MOLECULAR CHARACTERIZATION OF GROUP M HIV-1 SUBTYPES CIRCULATING IN MBEYA REGION OF THE UNITED REPUBLIC OF TANZANIA

By

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A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy (Medical Microbiology) of the Muhimbili University of Health and Allied Sciences.

Muhimbili University of Health and Allied Sciences

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In fulfilment of the requirements for the degree of Doctor of Philosophy (Medical Microbiology) of the Muhimbili University of Health and Allied Sciences

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DEDICATION

This work is dedicated to my caring husband Mainen Moshi, our daughter Eunice and our son Clemence and my parents Daniel and Pauline Mloka.

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ABSTRACT

The objective of this work was the molecular characterization of the group M HIV-1 subtypes circulating in Mbeya region of the United Republic of Tanzania.

The Multiregion Hybridisation Assay (MHAacd) was used to determine HIV-1 subtype distribution and prevalence of HIV-1 multiple infections in a high risk cohort, consisting of 600 female "local brew shops" and bar workers. An in-house antiretroviral drug resistance genotyping assay was used to determine the prevalence of primary antiretroviral drug resistance mutations in ARV naive HIV sero-positive women living in Mbeya Municipality.The prevalence and persistence of primary Nevirapine (NVP) resistance mutations in a cohort of 121 HIV sero-positive pregnant women living in Mbeya municipality, given single dose NVP prophylaxis for prevention of mother to child transmission (PMTCT) of HIV was also determined using an in-house genotyping method.

The molecular epidemiology of HIV -1 subtypes circulating in Mbeya region Tanzania was found to be complex. A HIV sero-prevalence rate of 68.7% in the cohort of 600 bar workers at recruitment and a cumulative incidence of 17% over 36 months of follow up were observed. The HIV-1 subtype distribution was 38% clade C, 6 % clade A, 2% clade D , 54% recombinant (AC, AD, CD or ACD) and a putative multiple infection frequency of 15.4% was observed. The prevalence of primary genotypic drug resistance mutations in chronically infected ARV naive women living in Mbeya municipality was relatively low 0.045 (n=192). Primary resistance to protease inhibitors was observed in 0.031% (n = 192) of the samples tested. Sequence polymorphism coding for secondary resistance to protease inhibitors was detected in over 50 % of analyzed samples. Primary NVP resistance mutations were shown to develop and persit for 24 weeks in the PBMC's of 5% of women given SD-NVP prophylaxis for PMTCT of HIV.

The study findings led to the conclusion that the molecular epidemiology of HIV-1 in Mbeya is complex, with individuals in this region prone to multiple HIV-1 re-infections. The findings of primary NVP resistance following its single use prophylactically for PMTCT, as well as the detection of primary resistance to protease inhibitors in ARV naive HIV infected women,

warrants a more extended and country wide surveillance of the emergence and spread of antiretroviral resistance in Tanzania.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
ARV	Antiretroviral
CD	Cluster Designation
DNA	Deoxyribonucleic Acid
HAART	Highly Active Antiretroviral Therapy
HISIS	HIV Superinfection Study
HIV	Human Immunodeficiency Virus
IAVI	International AIDS Vaccine Initiative
LMU	Ludwig Maximillian University
MHA	Multiregion Hybridisation Assay
MMRP	Mbeya Medical Research Program
MTCT	Mother To Child Transmission
MUHAS	Muhimbili University of Health and Allied Sciences
NVP	Nevirapine
PCR	Polymerase Chain Reaction
PWLA	People Living With HIV and AIDS
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TFDA	Tanzania Food and Drug Authority
UNAIDS	United Nations AIDS program
WHO	World Health Organisation

CHAPTER 1: GENERAL BACKGROUND

1.1. THE HIV EPIDEMIC

The first reports of homosexual patients suffering from previously rare diseases, such as, pneumocystis pneumonia (PCP) and Kaposi's sarcoma were published in May 1981 (MMWR rep 30 1981). However, it took almost two years until 1983, that the human immunodeficiency virus type I (HIV-1) was isolated and defined as the primary cause of the acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al 1983, Broder et al, 1984, Gallo et al, 1984, Shaw et al 1984).

