapartum transmission occurs during labor when the fetus comes into contact with the genital tract, maternal blood and/or vaginal secretions, containing infectious virions (Wolinsky, 1992). Women who elect to have a cesarean section instead of normal canal birth, have a reduced risk of perinatal transmission (Mandelbrot *et al.*, 1998). Postpartum transmission occurs mainly via infected breast milk (Taha *et al.*, 1999). Several researchers have shown that almost 30% of lactating HIV-1 positive mothers shed virions that are found in cell-free breast milk with the exclusion of colostrum (Gallaher *et al.*, 1989; Lewis *et al.*, 1998). Mothers given antiretroviral therapy at the time of delivery are discouraged from breastfeeding to prevent postpartum transmission. However, this puts infants particularly those in poor socio-economic conditions, at risk of contracting other diseases, including cholera, amoebiasis and other bacterial infections involving diarrhea, which are also life threatening (Kakai *et al.*, 1995).

Interestingly, a study carried out in Kenya revealed that MTCT of HIV-1 can occur intrapartum through the oral route (Gaillard *et al.*, 2000). The correlation between the presence of HIV-1 in maternal cervicovaginal secretions and in the infants' oro-pharyngeal secretions at birth, and the risk of MTCT were examined to obtain a better understanding of its mechanism. Results from this study indicated that infant exposure to HIV-1 in the birth canal and the presence of HIV-infected cells in the infants' oro-pharyngeal cavity are independently associated with intrapartum and early postpartum MTCT (Gaillard *et al.*, 2000).

Various other factors including the presence of STIs, poor general health, low levels or the complete absence of Nabs targeted at the infecting virus, reduced $CD4^+$ cell count, high viral load (VL) and the presence of high replicating, syncytium inducing (SI) strains in the infecting mother have also been cited as parameters that will impact on the risk of HIV-1 MTCT (Husson *et al.*, 1995).

Although HIV-1 can be found in other bodily fluids such as saliva, urine and sweat, the quantities of virus in these fluids are usually too low for successful transmission (Liuzzi *et al.*, 1996). However, several studies of individuals who engage in receptive oral intercourse suggest that this is an independent risk factor for HIV infection and may contribute to the high transmission rates (Rothenberg *et al.*, 1998). The antiviral properties of saliva may also limit the infectivity of HIV in the oral mucosa. *In vitro* infection of lymphocytes is reduced by saliva via mechanisms that include virus-specific-antibodies (Janoff *et al.*, 1999), aggregation of viral particles by salivary mucins (Bergey *et al.*, 1993), and competition for viral or cellular targets by inhibitory endogenous proteins (Robinovitch *et al.*, 1993).

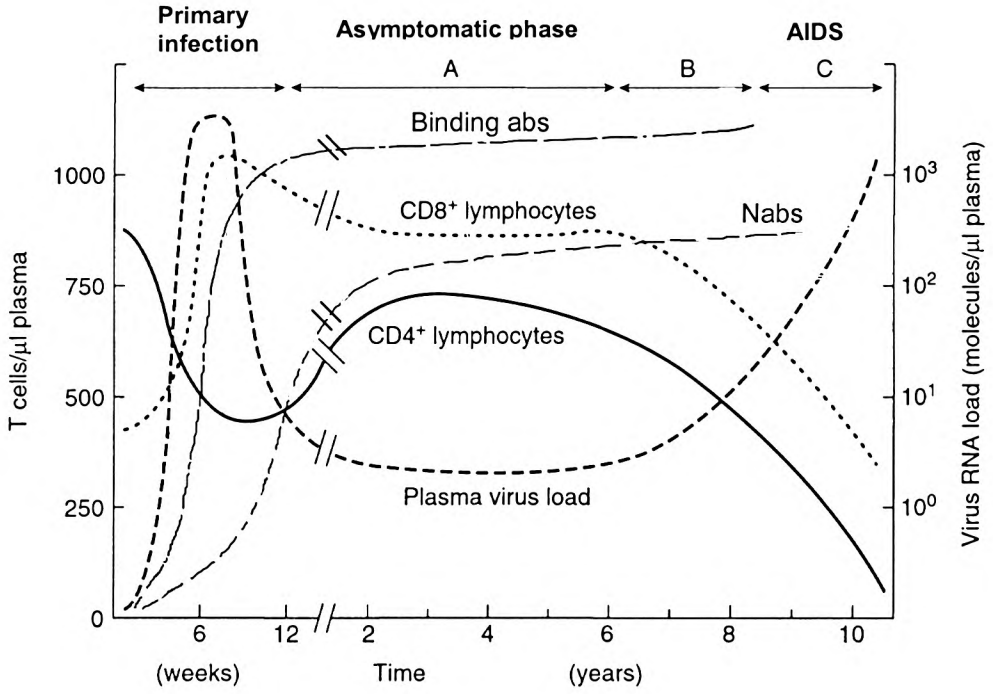
1.4 Natural history of HIV-1 infection

Following transmission of HIV-1 to a susceptible host, the viruses attach to DCs, and are transported to the lymph nodes where they infect activated $CD4^+$ T cells which subsequently become a life long reservoir of the virus (Lehner *et al.*, 1999). At this early infection stage before antibodies are produced, only sensitive molecular methods (such as polymerase chain reaction (PCR)) may be able to detect the virus in the blood. This stage of infection is commonly known as the window period, and represents a high risk for transmission via sexual contact, or infected blood.

Within four to eight weeks of infection, the majority of patients experience one to two weeks of sero-conversion illness also called acute/primary phase (Figure 1.1) which is characterized by mild symptoms that mimic those of other viral infections (Haase, 1999). These symptoms include fever, tiredness, localized skin rashes, a sore throat, muscle and joint aches and some swelling of the lymph nodes (Schacker *et al.*, 1996).

After the initial non-specific symptoms of HIV infection and sero-conversion, the patient progresses to Clinical Category A with a $CD4^+$ T cell count of >500 cells/μl, the normal being between 800-1200 cells/μl (Figure 1.1). At this stage, the symptoms range in severity, but may sometimes go unnoticed in certain individuals who remain well and asymptomatic for a period of up to ten years after primary infection. Alongside those illnesses seen in the primary stage, some succumbing patients have frequent bouts of candidiasis, fever and diarrhea lasting for a month or more (Kahn and Walker, 1998; Schacker *et al.*, 1996). Clinical Category A people are often in the clinical latency or 'silent phase' of HIV infection where the virus usually has a very low turnover number, and is characterized by a low viral load (VL). This VL is set at a point determined by host immune factors (Figure 1.1).

During asymptomatic infection, the virus is active in the body mainly in lymphoid tissues, causing progressive damage to the immune system (Fauci, 1988). With a weakened immune system, patients progress into Clinical Category B with a $CD4^+$ T cell count of 200-500 cells/µl (Figure 1.1). This stage is characterised by constant and severe infections, neoplasma, significant weight loss, myopathy, peripheral nervous system (PNS) and central nervous system (CNS) disease or dementia (Pantaleo *et al.*, 1995; Schacker *et al.*, 1996). This indicates a defect in cell-mediated immunity, and within two years the patient approaches the final stages of AIDS (McMichael and Rowland-Jones, 2001).



Fauci et al., 1996

Figure 1.1: The natural course of HIV-1 infection and host immune responses with time. Note the inverse relationship between infectious virus load/overt disease and T-cell concentrations in plasma. Clinical categories shown are described in the text with different stages of disease. Binding antibodies (abs) develop within weeks and are used in the diagnosis of HIV infection. CD8 T cells develop within weeks of infection and are involved in controlling viral load following primary infection. Neutralizing antibodies take considerably longer to develop.

The last stages of AIDS is Clinical Category C with <200 $CD4^+$ T cells/μl (Figure 1.1). Progression to full blown AIDS generally occurs within 10 years of infection, unless effective therapy is instituted (Fauci, 1988). If an AIDS patient does not die from infection with opportunistic micro-organisms, HIV infection progresses to cause further severe disease in the muscles, the PNS and CNS, ultimately leading to death (Fauci, 1988).

The complete removal of the virus from the circulation is currently an impossible task, because unlike the other simple lentiviruses, HIV-1 is able to infect resting $CD4^+$ / CD45RO memory T cells (Kuritzkes, 2000). These cells constitute a small but vitally important reservoir of latently infected $CD4^+$ T cells (10^3-10^4 cells per person), and it is possible that other reservoirs which have not yet been identified do exist (Kuritzkes, 2000; Stevenson *et al.*, 1992).

The presence of viral antigen causes the stimulation and proliferation of virus specific $CD4^+$ T cells and as virus infects and replicates in these cells, they are in turn preferentially destroyed (Mellors *et al.*, 1996). Alongside this, there is a dramatic expansion of virus specific $CD8^+$ T cells, which coincides with the dramatic suppression of viremia (Figure 1.1). However, the proliferation of $CD8^+$ T cells is highly dependent on the help from $CD4^+$ T cells (Garcia-Blanco and Cullen, 1991). Thus, during the acute stage of infection there is a fine balance between the destruction of virus specific $CD4^+$ T cells and the proliferation of helper/uninfected $CD4^+$ T cells (Fauci *et al.*, 1996; Rosenberg *et al.*, 1997). This balance determines the body plasma viral load at the end of the acute phase of primary infection, referred to as the viral set point. The viral set point is thought to predict the patients' rate of disease progression to AIDS (Mellors *et al.*, 1996). Patients having lower viral set point progress much slower to the chronic stage of AIDS, whilst those with a higher set point will progress faster to chronic AIDS and die earlier (Pantaleo *et al.*,

1993). Application of antiviral therapy during the primary infection helps preserve HIV-1 specific $CD4^+$ T cell function and hence reduce the ensuing plasma viral set point (Rosenberg *et al.*, 1997).

The natural history of HIV-1 infection shows that within weeks to months after infection, a detectable immune response associated with a decline in viremia occurs (Weiss *et al.*, 1985). A number of antibodies are made to all HIV-1 proteins, but only those against the envelope glycoprotein posses neutralizing activity. These Nabs can generally be detected in the plasma as early as nine months after infection, but may take years to develop (Burton and Montefiori, 1997; Moog *et al.*, 1997; Zwart *et al.*, 1994) (Figure 1.1). Some antibodies also bind to non-virion forms of the envelope (viral debris) and are generally termed binding antibodies, characteristically arising much earlier in infection. The relative amounts of different antibody types elicited vary between HIV-1 positive individuals.

The full duration between infection and death from AIDS varies enormously between different populations and individuals. In Africa, it is generally accepted that the period is shorter as compared to European countries, with factors including nutrition and access to primary health care arguably influencing the period (Grant *et al.*, 1997). Individuals who develop AIDS within three years of infection are referred to as rapid progressors (RPs) with typical progressors living between three and ten years (Figure 1.2). In long term non-progressors (LTNPs) or long term survivors an incubation period of beyond ten to twenty years has been recorded (Pantaleo *et al.*, 1995) (Figure 1.2). These individuals tend to have stable CD4 counts and low VLs, and the duration of clinical latency in LTNPs following HIV-1 infection is regulated by the interplay of at least three virus-host factors: (i) genetic susceptibility of the host, (ii) the ability of the host to elicit

effective immune responses and, (iii) the genotypic properties of the infecting virus (Fauci *et al.*, 1996).

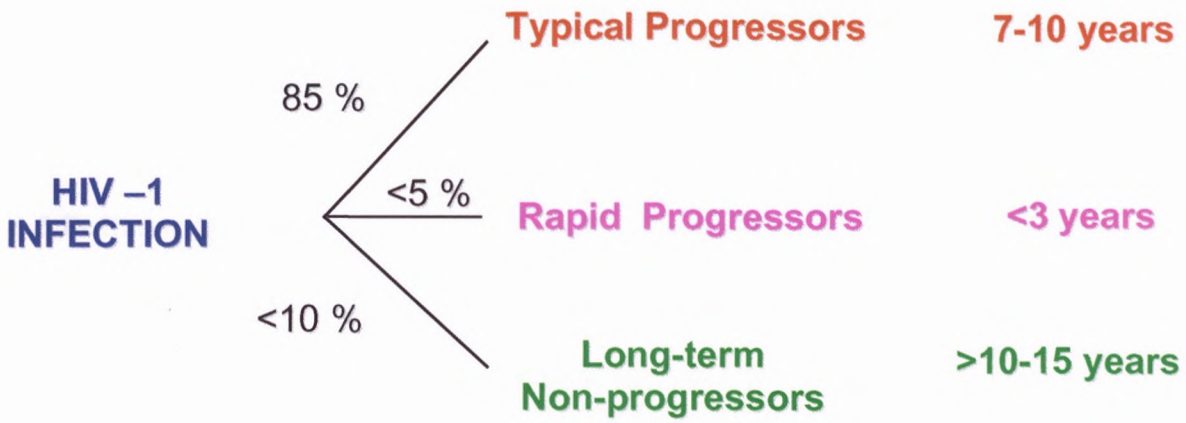


Figure 1.2: Three profiles that HIV-1 infection may follow in adults. The majority of HIV-1 infections result in typical progressors, and death is forecasted in 7-10 years (based on European cohorts). RPs die within 3 years, and LTNPs remain asymptomatic for more than 10 years. LTNPs form an important group that could answer vital questions between virus and host interactions.

Broad Nab responses have been observed in LTNPs, and with time, these LTNPs generally have higher Nab titres than RPs (Cecilia *et al.*, 1999). Although LTNPs remain symptom-free for many years without the assistance of antiretroviral therapy, the question of whether some individuals are able to present antigens and stimulate immune responses more effectively than others, or whether some envelope proteins are conformationally more sensitive to neutralization has presently not been answered.

Although the mechanisms underlying long-term non-progression have not been fully elucidated, slow progressors and LTNPs may harbor defective viral genomes that behave as a live attenuated vaccine. This would allow a robust immune response and

thereby allow viral suppression in infected individuals (Zaunders *et al.*, 1999). Interests in non-progressive HIV-1 infection has increased over time in the hope of understanding HIV-1 pathogenesis and the mechanisms of protection involved (Geffin *et al.*, 2000; Menzo *et al.*, 1998). Slow progressors and LTNPs provide a unique opportunity to study the complexity of virus-host interactions in detail with the aim of understanding the biological and molecular correlates of protection.

1.5 Pediatric HIV infection

Pediatric HIV-1 infection differs from infection in adults in that a third of vertically infected infants are RPs and develop AIDS by two years of age (Bobat *et al.*, 1998; Martin, 1996; Peckham and Gibb, 1995). HIV-1 RNA levels in infected infants are higher than in adults and decline only slowly during the first two years of life placing them at increased risk for rapid disease progression to AIDS (Shearer *et al.*, 1997). These children, because of an immature immune system are also at a greater risk of contracting and being unable to clear other viruses such as influenza, cytomegaloviruses, Epstein Barr virus (EBV), and bacterial infections such as *Pneumocystis carinii* pneumonia, and *Mycobacterium tuberculosis* (MTB) (Peckham and Gibb, 1995). The end stage of HIV infection affecting the brain and nervous system also occurs in untreated children (Peckham and Gibb, 1995).

The remaining two-thirds of children start to show symptoms of AIDS after two years of life, with approximately half-surviving beyond five years. Those that survive beyond eight years, showing no symptoms are termed LTNPs (Ganeshan *et al.*, 1997). The criteria for categorising vertically infected children as LTNPs have not been well defined, however, data suggests that asymptomatic vertically infected children older than 8-10 years of age are unusual (Paul, 1997). With proper nutrition and an immune system that has had

time to develop these children grow into adulthood (Peckham and Gibb, 1995). Similarly to adults, the natural history of HIV-1 infection is determined by multiple factors including the presence of Nabs, the inherent biological and genetic nature of the virus, VL and cell mediated immune responses (Shearer *et al.*, 1997; Wilson *et al.*, 1999). Investigations of these parameters will assist us in understanding the differences between rapid and slow disease progression in HIV-1 infected infants.

1.6 Molecular Biology of HIV-1

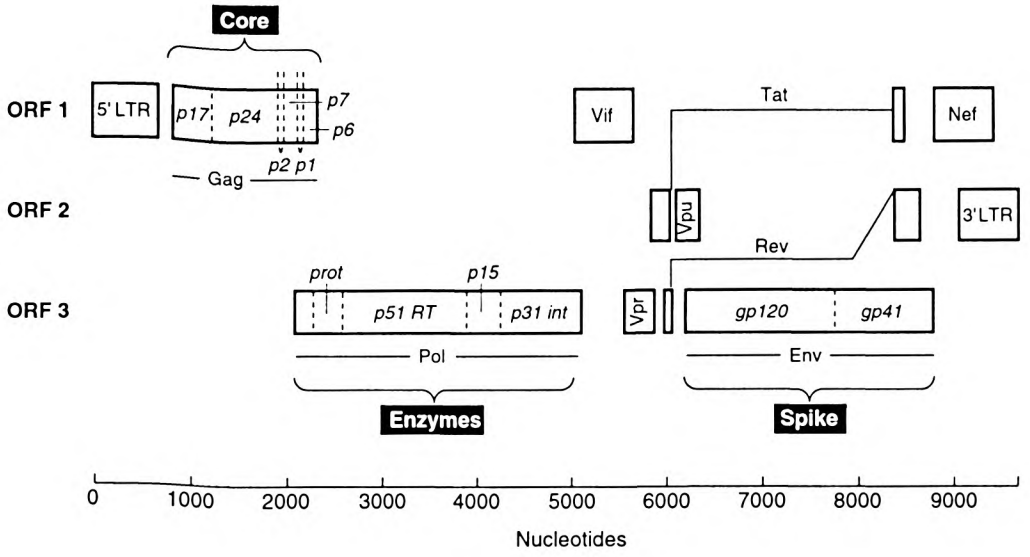
1.6.1 Structure and Genomic organization

HIV-1 belongs to the *Lentivirus* genus of the *Retroviridae* family (Coffin *et al.*, 1986). Characteristically, this family has slow acting cytopathic viruses, associated with long incubation periods and infection of the CNS (Hehlmann, 1991). Electron microscopy has revealed that the HIV-1 virion has an icosahedral structure measuring 100 to 150nm in diameter, containing 7 to 14 external spikes (which form a trimer) embedded in a lipid bilayer that is acquired as the virion buds from an infected host cell (Chertova *et al.*, 2002; Gelderblom *et al.*, 1987). Other structural proteins of HIV-1 include the matrix protein p17, located underneath the lipid bilayer envelope membrane and the p24 major capsid protein. Within the capsid is a diploid single stranded ribonucleic acid (ssRNA) genome held within a distinct p7 nucleocapsid and three viral enzymes: p11 protease, p32 integrase and the p51 reverse transcriptase (RT) alongside ribonuclease H (RNaseH). The diploid ssRNA genome is approximately 10kb in length, with several open reading frames.

The HIV-1 genome conforms to other basic retroviruses, such as human T-cell leukemia virus, with additional regulatory genes. Retroviruses contain the structural genes

5'-*gag-pol-env*-3' and are flanked by the characteristic long terminal repeats (LTRs) (Figure 1.3). During the early stages of replication, one double stranded deoxyribonucleic acid (dsDNA) provirus molecule is produced from the RNA template using the viral RT (Connor, 1997a). RNaseH selectively degrades the original RNA and the dsDNA (provirus) is transported into the nucleus, where it becomes integrated into the host genome with the help of the integrase enzyme. This integration is a random event, and currently no "hotspots" are known as targets for the integration of HIV-1 DNA into the host genome. From the provirus, HIV-1 synthesizes approximately 30 types of RNA, two of which encode the viral genome (which doubles as messenger RNA (mRNA) for the Gag and Gag-Pol proteins) and another that's a singly spliced mRNA (that encodes the Envelope glycoprotein (Env gp160)) (Peterlin and Trono, 2003). Thus, this very simple lentivirus has proteins that have both structural and enzymatic functions. Gene expression is controlled by *cis*-acting DNA or RNA sequences and by host-encoded factors acting in *trans*. On this basic plan, HIV-1 is able to make a number of proteins namely: Tat, Rev, Vif, Vpr, Vpu (or Vpx in HIV-2) and Nef, with positive or negative regulatory activity. In total, the 9 HIV-1 genes encode for 16 proteins (Connor *et al.*, 1997b; Peterlin and Trono, 2003).

In the presence of Rev, unspliced and singly spliced viral RNA transcripts (encoding structural and enzymatic proteins) as well as viral genomic RNA are transported to the cytoplasm where the products of *env*, *gag* and *pol* genes are translated (Barre-Sinoussi, 1996). The Env gp160 undergoes post-transcriptional modification that involves N-linked glycosylation, disulphide bond formation and oligomerization in the endoplasmic reticulum to generate the gp160 precursor protein. The gp160 passes through the golgi complex where it is cleaved by cellular proteases to form the gp120 and gp41 subunits.



Modified from J.A. Levy (1998).

Figure 1.3: Genomic structure of HIV-1. The positive-sense single stranded (ssRNA) is approximately 10kb in length. The scale indicates the genome size in nucleotides. Note that all three open reading frames (ORFs) are used, but only simultaneously at around 8500 nucleotides. *Gag*, *pol* and *env* are transcribed and translated to give polyproteins which are then cleaved by proteases. Genes not aligned require a frame shift for expression. Tat and Rev proteins are expressed from spliced RNAs.

The non-covalently associated oligomeric gp120-gp41 complex is transported to the surface of infected cells along lipid rafts where it is incorporated into budding virions which have already assembled HIV-1 RNA, Gag proteins and enzymes of the *pol* gene (Connor *et al.*, 1997b).

Interestingly, in resting lymphocytes, there are several barriers that preclude the completion of the replication cycle. For example, RT is too inefficient and energy levels are probably too low for effective nuclear import of complementary DNA (cDNA), thus double stranded viral genomes accumulate in the cytoplasm without integrating (Korin and Zack, 1999). The budding of mature virions with the host acquired lipid membrane complete with viral external envelope spikes (gp120) and transmembrane gp41 marks both the end stage of an infecting virus, whilst a new infectious virus is ready to initiate a new cycle of infection.

1.6.2 HIV-1 genetic diversity

HIV-1 shows a high level of genetic variability primarily as a result of the highly error prone RT enzyme (Ricchetti and Buc, 1990). RT has an estimated error rate that is approximately 10^5 –10^6 fold higher than the DNA polymerase of animal cells, which occurs at about one substitution in 10^3 –10^4 nucleotides synthesized. Therefore, a population of HIV-1 viruses will contain few if any identical genomes (Subbarao and Schochetman, 1996). Alongside the errors introduced to the HIV-1 genome from the error prone RT, the ability of the enzyme to switch templates during replication, can lead to recombination of two or more viruses (Ho *et al.*, 1995; Robertson *et al.*, 1995). The replication process of HIV-1 is further complicated by deletions, substitutions and insertions, which produce extensive genetic changes to the virus. The extremely high continuous turnover number of virus, sometimes reaching an estimated level of 10^9 virions per day results in a substantial viral burden with a high level of genetic diversity with which the immune system needs to contend (Ho *et al.*, 1995; Learn *et al.*, 2002; Lukashov *et al.*, 1995; Robertson *et al.*, 1995) (Figure 1.4).

The selective pressure being constantly exerted by the immune system facilitates an increase in genetic diversity (Coffin, 1986). This coupled with the ability of the virus to inadvertedly introduce errors to its genome results in as much as 30% differences between the *env* genes of different genetic subtypes (Myers and Myers, 1993). Significant variation also occurs within an individual, where HIV-1 undergoes genetic changes of approximately 1% per year in *env* (Myers *et al.,* 1994). Consequently, studies have shown that HIV-1 isolated from a single individual at any given time may contain a population or swarm of quasispecies composed of a heterogeneous genetic mixture (Hahn *et al.*, 1986; Myers and Myers, 1993) (Figure 1.4). As quasispecies increase with disease progression,

the biological phenotype of the virus may also change requiring a modified immune system to contain its versatile evolution (Miedema *et al.*, 1994). The high degree of genetic variability of HIV-1 poses several challenges to researchers, including the need to regularly change and update diagnostic tests since these may become inaccurate with time. Genetic variation may affect the responses of HIV-1 to chemotherapeutic agents, since selective inhibition has been demonstrated with the onset of selective chemotherapy (Janssens *et al.*, 1997). Genetic variation also has implications for transmission, and although there is no conclusive evidence from past studies, it is likely that transmission is influenced by genetic variation (see section 1.8).

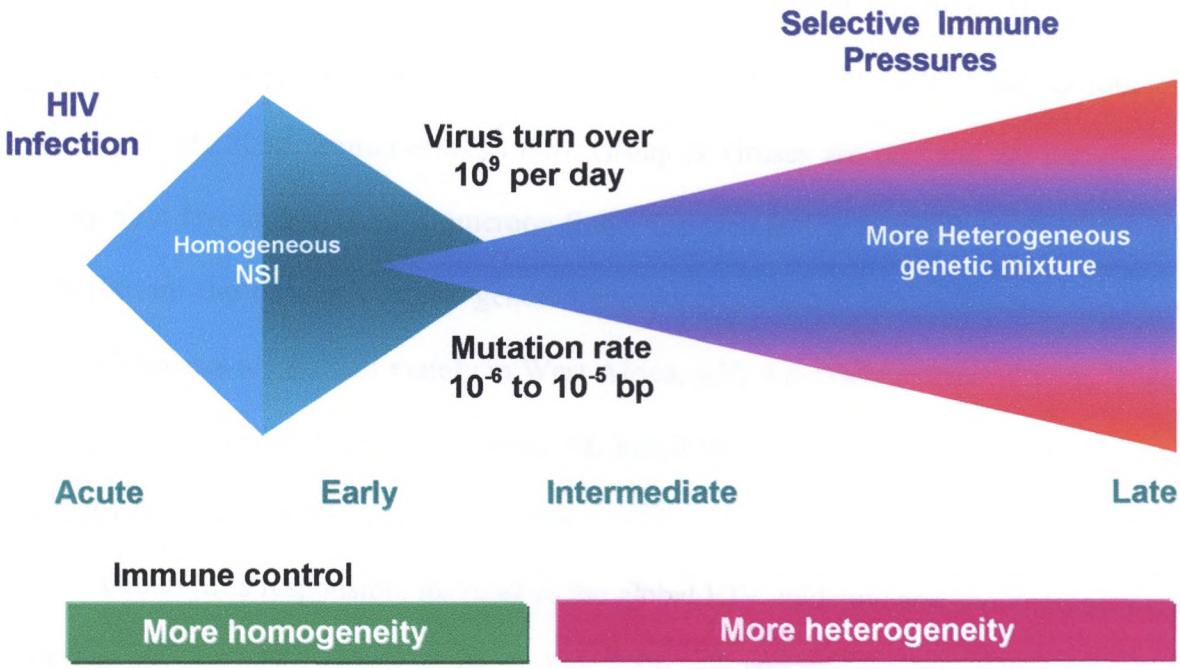


Figure 1.4: Genetic diversity during different stages of HIV-1 infection. A limited number of quasispecies or a homogeneous mixture are transmitted. In the early phase, an increase in the number of virus and quasispecies is pushed by the rapid turnover number, which exhibits a high rate of mutations, thus over time usually 7-10 years, a heterogeneous mixture evolves within the infected host.

The greatest challenge to HIV-1 vaccine development is genetic variability (Letvin, 2002). Candidate vaccines face the challenge of antigenic variation within the principal epitopes that elicit both humoral and T- cell-mediated immunity. Genetic variation results in antigenic diversity of HIV-1 proteins, which in turn diminish the binding of Nabs and the effectiveness of cytotoxic T-lymphocytes.

1.7 HIV-1 subtypes and global epidemiology

Extensive sequencing and analysis of HIV-1 isolates from different geographical regions has led to the classification of viruses into evolutionary linked groups, clades or subtypes and circulating recombinant forms (CRFs) (Los Alamos National Laboratory HIV Sequence Database, http://hiv-web.lanl.gov). The three related groups of HIV-1 viruses that have been described are M (major or main), N (non-M, non-O), and O (outlier) (Ayouba *et al.*, 2000; Simon *et al.*, 1998). Group N viruses are rare and are represented mainly by a few isolates from Cameroon (http://hiv-web.lanl.gov). Of these, YBF-30 and YBF-106 are the only full-length genome sequences available (http://hiv-web.lanl.gov). Group O isolates are present mainly in West Africa, with a prevalence rate of 2-5%. These viruses are highly divergent from group M, exhibiting only 50% amino acid sequence identity in the *env* gene (Gurtler *et al.*, 1994).

Group M is responsible for most of the global HIV epidemic and encompasses nine subtypes, A, B, C, D, F, G, H, J and K (http://hiv-web.lanl.gov), which differ from each other by more than 30% in their *env* sequences and 14% in their *gag* coding sequences (Myers *et al.,* 1994). Subtypes A and F are further divided into subsubtypes (subclades) including A1, A2, F1 and F2, illustrating a further increase in group M diversity (Peeters and Sharp, 2000; Triques *et al.*, 2000). The geographical distribution varies enormously

with subtype A being reported in most parts of East, West and Central Africa (Janssens *et al.*, 1997) and to a lesser extent in Europe (Brodine *et al.*, 1995). Subtype B has dominated the developed countries of Europe, Australia and North and South America and has been the most extensively studied subtype (Weniger *et al.*, 1994). Subtype C is associated with the rapidly growing epidemic in southern Africa, India and China, but has also been reported to occur in other parts of the world including Europe, Russia and Brazil (Leitner, 1996; Morris *et al.,* 2000). All subtypes have been found in Central Africa, including a high frequency and a wide variety of CRFs and novel recombinants, consistent with the region being the origin of the pandemic (Janssens *et al.*, 1997; Kanki *et al.*, 1994).

CRFs of HIV-1 subtypes have also been described and to date 15 have already been defined (http://hiv-web.lanl.gov/CFRs/CRFs.html). For example, CRF01_AE, a subtype A/E recombinant form circulating in intravenous drug users (IVDU) in Thailand (Carr *et al.*, 1996), CRF02_AG, a subtype A/G recombinant isolated in both East and West Africa (Carr *et al.*, 1998), and CRF03_AB a subtype A/B recombinant form isolated almost exclusively in IVDU in Kaliningrad, Russia (Liitsola *et al.*, 1998; Naganawa *et al.*, 2002). Several instances of B/F (CRF12_BF) recombinants have also been reported from Argentina and Uruguay (Marquina *et al.*, 1996). The CRF04_cpx, which represents a Cypriot/Greek recombinant form, that was previously classified as an A/G/I recombinant, has recently been found to be a complex mosaic comprised of subtypes A, G, H, K and unclassified regions (Nasioulas *et al.*, 1999).

The serologically distinct HIV-2 is much less endemic. Global distribution of HIV-2 indicates that the problem is essentially restricted to West Africa, and even within this region, there are great differences between countries and different groups (http://hiv-web.lanl.gov). The highest rates have been found in Guinea-Bissau, the Gambia, and

Senegal (Kanki *et al.*, 1994). HIV-2 shares approximately 40% homology with HIV-1 and has been classified into five genetic groups designated A,B,C,F and G (http://hiv-web.lanl.gov).

The global spread of HIV-1 illustrates that the distribution of different forms of the virus is a dynamic process. As more HIV-1 variants inevitably mix, so the likelihood of generating more complex viruses increases.

Although some investigators have argued that the rate of disease progression amongst individuals infected with HIV-1 genetic subtypes A to D are different from each other, no conclusive evidence exists (Alaeus *et al.*, 1999; Kanki *et al.*, 1999). To account for the rapid spread of subtype C, some investigators have suggested that molecular evolutionary changes are adapted by the virus. These changes include receptor affinity, mediated by the *env* gene, and increased transcriptional activation, mediated by changes in the LTR and the *tat* gene (Essex, 1999). Immune activation and opportunistic infections such as MTB, *Candida albicans* and *Pneumocystis carinii* may also affect rates of spread by fueling viral replication and enhancing transmission (Anzala *et al.*, 1995; Koito *et al.*, 1994; Morris L, 2000). Whether the spread of subtype C viruses is related to viral factors or environmental influences remains to be determined, although poor health care systems, limited access to health care, and concomitant infections including syphilis, gonorrhea, and lack of circumcision, may contribute to the rapid spread of the virus in endemic subtype C regions (Lagarde *et al.*, 2003).

1.8 Mother to child transmission

A number of studies have attempted to investigate whether there is selective transmission of a specific, homogeneous maternal viral population to the infant during *in*

utero and/or intrapartum transmission, similar to that found in heterosexual transmission. Several studies have shown that there is a random pattern of transmission of multiple and/or major maternal HIV-1 variants (Lamers *et al.*, 1994; Narwa *et al.*, 1996). However, some studies have indicated that single or few viral variants are transmitted from mother to child and thereafter a genetically defined viral diversity increases over time in the infected infant (Bjorndal *et al.,* 2000; Scarlatti *et al.*, 1997; Wolinsky, 1992). Studies by Casper *et al.* (2002a and b) suggests that in almost all subtypes of HIV-1, infants were usually infected with the predominant strain irrespective of the variants present in the mother. With the use of heteroduplex mobility assays (HMA), Dickover *et al.,* (2001) showed that *in utero* transmitters were more likely to transmit single or multiple major maternal viral variants, whilst intrapartum transmitters were more likely to transmit minor HIV-1 variants. However, whether this is due to selective transmission or the inability of the infant's immature immune system to support growth of all variants remains uncertain.

Other factors that could contribute to selective transmission include: a) compartmentalization of viruses with different properties in the cervical secretions or other parts of the birth canal (Meng *et al.*, 2002), b) preferential replication of certain viruses in cord blood (Ho *et al.*, 1992), and c) the presence of certain cytokines and chemokines during pregnancy that could encourage a subset of viruses to outgrow their competitors (Kollmann *et al.*, 1996; Patterson *et al.*, 2001; Verhofstede *et al.*, 2003). More so, the pressure exerted by maternal autologous Nabs (Kliks *et al.*, 1994) and cell mediated immune pressure (Wilson *et al.*, 1999) could also result in the selective transmission of HIV-1 escape variants to the infant. The lack of consensus amongst these studies may be due to the fact that a limited number of mother-to-child pairs have been analyzed and that the results were not correlated to the timing of transmission.

1.9 Virus-host interactions: viral attachment and chemokine receptors

In order to gain entry into a cell, the virus needs to bind to specific target cells expressing the CD4 molecule. Although *in vivo* many more cells such as DCs express this molecule and would thus be potential targets for the virus, macrophages and lymphocytes (amongst others) are the major targets *in vitro* (Haase, 1999). Other surface molecules involved with viral capture and transmission have been identified especially on DCs, where a variety of receptors and lectins are available (Turville *et al.*, 2002). Among them, DC-SIGN is believed to play a particular role during transmission enabling HIV-1 dissemination within an individual. Immature DCs residing in the skin and mucosa are thought to be the first cells targeted by HIV-1 during transmission, and DC migration from the periphery and recruitment of T cells within lymphoid tissues is likely exploited by HIV-1 to ensure its propagation, a step that would otherwise be rate limiting for infection (Frank and Pope, 2002).

Binding of the Env gp160 to CD4 induces conformational re-arrangements in the gp120 complex that increase the exposure of a co-receptor binding site and facilitates the interaction of gp120 with the co-receptor (Wyatt and Sodroski, 1998). The co-receptor is a seven-transmembrane chemokine receptor, 12 of which have been discovered (Doms and Trono, 2000). These receptors can function as HIV co-receptors in cultured cells, but only two are known to play a role *in vivo* (Scarlatti *et al.*, 1997). One of these, the β chemokine receptor 5, CCR5, is used by macrophage-tropic, non-syncytium inducing (NSI) or R5 viruses (Deng *et al.*, 1996; Dragic *et al.*, 1996). The other, an α chemokine receptor 4, CXCR4, is used by T-cell-tropic, syncytium-inducing (SI) or X4 viruses (Berger, 1997; Feng *et al.*, 1996) (Figure 1.5). Dual tropic viruses although rare, are able to use a combination of CCR5 and CXCR4 co-receptors for cell entry. The use of other minor co-

receptors has also been reported and include CCR2b or CCR3, BOB and Bonzo (STRL33) (Choe *et al.*, 1996; Connor *et al.*, 1997b; Feng *et al.*, 1996). However, their relevance *in vivo* has not been established.

In up to 13% of individuals of northern European descent, a naturally occurring deletion of 32 base pairs in the CCR5 gene results in a mutant CCR5 receptor that never reaches the cell surface (Liu *et al.*, 1996). This makes individuals homozygous for this mutation (1-2% of the Caucasian population) almost completely resistant to HIV infection by R5 viruses (Liu *et al.*, 1996; Martinson *et al.*, 1997).

The biological importance of co-receptor usage is highlighted by changes in the phenotypes of HIV-1 strains that predominate *in vivo* during progression of HIV-1 disease to AIDS. HIV-1 transmission appears to be mediated by NSI/R5 viruses which predominate throughout the early stages of HIV-1 disease (Connor *et al.*, 1997b; Scarlatti *et al.*, 1997). Later in the course of HIV-1 disease, viruses that use CXCR4, either exclusively or in addition to CCR5, often evolve and become dominant (Connor *et al.*, 1997b; Scarlatti *et al.*, 1997). The evolution of SI variants is associated with disease acceleration, in particular with a rapid drop in T cell counts (Richman and Bozzette, 1994). Such a switch of viral phenotype and co-receptor tropism occurs typically in RPs and in some normal progressors (Connor *et al.*, 1997b), whereas NSI viruses remain dominant in LTNPs infected with subtype B (Goudsmit, 1995).

With particular reference to subtype C viruses from South Africa, it has been shown that the majority of isolates use the CCR5 co-receptor even in patients with advanced disease, thus suggesting that preferences in co-receptor usage may differ not only with different stages of disease, but also with compromised immune systems (Cilliers and

Morris, 2002; Morris *et al.*, 2001). CXCR4 using subtype C isolates have also been described albeit at low frequency (Cilliers *et al.*, 2003; van Rensburg *et al.*, 2002).

About four to six co-receptors are required for viral fusion and after co-receptor binding, further conformational changes are induced that activate the fusion machinery of gp41 (Wyatt *et al.*, 1995). These changes include amongst others the formation of a coiled coil structure domain of gp41 allowing fusion of the viral membrane to the cell host membrane to take place by insertion of the gp41 subunit into the host cell lipid bilayer (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997) (Figure 1.5).

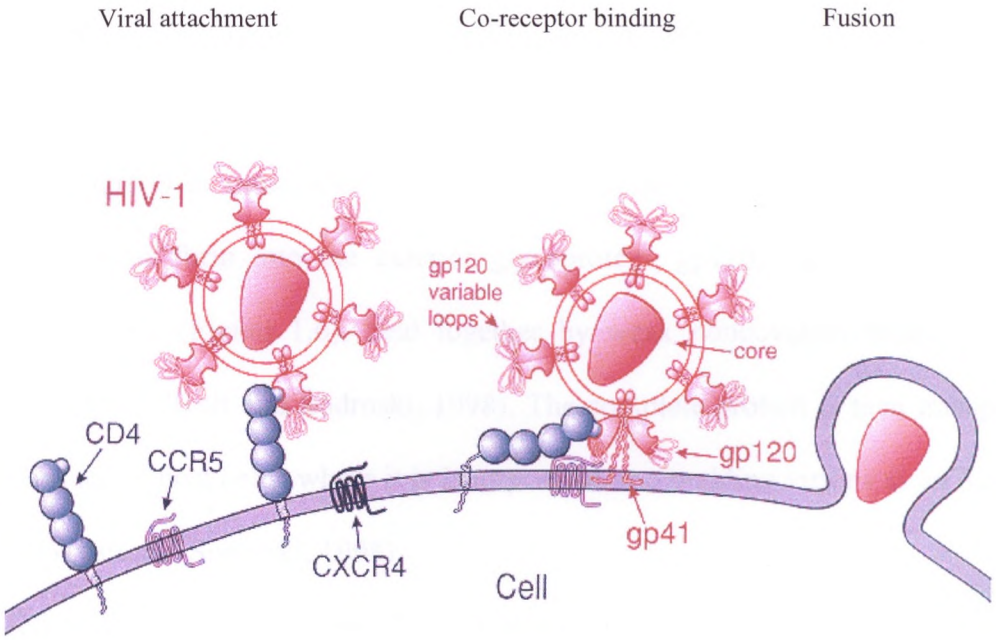


Figure 1.5: Mechanism of HIV-1 attachment and entry. Initial contact of HIV-1 (pink) to the cell is through the CD4 receptor, which undergoes further steric changes to allow the co-receptor molecule CCR5 or CXCR4 to form a second and stronger bond. After gp120 conformation changes, the gp41 transmembrane molecule anchors the virus, and fusion of the virus membrane occurs with that of the cell. The core Gag membrane matrix is then incorporated into the cell cytoplasm completing the attachment phase.

Once the virus has successfully attached to the target cell via the receptors, entry into the cell is facilitated by fusion of the lipids bilayers and release of the viral capsid into the cytoplasm. The viral capsid is then uncoated, releasing the pre-integration complex.

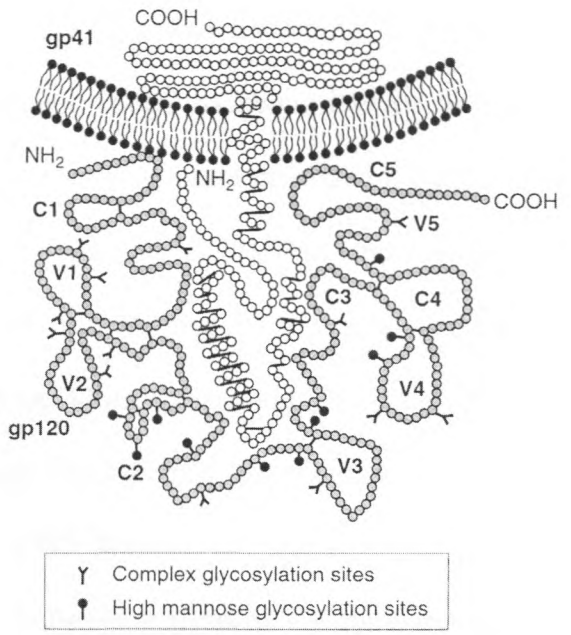
1.10 The envelope glycoprotein

1.10.1 Structural organisation of the envelope glycoprotein

The Env gp160 is synthesized as a precursor of 835 to 870 amino acids in the endoplasmic reticulum (Wyatt and Sodroski, 1998). Processing of the precursor protein includes posttranslational folding, disulfide bond formation, addition of N-linked high-mannose core oligosaccharides to particular amino acid, and dimerization, which occur in the rough endoplasmic reticulum (Stein and Engleman, 1990). Following oligomerization, and further processing of carbohydrates by the addition of complex sugars to form the mature Env gp160 is transported to the Golgi apparatus for cleavage by extracellular proteases such as furin into the exterior glycoprotein, gp120, and the transmembrane glycoprotein gp41 (Figure 1.6), held together by weak noncovalent bonds (Stein and Engleman, 1990; Wyatt and Sodroski, 1998). The complete protein is then transported to the cell surface of host cells, where it is incorporated into budding virions as an oligomeric complex (Wyatt and Sodroski, 1998).

The Env glycoprotein forms 7 to 14 oligomeric spikes on the surface of the virion (Chertova *et al.*, 2002). Physical and crystallographic studies have shown that the oligomeric Env gp160 complex is a trimer, consisting of three gp120 subunits non-covalently bound with three gp41 moieties (Center *et al.*, 2000). The trimeric Env gp160 complex sequentially binds HIV-1 to the host cell surface CD4 receptors and co-receptors, and mediates fusion between the viral and cellular membranes.

Within gp120 are five discrete hypervariable regions, named V1 to V5, which are interspersed between five conserved regions, named C1 to C5 (Modrow *et al.*, 1987) (Figure 1.6). The third hypervariable region (V3 loop) has been one of the most commonly studied regions for phylogenetic analysis and has been shown to contain the principal neutralization domain (Spenlehauer *et al.*, 1998), determinants of co-receptor usage (Shimizu *et al.*, 1999), and T cell mediated responses (Takahashi *et al.*, 1992).



Assembled from Leonard (1990) and Gallaher,(1989)

Figure 1.6: A monomer of the trimeric envelope spike protein. Each circle represents an amino acid residue. Gp160 is cleaved to form the gp41 (open circles) with the carboxyl-terminal transmembrane anchor, and the distal gp120 (shaded circles). The gp120 contains five antigenically variable (V) regions and five intervening conserved (C) regions; it is heavily glycosylated and the glycosylation sites are indicated in gp120; gp41 is also glycosylated. Intermolecular disulphide bonds in gp120 are also shown.

Because of its highly immunogenic nature, the V3 region is subject to strong selective immune pressures that lead to an increase in diversity. This is associated with an increase in antigenic variation (Kuiken *et al.*, 1993). Furthermore, functions such as infectivity (Freed *et al.*, 1992), viral fusion (Freed *et al.*, 1991), and cytopathicity

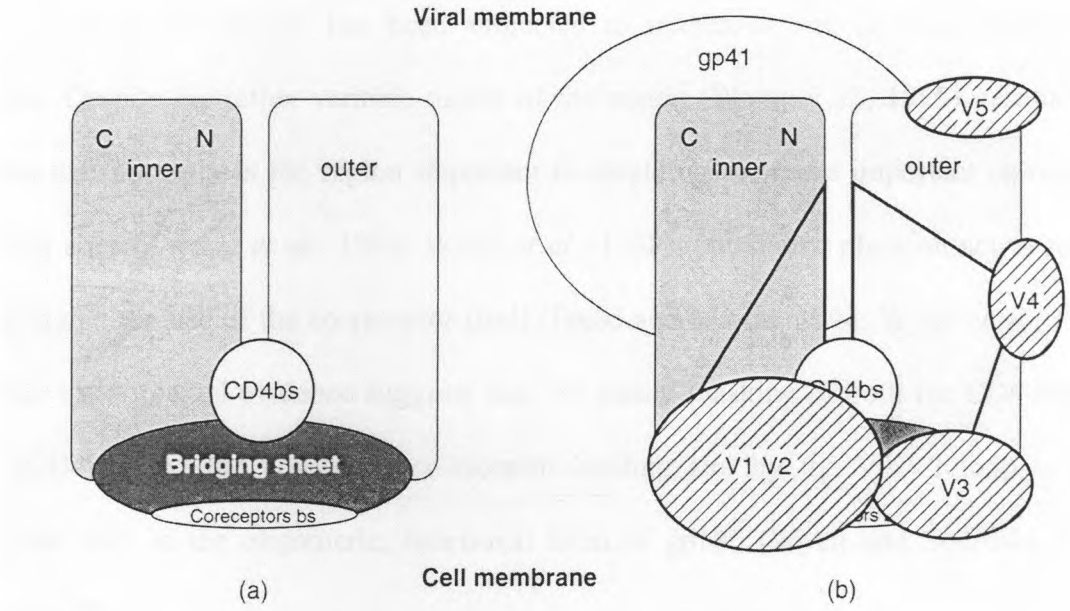
(Fouchier, 1992) have also been mapped to the carboxyl half of gp120, where the V4 and V5 regions are localized in close proximity to V3 (Figure 1.6). Infection by HIV-1 is mediated by complex interactions between distinct regions of the Env protein that include V1/V2 as well as V3.

1.10.2 The V1/V2 region

Analysis of the *env* region of HIV-1 subtype B isolate HXB2, has shown that the V1/V2 region is approximately 70 amino acids long. The disulfide attachments that link the V1/V2 loops to the genetic HXB2 backbone include the second major cysteine and encompass a complex double loop structure involving three disulfide bonds with cysteine residue pairs 119 / 205, 126 / 196, and 131 / 157 (Leonard *et al.*, 1990; Ratner *et al.*, 1987) (Figure 1.6). The V3, V4 and V5 regions are also separated from each other by a minimum of 50 conserved aa, whilst the V1 and V2 are closely linked, with no more than 12 conserved amino acids interspersed between the two clusters of hypervariable sequences (Modrow *et al.*, 1987; Myers *et al.,* 1994).

In contrast to the exhaustive analysis of V3, comparable genetic analysis of V1 and V2 sequences has been limited (Lamers *et al.*, 1993; Wang *et al.*, 1995). One reason for this is that the V1/V2 domain displays extensive length variation resulting from in-frame duplications, insertions and deletions, which makes it difficult to analyze. Interestingly, V1/V2 length variation is not restricted to epidemiologically unrelated viruses, but can occur within populations of epidemiologically linked viruses infecting groups of individuals (Goodenow *et al.*, 1989; Lamers *et al.*, 1993). Variants of HIV-1 with V1 or V2 domains of different length can be found concurrently and over time within infected individuals, suggesting that length variation is involved in virus-host interactions.

Extensive analysis of the V1/V2 domains has led to speculation that the loops may serve as an antigenic shield, screening more conserved, functionally important residues in the core of the envelope complex from immune recognition (Coffin, 1986; Fung *et al.*, 1992; Koito *et al.*, 1994).



Taken from Burton and Parren, 2000

Figure 1.7: Model of a gp120 monomer of HIV-1 based on the atomic structure determined by Kwong *et al.* (1998). (a) A simplified skeleton structure and (b) with loops added. The model is orientated with gp41 and the virus membrane at the top. The monomers meet in a trimer and obscure the inner face whilst the outer face is obscured with carbohydrates. Only the hatched surface of gp120 has epitopes that interact with Nabs (see text), although there are others on gp41.

Early evidence from structural experiments on HXB2 has shown that the V1/V2 region can be removed from the HIV-1 gp120 glycoprotein without globally disrupting the conformation of the protein (Wyatt *et al.*, 1995). This deletion encompassing approximately 70 amino acids gave rise to a replication-competent variant with markedly increased sensitivity to neutralization by monoclonal antibodies (MAbs) directed towards

epitopes within the core of the envelope complex (Cao *et al.*, 1997). Stamatatos and Cheng-Mayer (1998b) reported similar results for an HIV-1 M-tropic primary isolate, SF162, from which only the V2 loop had been removed. In another study, the complete deletion of the V1/V2 region resulted in a non-infectious virus (Wyatt *et al.*, 1993). Thus these studies support the hypothesis that the structural function of the HXB2 V1/V2 region is one of protection and infectivity.

The V1/V2 region has been subjected to numerous site directed mutagenesis studies. Despite the rather variable nature of the region (Wang *et al.*, 1995), results have shown that not only is the region important in shielding the more important co-receptor binding sites (Kwong *et al.*, 1998; Wyatt *et al.*, 1998), but it also plays an active role in determining the use of the co-receptor itself (Freed and Martin, 1994; Wyatt *et al.*, 1995). Further experimental evidence suggests that the partial masking of both the CD4 binding site (CD4bs) and the conserved co-receptor binding site by the V1/V2 loop is fully manifest only in the oligomeric, functional form of gp120 (Wyatt and Sodroski, 1998) (Figure 1.7).

Within an envelope oligomer, gp120 probably fluctuates between CD4bs 'open' and 'closed' conformations as a result of movements of the V1/V2 loops (Wyatt *et al.*, 1995). Multivalent binding between an 'open' gp120 oligomer and a cluster of CD4 molecules further displaces the V1/V2 and V3 loops, including the co-receptor binding site and loosens the gp120 association with gp41 prior to binding (Figure 1.7).

Several reports have suggested the importance of an interaction between the V1/V2 and the V3 regions of Env. A mutation at amino acid 198 (Thr Pro) located within the V1/V2 stem region that forms part of the third β strand (β3) of the bridging sheet of gp120 has been shown to alter the neutralization sensitivity of the primary isolate DH012 (Zhu *et*

al., 2001). This mutation has been shown to affect antibodies against multiple neutralization epitopes that include the V3 loop, CD4-induced, and CD4bs in gp120 (Zhu *et al.*, 2001). This can be seen in the crystal structure of the gp120 core which displays the bridging sheet that is situated in proximity to both the V1/V2 and V3 loops, (Kwong *et al.*, 1998). The single amino acid mutation Thr Pro in the β3 severely compromises the interaction between the V1/V2 and V3 loops and in turn this masks the neutralization epitope (Zhu *et al.*, 2001).

The role of the V1/V2 region in conjunction with the transmembrane molecule gp41 has also been investigated. Johnson *et al.* (2002) have shown that although the removal of a 100 amino acid fragment encompassing the V1/V2 region of SIVmac239 severely impaired viral replication, passaging of the deletion mutant in culture gave rise to variants bearing compensatory changes in the gp41 transmembrane protein. This V1/V2 deletion mutant was shown to be capable of CD4-independent fusion, and more importantly, it was extremely sensitive to antibody-mediated neutralization (Johnson *et al.*, 2002).

1.10.3 Antibody epitopes and neutralization

Several workers in this field have identified linear and conformation-dependent neutralizing antibody epitopes which map to the V1/V2 region. Of note, Moore and colleagues, have demonstrated that linear sequences from the V2 region are immunogenic in HIV-1-infected individuals, suggesting that the primary neutralizing antibody response may be directed to both the V2 and V3 epitopes (Moore *et al.*, 1993). McKeating and other reported that the MAbs 11/68b and CRA-4 bind to amino acid residues 162 to 171 in V1/V2 of HXB2, but changes at different loci of residues 183/184 (Pro/IIe Ser/Gly) and

191/192/193 (Tyr/Ser/Leu Gly/Ser/Ser) within the V2 domain abrogate this binding. They further showed that both of these MAbs were able to bind V1/V2 chimeric fusion proteins in the absence of the C4 region, thus suggesting that although residues in C4 are not components of the epitopes, amino acid changes and/or the complete removal of C4 may affect the structure of the V1/V2 domains within gp160 (McKeating *et al.*, 1993).

The highly divergent V1/V2 region has been suggested to play an active role in immune antibody neutralization resistance, constituting "viral debris" which does not antigenically mimic any given native trimeric complex (Parren *et al.*, 1997). So although the immune response to viral antigens is strong, that against infectious virus is weak. Viral debris might not just create an irrelevant immune response, but it may even decoy antibody production away from the functionally important forms of the envelope glycoprotein thus creating compromised neutralization responses (Moore and Sodroski, 1996; Parren *et al.*, 1997). Neutralization resistance can be acquired either directly by a point mutation within the antibody binding site that renders or abrogates the binding of the antibody or indirectly by a point mutation elsewhere in the *env* gene that alters the conformation of the antibody binding site (McKeating *et al.*, 1989).

1.10.4 Role of glycosylation in the envelope glycoprotein

The Env gp120 is an extensively glycosylated protein (Myers and Lenroot, 1992). It contains 23 to 24 N-linked glycosylation sites, and the glycans attached to these sites account for approximately one half of the protein's total mass (Myers and Lenroot, 1992) Figure 1.6). Numerous studies using glycosylation and glycosidase inhibitors have revealed the importance of carbohydrate moieties in determining the conformation of the HIV-1 envelope glycoprotein, a property that undoubtedly affects its processing,

intracellular transport and the ability to interact with CD4 (Fennie and Lasky, 1989; Ogert *et al.*, 2001; Pal *et al.*, 1989). In addition to being important for the functional and structural integrity of the protein, these sugar residues affect the antigenicity and immunogenicity of the protein (Reitter *et al.*, 1998), thus in effect, the carbohydrate structures shield the virus from Nabs and could create resistance by epitope masking (Chackerian *et al.*, 1997; Schonning *et al.*, 1996). The addition or removal of potential glycosylation sites located within the V1/V2 region has resulted in either increased or decreased susceptibility of these viruses to neutralization by certain MAbs, by sera collected from HIV-1 infected humans or SIV-infected macaques (Cao *et al.*, 1997; Johnson *et al.*, 2002; Reitter *et al.*, 1998; Stamatatos and Cheng-Mayer, 1998a). One possible explanation for this is that such environmental modifications affecting glycosylation increase the number and/or exposure of available neutralization epitopes within the virion-associated oligomeric viral envelopes (Wei *et al.*, 2003).

Other investigations on the removal and or addition of glycosylation sites in V1/V2 have shown the effects of interruption to replication (Palmer *et al.*, 1996), folding and oligomerization (Shioda *et al.*, 1991; Sullivan *et al.*, 1993; Wyatt *et al.*, 1995) of the virus. The use of site directed mutagenesis has also revealed that the N-linked glycosylation sites near the V1/V2 and V3 variable region are critical for the induction of membrane fusion and virus entry (Ogert *et al.*, 2001). Furthermore, in a recent investigation by Quinones-Kochs *et al.* (2002), it was demonstrated that the sequential removal of as many as three glycosylation sites in the V1/V2 region did not disrupt syncytium formation. However, mutations of four or five glycosylation sites reduced fusion, and mutation in all six glycosylation sites completely eliminated all fusion activity (Quinones-Kochs *et al.*, 2002).