This once unheard of virus was during the year 2007 infecting 33.2 (30.6- 36.1) million people and was the cause of 2.1 (1.9 – 2.4) million deaths worldwide (UNAIDS Global Report 2008). There are currently 22.5 (20.9- 24.3) million people living with HIV and AIDS (PLHA) in Africa of whom 61% are females. South Africa with an estimated 5.1 (4.6 -5.6) million PLHA by the end 2005, is the most affected nation worldwide.

The dissemination of HIV-1 in humans over the last two decades represents a catastrophic example of genome transmission and expansion (Ho et al, 2002, Stebbing et al, 2003). The global HIV epidemic can be compared to a 'molecular arms race'. However, in this particular competition, our lentiviral opponent appears able to run very fast indeed. Its reverse transcriptase error frequency, and the ability to produce in excess of 109 virions per day in vivo, provides HIV-1 a tremendous advantage of giving rise to new strains and transmitting them (Ho et al, 1995).

For those for whom it is available, Highly Active Antiretroviral Therapy (HAART) has reduced short-term mortality and markedly increased the quality of life by preventing opportunistic diseases. Despite this initial euphoria of finding drugs that selectively target the HIV virus; HAART has been unable to target the latent reservoir of the tisuses. Furthermore, the drugs are associated with well known clinical side effects and are plagued with the rapid development of drug resistance.

Despite international efforts to control the HIV/AIDS pandemic, through behavioural modification and other biomedical interventions, more than 8400 people become

infected with HIV every day. The joint United Nations Programme on HIV/AIDS (UNAIDS) estimates that to date 25 million people worldwide have died from HIV/AIDS (UNAIDS Global Report, 2006). Tanzania like many of its African neighbours has been heavily affected by the HIV/AIDS pandemic. It is estimated that there are over 1.6 million Tanzanians who are living with HIV/AIDS and that the national adult prevalence rate stands at 6.5% (UNAIDS Global Report, 2006). The epidemic in Tanzania like in many other African countries continues to spread, despite the use of multiple interventions.

The discovery of HIV as the aetiological agent of AIDS in 1983–84, raised hopes that a preventive vaccine would soon be developed and, in fact, the first human trial of a candidate HIV Vaccine was carried out in 1987 in the USA (IAVI AIDS Vaccine Trials Database report, 2006). At that time, however, the magnitude of the scientific challenge presented in developing of an AIDS vaccine was not appreciated. Since then, 95 candidate vaccines have been tested in human trials and efforts to develop an HIV vaccine are increasing (IAVI AIDS Vaccine Trails Database report, 2007). Depending on the results of the ongoing and planned large-scale phase III trials, the first HIV preventive vaccine could be available within the next 10-15 years. Even if these first-generation HIV vaccines work, they may not be as effective as desired, especially when one takes into consideration the scientific challenges posed by the virus to the human immune system. Nevertheless, a HIV vaccine with moderate efficacy could still play a significant role in preventing new infections. In any case, one should not expect that a future HIV vaccine, especially one with only moderate efficacy, will be a ''magic bullet'' that replaces other preventive interventions. Instead, vaccines will be part of a comprehensive HIV prevention package that also includes health promotion, behavioural change and biomedical interventions.

1.1.1 The Origin and History of HIV-1 virus

HIV and its subtypes are retroviruses. Human retroviruses were unknown until the 1980's, though animal retroviruses such as feline leukaemia virus had been detected previously (Lappin, 1995, Sparger et al, 1989). HIV belongs to a large family of ribonucleic acid (RNA) lentiviruses that are characterized by association with diseases of immunosuppression, central nervous system involvement, and long incubation periods following infection before manifestations of illness. Phylogenetic studies of primate lentiviruses provide compelling evidence that HIV-1 is closely related to a virus that naturally infects chimpanzees (SIVcpz) (figure 1-1), while HIV-2 is closely related to a virus that naturally infects sooty mangabey monkeys (SIVsmm). It is believed that zoonotic (or trans-species) transfer of these simian immunodeficiency viruses resulted in the emergence of HIV-1 and HIV-2 an argument that is strengthened by the fact that, sooty mangabeys are resident in the same region of West Africa in which HIV-2 is most prevalent. HIV-1 is thought to have evolved following either one or more initial infection events from monkeys, followed by subsequent human-to-human transmission events that resulted in widespread dissemination of the virus. The latter events most likely included changes such as urbanization, rapid mass transit, and new trade opportunities.