1.11 Neutralizing antibodies

Nabs are a principal component of an effective human immune response to many pathogens, yet their role in HIV-1 infection is unclear. Various mechanisms of HIV-1 antibody neutralization have been debated over the years with the prominent hypothesis suggesting neutralization occurs extracellularly by the binding of one or a few antibody molecules to Env. According to the extracellular model, neutralization occurs after a large proportion of the available sites on the virion are occupied by neutralizing antibodies, which leads to the inhibition of virus attachment to host cells or interference with the entry and fusion process. Other hypotheses of antibody neutralization include the intracellular model, and highlight the importance of conformational changes in Env molecules, or that viral inactivation occurs after entry into infected cells by blocking virus uncoating (Dimmock, 1995).

Some patients develop Nabs against infecting viruses (autologous neutralization) as early as 52 days after infection (Arendrup *et al.*, 1992; Richman *et al.*, 2003). Unfortunately, the emergence of escape viruses with a reduced sensitivity to neutralization by autologous sera has also been observed (Tremblay and Wainberg, 1990; Wei *et al.*, 2003). The process of genetic diversity engenders such a genetically heterogeneous population of virions that an antibody that can neutralize one HIV-1 isolate may fail to neutralize another isolate from the same individual. However, in a few patients studied, heterelogous neutralization occurs when the infected person develops Nabs that are able to neutralize their own virus alongside additional viruses, thus suggesting that specificity and potency of these Nabs, is more broad and of a higher standard (Moog *et al.*, 1997; Moore *et al.*, 1996). Althought the V3 loop was previously proposed to be the principle neutralizing determinant (Javaherian *et al.*, 1989), supporting data was only obtained from

T-cell line adapted (TCLA) strains. Failure to neutralize primary isolates was associated with poor exposure of the V3 loop and hence its rarely a target for neutralization (Javaherian *et al.*, 1989; Spenlehauer *et al.*, 1998).

Several broadly neutralizing MAbs against HIV-1 have been identified. Two of these MAbs bind to gp120. These are IgG1b12, which recognizes an epitope overlapping the CD4 receptor site (Roben *et al.*, 1994), and 2G12, which recognizes an epitope based around the C4/V4 region and is highly sensitive to the presence of N-linked glycans in this region (Calarese *et al.*, 2003; Sanders *et al.*, 2002). The third MAb, 2F5, has been mapped to the region overlapping the conserved motif ELDKWA on the membrane proximal region of the transmembrane envelope protein gp41 (Parker *et al.*, 2001; Zwick *et al.*, 2001). More recently, two MAbs Z13 and 4E10 have also been described which recognize an epitope involving the sequence NWF(D/N)IT located at the carboxyl terminus of the 2F5 epitope (Buchacher *et al.*, 1994; Zwick *et al.*, 2001). Another MAb with broad neutralizing ability, X5, recognizes a region close to the co-receptor binding site on gp120 and overlapping the epitope recognized by CD4-inducing MAbs, such as 17b (Moulard *et al.*, 2002).

HIV-1 neutralization escape is not always a simple matter of changing contact amino acid in the cognate epitope. Some epitopes need to be preserved to maintain viral fitness. Thus, the virus has evolved several mechanisms to mask these conserved epitopes. These include: a) shielding by tertiary folds that are conformationally flexible thus producing a masking effect, b) the positioning of the receptor binding domain in recessed pockets where access to antibody is limited, and more recently, c) asparagine (N)-linked 'glycan shields' have been described (Wei *et al.*, 2003). Selected changes in N-linked glycosylation has led to the hypothesis that an evolving 'glycan shield' mechanism of

neutralization escape may exist whereby selected changes in glycan packaging prevents Nab binding, but not receptor binding (Wei *et al.*, 2003). This hypothesis is supported by mutational substitution analysis showing that Nab-selected alterations in glycosylation, including regions in V1/V2, conferred escape from both autologous antibody and epitope specific MAbs.

These observations have been supported by data from Nab responses obtained from recombinant virus assays used to characterize circulating autologous HIV-1 in plasma (Richman *et al.*, 2003). The group studied primary HIV-1 infection and found that a significant Nab response to autologous viruses developed within 39 months (Richman *et al.*, 2003). Despite this evolution in Nabs, plasma viruses mutated at a very high rate, probably due to the selective pressures exerted by Nabs. As a result, the virus acquired the ability to rapidly change into forms that are not sensitive to neutralization by autologous antibodies (Richman *et al.*, 2003). They further showed that although prophylactic, eliciting a narrowly focused but potent Nab response is able to tackle very high levels of viral replication. However, heterologous antibody responses failed to develop during the first year of infection (Richman *et al.*, 2003). This lack of cross-neutralization creates a problem particularly for vaccine research, since the focus here is on Env immunogens that can generate a broadly reactive antibody response.

The V1/V2 and V3 regions represent the major targets for antibody-mediated neutralization. It is hypothesised that because prenatal transmission is via a limited number of variants, selective immune responses from slow progressing children will control viral pathogenesis, whilst responses from rapid progressors will have failed. Because Nabs have subjected selective pressure on the V1/V2, and the C2-V5 regions, and these include diversifying and/or purifying selective pressure, the identification of neutralization

sensitive and resistant variants in these regions may be paramount in identifying particular sites involved in eliciting these antibodies. In addition, this data contributes to the limited scientific information available on genetic variation in HIV-1 subtype C pediatric isolates. Few studies have correlated genetic changes with Nab sensitivity in V1/V2 and V3 among HIV-1 subtype C isolates.

1.12 AIMS OF THE PROJECT

This project will explore the genetic variation and antibody neutralization sensitivity in the V1/V2 and V3 regions of gp120 among pediatric isolates from rapid and slow disease progressors. HMA and HTA techniques will be used to determine which quasispecies are resistant or sensitive to autologous antibody neutralization.

1.13 SPECIFIC OBJECTIVES:

1) To establish a V1/V2 heteroduplex mobility assay for genetic subtyping and quasispecies identification
2) To compare the degree of genetic variation in V1/V2 and V3 regions of gp120 among HIV-1 isolates from rapid and slow progressing children
3) To establish a V1/V2 heteroduplex tracking assay
4) To determine whether viral isolates contain mixtures of antibody neutralization sensitive and neutralization resistant V1/V2 quasispecies.

CHAPTER TWO

EVALUATION OF A GP120 V1/V2 HETERODUPLEX MOBILITY ASSAY FOR GENETIC SUBTYPING AND VIRAL VARIATION ANALYSIS OF HIV-1 SUBTYPE C ISOLATES FROM INFANTS AND CHILDREN WITH RAPID AND SLOW DISEASE PROGRESSION

2.1 INTRODUCTION

HIV-1 genetic diversity is most prominent in the *env* gene (gp160), which encodes the surface exposed envelope glycoprotein gp120, and the transmembrane anchor gp41. Within gp120 are five hypervariable regions, V1 to V5, interspersed between five conserved regions, C1 to C5 (Modrow *et al.*, 1987). The major variable loops V1/V2 and V3 of the gp120 are prime targets of neutralizing antibodies. As a result of strong selective pressure there is an increase in genetic diversity in these regions with time (Pulsinelli and Temin, 1994). The rapid genetic evolution of HIV-1 is guided by several inherent factors including error-prone replication by reverse transcriptase which occurs at least once every replication cycle, recombination, deletions, substitutions (especially G to A) and insertions occurring almost simultaneously during replication (Goodenow *et al.*, 1989; Ho *et al.*, 1995; Simmonds *et al.*, 1990; Vartanian *et al.*, 1991). The net result is the occurrence of a heterogeneous population termed quasispecies or variants, and monitoring these changes has provided an unprecedented challenge to researchers.

Several methods have been developed to monitor genetic polymorphisms in the *env* gene. These include restriction fragment length polymorphisms, serotyping, heteroduplex mobility and tracking assays (HMA/HTA) and subtype-specific polymerase chain reaction (PCR; Cheingsong-Popov *et al.*, 1998; Nelson *et al.*, 1997; Peeters *et al.*, 1998; van Harmelen *et al.*, 1999a; White *et al.*, 2000). Of these, the HMA has proven to be the most efficient, inexpensive and standardizable. The use of HMA has been useful in

understanding the origin and spread of HIV, and has also helped to determine the diversification (subtype), recombination events, transmission and even compartmentalisation of the virus within and between individuals. In particular, the C2-V5 *env* HMA has been widely used to monitor genetic subtypes world-wide, including South Africa (Bredell *et al.*, 2002; van Harmelen *et al.*, 1999b).

Extensive analysis of the V1/V2 loops has led to speculation that they may serve as an antigenic shield screening more conserved and functionally important residues in the core of the envelope complex from immune recognition (Coffin, 1986; Fung *et al.*, 1992; Koito *et al.*, 1994). The exposed V1/V2 domain is therefore in direct contact with the immune system and so becomes its major target. The result of continuous exposure is especially evident in individuals with more robust immune responses, such as slow progressors (SPs) and long-term non-progressors (LTNPs), where it is speculated that these genetic changes may translate into virions that are less efficient at evading the immune response.

Cohorts of pediatric HIV-1 infected patients have in the past proven to be of particular importance not only because the timing of transmission is known, but they also have well defined disease profiles (Dickover *et al.*, 2001). Pediatric HIV-1 infection is typified by a trimodal pattern of disease progression (Duliege *et al.*, 1992). Approximately 30% of vertically infected infants have accelerated immunological deterioration with a precipitously declining CD4 T-cell count and develop AIDS by two years of age, termed rapid progressors (RPs) (Martin, 1996; Peckham and Gibb, 1995). For the remaining 70%, defining guidelines are not clear, but it has been suggested that about half of these children typically develop AIDS related illnesses at around four years of age, whilst the remaining proportion have a relatively slower development of disease (Ganeshan *et al.*, 1997). In

general asymptomatic children who are four years or older are termed slow progressors (SPs). A minority of this latter group of children have survived into adolescence with limited clinical sequela of HIV-1 disease and form a vitally important group known as LTNPs (Grubman *et al.*, 1995). Considering that a minimal number of quasispecies are passed on to the infant during mother to child transmission (MTCT), conclusive evidence addressing reasons why some children are SPs whilst others die within two years of birth have not been forthcoming (Delwart *et al.*, 2002; Wolinsky, 1992). Genetic changes occurring in the virus as a consequence of a strong immune system may thus provide useful information on why vertically infected children have different disease profiles.

An HIV-1 V1/V2 HMA to detect quasispecies within an individual has not previously been developed. The aim of this work was therefore to develop a V1/V2 HMA that could be used to detect viral quasispecies in pediatric HIV-1 isolates from both SPs and RPs, where SPs are expected to have a stronger immune response. This project also investigates the possibility of using the V1/V2 region for subtyping of HIV-1 samples, and compares this to the more widely used C2-V5 HMA subtyping protocol.

2.2 MATERIALS AND METHODS

2.2.1 Viral isolates

Ethical clearance for this study was obtained via the University of the Witwatersrand Research committee for Research on Human subjects (M00/03/39, appendix C). Viral isolates were obtained from 25 HIV-1 sero-positive perinatally infected children attending a clinic at Johannesburg Chris Hani Baragwanath Hospital (CHBH) between June 1999 and July 2000 (Table 2.1). They were between 4 and 9 years of age and

therefore categorized as SPs (TM cohort). The clinical status for the majority of the children (n=18) was moderately symptomatic (Clinical Category B). Four had mild symptoms (Clinical Category A), two had severe symptoms (Clinical Category C) and one child was asymptomatic (Clinical Category N). A second group (n=16) comprised of rapid progressing infants with Clinical Category C definition of AIDS, all of who died between 2 and 25 months after birth (PETRA, COT, RP and BLM cohort). These samples were from children attending clinics in CHBH and Bloemfontein. Viral loads were determined using the Chiron bDNA assay (Chiron, San Francisco, CA), whilst CD4 counts were carried out using a FACScount (Becton Dickinson, San Jose, CA).

Viral isolates were generated by standard co-culture techniques and MT-2 and co-receptor assays performed by infecting U87.CD4 cells expressing either the CCR5 or CXCR4 co-receptors as previously described (Cilliers *et al.*, 2003).

2.2.2 HIV-1 viral RNA extraction

HIV-1 viral RNA was extracted from supernatant of co-cultured primary isolates with the use of the QIAamp® Viral RNA mini isolation kit (QIAGEN, GmbH, Germany). Briefly, 560µl of lysis buffer AVL containing carrier RNA was added to 140µl of cultured virus supernatant. The mixture was vortexed for 15 seconds and incubated at room temperature for 10 min. To the mixture, 560µl of 100% ethanol was added before vortexing again for 15 seconds. The mixture was then filtered through a QIAamp spin column at 8,000 rpm (4300 xg) for 1 min. The RNA was then cleaned with 540µl of Buffer AW1 and centrifuged at 8,000 rpm (4300 xg) for 1 min. Five hundred microlitres of buffer AW2 was also used to rinse the RNA. The spin column was dried by centrifuging at

Table 2.1: HIV-1 isolates from children with slow (a) and rapid (b) disease progression. Epidemiological and clinical data from the two cohorts have also been included alongside biological characteristics of the isolates.

Table 2.1a Isolates from Slow progressing children

Number	Sex	Age (Years)	CD4 (Cells/µl)	VL (RNA copies /ml)	CD4 %	Clinical Category	MT-2 Assay	Co-receptor Usage
TM 1	M	8	4	16,157	0.2	C	SI	R5X4
TM 2	F	8	nd	25,156	nd	B	SI	X4
TM 3	F	6	329	11,178	18	B	NSI	R5
TM 4	F	4.5	692	25,815	30	B	NSI	R5
TM 5	F	4.5	1,378	22,488	nd	B	NSI	R5
TM 6	F	6	846	108,716	40	B	NSI	R5
TM 7	M	9	430	66,774	10	B	NSI	R5
TM 9	M	8	11	296,865	1	C	SI	X4
TM 10	F	7	1,158	685	62	N	NSI	R5
TM 12	M	5.5	976	21,976	36	B	NSI	R5
TM 18	M	5	1,239	699,740	20	B	NSI	X4
TM 19	M	9	135	114,295	12	B	NSI	R5
TM 20	F	7	334	34,310	10	B	NSI	R5
TM 21	F	6	419	13,104	19	B	NSI	R5
TM 22	F	6	339	39,266	21	A	NSI	R5
TM 23	F	5.5	338	106,377	16	A	NSI	R5
TM 24	F	6.5	1,107	2,455	21	B	NSI	R5
TM 25	M	9	150	150,208	11	B	NSI	R5
TM 26	M	8	38	37,586	3	B	NSI	R5
TM 28	M	4.5	216	359,060	18	B	NSI	R5
TM 29	F	4	2,300	26,370	26	B	NSI	R5
TM 30	M	4	911	nd	38	A	NSI	R5
TM 31	M	4	779	191,946	22	B	NSI	R5
TM 32	M	5.5	715	208,804	29	B	NSI	nd
TM 33	M	6.5	1,057	12,275	30	A	NSI	nd

Table 2.1b Isolates from Rapid progressing children

Number	Sex	Age (Months)	CD4 (Cells/µl)	VL (RNA copies/ml)	CD4 %	Clinical Category	MT-2 Assay	Co-receptor usage
PETRA 44	M	4.7	nd	nd	nd	C	NSI	R5
PETRA 60	M	5	nd	nd	nd	C	NSI	R5
PETRA 100	M	3	nd	nd	nd	C	NSI	R5
PETRA 137	F	9.3	nd	nd	nd	C	NSI	R5
PETRA 192	F	7	nd	nd	nd	C	NSI	R5
COT 1	M	2	1,229	>500,000	19	C	NSI	R5
COT 2	F	12	2,033	>500,000	20	C	NSI	R5
COT 6	F	25	407	267,999	8	C	NSI	R5
COT 9	M	12	631	>500,001	13	C	NSI	R5
RP 1	F	12	7	178,830	1	C	SI	R5X4
RP 3	M	6	654	98,440	23	C	NSI	R5
RP 4	M	4	1469	>500,000	38.2	C	NSI	R5
RP 5	F	4	385	>500.000	43	C	NSI	R5
RP 6	M	4	726	>500,000	45.7	C	NSI	R5
BLM 84	UN	6	nd	nd	nd	C	NSI	R5
BLM 106	UN	3	nd	nd	nd	C	NSI	R5

UN = Unknown, nd = not done, VL = Viral load, SI = syncytium inducing, NSI = non-syncytium inducing, R5 = CCR5, X4 = CXCR4, R5X4 = CCR5 and CXCR4. Clinical Categories are as follows: N = Non-symptomatic, A = mildly symptomatic, B = moderately symptomatic and C = severely symptomatic (according to the Centre for Disease Control pediatric HIV classification system).

14,000 rpm (13100 xg) for 3 min. Final elution was done with 60µl of RNase-free buffer (AVE) with incubation on the silica-gel column for 1 min before centrifuging at 8,000 rpm (4300 xg) for 1 min. The filtrate contained the extracted RNA and was stored at –20°C until used in a reverse transcription-polymerase chain reaction (RT-PCR).

2.2.3 Reverse transcription and polymerase chain reaction

Viral RNA was transcribed into complementary DNA (cDNA), with the use of reverse transcriptase. The RT-PCR step was combined with the first of two PCR reactions using the primer ED5 (sense) 5'-ATGGGATCAAAGCCTAAAGCCATGTG-3' from positions 6556-6591 and primer ED12 (antisense) 5'-AGTGCTTCCTGCTGCTCCCAAG AACCCAAG-3' from positions 7822-7792 on the genome of the HXB2 subtype B virus isolate (Delwart, 1995). The final RT-PCR mixture contained a total of 50µl and comprised of 10pmol of each primer, 8µmol di-nucleotide phosphates (dNTPs), 0.7mM $MgCl_2$, 10U RNase inhibitor, 4.8U AMV RT and 0.625U Super-Therm Taq DNA polymerase, with 5µl of the recommended 10 X buffer (Advanced Biotechnologies Limited, UK). The final RT–PCR mixture was incubated at 42°C for 1 hour. All PCR amplifications were done in a GeneAmp® PCR system 9700 thermocycler.

The first round PCR was performed under the following cycling conditions: three cycles of 94°C for 90 seconds, 60°C for 60 seconds and 72°C for 120 seconds; 30 cycles of 94°C for 60 seconds, 60°C for 45 seconds, and 72°C for 120 seconds, and a final extension of 72°C for 10 min. The second round PCR was performed in final volumes of 100µl using twice the volume of reagents as those used in the first round PCR, with 10µl of the first round PCR product as template. The PCR mixture was made to a total of 100µl with sterile sabax water and comprised of 20pmol of each primer, 8µmol dNTPs, 1.4mM $MgCl_2$, and

0.625U Super-Therm DNA polymerase, with 10µl of the recommended 10X buffer. For the C2-V5 region primer ES7 (sense) 5'-CTGTTAAATGGCAGTCTAGC-3' from positions 7001-7020, and primer ES8 (antisense) 5'-CACTTCTCCAATTGTCCCTCA-3' from positions 7359-7380 on the HXB2 genome were used for the amplification, with the following cycling conditions: three cycles of 94°C for 90 seconds, 55°C for 45 seconds and 72°C for 90 seconds; 32 cycles of 94°C for 60 seconds, 55°C for 45 seconds, and 72°C for 90 seconds, with a final extension of 72°C for 10 min.

For the V1/V2 region the following primer set: A589 (sense) 5'-GTGTAAAGTTA ACCCCACTCTG-3' from positions 6580-6601 and primer A590 (antisense) 5'-AATCGCGAAACCAGCCGGCGCACAAT-3' from positions 6874-6899 on the HXB2 genome were used for the amplification (Palmer *et al.*, 1996). The PCR reagents remained as those above for the nested PCR, and cycling conditions were as follows: three cycles of 94°C for 90 seconds, 56°C for 90 seconds and 72°C for 90 seconds; 32 cycles of 94°C for 45 seconds, 56°C for 40 seconds, and 72°C for 90 seconds, with a final extension of 72°C for 10 min.

The reference amplicons used to compare the subtypes of interest were also PCR amplified under similar conditions. However as template DNA, 10µl of diluted 1:10 reference plasmid (NIH AIDS Research and Reference Reagent Program, Bethesda, MD) was added to the PCR mix. The following reference subtype plasmids were used to provide a template for the ~700bp C2-V5 region and the ~320bp V1/V2 region: A_1 (SF170 – Rwanda), A_2 (IC144 - Ivory Coast), B_1 (BR20 - Brazil), B_2 (TH14 - Thailand), C_1 (MA959 - Malawi), C_2 (ZM18 - Zambia), D_1 (UG21 - Uganda), and E_2 (TH06 - Thailand).

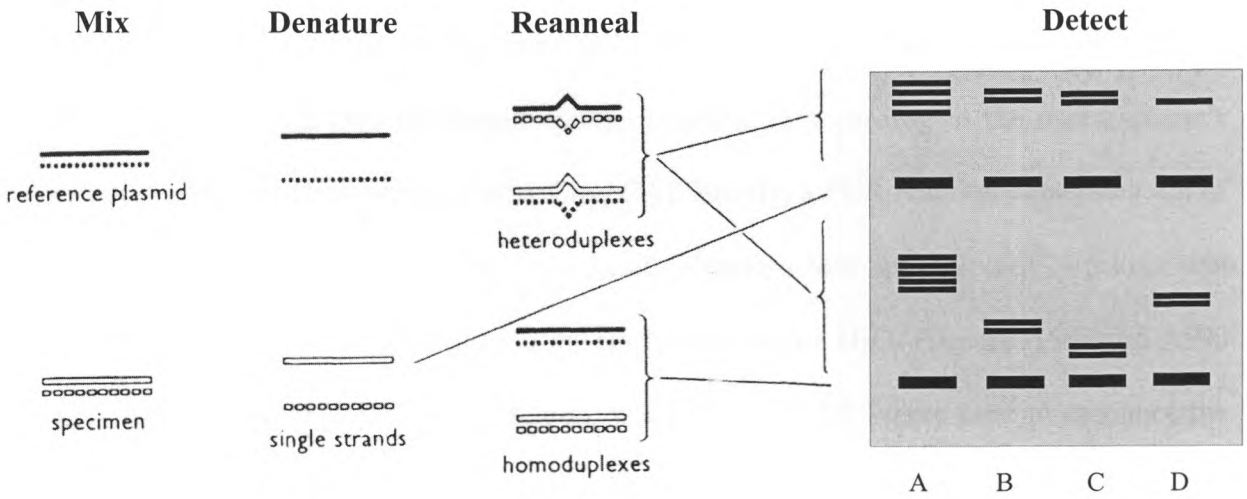
2.2.4 Agarose gel electrophoresis

PCR amplicons were separated and analyzed using agarose gel electrophoresis. In all cases a 1% agarose (ultraPURE, Life Technologies Scotland) gel was made with 1 X TBE (Tris-borate EDTA) buffer containing 0.5mg/ml ethidium bromide (EthBr; Promega, USA). For electrophoresis, 10µl of amplicon was mixed with loading dye that contained 0.25% bromophenol blue, 0.25% xylene-xyanol loading dye and 30% glycerol (Fluka AG, Switzerland), prior to loading on the gel. Electrophoresis was done at 100volts until the xylene-xyanol dye had reached the end of the gel. Amplicons were visualized using an ultraviolet (UV) trans-illuminator (Sigma, Germany) and photographed.

2.2.5 C2-V5 *env* HMA

The C2-V5 *env* HMA protocol and kit was supplied by the NIH AIDS Research and Reference Reagent Program (Bethesda, MD), and the methodology followed as recommended. The principle of HMA is shown graphically in Figure 2.1. Briefly, heteroduplexes were generated by combining 1.1µl of 10X heteroduplex annealing buffer (1M NaCl, 0.1M Tris, pH 7.8, 20mM EDTA; (Delwart, 1995)), 7.5µl of the second round C2-V5 PCR product of each test isolate with 2.5 µl of the PCR product of each reference subtype plasmid A1, A2, B1, B2, C1, C2, D1 or E2 in a thin walled 0.2ml PCR tube. The mixture was then heat denatured at 94°C for 2 mins, and snap cooled on wet ice for 10 mins. Loading buffer, 3µl (0.25% bromophenol blue, 0.25% xylene-xyanol, 30% glycerol) was added to the heteroduplex mixture, and resolved on a 160 X 160 X 1.5mm (protein II XI cell; Bio-rad, Hercules, CA) 5% polyacrylamide gel (30:0.8 acrylamide:bisacrylamide) by electrophoresis in 1XTBE (Bredell *et al.*, 1998; Delwart, 1995). The gel was

electrophoresed at 250 volts, until the xylene-xyanol dye had run off the bottom of the gel (approximately 5½ hours). The gels were post stained in EthBr (0.5mg/ml in 1 X TBE), viewed under the UV light, and photographed.



Reviewed by Barlow *et al.*, (2000)

Figure 2.1: Schematic representation of the HMA. After mixing the reference subtypes and test sample, denaturing and annealing on ice, homoduplexes and heteroduplexes form. Homoduplexes migrate ahead and single stranded DNA migrate behind the heteroduplexes. Heteroduplexes with the closest homology migrate the fastest and are indicative of the subtype (Lane C). Multiple bands in the heteroduplex zone are indicative of a large number of quasispecies (Lane A).

2.2.6 V1/V2 *env* HMA

A novel *env* HMA encompassing the V1/V2 region was developed and optimized. The V1/V2 HMA protocol followed that of the C2-V5 region. Briefly, isolate amplicons from the nested V1/V2 PCR reaction (7.5µl) were mixed with 2.5µl of the amplified V1/V2 PCR fragment of each reference subtype plasmid. This was loaded onto the resolving gel, of which dimensions and composition remained the same as that used to analyze the C2-V5 region with slight modifications (Bredell *et al.*, 1998; Delwart, 1995).

The 5% polyacrylamide gel was electrophoresed for only 3½ hours before post-staining. Post-staining in EthBr (0.5mg/ml in 1XTBE) was allowed for only 15 min before viewing and photographing with UV transillumination.

2.2.7 Sequencing of V1/V2 and C2-V5 fragments

Sequencing of the V1/V2 and C2-V5 fragments of the isolates was done with the use of the ABI Big Dye terminator system (version 2) according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Briefly, a PCR reaction containing 6µl of amplified PCR product, 8µl Terminator Ready Reaction Mix and 3.2pmol of primer was mixed in a total volume of 20µl with the addition of sterile H_2O. Primers A589 and A590 were used to sequence the V1/V2 region, whilst ES7 and ES8 were used to sequence the C2-V5 region. Sequencing conditions on the GeneAmp® PCR system 9700 thermocycler were as follows: 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The reaction was held at 4°C until it was cleaned.

Excess dye and dNTPs were removed from the sequencing reactions with CENTRI-SEP columns (Princeton Separations, Adelphia, New Jersey). The columns were hydrated in 800ml sterile H_2O at least 1 hour prior to use, and the water allowed to drain by gravity for 20 min. The columns were centrifuged at 3,000 rpm (600 xg) for 2 min in a standard centrifuge at room temperature. The filtrate was discarded before 20µl of the reaction mix was loaded on the gel matrix. After centrifuging at 3,000 rpm (600 xg) for 2 min, purified sample was collected and vacuum dried for 45 min without heat (Savant, Halbrook NY). Samples were resuspended in 10µl HiDi-formamide (Applied Biosystems) before loading onto the ABI 3100 automated capillary sequencer (Applied Biosystems).