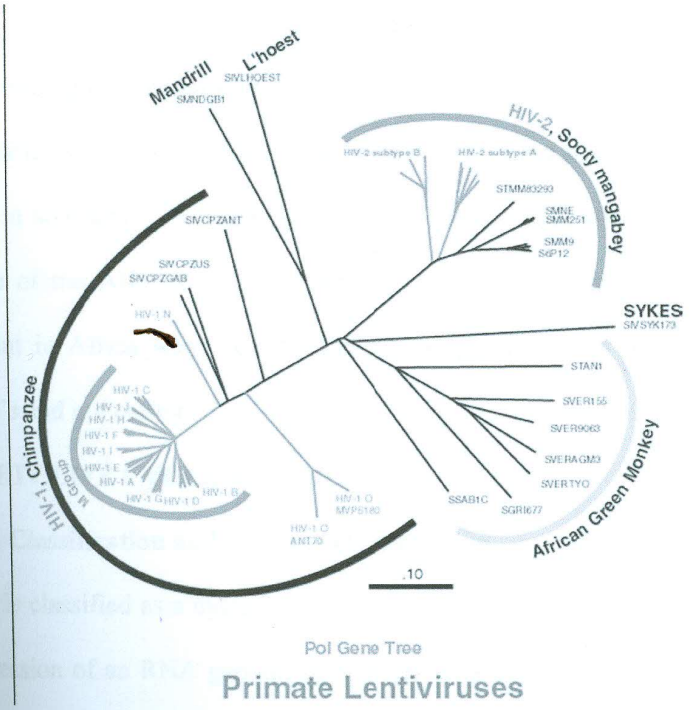


Figure 1-1. Neighbouring phylogenetic tree of fulllength genome isolates demonstrating relationship between human and animal lentiviruses. Source; Gao etal Nature 1999 vol.397 pg 436.

In the case of HIV-1 group M viruses (for majority), it is currently believed that these viruses first entered human populations in the early 1930s (Krober et al, 2000). The viruses diversified into well-recognized and distinct subtypes (or clades) by the 1950s. It is believed that at least 3 separate monkey-to-human transfers occurred, to give rise to the 3 genetically distinct HIV-1 groups - groups M (majority/ major), N (novel) and O (outlier). These groups are genetically distinct and have unique geographic distributions, with M being responsible for the global HIV-1 epidemic. Group O is represented by fewer strains and is localized to Cameroon, Gabon and Equatorial Guinea; group N was discovered more recently and is represented by very few strains all from Cameroon (Peters et al, 2000, Lemey et al, 2004, Lal et al, 2005).

While a discussion of the origins of HIV is an interesting and potentially an important scientific debate, it is necessary to separate origin from cause. The identification of the origin of HIV in African primates, does not in anyway explain the disparity between the

molecular evidence presented by scientists all over the world and the current epidemiological features of the current AIDS epidemic. Furthermore, much of the scientific evidence presented so far; such as the recent study done on *Pan. t. troglodytes* apes in southern Cameroon (Keele et al, 2006); raises even more questions about the cause of the AIDS epidemic, such as, if the virulent form of the virus was always present in Africa why then did the AIDS epidemic only manifest itself in the early 1980's and not earlier?

1.2. HIV LIFE CYCLE AND BIOLOGY

1.2.1 Classification and Genome Organization

HIV is classified as a cytopathic retrovirus. The defining feature of retroviruses is their possession of an RNA genome and the enzyme reverse transcriptase, which facilitates the conversion of RNA into DNA. Other distinguishing traits include a modified chemical structure to enable the different mode of RNA synthesis, a dimeric genome (two subunits which are almost identical, held together by base pairing), and a unique association with a specific molecule of lysl tRNA, which serves as the primer for reverse transcription (Renda et al, 2004) .

The HIV genome is about 9.2 kb in size and consists of nine genes (Thomson et al, 2002). Three of these, gag, pol, and env are common to all other retroviruses, the remaining 6, vif, vpr, tat, rev, nef, and vpu, are unique to HIV (Figure 1-2).

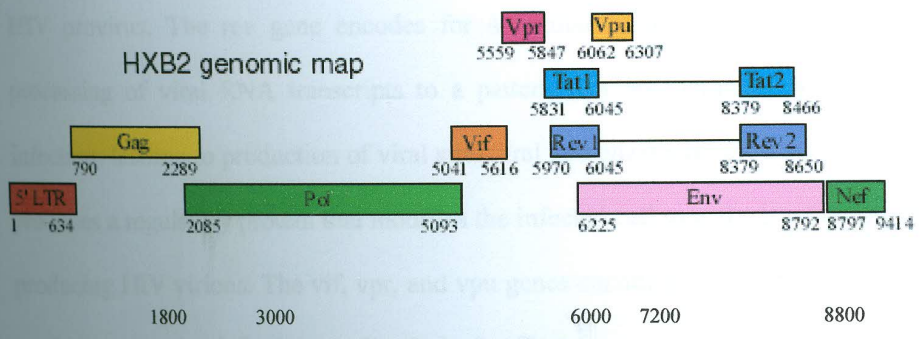


Figure 1-2. HIV genome organization. Source; www.lanl.gov/content /immunology/pdf/2000/intro/genomemaps.pdf

The mature virus consists of a bar-shaped electron dense core containing the viral genome of two short strands of RNA of about 9200 nucleotide bases long. Within the core are a number of viral specific enzymes including the viral reverse transcriptase, protease, ribonuclease, and integrase enzymes. On the outside, the viral core is encased in an outer lipid envelope, which has 72 surface projections containing an antigen, gp120, which aids in the binding of the virus to the target cells with CD4 receptors. When seen by electron microscopy, infected CD4+ cells exhibit budding virus particles approximately 90 to 100 nm in diameter.

The genome of HIV, contains three major genes—gag (group specific antigen), pol (polymerase), and env (envelope). These genes code for the major structural and functional components of HIV, including envelope proteins and reverse transcriptase. The major structural components coded by the env genes include the envelope glycoproteins, including the outer envelope glycoprotein gp120 and transmembrane glycoprotein gp41 derived from glycoprotein precursor gp160,nucleocapsid proteins p55, p40, p24 (capsid, or "core" antigen), p17(matrix), and p7 (nucleocapsid). The important proteins coded by pol are the enzyme proteins p66 and p51 (reverse transcriptase (RT)), p11 (protease), and p32 (integrase) (Brayant et al, 1990, Franke et al, 1994, Thali et al, 1994). Accessory genes carried by HIV include tat, rev, nef, vif, vpr, and vpu (for HIV-1) or vpx (for HIV-2). The functions of some of these genes are known. The tat gene produces a regulatory protein that speeds up transcription of the HIV provirus. The rev gene encodes for a regulatory protein, which switches the processing of viral RNA transcripts to a pattern that predominates with established infection, leading to production of viral structural and enzymatic proteins. The nef gene produces a regulatory protein that modifies the infected cell to make it more suitable for producing HIV virions. The vif, vpr, and vpu genes encode proteins that appear to play a role in generating infectivity and pathologic effects.

1.2.2. HIV Transmissison and Pathogenesis

The transmission of HIV is a function of where the virus appears in the body and how it is shed. HIV can be present in a variety of body fluids and secretions, but the presence of HIV in genital secretions, in blood, and to a lesser extent in breast milk, is significant for spread of HIV. The presence of HIV in saliva, urine, tears, and sweat seems to be of no major clinical importance, as transmission of HIV through these fluids is rare, primarily because of the low concentration of virus in these fluids.