2.2.8 Nucleic acid sequence analysis

Sequence data was edited with the Sequencing Analysis 3.3 program (Applied Biosystems), and assembled using Sequencher 3.1 (Genecodes, Ann Arbor, MI). Alignment of the sequences was done using Clustal X (Thompson *et al.*, 1997) and further manual alignment of the sequences was done using software from BioEdit (Hall, 1999). For the V1/V2 region, the amino acids were aligned using the glycosylation sites. Although we attempted nuclcleotide alignment, these were less clear. Nucleotide sequences were aligned with reference sequences, from HIV-1 group M subtype A to K, CRFs CRF01-AE and CRF02-AG, group N, group O and SIV_{CPZ}. All reference sequences including the consensus subtype C were obtained from the Los Alamos database (http://hiv-web.lanl.gov). Neighbor-joining trees were generated using the Kimura two-parameter method and the reliability of the branches was assessed with 1000 bootstrap trials. Values of 70% and above were taken as significant and final trees were viewed using TreeView (Win32; http:taxonomy.zoology.gla.ac.uk/rod/rod.html). Amino acids sequences were analysed using pepstats from EMBOSS (http://hamlyn.sanbi.ac.za/EMBOSS) for charge analysis, whilst potential glycosylation sites were predicted by PROSCAN (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.plpage=npsa_prosite.html). Aligned reference sequences of different subtypes included: 26 HIV-1 group M A1-Q23, A1-U455, A2-KS10, A2-CY017, B-MBC200, B-HXB2, C-8ZA151Du, C-98ZA179Du, C-92BW1210, C-92BR025, C-ETH220, C-IN068, D-ELI, D-MB2059, F1-93BR02, F1-IN9363, F2-MP255, F2-MP257, G-92NG083, G-SESE6165, H-V1991, H-V1997, J-JE92809, J-SE91733, K-MP535C, K-EQTB11C, 4 HIV-1 recombinants CRF01AE-CF11697, CRF01AE-CM240, CRF02AG-MP807, CRF02AG-IBNG, group N-YBF30, group O-MVP5180 and CPZ-CPZANT. The relationship between positive selection

pressure for change and disease progression was analysed with phylogenetic reconstruction of the V1/V2 sequences. Sequences were manually aligned using the Se-Al program (http://evolve.zoo.ox.ac.uk). Maximum likelihood (ML) trees for each data set were estimated using the PAUP* package (Swafford, 2001). We employed the General Time Reversible (GTR) model of nucleotide substitution, incorporating empirical estimates of base composition and the maximum likelihood estimate of the shape parameter of a gamma model of rate variation among sites (with four rate categories). The gamma model was chosen over a codon position rate model as it consistently gave much higher likelihood. The substitution rate matrix, relative base frequencies, proportion of invariable sites and the gamma shape parameter were estimated from the data, and TBR branch swapping was carried out to find the ML tree. To determine positive selection pressures in each cohort, the ratio (ω) of non-synonymous (d_n) to synonymous (d_s) substitutions (d_n/d_s) at each site was analysed using a maximum likelihood technique (Yang and Bielawski 2000), and using the program CODEML (Yang, 1997). This method involves the application of two models of codon substitution to the data sets; in model M7 all categories of substitution are either neutral (ω = 1) or invariant (ω = 0), whereas in model M8 an additional category of positively selected sites exsist in which ω can be greater than 1. The likelihood scores of these two analyses can then be compared and a P value calculated using a likelihood ratio test for positive selection with the following equation:

$$\text{Chi dist } (2 (\Delta \text{ M7} - \text{M8}), 2)$$

P values of less than 0.05 are considered significant evidence for selection. The program then uses Bayesian methods are then used to identify the codons under positive selection, and estimates a significant statistic of that finding. The results of the analysis are expressed

here as P (proportion of sites identified by Bayesian probability statistics as belonging to a selected site class with a probability of >0.9).

2.3 RESULTS

2.3.1 PCR amplifications and HMA

PCR amplification products from the V1/V2 and C2-V5 regions were obtained for all 41 isolates. Agarose gel electrophoresis analysis of the V1/V2 region showed that the amplicons ranged in size from 250 to 350bp (Figure 2.2a & b). In particular, the reference strains showed differences in size, suggesting that the overall length of the V1/V2 region differed markedly between and within different subtypes. Agarose gel electrophoresis of the C2-V5 PCR products revealed more consistently sized amplicons for all isolates and reference amplicons (Figure 2.3a & b).

The V1/V2 and C2-V5 amplicons were used to perform HMA analysis on all isolates, except two BLM isolates. Data was therefore available on 39 isolates. The ~320bp V1/V2 homoduplex and heteroduplexes were observed to migrate faster in the 5% polyacrylamide gel compared to the larger C2-V5 fragments. However, the V1/V2 HMA profiles were often difficult to interpret particularly with the subtype A reference that migrated alongside the subtype C in many investigated isolates. Samples with concordant V1/V2 and C2-V5 HMA profiles are shown in Figure 2.4 and those with discordant profiles are shown in Figure 2.5. Some isolates could not be subtyped using the V1/V2 HMA, whilst others showed bands that migrated equally with both subtypes A and C (Figure 2.5).

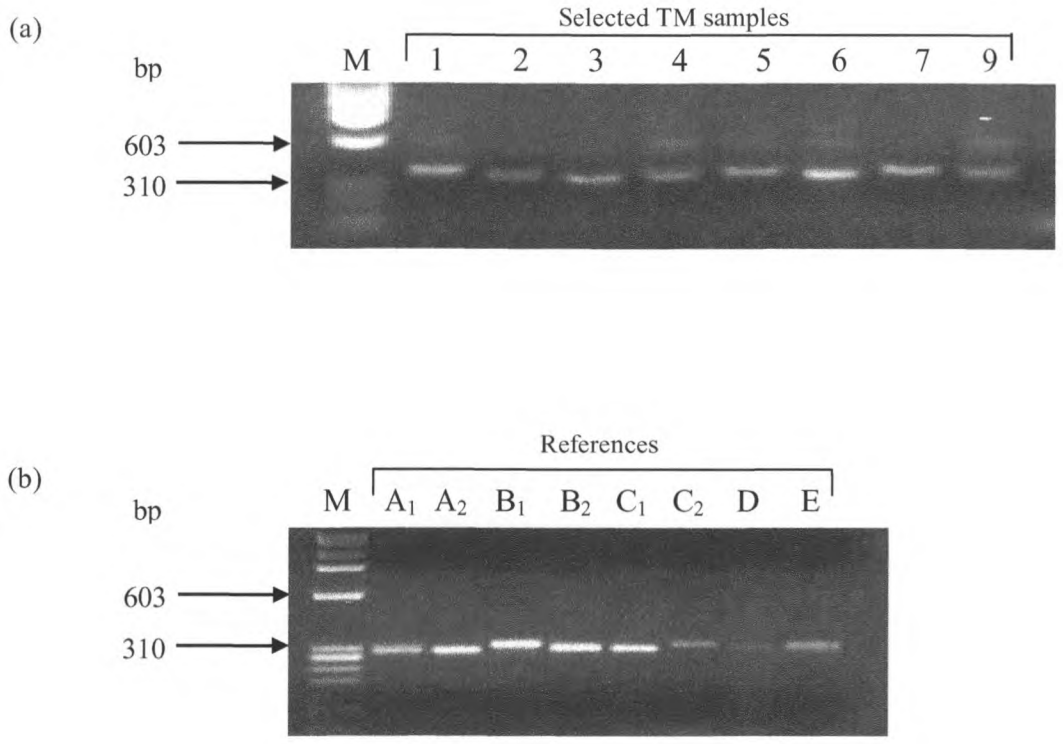


Figure 2.2: DNA agarose gel electrophoresis of the ~320bp V1/V2 PCR products from (a) selected isolates and (b) reference plasmids. Molecular weight marker (M, number IX, Roche) is shown on the left hand side and was used to identify the amplicon sizes. The 310 and 603bp bands are indicated. Only A_1 was subsequently used in the HMA analysis giving similar results as A_2.

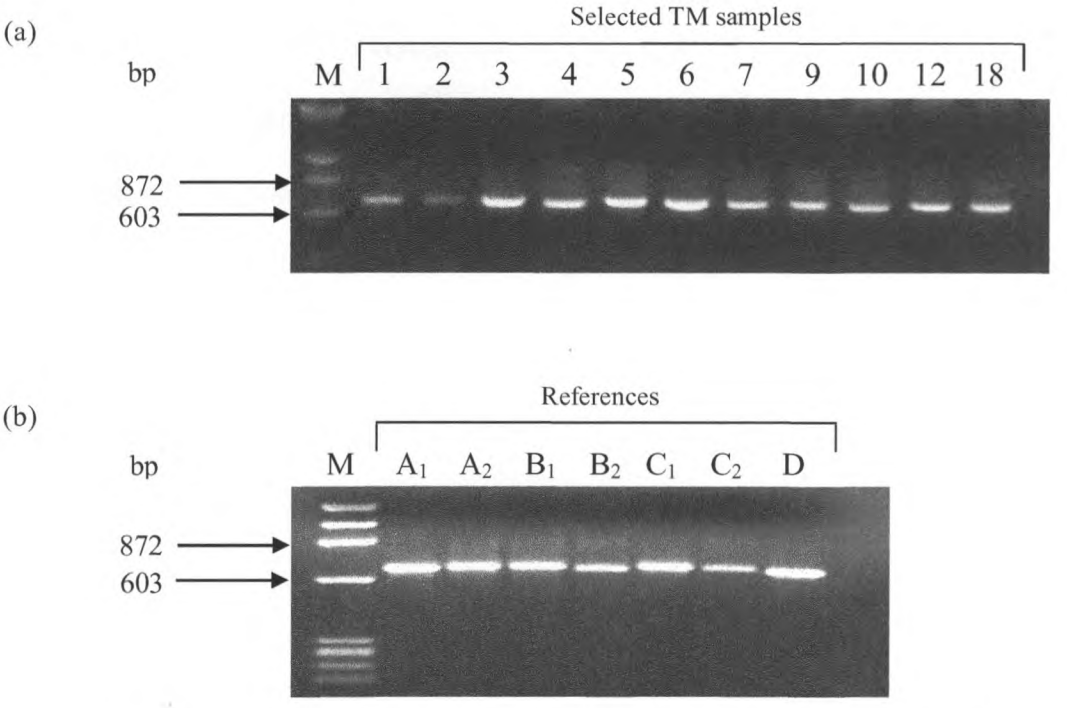


Figure 2.3: DNA agarose gel electrophoresis of the ~700bp C2-V5 PCR products from (a) selected isolates and (b) reference plasmids. Molecular weight marker (M, number IX, Roche) is shown on the left hand side with the 872 and 603bp bands indicated. Only A_1 was subsequently used in the HMA analysis giving similar results as A_2.

Overall, the V1/V2 HMA subtyping results suggested that 25 of the 39 pediatric isolates were subtype C, three were subtype A, one was subtype B, four were A-C and six were unclassified (Table 2.2).

In comparison, 38 of the 39 isolates were subtype C using the standardized C2-V5 HMAs (Table 2.2). One of the isolates, PETRA 44, subtyped as B (Table 2.2), and was consistent in both HMA protocols (Figures 2.6). A mother and child pair (PETRA 192 and 192M) was also included in this study, and the similarity of the V1/V2 profiles suggested the possibility of using this assay in transmission studies. However, in both cases, the isolates were incorrectly subtyped as A (Figure 2.7). In total 13 of the 39 isolates (33%) showed discrepant results between the V1/V2 and C2-V5 regions, suggesting that the V1/V2 HMA is not a reliable subtyping tool.

In all the investigated isolates, the V1/V2 HMA profiles indicated a lot of genetic variation illustrated by an array of bands on the 5% polyacrylamide gels (Figures 2.4 & 2.5). This was not seen in the corresponding C2-V5 HMA profiles, which showed sharp bands (Figure 2.4). When the HMA profiles of the TM cohort were compared to those of the RP cohort, there was no evidence of an increase in number of bands in either the V1/V2 profiles or the C2-V5 profiles.

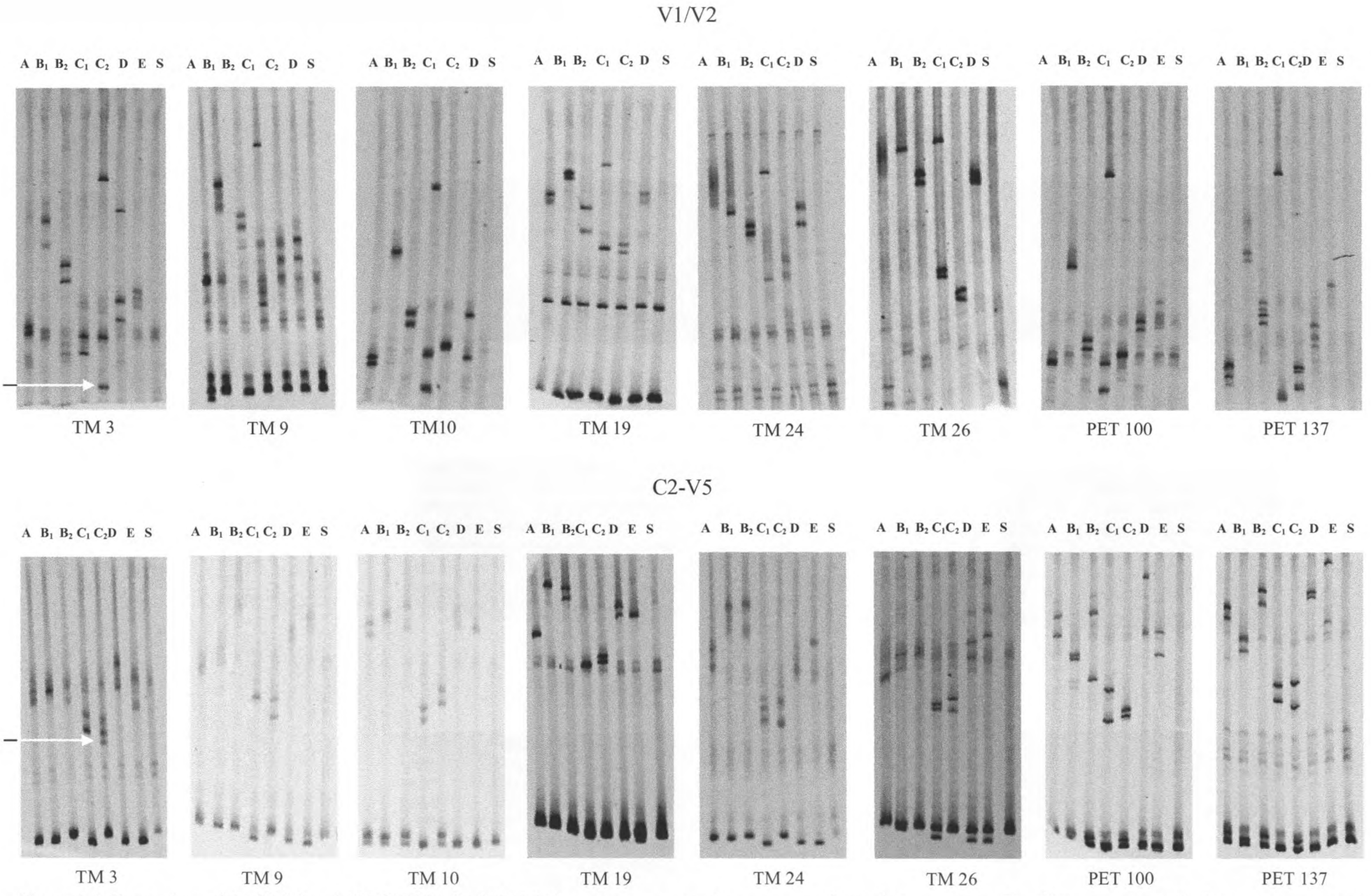


Figure 2.4: Comparison of the V1/V2 and C2-V5 HMA for HIV-1 infected pediatric samples with concordant subtyping results. For all samples, heteroduplexes migrated fastest with subtype C references (see arrow in first gel for example). In some cases homoduplexes are not shown. Different reference strains were used including A_2 but had no effect on the interpretation of the results.

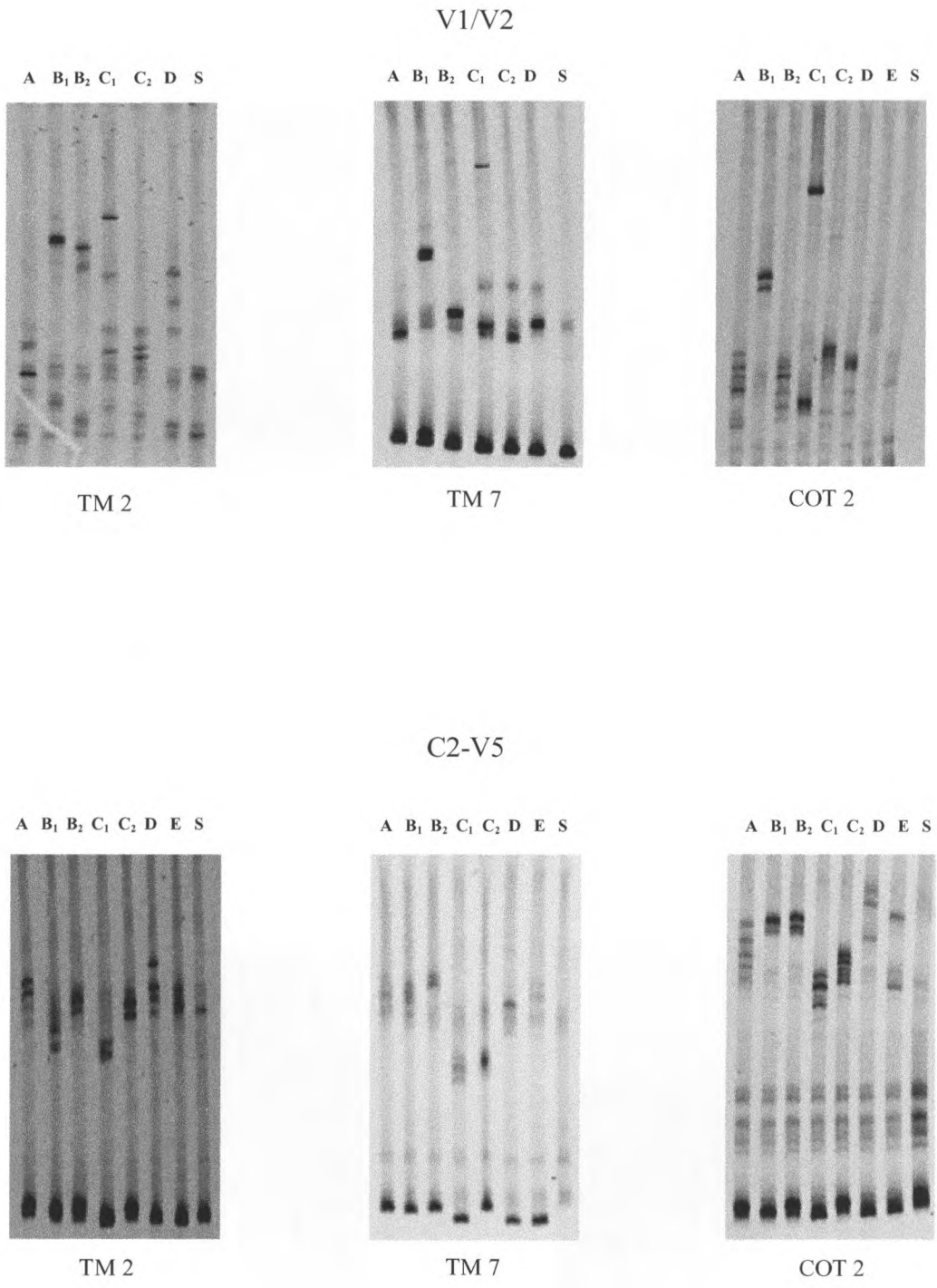


Figure 2.5: V1/V2 and C2-V5 HMA profiles for HIV-1 pediatric samples with discordant results. For all three samples, the C2-V5 HMA indicated a subtype C virus whilst the V1/V2 HMA profiles were inconclusive and listed as unclassified.

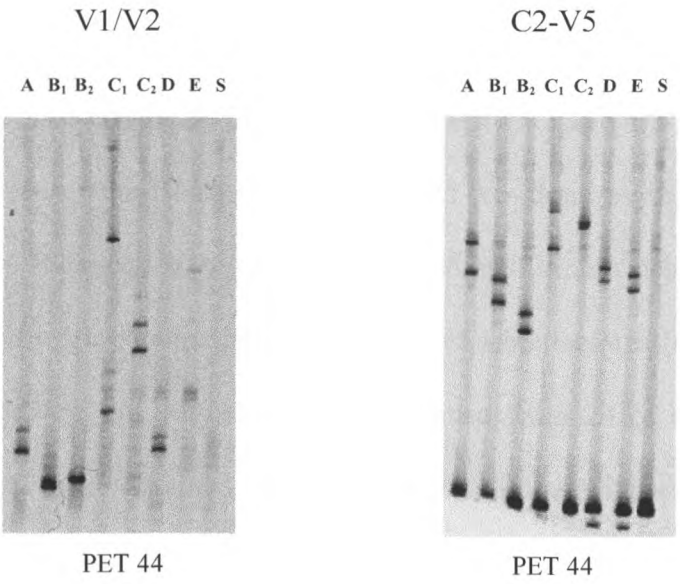


Figure 2.6: V1/V2 and C2-V5 HMA profiles from PETRA 44. Both profiles identified this patient as having a HIV-1 subtype B infection.

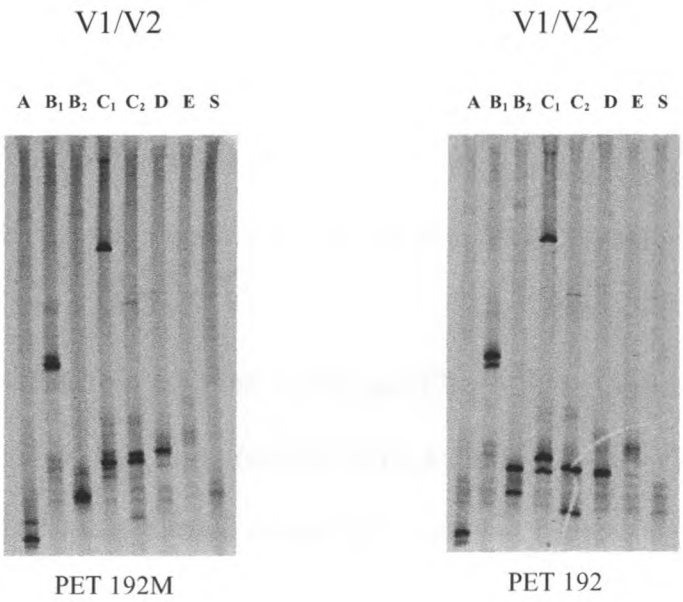


Figure 2.7: V1/V2 HMA of isolates from an HIV-1 infected mother and infant pair. Although the V1/V2 HMA identified these incorrectly as a subtype A virus, the patterns were similar suggesting this method may be useful in transmission studies.

2.3.2 Sequencing and phylogenetic analysis of the V1/V2 and C2-V5 regions

Authentication of the HMA subtyping results was done by sequencing the V1/V2 and C2-V5 regions. Nucleotide sequences and predicted amino acid sequences for the entire V1/V2 region were obtained from 34 of 41 available isolates (Figures 2.8 & 2.10). For the entire C2-V5 region, sequences were obtained from 24 of 41 isolates, whilst a further 10 sequences were obtained for the V3 region alone (Figures 2.9, 2.11 & 2.12). This included PETRA 192M (mother sequence) was included as an internal control for our phylogenetic analysis. After phylogenetic tree analysis, it was established that the V1/V2 (except TM19) and the C2-V5 nucleotide sequences clustered within the HIV-1 subtype C branches (Figures 2.8 and 2.9, respectively). Significant bootstrap values were however not achieved for the V1/V2 region (Figure 2.8), whilst significant values of 97% were recorded for the C2-V5 region (Figure 2.9). TM19 did not cluster together with the other sequences in the V1/V2 tree, and this isolate could not be subtyped by HMA in the V1/V2 region (Figure 2.8 and Table 2.2). It however clustered together with the other subtype C isolates in the C2-V5 tree. PETRA 44, identified as subtype B by HMA, could not be sequenced in either the V1/V2 or C2-V5 regions.

The data obtained from both the V1/V2 and C2-V5 HMA profiles were compared against their respective sequence data (except PETRA 192M). Thus for the V1/V2 region, only 20 of 33 (60.6%) isolates showed concordance between the HMA and sequence data, whilst all 33 isolates showed 100% concordance in the C2-V5 region, although for some isolates sequence data from the V3 region alone was available. The isolate *env* subtype was thus designated from the concordance of the HMA and sequence data. Overall, 40 isolates were C2-V5 subtype C and one was subtype B as determined by HMA and/or sequence analysis. A summary of HMA and sequence data is shown in Table 2.2.

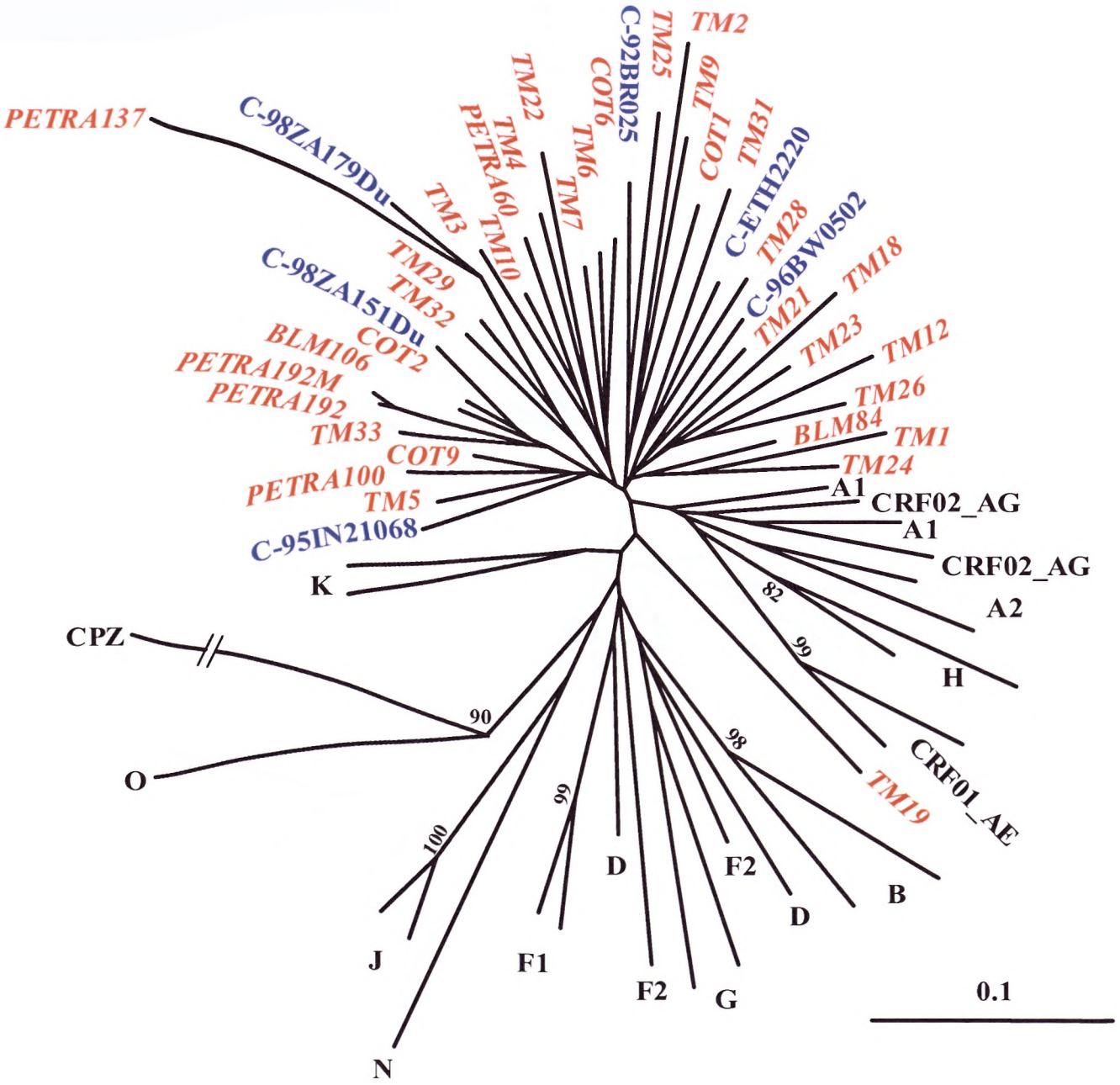


Figure 2.8: Phylogenetic tree analysis of the V1/V2 regions of the newly characterized pediatric HIV-1 viruses. New isolates from the RP and SP cohorts are highlighted in red, whilst those of known subtype C reference sequences from Los Alamos are indicated in blue. Bootstrap values above 70% are significant. The new isolates charecterised in this study cluster together with those of subtype C references, but without significant bootstrap values. TM19 was outside of the subtype C cluster.