HIV is primarily spread as a sexually transmissible disease. Transmission of HIV can occur from male to male, male to female, and female to male. Female to female transmission remains extremely rare, though women with same-sex contact are often bisexual and have additional risk factors for HIV infection (Manavi, 2006). Reported rates of HIV transmission through unprotected sexual intercourse ranges between 0.05 – 3.2 % per sexual contact with an HIV-infected person. Thus, it is true, that some individuals have become HIV-infected after a single unprotected sexual contact, while others have remained uninfected after hundreds of unprotected sexual contacts (Nwokolo et al, 2001).

Sexual contact with persons with high HIV viral loads, either within the early infection period or in the late stage of clinical AIDS, increases the transmission risk. Other factors such as cervical neoplasia, oral contraceptive use, in uncircumcised men, and genital ulcer disease in either sex partner increase the risk for HIV infection (Manavi, 2006).

HIV can also be transmitted by the parenteral route of exposure. Peripheral blood mononuclear cells are capable of either harbouring or becoming infected by HIV in blood or in other body fluids or secretions. The primary risk group for HIV transmission via blood are intravenous drug users sharing contaminated syringes and needles. Common practices such as tattooing and female genital mutilation where the use of non sterile needles or razors is common, are assumed to potentially increase the risks of HIV transmission.

Mother-to-child transmission (MTCT) is by far the largest source of HIV infection in children under the age of 15years. Congenital AIDS occurs, on average, in about one fourth of babies born to HIV-1 infected mothers, with actual rates of transmission varying from 30-40% in breastfeeding women and 15-20% in non breasting feeding in the first year of life without intervention (Manavi, 2006). HIV transmitted 'vertically" or "perinatally", is the primary means by which infants acquire HIV. Paediatric HIV infections may occur in utero, intrapartum or through breastfeeding. An infant is considered to have in utero infection if virologic tests (HIV DNA or RNA PCR or culture) are positive within 48 hours of life. Infants are considered to have acquired an intrapartum HIV infection if diagnostic tests within the first 48 hours of life are negative but further virologic testing after 1 week of life is positive. Infants with in utero infection appear to have a more rapid disease course when compared to infants who acquire HIV infection intrapartum (Dickover et al, 1994, Mayaux al,1996). The pathogenesis of in utero HIV infection is still not well understood but infection appears to result from transplacental passage of virus in the presence of chorioamnionitis, or ascending viral infection in patients with prolonged rupture of membranes (Minkoff et al, 1995). HIV-2 can also be vertically transmitted; however the risk of acquiring MTCT HIV-2 is much lower than for HIV-1 (1.2% [95% CI, 0.0% to 3.5%]) (Adjorlolo-Johnson et al, 1994). The lower MTCT rates for HIV-2 infections are probably a result of the natural pathogenesis of the virus that results in low maternal HIV-2 RNA levels, which are on average 37-times less, in comparison to HIV-1 infections (O'Donovan et al, 2000, Schim van der Loeff et al, 2003).

The pathogenesis of HIV infection is a function of the virus life cycle, host cellular environment, and quantity of viruses in the infected individual. The progression of the HIV infection may be determined through various outcome measures such as the plasma virus load, the rate of decline in CD4 count, increase in plasma virus load and the HIV associated opportunistic infections. However, a number of factors may influence the course of disease progression, a balance between the host factors and the virus factors

are major determinants of the outcome of HIV infection. At one end of the spectrum are the persons who are able to mount an immune response that is able to control the virus multiplication successfully and such individuals may remain clinically normal with minimal loss of CD4 cells and very low to undetectable plasma virus load. These are individuals are referred to as "long term survivors". At the other end of the spectrum are individuals who are unable to control virus multiplication successfully and show rapid decline in CD4 cell counts and may develop AIDS within 2-3 years of acquiring HIV infection. These individuals are referred to as "rapid progressors". In contrast, to "typical progressors" who have a median period of 10 years before development of symptoms.