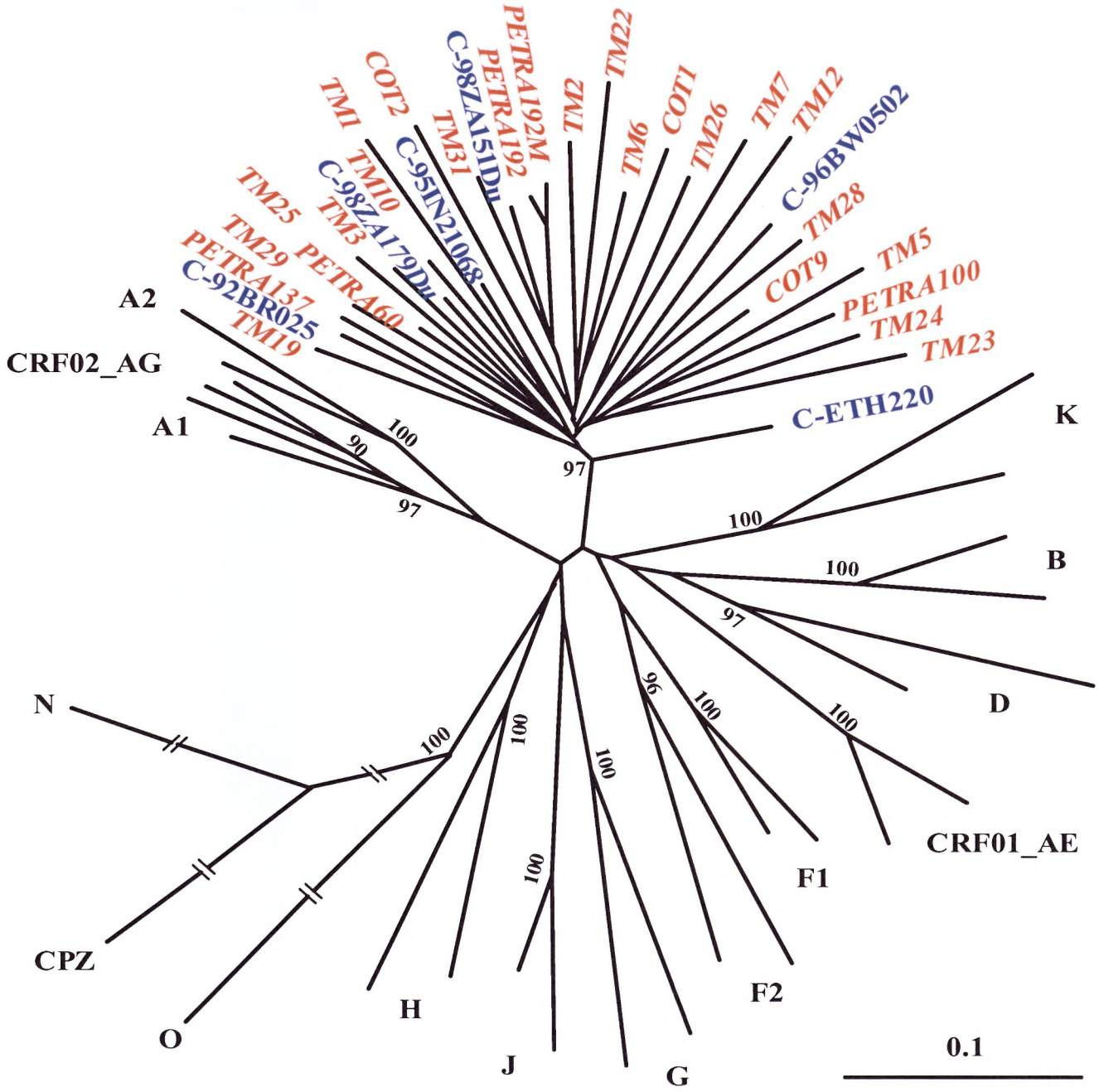


Figure 2.9: Phylogenetic tree analysis of the C2-V5 regions of the newly characterized pediatric HIV-1 viruses. New samples from the RP and SP cohorts are highlighted in red, whilst those of known subtype C reference sequences from Los Alamos are indicated in blue. Bootstrap values above 70% are significant. The isolates from the two cohorts cluster together with those of subtype C references with a significantly high bootstrap value of 97%.

Table 2.2: Comparison of HMA and sequence subtype data for V1/V2 and C2-V5 regions from slow (a) and rapid (b) disease progressing HIV-1 infected children.

Table 2.2a Slow progressors

Number	V1/V2			C2-V5			Isolate Subtype
	HMA	Sequence	Concordance	HMA	Sequence	Concordance	
TM 1	C	C	YES	C	C	YES	C
TM 2	**U**	C	NO	C	C*	YES	C**
TM 3	C	C	YES	C	C	YES	C
TM 4	C	C	YES	C	C*	YES	C
TM 5	C	C	YES	C	C	YES	C
TM 6	C	C	YES	C	C	YES	C
TM 7	**A-C**	C	NO	C	C	YES	C**
TM 9	C	C	YES	C	C*	YES	C
TM 10	C	C	YES	C	C	YES	C
TM 12	**A**	C	NO	C	C	YES	C**
TM 18	C	C	YES	C	C	YES	C
TM 19	C	**U**	NO	C	C	YES	C**
TM 20	C	C	YES	C	C*	YES	C
TM 21	**A-C**	C	NO	C	C*	YES	C**
TM 22	**U**	C	NO	C	C	YES	C**
TM 23	C	C	YES	C	C	YES	C
TM 24	C	C	YES	C	C	YES	C
TM 25	C	C	YES	C	C	YES	C
TM 26	C	C	YES	C	C	YES	C
TM 28	**U**	C	NO	C	C	YES	C**
TM 29	C	C	YES	C	C	YES	C
TM 30	**U**	C	NO	C	C*	YES	C**
TM 31	**U**	C	NO	C	C	YES	C**
TM 32	C	C	YES	C	C*	YES	C
TM 33	C	C	YES	C	C*	YES	C

Table 2.2b Rapid progressors

Number	V1/V2			C2-V5			Isolate Subtype
	HMA	Sequence	Concordance	HMA	Sequence	Concordance	
PETRA 44	**B**	nd	na	B	nd	na	B
PETRA 60	**A**	C	NO	C	C	YES	C**
PETRA 100	C	C	YES	C	C	YES	C
PETRA 137	C	C	YES	C	C	YES	C
PETRA 192	**A**	C	NO	C	C	YES	C**
COT 1	C	C	YES	C	C	YES	C
COT 2	**U**	C	NO	C	C	YES	C**
COT 6	C	C	YES	C	C*	YES	C
COT 9	**A-C**	C	NO	C	C	YES	C**
RP 1	C	nd	na	C	nd	na	C
RP 3	C	nd	na	C	nd	na	C
RP 4	C	nd	na	C	nd	na	C
RP 5	**A-C**	nd	na	C	nd	na	C
RP 6	C	nd	na	C	nd	na	C
BLM 84	nd	nd	nd	nd	C*	na	C**
BLM 106	nd	nd	nd	nd	C*	na	C**

U = undetermined, nd = not done, na = not available, * = sequence data available from V3 region alone, ** = Isolate subtype based on sequence data.

2.3.3 Amino acid analysis

The predicted amino acid sequences of the V1/V2 and C2-V5 regions are shown in Figures 2.10 and 2.11. Analysis of V1/V2 region shows that the three cysteines at the beginning, middle and end of the loops were highly conserved in all isolates. There was a large amount of genetic polymorphisms as a result of substitutions, insertions and deletions particularly in the V1 loop. The few loci that were highly conserved were mainly in the V2 region although there was a highly polymorphic region near the C-terminus. This extensive polymorphism resulted in significant length variations between the different isolates with almost all having regions longer than that of the consensus subtype C sequence. One isolate TM19 had a large insertion in V2 with an overall length of 86 amino acids compared to the consensus, which had 58. Three isolates had short V1/V2 fragments; in particular TM31 had only 53 amino acids.

Twenty randomly selected subtype C isolates from southern Africa were selected to serve as reference values for a comparative statistical analysis of sequences from SPs and RPs. Analysis of the V1/V2 sequences is shown in Table 2.3. The mean lengths of the V1/V2 region (number of amino acids) did not differ significantly between the 2 groups ($P<0.05$, unpaired t-test; Table 2.3) and was within range of the reference values. The SP however had the widest range between 53 and 86 amino acids. The overall charge for the 20 reference sequences was +1 while for the SP it was 0 and –0.5 for the RP. These values were not significantly different ($P<0.05$, unpaired t-test). However SP had the largest range of charges in the V1/V2 region possibly due to the larger sample size of this group.

Potential N-linked glycosylation sites (NXT or NXS, where X is any amino acid) were determined using PROSITE and are highlighted in yellow in Figures 2.10 & 2.11. Half of the isolates had a higher number of predicted N-linked glycosylation sites than the

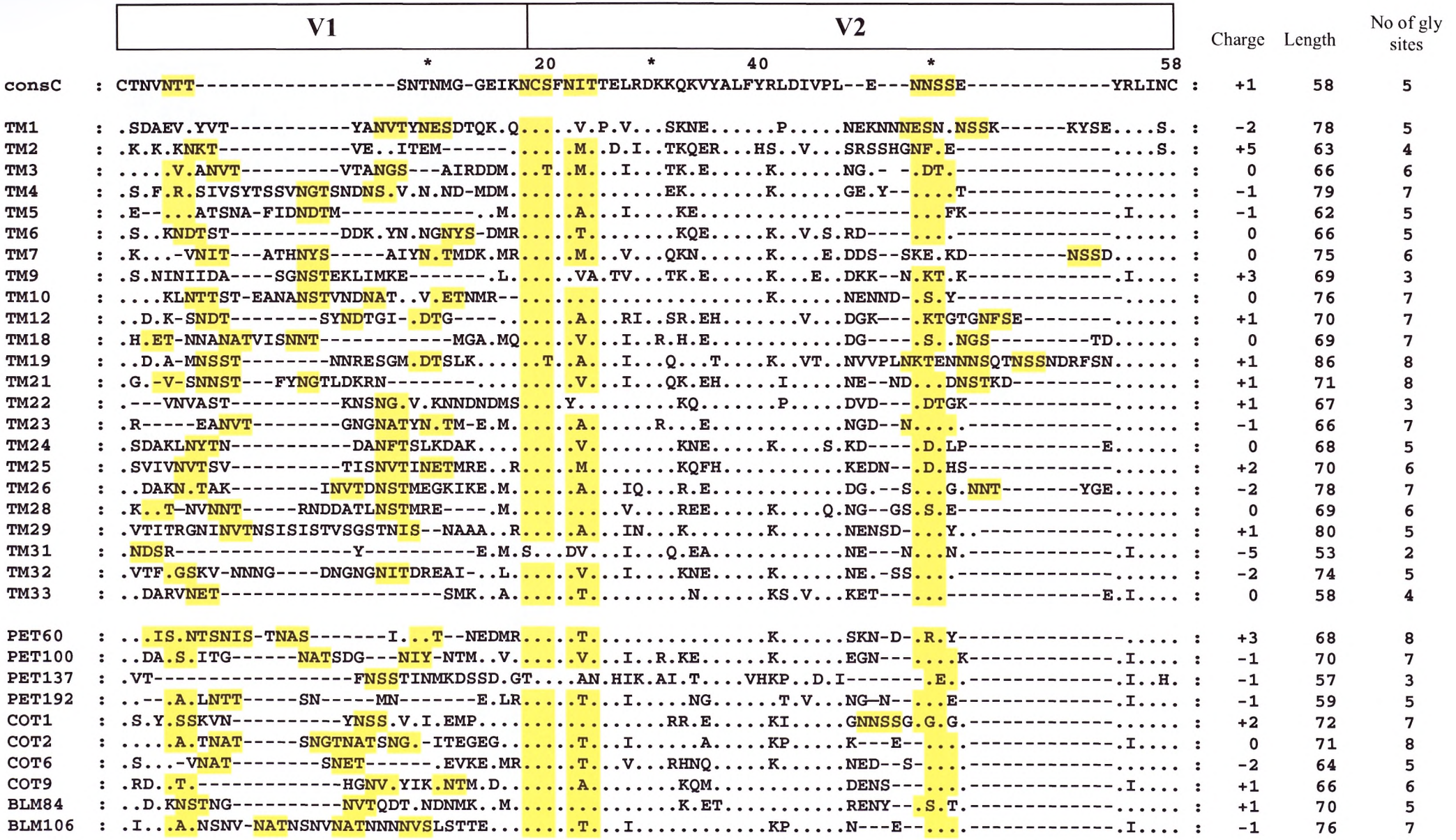


Figure 2.10: Predicted amino acid sequence alignments of the V1/V2 regions of isolates from slow (TM) and rapid (PETRA, COT, BLM) progressing HIV-1 infected children. The consensus C sequence is shown on top with all amino acids, and (-) indicates a deletion, (.) indicates similar amino acids as that in the consensus bar. Potential N-linked glycosylation sites (gly) have been highlighted in yellow. The overall charge, length and number of glycosylation sites are indicated on the right.

subtype C consensus sequence, which had 6 sites. Four isolates, which had longer sequences, had 8 potential N-linked glycosylation sites, and thus it appears that the number of N-linked glycosylation sites increased with longer sequences. The median number of glycosylation sites did not differ significantly between the SPs and RPs (Table 2.3, $P<0.05$, unpaired t-test).

The SPs and RPs were compared for genetic relatedness (Table 2.3). The V1/V2 mean genetic distance was higher in the SP than the RP cohort, indicative of lower genetic relatedness amongst infections in this cohort. This difference was statistically significant (Mann-Whitney 2 tailed t-test, $P<0.0001$). The ratio of non-synonymous to synonymous base substitutions (d_n / d_s) was higher for the SPs than the RPs, and when the two means were compared, the different ratios' were statistically significant (Mann-Whitney 2 tailed t-test, $p = 0.0233$). The different d_n/d_s ratios suggest that there is immune selection for change in the V1/V2 region in both cohorts. However, the selection is stronger amongst

Table 2.3: Statistical analysis of the V1/V2 region among HIV-1 subtype C reference isolates, slow progressors (SPs) and rapid progressors (RPs).

	References (n=20)	SPs (n=23)	RPs (n=10)	P - Value	Significance
Mean length (Range)	68.2 (58-84)	70.1 (53 - 86)	67.3 (57 - 76)	t-test 0.3013 f-test 0.2269	ns ns
Median charge (Range)	+1 (-1- +3)	0 (-5 - +5)	-0.5 (-2 - +3)	t-test 0.9362 f-test 0.2770	ns ns
Median no of Gly sites (range)	6 (4 - 9)	6 (2 - 8)	6.5 (3 - 8)	t-test 0.3879 f-test 0.5109	ns ns
Mean genetic distance	0.688	0.758	0.517	t-test 0.0001 f-test 0.0180	Yes P <0.0001
ω*	-	1.435	0.981	t-test 0.0001 f-test <0.0001	Yes P = 0.0233
No of selected sites	-	24	16	-	-

t-test was unpaired and variance was compared using f-test; ns = not significant; * = d_n/d_s ratio

slow progressing infants.

Analysis of the C2, V3 and C4 loops revealed that the regions were relatively conserved, whilst the C3, V4 and V5 regions were more genetically diverse (Figure 2.11). There was no difference in the polymorphisms observed between the SP and RP cohorts, but this may have been due to the small numbers in the RP group.

The mean length of the C2-V5 region among the 20 reference isolates was 197 amino acids, which was similar to the SP (198 amino acids) and the RP (195 amino acids) (Table 2.4). The RPs had a higher median charge of +10 compared to the SPs which had a charge of +8 but this was not significantly different ($P<0.05$, unpaired t-test). Both the SPs and RPs had a median number of 13 potential N-linked glycosylation sites, suggesting that the rates of insertions and/or deletions were similar in both cohorts.

Comparison of the d_s / d_n ratios indicated that RPs had a significantly higher ratio than the SP children (Mann-Whitney 2 tailed t-test, $p = 0.0029$; Table 2.4). This suggests that the region is undergoing more change in the RP children than in their SP counterparts. These sites were statistically significant with a probability of >0.9 (data not shown). However, the number of positively selected sites in the C2-V5 region was higher in the SP (24 sites) than in the RP (16 sites) indicating a broader and stronger immunue pressure across the C2-V5 region. These results are however limited by the small sample size of the RP pediatric cohort.

The number of amino acids in the V3 loop remained relatively conserved ranging from 34 to 37 amino acids (Figure 2.12). The isolates TM1, TM2, and TM9 contained insertions after the tetra peptide motif GPGQ, and TM23 contained an insertion at position 6, which was a Proline (based on V3 loop numbering; Figure 2.12). The isolates TM3,

TM10, TM12 and TM31 all contained deletions, thus reducing their total number of amino acids to 34 in the V3 region (Figure 2.12).

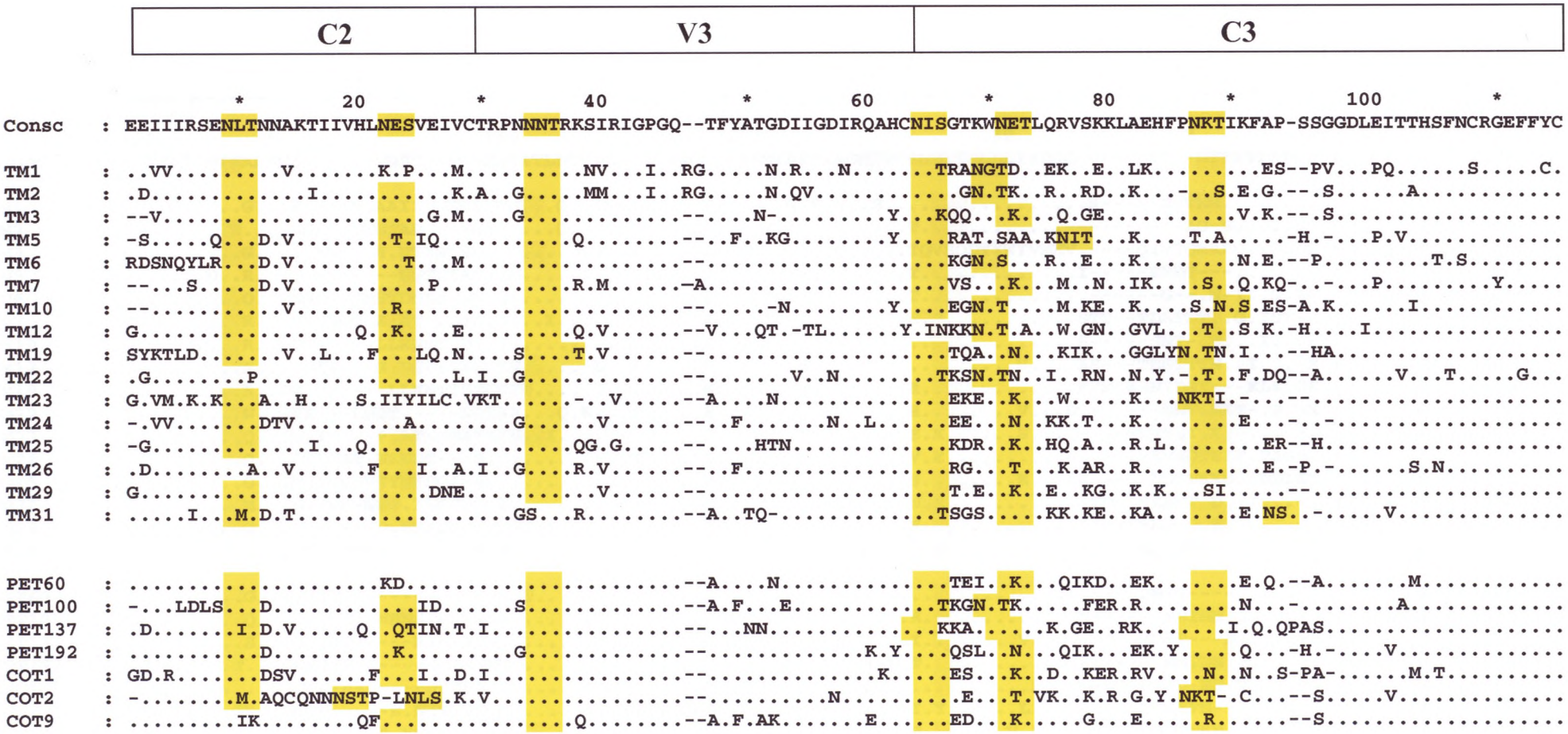


Figure 2.11: Predicted amino acid sequence alignments of the C2-V5 regions of isolates from slow and rapid progressing HIV-1 infected children. The consensus C sequence is shown on top with all amino acids, (-) indicates a deletion and (.) indicates similar amino acids as that in the consensus sequence. Potential glycosylation sites (gly) have been highlighted in yellow. The overall charge, length and number of glycosylation sites are indicated on the right.

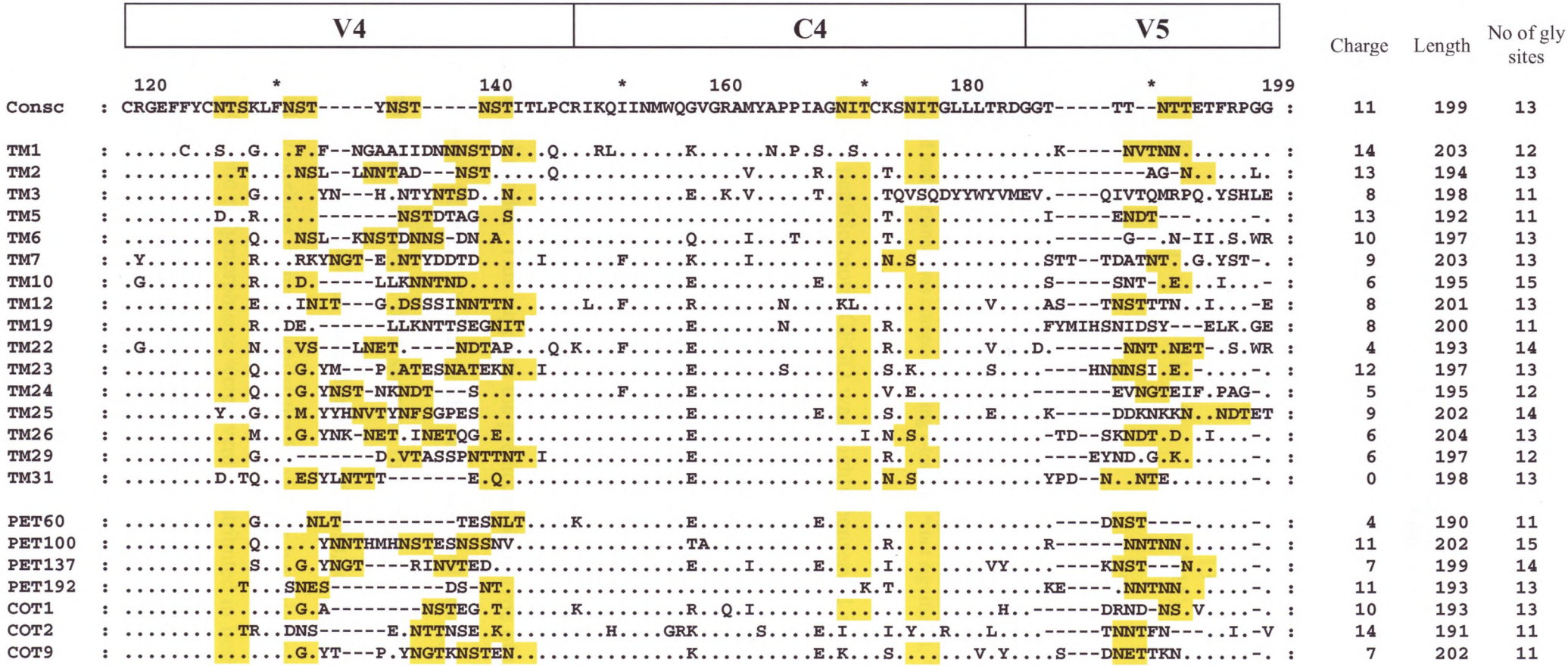


Figure 2.11. Cont.

Table 2.4: Statistical analysis of the C2-V5 region among HIV-1 subtype C reference isolates, slow progressors (SPs) and rapid progressors (RPs).

	References (n=20)	SPs (n=16)	RPs (n=7)	P - Value	Significance
mean length (range)	197 (188-205)	198 (192-204)	195.7 (190-202)	t-test 0.2073 f-test 0.1273	ns ns
median charge (range)	+8.5 (+4 -+7)	+8 (-14 - 0)	+10 (-14- +4)	t-test 0.6285 f-test 0.4334	ns ns
median no of Gly sites (range)	13.50 (9-16)	13 (11-15)	13 (11-15)	t-test 0.8152 f-test 0.1042	ns ns
mean genetic distance	0.278	0.324	0.254	t-test <0.0001 f-test 0.1709	Yes P < 0.0001
ω*	-	1.054	1.651	t-test 0.0029 f-test <0.0001	Yes P = 0.0029
No of selected sites	-	28	24	-	-

t-test was unpaired and variance was compared using f-test; ns = not significant; * = d_n/d_s ratio

The tetra peptide motif GPGQ was highly conserved in all isolates except TM1, TM2 and TM9, which had the motif GPRG. These isolates have been shown to use the CXCR4 co-receptor for cell entry (Table 2.1). An uncharged amino acid was present at position 11 in the V3 region with the exception of TM32 that contained a negatively charged Glutamic acid (Figure 2.12). Position 25 had more changes although many of the isolates substituted a Glutamic acid residue for an Aspartic acid (also negatively charged) or an uncharged residue. This supports the predicted phenotype of the isolates as NSI and macrophage tropic (Table 2.1). Only TM1 had a positively charged Arginine at position 25, supporting the phenotypic data showing this to be an SI, CXCR4-using virus. The overall V3 charge of the isolates ranged from +2 (COT9), to +8 (TM1). In general the NSI viruses had low positive charges, whilst the SI viruses had high charges in the V3 loops (Table 2.1). Genetic polymorphisms were evenly distributed in both cohorts and the only noticeable difference was the occurrence of an uncharged Valine at position 12 at a higher frequency in the SP cohort than the RP cohort.