1.2.3. Viral Entry

Infection is a probability function of both the number of infective HIV virions in the body fluid which contacts the host as well as the number of cells available at the site of contact that have appropriate receptors. Cells susceptible to HIV infection may include those of the mononuclear phagocyte system, principally blood monocytes and tissue macrophages, T- helper lymphocytes, dendritic cells (Langerhans cells of epithelia and follicular dendritic cells in lymph nodes), haematopoietic stem cells, endothelial cells, microglial cells in the brain, gastrointestinal epithelial cells as well as cells with galactosylceremide receptors (Li, 2005). After entering the body, the viral particle attaches to cells with the appropriate CD4 receptor molecule. Following attachment, it enters by endocytosis. In addition to the CD4 receptor, coreceptors known as chemokines are required for HIV to infect cells. The chemokine coreceptors include the receptors of CXC family (CXCR1 to CXCR5) and the CC family (CCR1 to CCR9). Initial binding of HIV to the CD4 receptor is mediated by conformational changes in the gp120 subunit, but such conformational changes are not sufficient for fusion (Clapham et al, 2001, Cilliers et al, 2003). The chemokine receptors produce an additional conformational change in the gp41 subunit of HIV that allows fusion. The diversity of

chemokine co receptors on different types of cells may explain how different strains of HIV may infect cells selectively.

A novel classification system has been devised, that distinguishes three main viral variants on the basis of their ability to use selectively CCR5 (R5) or CXCR4 (X4), or either co receptor interchangeably (R5X4). This classification has replaced the old and obsolete nomenclature based on cellular tropism (Tcell- versus macrophage-tropic) or giant multinucleated cell formation (syncytia versus non-syncytia inducer). It is now increasingly recognized that such distinctions were essentially laboratory artifacts: all primary HIV-1 isolates can indeed grow in primary CD4þ T cells and readily induce the formation of syncytia in cells that express the appropriate coreceptors; furthermore, despite a persisting prejudice, most primary HIV-1 isolates have the ability to replicate to some extent in primary macrophage cultures, regardless of their coreceptor preference (Lusso et al, 2006). The HIV-1 strains that are most commonly responsible for transmission and predominate during the long asymptomatic phase are almost invariably restricted to CCR5 usage (R5). They replicate efficiently in activated/memory cells, which express high levels of CCR5 and are particularly abundant in the gut associated lymphoid tissue, which is now recognized as a primary site of HIV and SIV replication (Lusso et al, 2006). Later in the infection course, concomitant with immunologic and clinical signs heralding disease progression, HIV-1 strains may emerge that use CXCR4, a homeostatic chemokine receptor expressed on a broader range of cells, including naive/resting T cells and thymic precursors.

There is a strong association between the human genetic polymorphism, HIV susceptibility, and disease progression, as seen in homozygous individuals with a 32 base pair deletion in the CCR5 receptor gene (CCR5Δ32). Homozygous individuals with this deletion have been shown to have decreased susceptibility to HIV infection, whereas, heterozygous persons with the same deletion, are not necessarily less susceptible to HIV infection; but tend more likely to become "long term survivors". The CCR5 gene promoter region has been shown to have nine stable haplogroups some of

which may influence the HIV disease progression. The CCR5 haplogroup E has been shown to speed up the decline in CD4 counts and is associated with accelerated disease progression (Li et al, 2005). HIV infected individuals harbouring either the CCR2-V64I mutation or a mutation in stromal derived factor 1 (SDF-1), ligand for CXCR4, are thought to progress to AIDS more slowly.

1.2.4. Viral Replication and Host Immune System Destruction

Viral multiplication follows membrane fusion of the virus core, which results in the "uncoating" of the viral genome into the cytoplasm of the target cell. The conversion of viral RNA into proviral DNA is the most important step in establishing cellular HIV infection. This process is mediated by the viral RT enzyme, and results in the transcription of full-length double-stranded HIV DNA with Long Terminal Repeats (LTR) at each end. HIV-1 entry into quiescent CD4 cells is comparable to HIV-1 entry in activated CD4 cells; however, synthesis of HIV-1 DNA remains incomplete in quiescent cells (Zack et al, 1990). In activated CD4 cells, transcription occurs and results in the integration of the proviral DNA into the host nucleus while in quiescent CD4 cells, reverse transcription results in the accumulation of non-integrated viral DNA. The accumulation of non integrated viral DNA in infected quiescent, CD4 cells, monocytes, macrophages and microglial cells represent a latent reservoir of the virus. This reservoir is responsible for inability to completely eradicate the virus from infected individuals even if these individuals are put on HAART, as HAART only affects activated cells (Koup, 2001).

In activated infected cells, integration of the proviral DNA by viral host enzyme integrase, results in cellular transcription factors such as Nuclear Factor Kappa B (NF-kB) which binds to the LTR regions. The binding of these factors facilitate the binding of the RNA polymerase II and results in the transcription of HIV genes (Karin et al, 2000). Transcription initially results in the early synthesis of regulatory HIV-1 proteins such as tat or rev. Tat binds to the "Transactivation Response Element"(TAR) site at the

beginning of the HIV-1 RNA in the nucleus and stimulates transcription and the formation of longer RNA transcripts. Rev activates the expression of structural and enzymatic genes and inhibits the production of regulatory proteins, therefore promoting the formation of mature viral particles. These include proteins coded for by pol, gag and env. The gp120 spikes of the envelope are synthesized as large gp160 precursor molecules and are cleaved by the HIV-1 protease into gp120 and gp41. The gag proteins are also derived from a large 53 Kd precursor molecule, from which the HIV-protease cleaves the p24, p17, p9 and p7 gag proteins. The formation of new viral particles is a stepwise process: HIV-1 RNA, gag proteins and various pol enzymes, form a new virus core. The large precursor molecules are cleaved by the HIV-1 protease, which results in the infectious viral particles budding through the host cell membrane.

After initial entry of HIV into host cells and the establishment of infection, newly formed HIV virions released from infected cells by budding or lysis may then enter the systemic circulation and be carried to various sites within the body. Cells of the mononuclear phagocyte system, including those in lymph nodes, spleen, liver, and bone marrow can then become infected with HIV. Besides lymph nodes, the gut associated lymphoid tissue in the gastrointestinal submucosa provides a substantial reservoir for HIV. Primary HIV infection is usually followed by a burst of viremia in which virus is easily detected in peripheral blood in mononuclear cells and plasma. Initial infection is usually followed by a period of clinical latency, where there is little detectable virus in peripheral blood, but viral replication actively continues in lymphoid tissues.

1.2.4.1 Host Cell Death

The principal impact of HIV infection on the immune system is the gradual destruction of the CD4+ T lymphocytes. The half-life of most HIV infected T cells in vivo is 12-36 hours (Paranjape et al, 2005). HIV uses multiple mechanisms for destroying CD4 T lymphocytes; not only HIV infected cells but HIV uninfected CD4 cells as well. Host cell death ensues as a result of several diverse viral and host mechanisms including:

direct viral cytopathic effects, fusion to form multinucleated giant cells (syncytia formation), cytotoxic immune response by other lymphocytes (CD8+ cytotoxic T-lymphocytes), autoimmune mechanisms, disruptive interaction of HIV envelope proteins with the cell membrane, immune clearance from alteration of antigenicity of the host cell, activation of apoptosis (programmed cell death), toxic accumulation of viral DNA, RNA, or proteins, in conjunction with a variety of host factors.

1.2.4.2. Direct Killing

Host cell plasma membrane destruction is a major mechanism that results in host cell CD4+ T lymphocyte depletion. The continuous budding of the viruses from infected cells causes membrane disruption and increased permeability resulting in ultimate death of the cell.