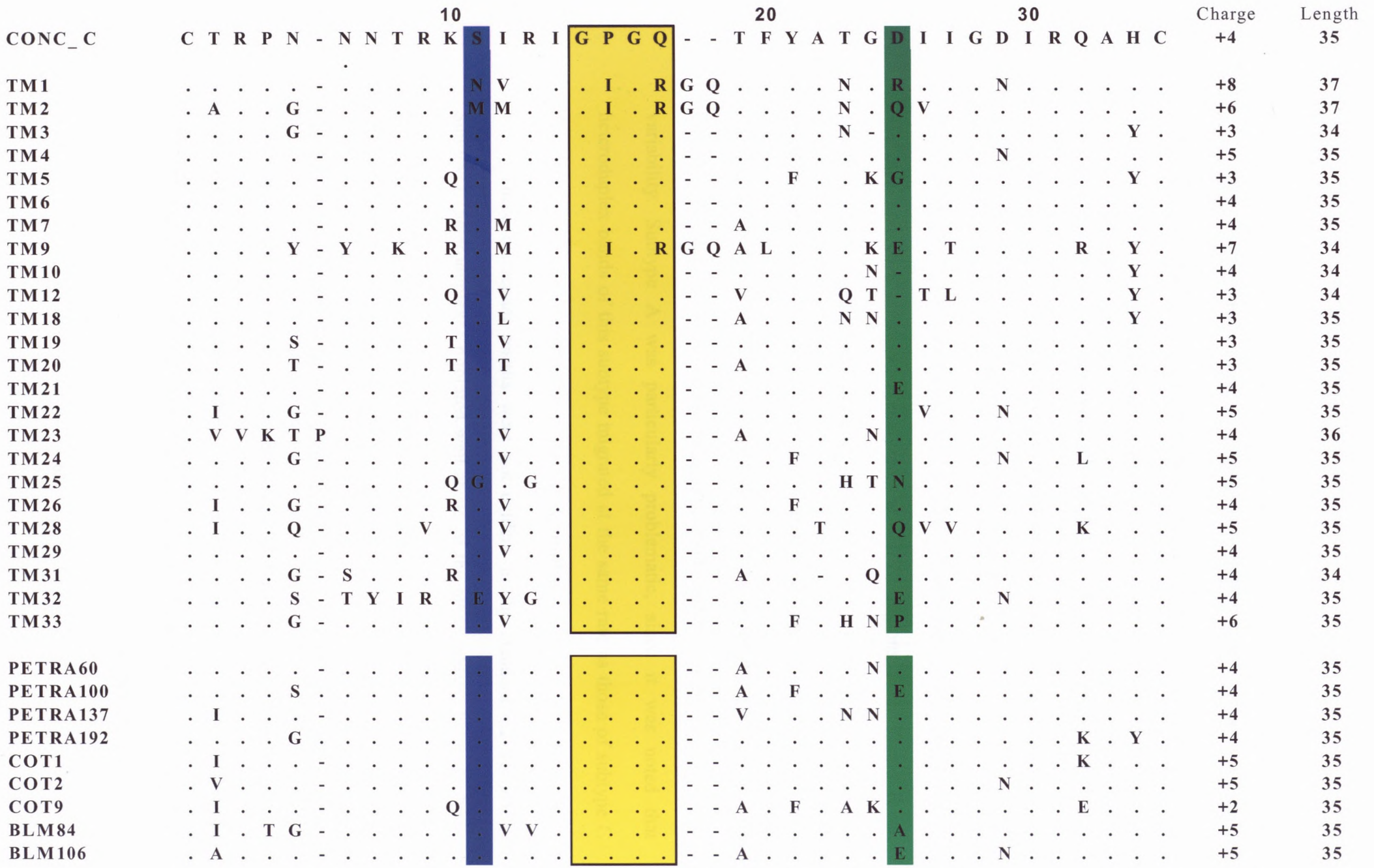


Figure 2.12: Analyzed V3 loop regions from HIV-1 infected slow and rapid progressing children. The tetra motif GPGQ is boxed in yellow and amino acid numbers 11 and 25 are in blue and green respectively. The overall charges and lengths of the region have also been included.

2.4 DISCUSSION

This study contains valuable information for HIV-1 subtyping of viral isolates and analysis of genetic variation between isolates. From this study it is clear that the V1/V2 HMA is not a reliable assay for genetic subtyping of HIV-1 subtype C isolates from pediatric patients. It was realized that the region is too small (320bp) and contained too much genetic and length variation for accurate subtyping. Extensive length variation is evident in the V1/V2 PCR, where the amplicons can be seen to be of different lengths (Figures 2.2a and b). These observations were also noted by Martinez and co-workers who analyzed the V1/V2 regions from Puerto Rican pediatric subtype B patients (Ramos Martinez *et al.*, 1996). During HMA gel electrophoresis these short fragments re-anneal and migrate at different rates indicative of genetic relatedness. The results observed for the V1/V2 region shows that the HMA profiles gave unclear subtyping results because of genetic and length variability. Subtype A was particularly problematic, since it was noted that heteroduplex bands of this subtype migrated at the same rate as those of subtype C thus prohibiting clear interpretation of the HMA profiles.

These discrepant results were not evident in the standard C2-V5 HMA. Subtype C heteroduplexes migrated well ahead of the other subtype heteroduplexes and were clearly distinguishable. Results showed that all isolates were subtype C except one, PETRA 44, which was a subtype B. The V1/V2 HMA accurately subtyped the B isolate but failed to accurately subtype 13 of 38 subtype C samples. This translates to 34% of the isolates and is too high for the V1/V2 HMA protocol to be relied upon for accurate subtyping.

However, the V1/V2 HMA profiles, from an infant and mother pair (PETRA192 and 192M) showed migration patterns with the different reference

subtypes. This strongly suggests that the V1/V2 HMA can be used in transmission studies, particularly because it is fast. Another advantage of the V1/V2 HMA is the ability to detect variant (quasispecies) within an isolate. This was evident in many of the profiles that had multiple bands suggesting numerous V1/V2 quasispecies. These observations suggest that the V1/V2 HMA can be used as a rapid and inexpensive method to screen isolates for quasispecies analysis.

The V1/V2 nucleotide sequences were subjected to extensive phylogenetic analysis and data showed that the isolates grouped together with subtype C reference strains with the exception of TM19. However, bootstrap values of the tree were not significant (below 70%; Figure 2.8). This suggests that although the isolates and even the different subtypes and six CRFs grouped together, the V1/V2 region did not contain enough informative sites to certify the relatedness of the isolates to the reference strains (Figure 2.8). This was attributed to the length variations from extensive insertions and deletions in this region. These observations are further evidenced by the extensive amino acid variations seen in the predicted amino acid sequence of the region relative to the consensus C (Figure 2.10). The results also show that TM19 had a large insertion of 14 amino acids at the carboxyl terminus of the V2 region, which could have accounted for it being an outlier of subtype C on the phylogenetic tree (Figure 2.10) despite it being a clear C on the HMA (Figure 2.4). Thus in this unusual case the HMA was more reliable that phylogenetic analysis. Interestingly, phylogenetic tree analysis showed that the V1/V2 regions of subtypes A, CRF02_AG and C are closely related (Figure 2.8). This genetic relatedness may account for the poor resolution of subtypes A and C seen on the V1/V2 HMA (Figure 2.5).

Phylogenetic analysis of the C2-V5 nucleotide sequences accurately grouped the isolates with the reference subtype C strains with 97% bootstrap values confirming the HMA results. The other subtypes and CRFs also grouped with significant bootstrap values. This shows that the C2-V5 region contains enough informative sites for accurate and reliable phylogenetic analysis. Overall, based on these results, 40 of the isolates were *env* subtype C, and one was subtype B. It is highly unlikely that there are 10% recombinants in the V1/V2 and C2-V5 regions, since the proportions of non-C HIV-1 subtypes circulating in South Africa are extremely low (<1%, C. Pillay, unpublished data). However, the possibility that some of the isolates may be recombinants cannot be ruled out. This can only be confirmed by full-length genome sequence analysis.

Inspection of the V1/V2 amino acid sequences (Figure 2.10) showed that there were no obvious genetic polymorphisms that distinguished the SPs from the RPs, although the latter did have a slightly more conserved V2 region (relative to the consensus subtype C sequence). However, we found significant differences in the genetic distance and d_n/d_s ratios when comparing the SPs and RPs. Higher positive selective pressure was observed in the SP cohort. The higher d_n/d_s ratio for children who had slow progression to disease suggests that the V1/V2 region in these children is under greater selective pressure for change possibly due to neutralizing activity, than is the same region amplified from isolates obtained from children who had rapid disease progression to AIDS. The d_n/d_s ratios observed between the rapid and slow progressors V1/V2 region was greater than that observed for the C2-V5 region, and indeed the RPs value was higher than that of the SPs in the C2-V5 region suggesting adaptive evolution in this region is less strongly associated with immune pressures. The C2-V5 region is responsible for viral infectivity and these RP infants have been

shown to carry higher infectious virus (P.Walker *et al.,* in preparation). This slightly higher d_n/d_s amongst RPs may indicate selection for higher replicative capacity.

The greater genetic pressure for change being observed in the slow progressing children supports the hypothesis that the V1/V2 region does indeed shield the inner conserved regions of gp120, and that this region would thus be in direct contact with the immune system (Wyatt *et al.*, 1993). Observations recorded by Fung and co-workers suggest that the V1/V2 region has an important role to play in important biological properties *in-vitro* including antibody neutralization sensitivity, particularly from LTNPs (Fung *et al.*, 1992). The ability of the slow progressing children to control viral pathogenicity suggest that their immune system is possibly producing antibodies targeted to the exposed and immunogenic V1/V2 gp120 region. This in turn is associated with an increase in antigenic variation (Koito *et al.*, 1994).

The occurrence of N-linked glycosylation sites in the V1/V2 region continues to engage scientists in the understanding of the relationship between virus and host defense mechanisms. The V1/V2 region is heavily glycosylated and is thought to cover the conserved inner regions of the gp120. This shielding effect has been observed in many studies with different subtypes including subtype C (Klevytska *et al.*, 2002). Our results suggest that the median number of potential N-linked glycosylation sites in both cohorts was not statistically significant, indeed they were 6 and 6.5 for the SPs and RPs respectively. The lack of evidence to suggest differences in the median number of potential N-linked glycosylation sites in our study could have stemmed from the small sample size of the RPs cohort, and/or obtaining isolates from patients too late in infection. This warrants the need for longitudinal studies to be performed. An ideal study cohort would consist of infants grouped together at the time of birth. Regular blood sampling would be required, and the infants who died

within 2 years would be grouped as RPs, those who lived beyond that to 5 years would be grouped as slow progressors and still further, those who survived beyond 5 years, would be termed long term survivors or non-progressors. This would account for and normalize most of the differences expected between the children. Finally, three important points of examination would be of emphasis; the first being samples from the two cohorts representing RPs and SPs, and a second from children who are SPs. Understandably, such a study would take between 8 and 10 years, and this would be a difficult study to set up, thus what has been presented here, is to the best of our knowledge, a representative of an ideal study.

In conclusion, understanding changes at the V1/V2 region of gp120 has presented unprecedented challenges to researchers. Structural data suggest that the V1/V2 loops cover the underlying conserved region of gp120 (Wyatt *et al.*, 1998). The extensive genetic variation seen in the V1/V2 regions in the SP cohort imply that this region is under constant immune pressure and thus constantly undergoing genetic evolution and giving rise to numerous quasispecies. Understanding the role V1/V2 plays in HIV-1 disease pathogenesis is of notable importance since this region plays a key role in cell entry and the ability of the virus to escape the host immune response. Here we show that these genetic changes can be tracked in the V1/V2 HMA protocol. At any given time, in an infected individual, there can be neutralizing antibody resistant and susceptible quasispecies and this needs to be investigated further.

CHAPTER THREE

DEVELOPMENT OF A V1/V2 HETERODUPLEX TRACKING ASSAY FOR ANALYSIS OF ANTIBODY NEUTRALIZATION SENSITIVE HIV-1 VIRAL VARIANTS

3.1 INTRODUCTION

HIV-1 is characterised by diverse populations of quasispecies as a result of rapid turnover, high mutation rates and high frequency of recombination (Ho *et al.*, 1995; Wain-Hobson, 1992). Several studies have shown that soon after infection, most adults and children harbour a single variant from which quasispecies evolve during the course of infection (Delwart *et al.*, 1997; Lukashov *et al.*, 1995; Zhu *et al.*, 1993). With time, quasispecies diversity increases both within the individual as well as within a given geographical area (Delwart *et al.*, 1993; Wolinsky *et al.*, 1996). This extreme genetic diversity has advantages for most RNA viruses, including altered pathogenesis, development of new host (tissue, organ or species) tropism, and evasion of antiviral therapy and the host immune responses (Domingo and Holland, 1997). With time, these rapid changes in the quasispecies pool also result in mutants with a higher genetic fitness, which sometimes outgrow less fit variants (Domingo and Holland, 1997).

Long term non-progressing (LTNP) and slow progressing (SP) HIV-1 infected individuals have been shown to have vigorous humoral and cellular antiviral immune responses. These responses drive the emergence of new variants as the virus evolves to escape immune selection pressure (Schreiber *et al.*, 1997; Shankarappa *et al.*, 1998). Studies on these unusual individuals may yield important information regarding HIV-1 pathogenesis and genetic diversity.

Several methods have been used to investigate HIV-1 quasispecies within populations. The heteroduplex mobility assay (HMA) technique has been widely used, although it remains insensitive to study small viral populations within infected individuals (Delwart and Gordon, 1997; White *et al.*, 2000). Other methods include direct sequencing, RNase A mismatch cleavage, DNA fragment length polymorphisms and single-stranded conformational polymorphisms, amongst others (Boden and Markowitz, 1998; Hynes *et al.*, 1993; Lopez-Galindez *et al.*, 1991; Simmonds *et al.*, 1990). Whilst these methods are able to distinguish some variants, their use in quasispecies analysis have been greatly limited mainly due to their limitations in sensitivity and/or artifactual variant frequencies (Hynes *et al.*, 1993).

The development of the heteroduplex tracking assay (HTA) as an investigative tool has greatly enriched research in quasispecies investigation/identification. DNA HTA using single-stranded probes provides a rapid means of resolving genetic variants co-amplified by PCR and measuring the level of particular variants in complex populations. The sensitivity of detection of rare variants within a genetically mixed population using single-stranded DNA (ssDNA) probes has been shown to be as high as 1:500 (Delwart and Gordon, 1997). HTA has been used to investigate HIV-1 quasispecies in different organs and within the same organs (Freel *et al.*, 2001; Resch *et al.*, 2001). In the study by Freel *et al.* (2001), HTA was successfully used to phenotypically characterise HIV-1 viral isolates in saliva and showed that it can be used as a non-invasive method for evaluating and monitoring viral evolution in infected persons (Freel *et al.*, 2001). The V3 HTA has been used to examine populations of HIV-1 variants in blood plasma (Buckner *et al.*, 2002; Nelson *et al.*, 1997), and also to investigate HIV-1 variants in cell-free plasma fractions of semen in different patient cohorts over time both before and after antibacterial therapy for

urethritis (Ping *et al.*, 2000). Other investigators have also used the technique to measure the impact of HIV-1 fitness on disease progression (Quinones-Mateu *et al.*, 2000). In this investigation, HIV-1 dual infection of peripheral blood mononuclear cells (PBMCs) was investigated with the use of HTA for both non syncytium inducing (NSI) and syncytium inducing (SI) isolates, and also to measure relative amounts of virus production (Quinones-Mateu *et al.*, 2000). More recently, Kitrinos and co-workers investigated variant changes in nucleotides and length differences using a HTA designed from the V1/V2 region of gp120. Analysis to detect longitudinal virus population changes in monthly samples from entry point yielded successful results especially when using a heterologous probe rather than one from the sample itself (Kitrinos *et al.*, 2003). These investigations suggest that the highly variable V1/V2 region can be useful in monitoring longitudinal changes with time in viral populations in infected individuals.

Few studies have used the V1/V2 region to investigate selective immune pressure in slow progressors in both pediatric and/or adult cohorts, despite the region having a recognized role in antibody neutralization (Etemad-Moghadam *et al.,* 1999; Jurkiewicz *et al.,* 1997). This chapter explores the development and use of the V1/V2 HTA method to evaluate genetic changes occurring in the gp120 region for individuals with strong neutralizing antibody titres. Analysis of the V1/V2 HMA profiles in the previous section (chapter two) formed the basis for selecting samples that would be screened with the more sensitive V1/V2 HTA.

3.2 MATERIALS AND METHODS

3.2.1 Patient samples

Three isolates were chosen for the V1/V2 HTA assay based on the large number of quasispecies visible on the V1/V2 HMA and good heterologous neutralizing titres (N. Taylor, unpublished data). The chosen isolates included TM6b, TM22b (Chapter two) and HIVNET 536-10-7. The HIVNET 536-10-7 isolate was part of another study in our laboratory, and was selected because it showed very high antibody neutralizing titres. All viral isolates were prepared as previously described in sections 2.2.1. Patient and viral isolate data is shown in Table 3.1.

Table 3.1: Demographic and clinical data from three patients whose viral isolates were chosen for HTA neutralization experiments. All three patients had high titres of heterologous neutralizing antibodies (not shown).

Isolate	Sex	Age (Years)	CD4 (Cells/ml)	VL (RNA copies/ml)	MT-2 assay	Co-receptor Usage	Envelope Subtype
HIVNET 536-10-7	F	23	373	1,250	NSI	R 5	C
TM6b	F	6	1,026	106,638	NSI	R 5	C
TM22b	F	6	339	39,266	NSI	R 5	C

3.2.2 Titration of serum and neutralizing antibody assays

Antibody neutralization of primary HIV-1 isolates was measured in PBMCs by a reduction in p24 gag antigen (Ag) production (Bures *et al.*, 2000). Autologous serum was used in all experiments. Briefly, PBMCs were prepared from buffy coats of healthy HIV-1 negative individuals and grown in RPMI 1640 growth medium, supplemented with 20% FCS (Sterilab, USA) and 5% interleukin 2 (IL-2, 50μg/ml) (Roche, GmbH, Germany). Viral isolates were generated from standard co-culture techniques and used to infect mitogen-stimulated cultures. Following infection cells

were washed in RPMI 1640 with 20% FCS (wash medium) (Sigma, Germany) to remove unbound virus.

For the chosen isolates, 15µl of undiluted virus stock was incubated with various dilutions of autologous serum in triplicate for 1 hour in a 96-well U-bottom culture plate (Costar, Corning, USA). All incubations were done at 37°C in a 5% CO_2 humidified incubator. PHA-stimulated PBMCs diluted to $5X10^6$ cells/ml in growth medium were added and plates incubated for a further 24 hours. The cells were washed twice with wash medium by removing 165µl and replacing it with 155µl with intermittent centrifuging at 1,200 rpm (100 xg) for 10 min. The cells were finally re-suspended in 155µl of fresh growth medium until day 3, 4, 5 and 6. Culture supernatants (10µl) were collected daily and mixed with 270µl of 0.5% Triton X-100 (Roche, GmbH, Germany), and stored at 4°C. The removed culture fluid was replaced with 10µl of fresh growth medium. For each experiment, virus control (VC) wells (virus plus cells but with no serum) were included and this served to monitor the normal growth rate of the virus. A pilot experiment was set up for each experiment to determine the best day to harvest supernatant, that is, when virus growth was at the exponential phase.

3.2.3 DuPont's p24 ELISA assay

Concentration of p24 Gag Ag in Triton X-100 lysates was measured in an Ag capture ELISA as described by the supplier (DuPont / NEN Life sciences, Boston, Massachusetts). The limit of detection in the p24 ELISA is 0.1ng of p24/ml and antibody neutralization titres were taken as a reciprocal of the minimum serum dilution that reduced p24 synthesis by 80% relative to a negative control serum sample. Briefly, the Ag ELISA was composed of a standard positive, which was

diluted from 10ng/ml to 0.1ng/ml in 0.5% Triton X-100. Ten micolitres of test sample was loaded to the same plate along the standard and incubated for one hour. The plate was washed six times in a standard plate washer with supplied wash solution before adding 100µl of detector antibody. After an hour of incubation, the plate was again washed before adding Streptavidine-horseradish peroxidase (HRP) diluted 1:100 in its diluent. This was followed by an incubation of 30 min at room temperature and another six washes. One *o*-Phenylenediamine dihydrochloride (OPD) tablet diluted in 11ml diluent was prepared and 100µl dispensed to each well. The kinetic curve was prepared and readings taken immediately at 450nm on the Versamax-pro microplate reader using Softmax Pro 3.1.2 software (Molecular devices, USA).

3.3 Cloning of V1/V2 98ZA151Du (Du151) probe

3.3.1 V1/V2 insert preparation

The insert gp120 V1/V2 region was PCR amplified from a previously cloned Du151 gp160 (obtained from Dr. Maria Papathanasopoulos) as described in sections 2.2.3 and 2.2.4. The Du151 amplicons were purified using a High Pure PCR Product Purification Kit (Roche, GmbH, Germany) according to the manufacturer's specifications. Briefly, 500µl binding buffer containing chaotropic salt guanidine thiocyanate, was mixed with 90µl PCR product and the mixture applied to the High Pure filter tube. This procedure allows the binding of DNA to special glass fibres pre-packed in the filter tube. The tube was centrifuged at maximum speed for 60 sec in a standard bench top centrifuge before discarding the filtrate. Wash buffer (500µl) was loaded to the top of the filter and the column centrifuged as before. A further 200µl of wash buffer was used to clean the DNA a second time, before eluting the DNA with 90µl of supplied elution buffer. The eluted DNA was again checked by

electrophoresis in a 1% agarose gel, and using the molecular weight marker, a visual estimate of the amount of clean DNA needed for subsequent cloning was done.

3.3.2 Probe cloning and transformation

The V1/V2 PCR product from section 3.3.1 was blunt end cloned into a linearised pMOS vector using the pMOS*Blue* blunt ended cloning kit (Appendix E; Amersham Pharmacia Biotech, UK). Successful cloning of the V1/V2 region PCR products depends on the interruption of the coding sequence of β-galactosidase (Appendix E). Within this region, there are multiple cloning sites, and insertion inactivation allowed recombinant clones to be directly identified by blue/white colony screening on the 5-Bromo-4-chloro-3-indolyl-β-D galactoside (X-gal) (Roche, GmbH, Germany) / isopropylthio-β-D-galactopyranoside (IPTG) (Roche, GmbH, Germany) indicator plates.

The pk reaction was undertaken to prepare the insert for ligation before transformation. This reaction systematically removes any overhanging nucleotides by the action of kinases on double stranded DNA, until the amplicon remains blunt ended. The pk reaction was carried out with 1µl of 10 X pk buffer, 0.5µl of 100mM dithiothreitol (DTT), 1µl of pk enzyme mix and 10µl of purified PCR product. The quantity of DNA used in this reaction was estimated from the 10µl loaded on the 1% agarose gel after the cleaning process (section 3.3.1). The optimum amount of insert required to obtain a molar vector: insert ratio of 1:2.5, using 50ng of vector, i.e. 125ng, was determined to be 8µl. The pk reaction was made up to 12µl with supplied water. Efficiency of the system was tested with 2µl of the provided control insert. The reaction was incubated at 22°C for 40 min before centrifuging. Kinase inactivation was achieved by incubating at 75°C for 10 min followed by a short incubation on wet

ice. Efficient ligation was achieved with 50ng of linearised pMOS*Blue* vector (50ng/µl) and a reaction containing 10µl of the pk reaction and 1µl T4 DNA ligase (4 Weiss units). Transformation was done overnight at 22°C. The supplied pMOS competent cells (20µl) were transformed with 1µl of ligation mix. A positive control to test the efficiency of the pMOS*Blue* competent cells was also tested using 0.2ng of the provided test plasmid and 20µl of the cells. The reaction tubes were placed on ice for 30 min, before being heat shocked for exactly 40 sec in a 42°C water bath. The mixture was again cooled on wet ice for two min, before adding 80µl of SOC medium. The reaction tubes were further incubated in a 37°C shaker at 225 rpm (3.4 xg) for one hour.

X-gal / IPTG Luria Bertani (LB) (GIBCO BRL, Life Technologies, Scotland) agar plates were prepared by spreading 1.75mg/ml X-gal and 0.33mg/ml IPTG onto LB agar plates containing 50µg/ml ampicillin and 15µg/ml tetracycline (Roche, GmbH, Germany). Fifty microlitres of each transformation mixture was spread onto plates before being incubated overnight at 37°C.

3.3.3 Identification of recombinant clones

Twenty white recombinant colonies were picked at random and each inoculated into 100µl of LB broth containing 50µg/ml ampicillin and 15µg/ml tetracycline. The mixture was incubated at 37°C for three hours with shaking at 225 rpm (3.4 xg). To determine which clones had inserts, the second round V1/V2 PCR was done. In the PCR test, 2µl of the cell culture was added instead of DNA, and tested using the second round parameters with primers A589/A590 (section 2.2.3). An initial step of incubation at 92°C for two minutes was also included to rupture the bacterial cell walls. The presence of the ~320bp fragment was visualised by

electrophoresis on a 1% agarose gel. PCR recombinant clones testing positive were expanded into 30mls of LB broth with the appropriate antibiotics and incubated overnight in a 37°C shaker at 225 rpm (3.4 xg).

3.3.4 Isolation of recombinant plasmids

Bacterial stocks were made by adding 300µl sterile glycerol to 700µl of cultured bacterial cells and stored at –70°C. To recover recombinant plasmids, bacterial cells were harvested from 5ml of the 30ml overnight cultures by centrifugation at 6,000 rpm (2400 xg) for 15 min and plasmids extracted with the ABI Prism Miniprep Kit (Applied Biosystems, USA). The plasmid isolation procedure followed that of the manufacturer. Briefly, the bacterial culture was re-suspended and lysed before the addition of ice-cold precipitation salt. Genomic DNA, proteins and cell debris were separated by centrifugation at 14,000 rpm (13100 xg) for 5 min before the addition of binding buffer to the extracted supernatant fluid containing the plasmid DNA. This was then poured onto the Miniprep column containing an anion-exchange resin and the apparatus centrifuged at 3,500 rpm (820 xg) for 30 seconds. Wash buffer was used to clean away the salts present from the precipitation, and a final wash ensured all other contaminants were removed. Plasmid elution was done using 60µl of TE buffer. Isolated plasmid DNA was visualised on a 1% agarose gel to determine and estimate the amount of DNA.

3.3.5 Restriction enzyme digestion and sequencing of recombinant plasmids

Recombinant plasmids were characterized to determine the size of the cloned insert. Double digest restriction enzyme analysis was carried out on 8µl of isolated plasmid, 2µl of buffer K, 10U each of the *Spe*I and *Bam*HI (Roche, GmbH,

Germany), and 8µl sterile water. The reaction was incubated overnight at 37°C in a water bath. A standard 1% agarose gel was used to verify if digestion of the recombinant plasmids had taken place.

The recombinant plasmids were also sequenced to determine the orientation of the insert and the exact number of nucleotides. A total of six clones were selected and 1µl plasmid DNA was added to each sequencing reaction mix alongside the other sequencing components mentioned in section 2.2.7. Sequencing was done in the ABI 3100 automated sequencer as described earlier. The entire V1/V2 region was targeted together with the adjacent 300 nucleotides on either side of the insert. In total approx. 1050bp of the insert and vector were sequenced containing enough information for analysis (Appendix F).

3.3.6 Radioactive labelling of Du151 probe with ^{35}S [ATP]

Prior to labelling, the V1/V2 region was scanned for potential restriction enzyme digestion sites using the Sequencher 3.1 program. The cutting sites for *Nde*I and *Kpn*I were not found on the Du151 V1/V2 region, thus any subsequent enzyme digestions would only cut the vector in the multiple cloning site. Digestion of the recombinant plasmid with *Nde*I enabled it to be linearised close to the cloned V1/V2 fragment. This reaction contained 10µg of eluted plasmid DNA from section 3.3.4, 14µl of sterile H_2O, 3µl of Buffer K, 0.3µl of BSA and 10U of *Nde*I (Roche, GmbH, Germany). Incubation was carried out at 37°C for three hours before using Klenow (Amersham Pharmacia Biotech, England) to add the adenosine and threonine to the 3' end of the digested fragment (Drlica *et al.*, 1980; O'Farrell *et al.*, 1980).

The adenosine is radiolabelled with ^{35}S and thus would allow the DNA strand to which it's being incorporated into to become radiolabelled for further analysis. This

reaction was completed with the addition of 1.5µl of dTTP (1mM), 2µl of ^{35}S [ATP], 0.3µl of DTT and 10U of Klenow (Amersham Pharmacia Biotech, England). The reaction was allowed to sit at room temperature for 15 min, before denaturing all enzymes at 70°C for 10 min.

The radiolabelled V1/V2 fragment was then released from the vector by a second digestion with 10U of *Kpn*I, and incubation at 37°C for two hours (Roche, GmbH, Germany). The "hot" V1/V2 fragment was then cleaned to remove all impurities with a High Pure PCR Product Purification Kit as in section 2.2.7. with the final product being eluted in 100µl of the recommended elution buffer.

3.3.7 Sample preparation

The V1/V2 HTA validation was done using V1/V2 PCR samples from recombinant gp160 plasmid DNA from Du151, Du179, CM9, TM2, TM9, TM12 (obtained from Dr. Maria Papathanasopoulos) and HXB2 (NIH AIDS Research and Reference Reagent Program, Bethesda, MD). The V1/V2 PCR products from the three isolates used in this study (TM6b, TM22b and HIVNET 536-10-7) were also included on the same gel for comparative purposes. Subsequent samples for HTA analysis were prepared from the pooled supernatant obtained from the antibody neutralization experiments (described in sections 3.2.2 and 3.2.3), and the RNA and/or DNA used in amplification, according to sections 2.2.2 and 2.2.3.

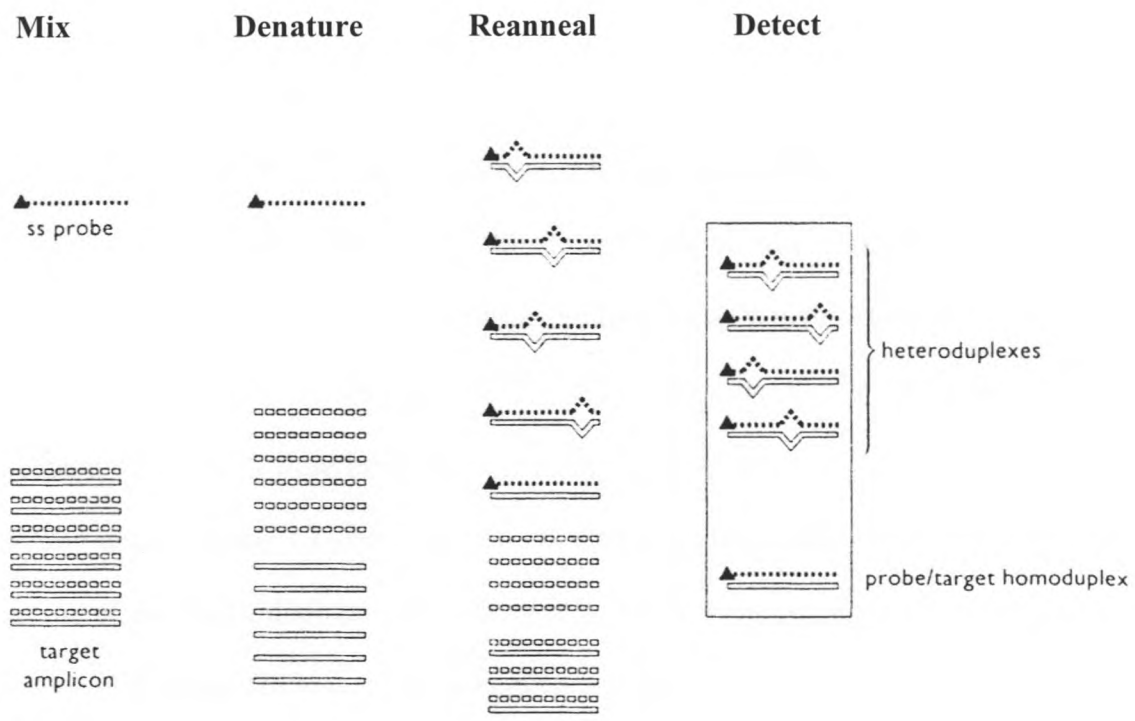
3.3.8 Heteroduplex Tracking Assay

Heteroduplex formation reactions contained the following in a master pre-mix: 1µl of 10 X annealing buffer, 2µl of HTA loading buffer (0.25% bromophenol blue,

0.25% xylene-xyanol, 15% Ficoll) in 1 X TBE, 1µl of radiolabelled probe (in excess) and 0.8µl of undiluted stock primer mix: 54pmol/µl A589 and 82pmol/µl A590 per reaction. This allowed the resolution of the single stranded labelled probes for use during analysis. The premix was aliquoted into thin walled PCR reaction tubes before the addition of 8µl of PCR product. A control reaction without any sample PCR product, i.e. probe alone was always included for each reaction. A schematic representation of the HTA method is shown in Figure 3.1.

The mixtures were heat denatured at 94°C for 2 min in a GeneAmp® PCR system 9700 thermocycler and the DNA mixture re-annealed by slowly allowing the cooling of the mixture to room temperature (~5min) without the use of wet ice. Resolution was on a 6% polyacrylamide gel measuring 160 X 160 X 1.5mm (protein II xi cell; Bio-rad, Hercules, CA; Figure 3.1). All apparatus are carefully washed with acetone and ethanol prior to assembling. Components for the 6% polyacrylamide gel included: 5ml of polyacrylamide (37:1 acrylamide:bisacrylamide) (Roche, GmbH, Germany), 17.5ml sterile H_2O, 2.5 ml 10 X TBE, 125µl of 10% fresh ammonium persulphate (Sigma, Germany) and 19µl TEMED (Sigma, Germany).

The gel was electrophoresed at 17mA, for approximately five or 14 hours. The radioactive gel was dried on Whatmann Chromatography filter paper (3mm Chr, England) in a Biorad gel dryer for two hours.



Reviewed by Barlow *et al.*, (2000)

Figure 3.1: Schematic representation of an HTA. A labelled single stranded (ss) DNA probe is mixed with an excess of double strand (ds) PCR amplicons (driver). The mixture is heat denatured and on cooling, heteroduplexes are formed between the ssDNA probe and its complementary strand from the driver. Complementary single strands from the test amplicon also re-anneal to form homoduplexes. Following gel electrophoresis and visualisation, only those molecules having a labelled strand will be visible. The fastest migrating band is the homoduplex, leaving the heteroduplexes to migrate more slowly at rates, which indicate the extent of diversity in the quasispecies. Unhybridised single strands will migrate more slowly.

3.3.9 HTA negative film development

One-sided Biomax MR X-ray film measuring 18 X 24 cm (Kodak, Eastman New York), was placed in direct contact with the dried gel and sandwiched in an X-ray cassette for a period of 60 hours before developing the image. The X-ray was developed in Kodak GBX Developer (diluted to the recommended amount of 103ml in 370ml distilled water), for five minutes before washing the film in distilled water for one minute. The negative film was fixed by soaking in Kodak GBX Fixer (diluted to the recommended amount of 103ml in 370ml distilled water), for five minutes before it was finally washed under running water for one minute, dried and viewed.

3.4 RESULTS

3.4.1 V1/V2 probe preparation

The isolate Du151 was chosen as a heterologous probe for the V1/V2 HTA because it is an HIV-1 subtype C, R5 virus, whose envelope sequence is the most representative of the viruses circulating in South Africa (Williamson *et al.*, 2003). The V1/V2 region of Du151 was successfully cloned into pMOS*Blue*, and a total of six recombinant clones were investigated for the presence and correct orientation of the 320bp insert. Figure 3.2 shows that all six clones contained the insert. However, one of the clones had a double insert indicated by the ~640bp amplicon (Figure 3.2; lane 4). Further investigations on this clone were terminated.

Sequence information established the orientation of the insert in all the five selected recombinant clones. Clone A sequence is shown in Appendix F. This clone was successfully labelled and used as the probe in all subsequent HTA experiments.

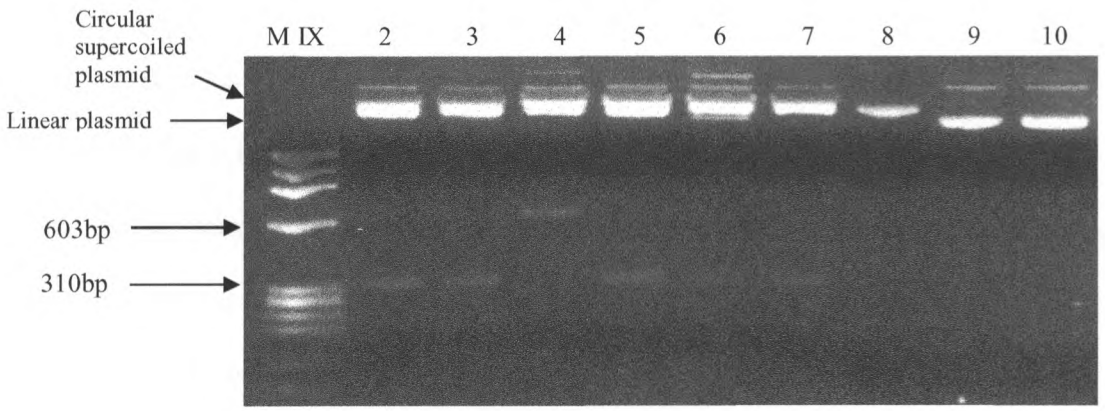


Figure 3.2: Selection of V1/V2 clones for probe preparation. Six clones of Du151 V1/V2 in pMOS*Blue* were digested with *Spe* 1 and *Bam* H1. Lane M IX is Molecular weight marker IX (Roche, GmbH, Germany) and shown on left in bp. Lanes 2,3,5,6 and 7 all contain the target 320bp V1/V2 region, whilst lane 4 contains a 640bp fragment possibly from two inserts ligated before incorporation into the vector. Lane 8 is undigested control vector, lane 9 and 10 contain, undigested recombinant plasmid.

3.4.2 Validation of HTA

The V1/V2 HTA was first validated using recombinant gp160 plasmid DNA from seven isolates (section 3.3.7). These included six subtype C clones (one of which was Du151) and one subtype B. The test PCR amplicons were mixed with an excess of probe and denatured at 92°C. After cooling to room temperature for five minutes, the mixture was run on a 6% HTA polyacrylamide gel for 5½ hours. The results are shown in Figure 3.3. The profile shows that the V1/V2 amplicons migrated as homoduplexes and heteroduplexes, the latter which migrated at different rates, confirming that they were genetically different. The two bands (homoduplex and heteroduplex) present in the profiles of the cloned isolates also confirm that only a single variant is present. Two bands are also observed in the probe lane, one respresents the homduplex and the other a heteroduplex. Both have been radiolabelled. This was in contrast to the profiles seen for the viral isolates HIVNET 536-10-7 represented in lane 11, TM6b in lanes 12 and TM22b in lane 13. These isolates are not clonal and the multiple bands suggest that more than one variant/quasispecies is present within the sample (see Figure 3.3).

The amplicons from viruses TM2, TM9, TM12, HIVNET 536-10-7, TM6b and TM22b migrated above the single stranded conformations of the probe. This slow migration warranted the need to extend the running conditions to allow for better resolution. Fourteen hours was found to be the optimal running time. The HXB2 (subtype B) band in particular runs very slowly in relation to the other plasmids that are envelope subtype C viruses. This may be caused by either the large insertion at the amino terminus of the V1 region of HXB2, or the heterologous probe that was from a subtype C isolate. There was no heteroduplex band in the Du151 lane, as expected given that the probe is from the same isolate.

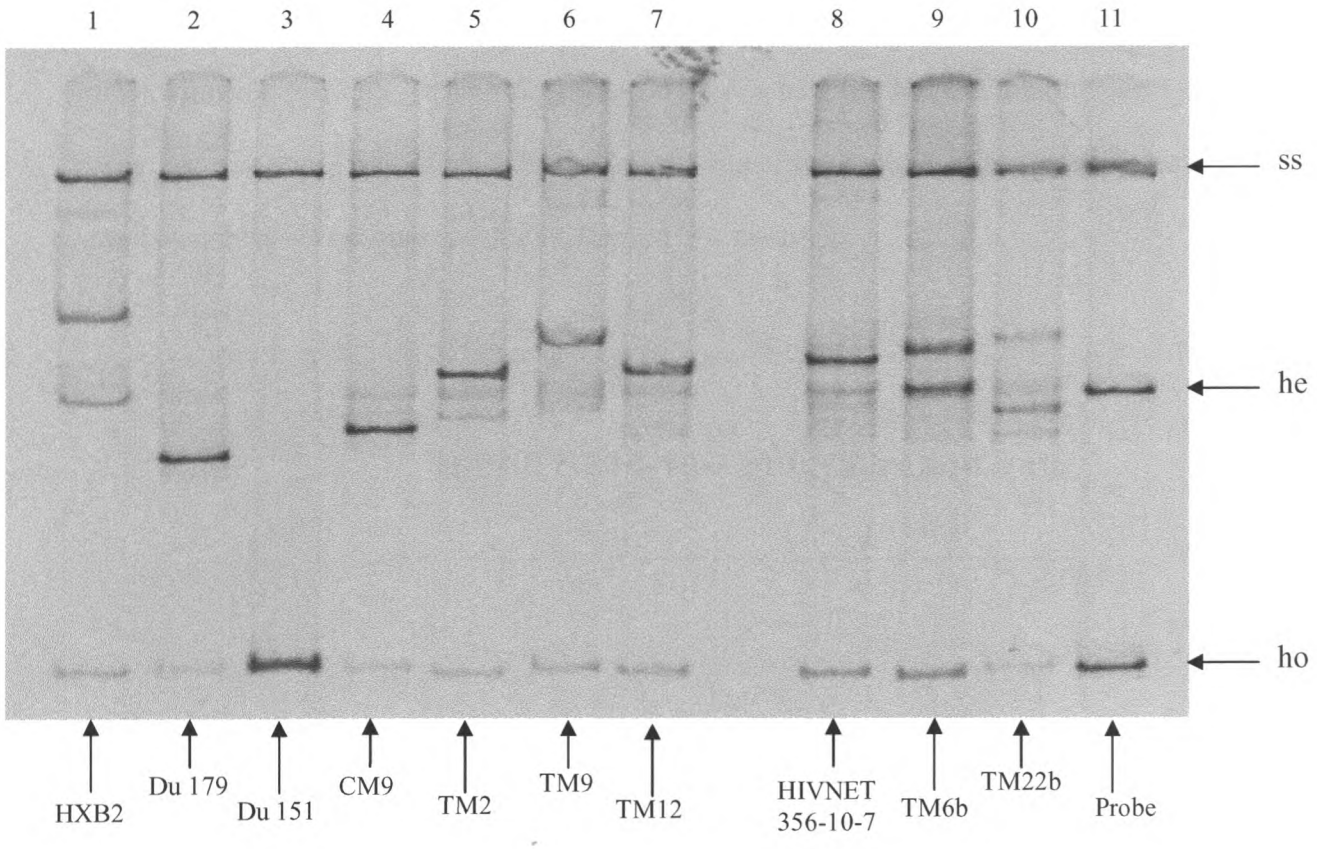


Figure 3.3: HTA profiles from clones and isolates used in this study. SS = single stranded; he = heteroduplex; ho = homoduplex. The recombinant gp160 plasmid lanes contain only one quasispecies and thus have only two bands. One from the sample annealing to itself (homoduplexes) and the other from samples annealing to the probe (heteroduplexes). Lane 3 is of particular importance because it shows a control lane containing plasmid from another cloned Du151 against the labelled probe Du151. This lane shows one band (homoduplex) in high quantity because all the heteroduplexes have been driven to complex with homoduplexes.

3.4.3 Neutralization and HTA of HIVNET 536-10-7 isolate

PBMC neutralization assays were set up using the HIVNET 536-10-7 viral isolate and autologous serum samples. Virus growth was measured between days 3-6 in the virus control (VC) wells (data not shown). Exponential virus replication was observed 4 days after infection (in the VC wells) and this day was chosen as the most suitable for further analysis (See Figure 3.4). In the presence of autologous serum there was significant p24 antigen production inhibition indicating good autologous

neutralizing activity. Figure 3.4 shows that at serum dilutions 1:32 and 1:108 there was significant inhibition of p24 antigen that exceeded the ID_{80} (dilution that inhibits 80% viral replication). There was a good dose response in that the inhibitory effect was lost as serum was diluted out to 1:324. Supernatants and cells from triplicate wells were harvested on day 4 for PCR and HTA analysis.

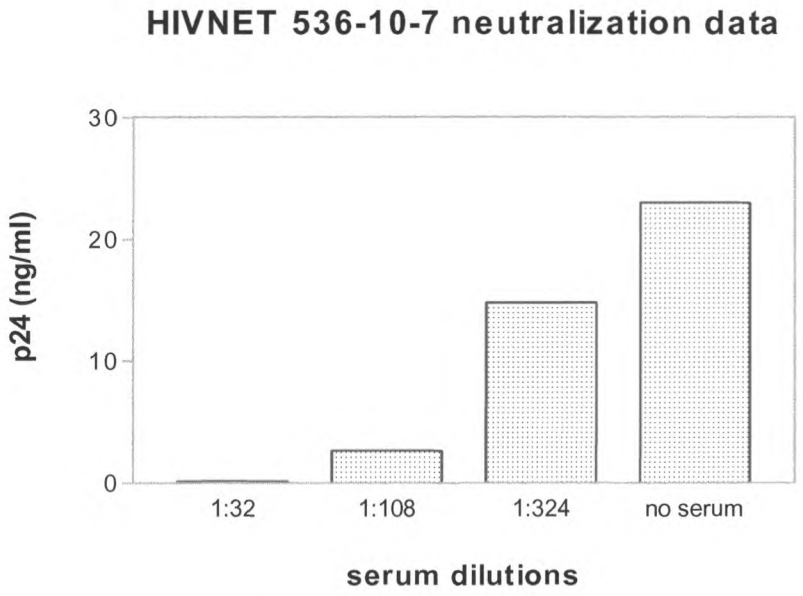


Figure 3.4: HIVNET 536-10-7 antibody neutralization assay. Day 4 p24 Ag production is shown against increasing serum dilutions. Good neutralization is observed in the virus/serum wells compared to the virus control with no serum.

Both DNA and RNA were successfully extracted from pooled wells as mentioned in section 2.2.3. A V1/V2 PCR was performed on both RNA and DNA samples, and samples run on a 1% agarose gel (Figure 3.5). Different quantities of amplicons can be seen in this picture, reflecting different levels of p24 Ag production and thus viral RNA/DNA copies (Figure 3.5). The gel also illustrates that no differences were observed with either DNA or RNA preparations.

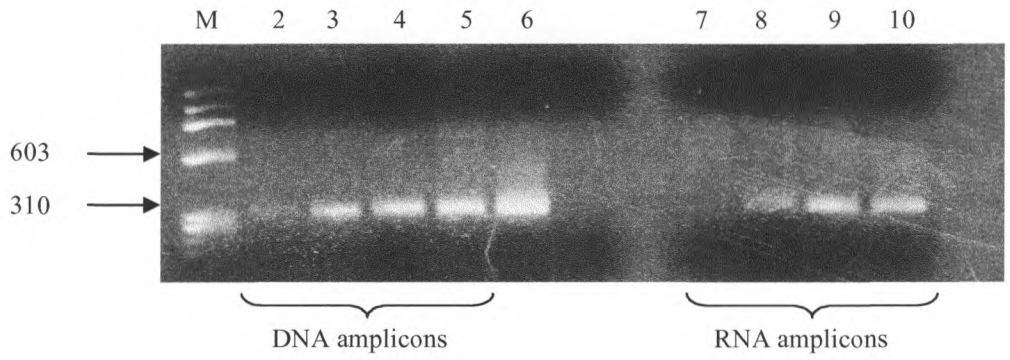


Figure 3.5: V1/V2 PCR amplicons obtained after antibody neutralization of HIVNET 536-10-7 samples. Lane 1 is molecular weight marker IX (shown in bp on the left), Lanes 2 and 7: 1:32 serum dilution, Lanes 3 and 8: 1:108 serum dilution, Lane 4 and 9:1:324 serum dilution, Lane 5 and 10:VC, Lane 6 is a positive control with ~100ng of DNA.

A V1/V2 HTA was performed for both the DNA and RNA samples from all serum dilutions, and the gel was electrophoresed for 14 hours. This extended time allowed sufficient separation of distinct bands in the mixture. The HTA photograph for both the DNA and RNA samples is shown in Figure 3.6. Neutralization resistant bands were present at high serum concentrations (1:32) in both the RNA and DNA extractions, whilst neutralization sensitive bands were visible only in more dilute serum concentrations (1:108 and 1:324). These neutralization resistant bands were also present in the VC, where there was a complete absence of serum. All bands were expected to be evident in this lane. The slight shift in bands can be seen in the different lanes, alongside the probe lane that has two distinct bands. These bands in the probe lane represent the labelled strands. This probe was from a different batch of Du151 and is an indication that both strands were labelled. This could have arisen from the presence of the Klenow enzyme which was not denatured fully after incubation at 70°C. As a result, Klenow incorporated radiolabelled ^{35}S [ATP] into the overhang created by *NdeI* during the release of the insert from the vector. Results could not be repeated due to limited sample, nonetheless, the data are still interpretable, and formed the basis for further optimisation of the V1/V2 HTA.

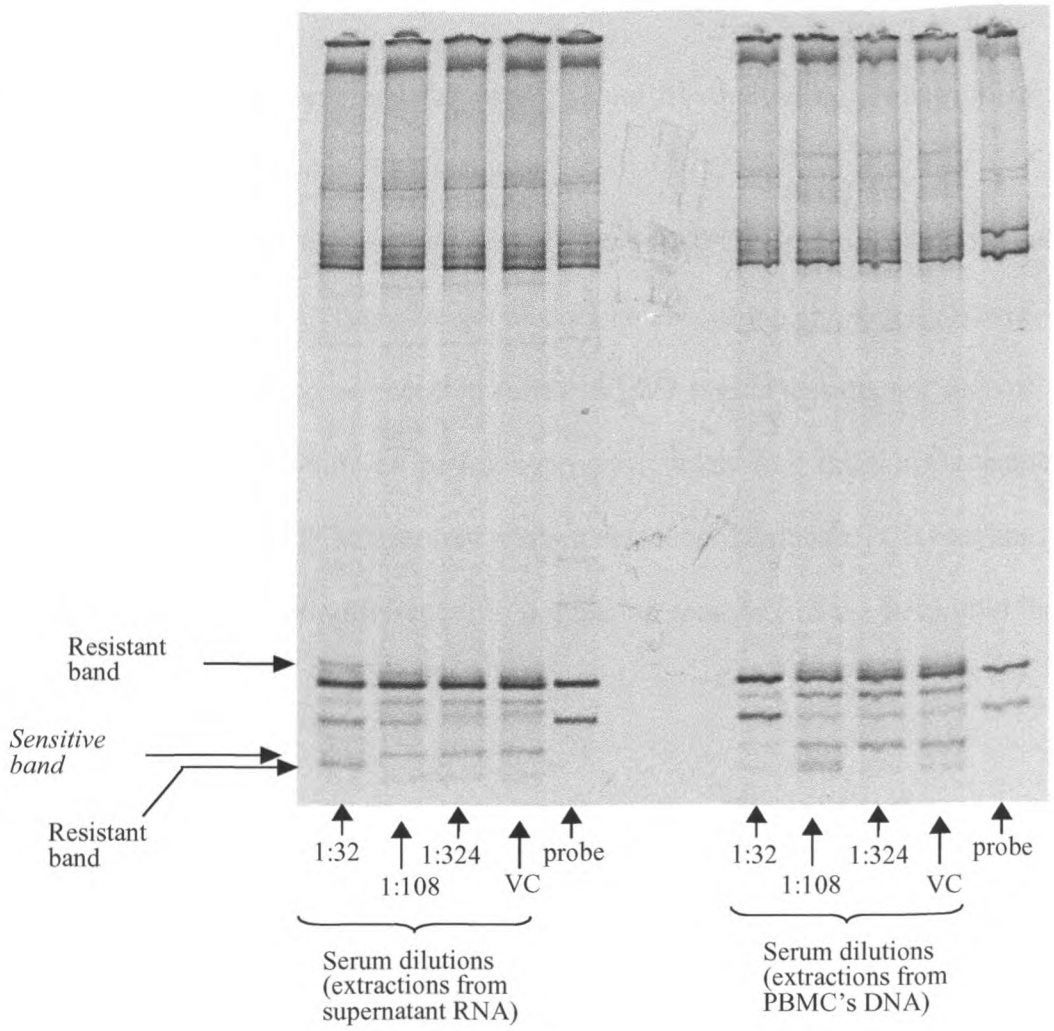


Figure 3.6: HIVNET 536-10-7 HTA profile after antibody neutralization assay. RNA and DNA lanes run alongside each other. No differences are observed between the two sets. Neutralisation resistant and sensitive bands are evident in the different serum lanes. The double lane in the probe lane indicates that both strands are labelled.

3.4.4 Neutralization and HTA of TM6b isolate

Autologous neutralization for TM6b is shown in Figure 3.7. There was good virus growth in the absence of serum that peaked on day 5½. This day was used to determine the extent of antibody neutralization. There was complete inhibition of virus replication at the lowest serum dilution of 1:4. As the serum was diluted virus replication increased except for the 1:972 dilution where there was unexplained inhibition. This may have been due to technical reasons, for example one of the

triplicate wells may have had the wrong serum dilution added. However, all wells were extracted and used in HTA analysis.

The RNA was successfully extracted and RT-PCRs performed on the samples as in section 2.2.3. A 1% agarose gel electrophoresis of the amplicons showed bands of equal size of ~320bp as expected for the V1/V2 region (results not shown). This included the sample where no p24 antigen was evident (1:4 dilution) indicating the superior sensitivity of PCR over p24 antigen detection. Duplicate PCR reactions were performed to investigate differences in population sampling of the RNA into the RT-PCR reactions.

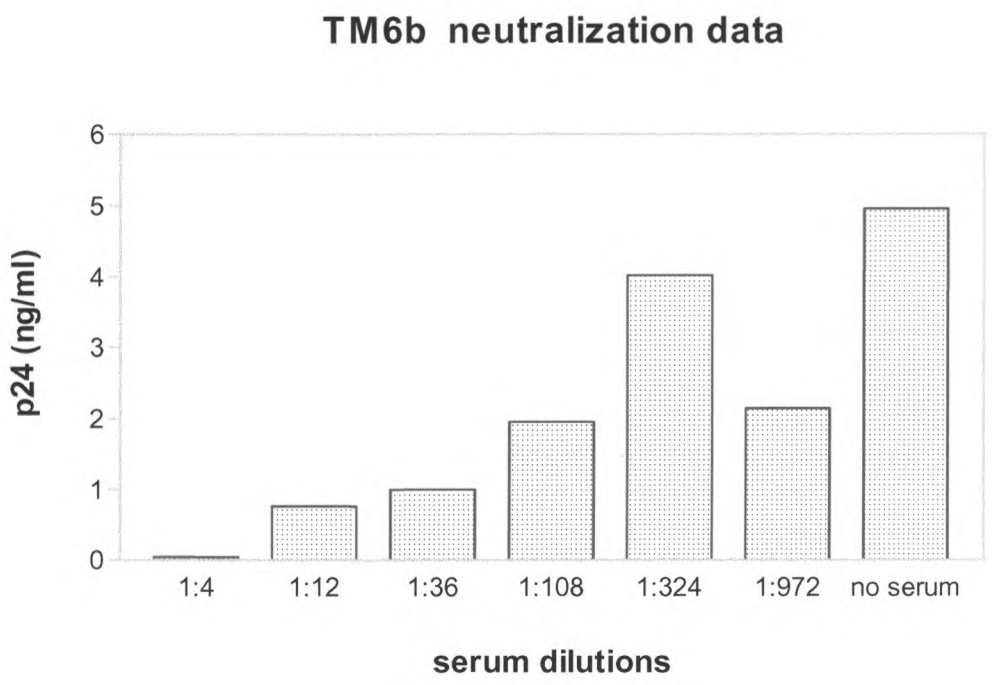


Figure 3.7: TM6b antibody neutralization assay. p24 Ag production on day 5½ at different serum dilutions. There was good virus growth in the absence of serum. In the presence of serum, virus replication was inhibited.

Successful HTA analysis was performed using V1/V2 PCR amplicons and 14-hour polyacrylamide gel electrophoresis (Figure 3.8). No differences were observed between the duplicate PCR reactions. This suggests that all PCR reactions had equal

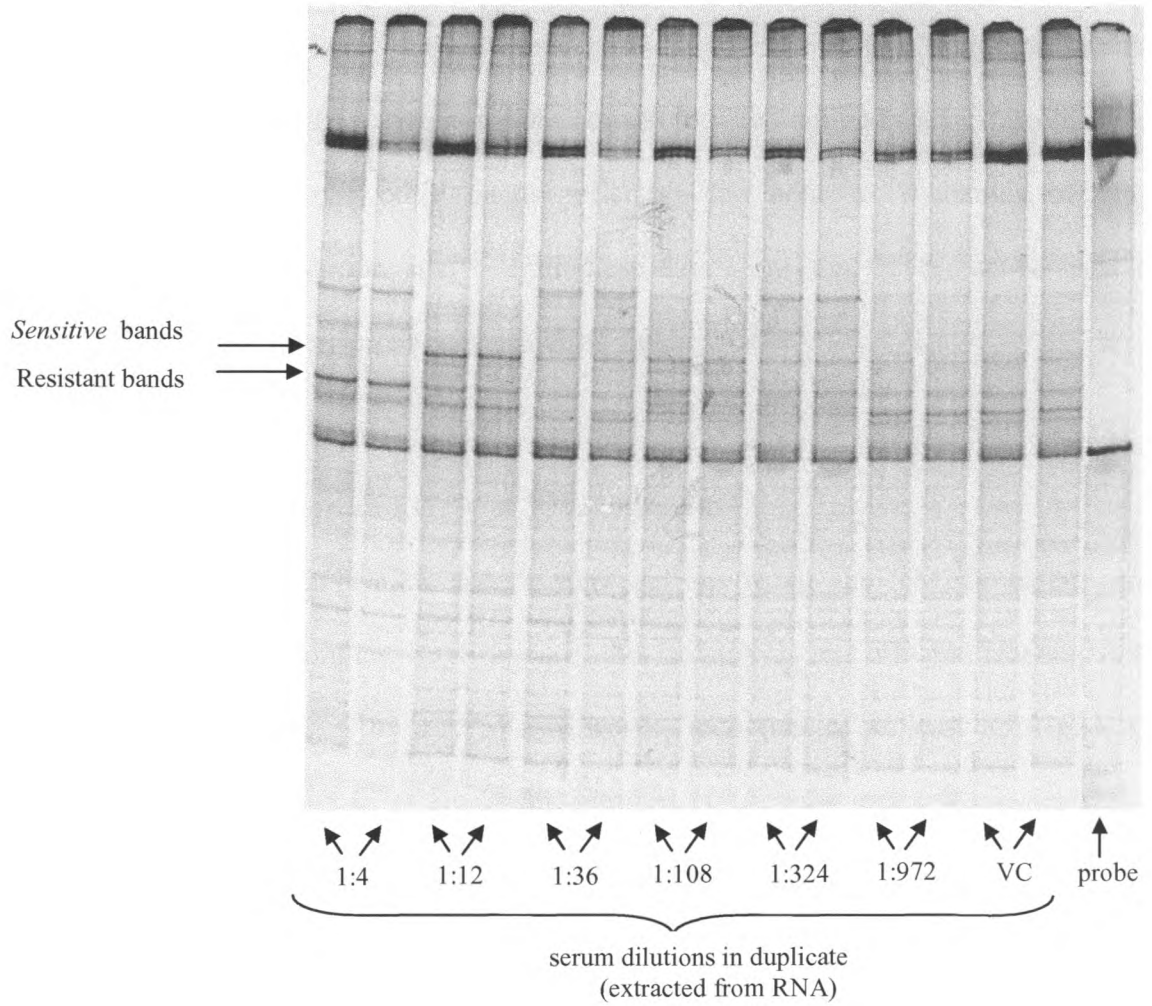


Figure 3.8: TM6b HTA profile after antibody neutralization assay. Neutralization sensitive and resistant quasispecies are shown in duplicate. At least one distinct band was noted to disappear in the high serum lanes (Lane 1 and 2) indicating antibody neutralization sensitive quasispecies, whilst another band was present in the third and fourth lane but disappeared in subsequent lanes (antibody neutralization resistant quasispecies).

proportions of Variant/quasispecies, and a reflection of correct population sampling in RT-PCR. This indicates that any differences observed are due to the impact of neutralization rather than possible PCR sampling errors. As seen in Figure 3.8, the HTA profiles of viruses neutralized with different dilutions of autologous serum were not identical. Some bands were absent in the 1:4 dilutions but were present in all other dilutions including the VC wells. This suggests that this variant is sensitive to antibody neutralization at high concentrations only. Similarly a prominent band in the

1:4 dilution disappeared with decreasing levels of serum. This suggests that the neutralization resistant variants are out-competed when antibody levels are limiting. There were numerous other bands reflecting the complex dynamics of virus replication and inhibition, and overall these data show that HTA can be used to investigate these interactions between virus and host cells.

3.4.5 Neutralization and HTA of TM22b isolate

PBMC neutralization assays were set up using the TM22b isolate and autologous serum samples. Replication of this isolate was less efficient reaching only 2.4 ng of p24 antigen by day 5½. However, reduction of at least 80% p24 Ag production was achieved at all serum dilutions (1:10, 1:30 and 1:90) indicative of good neutralization (Figure 3.9). Day 5½ was subsequently used for HTA analysis.

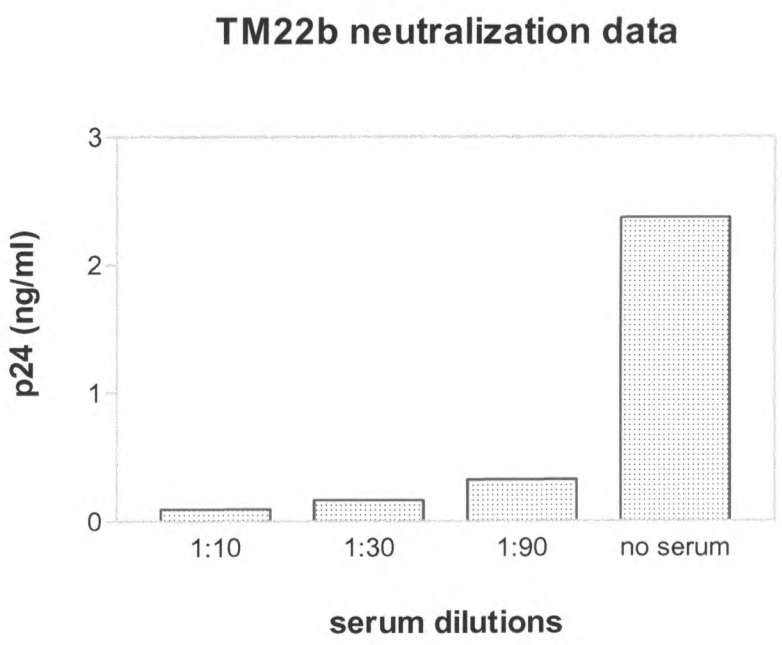


Figure 3.9: TM22b antibody neutralization assay. These results represent pooled day 5½ p24 Ag production against serum dilutions. The rise in virus growth with diminishing serum concentrations suggests good neutralizing activity with autologous serum.

extracted. V1/V2 RT-PCR was performed on all the samples, and in duplicate for the

1:10 and VC cultures. This was to investigate possible discrepancies due to sampling errors of the input RNA. A 320bp PCR amplicon was visualised on a 1% agarose gel (results not shown). No difference was however noted in band intensity. Electrophoresis was done on a 6% polyacrylamide gel for 14 hours and the HTA is shown in Figure 3.10. HTA analysis suggests that bands from all serum dilutions were identical, i.e. that no V1/V2 antibody neutralization sensitive bands were observed in this isolate.

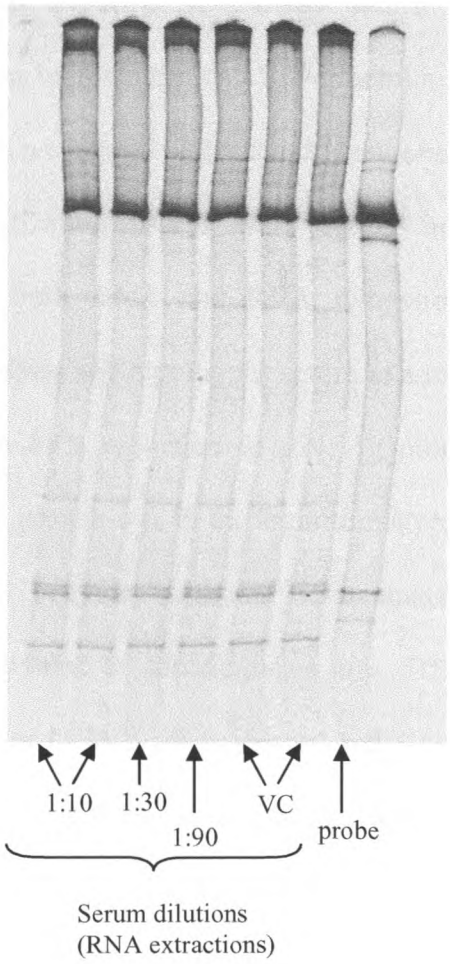


Figure 3.10: TM22b HTA profile after antibody neutralization assay. The probe lane shows the different forms of radioactively labelled fragments. Identical patterns were seen at all serum dilutions, similar to those seen in the VC, suggesting no neutralization sensitive quasispecies in the V1/V2 region.

3.5 DISCUSSION

In this project we have developed a sensitive HTA for exploring genetic diversity in the V1/V2 region of *env* and applied this to the study of antibody-mediated neutralization. This HTA allowed the quantity of quasispecies to be viewed in a rapid and inexpensive investigation with high sensitivity. Three isolates were selected for this study. Two of the isolates were chosen based on patterns seen in the V1/V2 HMA profiles (Chapter two). A third isolate was chosen because previous experiments showed that it had good neutralizing antibody titres (N. Taylor, unpublished data). We therefore biased our selection to favour isolates likely to have extensive genetic diversity in V1/V2.

PBMC-based neutralization assays were set-up in triplicate to provide sufficient material for both DNA and RNA extractions. Experiments were performed with virus isolates and autologous serum at a range of dilutions, which would allow at least an 80% reduction in p24 Ag production. Cultures were harvested when the virus growth was in the logarithmic phase in order to quantify virus neutralization and provide sufficient viral material for analysis. The triplicate samples were pooled to accommodate any differences between wells. The harvested RNA and/or DNA were processed and V1/V2 amplicons obtained following a nested PCR. These were run on a 6% polyacrylamide HTA gel using a radiolabelled heterologous probe from Du151, which is an R5 virus. Interpretation of the features on the HTA profiles suggest that mismatches and possible length differences affects the migration of the bands. The differing migration rates thereby correspond to differing genetic compositions of the neutralized and/or un-neutralized virus quasispecies.

The HTA was first validated using PCR amplicons from cloned envelope genes of different isolates. The polyacrylamide gel was run for 5½ hours, which allowed visualization of the whole frame and demarcated the migration patterns of homoduplexes and heteroduplexes (Figure 3.3). Using this protocol it was clear that PCR amplicons from recombinant plasmids contained one species, whilst the TM and HIVNET 536-10-7 isolates contained numerous quasispecies. Subsequent HTA gels were run for 14 hours. This allowed the less important homoduplexes to run off, whilst the heteroduplexes representing quasispecies were better separated thus allowing clearer identification. During the course of these studies it became evident that in some cases the probe gave 2 heteroduplex bands when only one was expected. This was due to both strands of the probe being radio-labelled as a result of incomplete inactivation of the Klenow enzyme during the labelling process. Subsequently the enzyme was heat-inactivated for longer and cleaned by filtering before use. This did not however compromise our results but did render interpretation slightly more difficult in one case (see Figure 3.6). HTA profiles were remarkably consistent between RNA (supernatant) and DNA (PBMC) suggesting that antibody neutralization is at the initial infection (entry) stage rather than the post-integration stage. Duplicate PCR reactions also gave identical HTA profiles suggesting that this assay is extremely robust.

All isolates were effectively neutralized by their respective autologous sera. However there was differential neutralization of the different quasispecies by these sera; thus some quasispecies which were initially absent in high serum concentrations were present in diluted sera suggesting that they were neutralization *sensitive,* whilst others which were present in high serum concentrations were neutralization *resistant.* Some of these resistant bands

subsequently disappeared as serum was diluted suggesting that they were out competed by neutralization sensitive variants. The TM6b and HIVNET 536-10-7 isolates both had V1/V2 antibody neutralization sensitive and resistant bands, whilst TM22b had no differences in the V1/V2 quasispecies following antibody neutralization. For TM22b, almost all viruses were neutralized but yet PCR products were amplified from RNA, suggesting that Nab determinants could be mapped to the V3 region or elsewhere in this particular isolate, or the patient did not make antibodies towards V1/V2. The possibility of technical difficulties including insufficient washing of the cells to remove input virus cannot also be ruled out. This residual non-neutralized virus may have been the source of the identical bands seen in this HTA profile (Figure 3.10). It would be important to repeat this experiment to determine if the V1/V2 region of this isolate is not a target of autologous antibodies.

This is the first study on a V1/V2 HTA on subtype C isolates where quasispecies can be tracked with an easy, cheap and standardizable technique. Interpretations of our results match those of Delwart and co-workers who showed that HTA could identify variants at 1:500 (Delwart and Gordon, 1997). We have thus identified quasispecies with possible single and/or multiple nucleotide substitutions at high sensitivity. Future work that will focus on sequencing of the individual bands that represent neutralization sensitive and resistant quasispecies will help define genetic determinants of antibody neutralization sensitivity. Such information could provide important clues for the future design of an *env* immunogen exposing neutralization sensitive V1/V2 epitopes.

Studies by other workers have shown antibody neutralization epitopes in V1/V2 and that genetic changes in this region affect neutralization sensitivity. For

both SIV and HIV, alterations in N-linked glycosylation sites in both V1 and V2 were shown to be associated with increased neutralization resistance (Cheng-Mayer *et al.*, 1999; Rudensey, *et al.,* 1998; Ly and Stamatatos, 2000). Fung and others demonstrated that amino acid residues 169 to 183 of the HIV-1 IIIB V2 region encompassed a neutralization determinant, which was recognized by antibodies in both a conformation dependant and independent manner (Fung *et al.*, 1992). Moore and co-workers also showed that linear sequences from the V2 region are immunogenic in HIV-1-infected individuals, suggesting that the primary neutralizing antibody response may be directed to both the V2 and V3 epitopes (Moore *et al.*, 1993). Sequential mutations introduced into HXB2 affecting the length, charge and number of potential N-linked glycosylation sites were investigated by Fox *et al.,* (1997) and results indicated that all insertions affected the sensitivity of the virus to neutralization by soluble CD4 and monoclonal antibodies specific for epitopes in the V3 and CD4bs regions (Fox *et al.*, 1997). Whether the resistant and/or sensitive V1/V2 quasispecies identified in this study will have similar changes as described by others remains to be explored.

Overall, this project suggests that the V1/V2 region is involved in antibody neutralization sensitivity of HIV-1 subtype C isolates. HTA has allowed us to investigate the V1/V2 changes occurring at the nucleotide level, and have tracked sensitivity of the isolates with differing autologous serum dilutions. Although these investigations are preliminary, they strongly suggest that changes in the V1/V2 region could be accommodating the evolution of neutralizing antibody epitopes in order to protect more important and conserved target sites. It is likely that one or more of the above events influencing the V1/V2 genetic environment could cause antibody resistant quasispecies to arise and thus be detected in the

HTA profiles. The V1/V2 HTA method described here coupled with sequencing of different quasispecies will provide a powerful tool that is cheap and standardizable to identify the exact epitopes involved in antibody neutralization sensitivity.

CHAPTER FOUR

GENERAL DISCUSSIONS AND CONCLUSIONS

The studies presented here add to the growing body of scientific knowledge on HIV-1 and the impact of neutralizing antibodies on quasispecies diversity. For this, a novel V1/V2 HMA assay was established and its performance was compared to the more widely used C2-V5 HMA. Despite unsatisfactory subtyping results from the V1/V2 HMA, the assay illustrated its usefulness in monitoring genetic variability and this in turn led to the establishment of a more sensitive V1/V2 HTA. With this assay, we demonstrated that viral populations contain mixtures of variants, some of which were resistant or sensitive to antibody neutralization.

Studies on LTNPs, including children with immature immune systems are of interest as they may provide insights and opportunities for HIV-1 vaccine design. Such individuals regulate HIV replication in the absence of anti-retroviral therapy through strong immune responses, including neutralizing antibodies. The selective advantage of being able to escape antibody pressure will favour outgrowth of certain variants, which will become the dominant quasispecies. In time, these too will become the targets for neutralizing antibodies and will be suppressed. This dynamic system results in a large turnover of genetically similar but different quasispecies, and the ability to monitor, and by extension predict, the changes that the virus will undertake could hold clues for vaccine design.

The V1/V2 region is a major target for neutralizing antibodies. This results in a collection of variants with extensive genetic polymorphisms and length variation. Here we show that these quasispecies have different sensitivities to neutralizing antibodies. Genetic analysis of these variants could hold clues as to why some

variants have a selective advantage i.e. increased fitness in the presence of strong immune responses. Studies have shown that antibody escape can be due to changes in one or two amino acids or changes in N-linked glycosylation sites, many of which have been mapped to the V1/V2 region. The changes in amino acid sequences may affect the structure so they are no longer targets for antibodies. The challenge is thus to identify those structures that are sensitive to antibody neutralization, characterize them further, and exploit these in the design of an HIV-1 vaccine.

CHAPTER FIVE

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APPENDIX A

COMPOSITION OF BUFFERS, MEDIA AND GELS

1. Reagents for agarose gel electrophoresis

1% agarose gel:
1g D-1 LE agarose (molecular grade) in 100mls 1 X TBE. Microwave until dissolved. Allow to cool to room temperature and add 5µl 10mg/ml EthBr before pouring the gel.

10 X TBE stock buffer:

Tris-HCL	108g
Boric acid	55g
0.5M EDTA	40ml

Make up to 1000ml with distilled water. Dilute 1:10 for 1 X TBE and 1:20 for 0.5 X TBX running buffer.

2. Reagents for HMA

10 X Heteroduplex annealing buffer:

1 M NaCl	5.84g
1 M Tris base (pH 7.8)	10ml
0.5 M EDTA	4ml

Make up to 100ml with sterile, deionized, distilled water.

3. Reagents for polyacrylamide gel electrophoresis

5% PAGE gel:

30% acylamide:bisacrylamide (30:0.8)	8.3ml
10 X TBE	5ml
Sterile, deionized, distilled water	36.7ml
10% ammonium persulphate	500µl
TEMED	33µl

4. Reagents for transformation

SOC medium:

Bacto-tryptone	20g
Bacto-yeast	5g
1 M NaCl	1ml
1 M KCl	250µl

Add 97ml distilled water and autoclave for 15 mins at 121°C. Allow to cool to room temperature and then add 1Ml 2M Mg^{2+} stock (1M $MgCl_2.6H_2O$/1 M $MgSO_4.7H_2O$) and 1Ml 2M sterile glucose (both filter sterilised). Make up to 100ml with distilled water. Filter sterilise the complete medium through a 0.2μm filter unit. Final pH should be 7.0.

LB agar plates:

LB agar (Luria Bertani agar)	32g

Make up to 1L with distilled water and autoclave for 15 min at 121°C. Allow to cool to 60°C and then add 1ml 50mg/ml ampicilin (and 3ml of 5mg/ml tetracycline in the case of pMOS*Blue* T-vector).

LB growth medium:

LB broth base (Lennox L broth base)	20g

Make up to 1L with distilled water and autoclave for 15 min at 121°C. Allow to cool to room temperature and then add 1ml 50mg/ml ampicillin (and 3ml 5mg/ml in the case of pMOS*Blue* T-vector).

5. Media for cell cultures

PBMC cultures:

RPMI 1640	80ml
FCS	20ml
IL-2	5ml

APPENDIX B

REAGENTS AND SUPPLIERS

ABI Prism Big Dye Ready Reaction Kit version 2.0	PE Applied Biosystems, USA
ABI 3100 Automated sequencer	PE Applied Biosystems, USA
Acrylamide	Sigma, Germany
Agarose	GIBCO Scotland
Alkaline phosphatase	Aalto Bio Reagents Ltd, Ireland
Ammonium persulphate	Sigma, Germany
Ampicillin	Roche, GmbH, Germany
^{35}S [ATP]	Amersham Pharmacia Biotech, England
Bacto-tryptone	Unipath Ltd., England
Bacto-yeast extract	DIFCO Laboratories, USA
Baxter water	Adcock Ingram, South Africa
Biometric TRIO-Thermoblock T1 cycler	Gottigen, Germany
Bis-acrylamide	Roche, GmbH, Germany
Boric acid	Merck, Germany
Bromophenol blue	Merck, Germany
Buffer K	Roche, GmbH, Germany
CENTRI-SEP columns	Princeton Separations Inc., USA
Chromatography paper	Whatman Scientific Limited, England
DNA Molecular Weight Marker (IX, X)	Roche, GmbH, Germany
dNTP's	Roche, GmbH, Germany
dRhodamine Terminator Cycle-Sequencing kit	Perkin Elmer Applied Biosystems, Great Britain
EDTA	Merck, South Africa
Ethanol	Associated Chemical Enterprises, South Africa
Ethidium bromide	Promega, USA
Fetal calf serum (FCS)	Sterilab, UK
Ficoll Hypaque	Amersham Pharmacia Biotech AB, Sweden
GeneAmp® PCR system 2400/9600-thermocycler	Perkin Elmer Applied Biosystems, Great Britain
Glycerol	NT laboratory suppliers, South Africa
High Pure PCR Product Purification kit	Roche, GmbH, Germany
Heteroduplex Mobility Assay kit	NIH AIDS Research and Reference Program, US
Hybaid Touch down PCR system	Hybaid, UK
Interleukin-2 (IL-2)	Roche, GmbH, Germany
IPTG	Roche, GmbH Germany
Klenow	Roche, GmbH, Germany
*Kpn*I	Roche, GmbH, Germany
LB agar	GIBCO BRL, Life Technologies, Scotland
LB broth	GIBCO BRL, Life Technologies, Scotland

*Nde*I	Roche, GmbH, Germany
Phosphate Buffered Saline	Merck, Germany
Phytohemagglutinin (PHA)	Roche, GmbH, Germany
pMOS*Blue* T-vector kit	Amersham Pharmacia Biotech, England
Polycarbonate microtiter plate	HYBAID Limited, UK
Primers	Dept. of Biochemistry UCT
Proteinase K	Roche, GmbH, Germany
QIAamp® Viral RNA isolation kit	QIAGEN, Germany
QIAGEN Plasmid isolation kit	QIAGEN, Germany
Reverse transcriptase	Roche, GmbH, Germany
RNase inhibitor	Roche, GmbH,Germany
RPMI 1640	Sigma, Germany
SpeI	Roche, GmbH, Germany
Super-Therm DNA polymerase-(including 10 X buffer and $MgCl_2$)	Advanced Biotechnologies Limited, UK
T4 DNA ligase	Roche, GmbH, Germany
TEMED	Amersham Pharmacia Biotech, England
Tetracycline	Roche, GmbH, Germany
Tris-base	Pharmacia, Biotech, Sweden
Tris-HCL	Roche, GmbH, Germany
Triton X-100	Roche, GmbH, Germany
X-gal	Roche, GmbH, Germany
Xylene Xyanol	Fluka AG, Switzerland

APPENDIX C

ETHICAL CLEARANCE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Ang'Ang'A Choge

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M01-06-01

PROJECT Genetic Variation In V1/V2 & Neutralisation Sensitivity of HIV -1 Subtype C Isolates From Infants With Rapid & Slow Disease Progression

INVESTIGATORS Mr I Ang'Ang'A Choge

DEPARTMENT Dept of Medical Virology, National Institute for Virology

DATE CONSIDERED 01-06-15

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 01-09-25 **CHAIRMAN**.....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr L Morris
Dept of Department of Virology, National Institute for Virology

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/~~we~~ fully understand the conditions under which I am/~~we are~~ authorized to carry out the abovementioned research and I/~~we~~ guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/~~we~~ undertake to resubmit the protocol to the Committee.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

ISAAC CHOGE

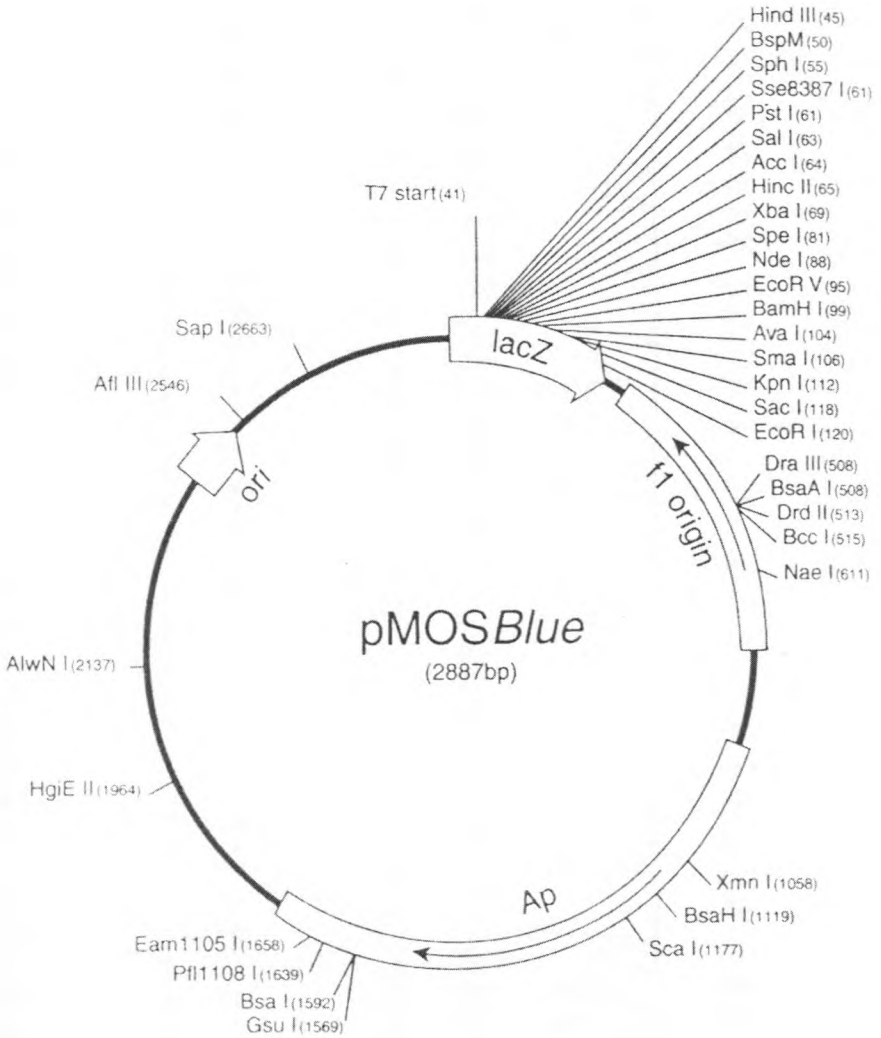
APPENDIX D

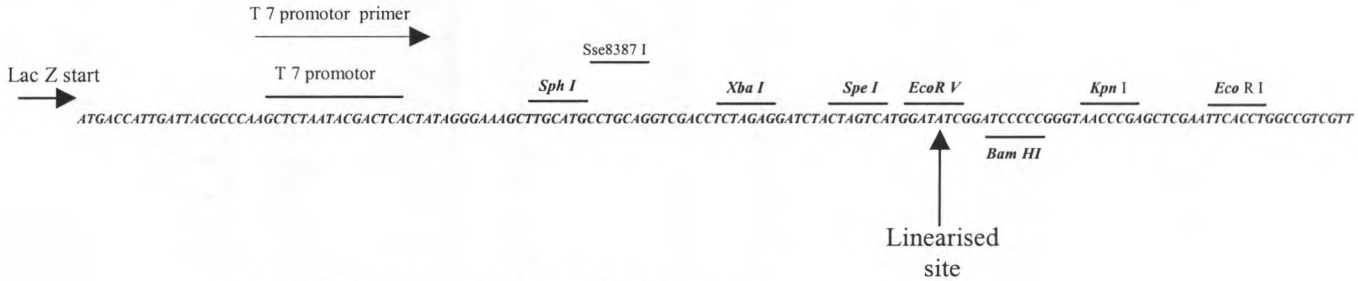
NAMES AND ABBREVIATIONS OF THE COMMON AMINO ACIDS

Amino acid	Three letter abbreviation	One letter abbreviation
Amino acids with unpolar side chains:		
Alanine	Ala	A
Glycine	Gly	G
Isoleucine	Ile	I
Leucine	Leu	L
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Tryptophan	Trp	W
Valine	Val	V
Amino acids with uncharged polar side chains		
Asparagine	Asn	N
Cysteine	Cys	C
Glutamine	Gln	Q
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Amino acids with charged polar side chains		
Arginine (+)	Arg	R
Aspartic Acid (-)	Asp	D
Glutamic Acid (-)	Glu	E
Histidine (+)	His	H
Lysine (+)	Lys	K

APPENDIX E

pMOS Blue vector as supplied





Linearised short segment of the pMOSBlue vector

APPENDIX F: 98ZA151Du nucleotide sequence