1.2.4.3 Apoptosis

Apoptosis plays a key role in the decline in T cell numbers, which is the hallmark of HIV infection. Apoptosis, a mechanism normally used by the body to eliminate redundant cell populations and defective cells, is utilized by the HIV virus to destroy both infected and uninfected cells. Elevated levels of apoptosis are found to correlate directly with disease progression and inversely with CD 4 cell counts (Paranjape et al, 2005). It is thought that HIV proteins, nef, env and tat up regulate Fas (also referred to as CD95 or APO-1) is a 48-kDa (319 amino acids) member of the TNF/nerve growth factor receptor family. Fas (CD95) and Fas ligand (FasL) are a type II transmembrane protein that belongs to the tumor necrosis factor (TNF) family levels. This in turn enhances the susceptibility of the HIV infected and uninfected host T cells to Fas mediated killing (Azad et al, 2000 and Teodoro et al, 1997). Soluble gp120 induces apoptosis in uninfected T- helper cells through cross-linking of the CD4 molecule (Banda et al, 1992, Anand et al, 2006). The binding initiates part of the T cell

activation pathway; however, in the absence of the T cell receptor (TCR) being specifically activated, this signal results in apoptosis or inhibition of antigen-induced cell activation (Marschner et al, 2002). Soluble and membrane-bound gp120 induce, through cell receptors such as CD4, CXCR4 and CCR5, the apoptotic pathways both through Fas-dependent (up regulation of Fas/FasL, decreased FLIP) and Fas-independent (increased Bax, decreased Bcl-2) mechanisms (Arthos et al, 2002).

1.2.4.4 Syncytia Formation

CXCR4-tropic HIV isolates, generally found in the later stages of HIV infection, preferentially infect T cells and induce membrane fusion between cells to form giant multinucleated cells called syncytia. Although syncytium formation is not necessary for the progression to AIDS, syncytia have shorter cellular life spans compared with normal cells. The emergence of CXCR4-tropic strains usually correlates with an increased depletion of T cells and results in host immunosuppression (Alimonti et al, 2003).

1.2.4.5 Anergy and Superantigens

Superantigens are microbial proteins that are potent activators of CD4+ T cells. Superantigens can have profound effects on the immune system, both acute and long-term. Acute effects include food poisoning and toxic shock syndrome. Long-term effects include autoimmune diseases and immunodeficiency. These effects have generally been considered ``bad" and ``ugly". However, if the burst of T-cell activation that occurs with superantigens could be harnessed and exploited, then superantigens would have ``good" effects for the host, such as enhancement of desirable immune responses (Figure.1-3) (Torres et al, 2001).

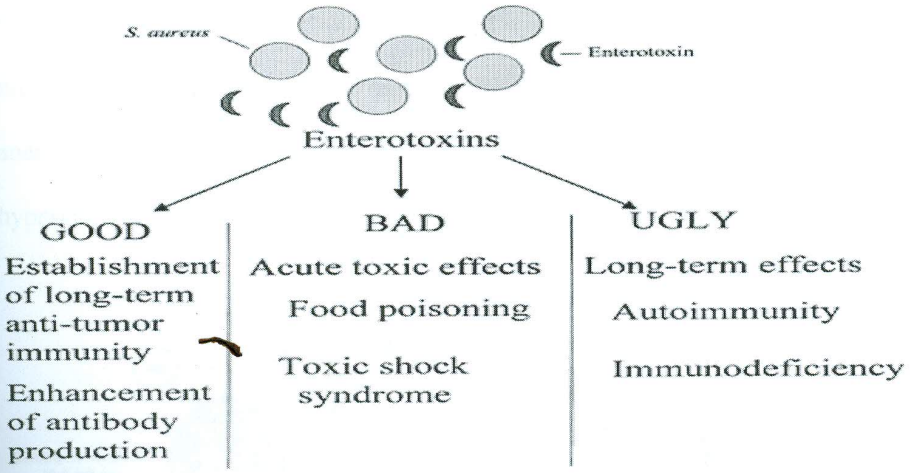


Figure 1-3. ``Good", ``bad", and ``ugly" effects of the staphylococcal enterotoxin superantigens on the host. Source; Torres et al; Experimental Biology and Medicine 2001; 226:164-176

Immunological perturbations speculated to be associated with HIV-encoded superantigen production in HIV-infected individuals are polyclonal activation of B cells with increased immunoglobulin production, reduced antigen and mitogen responses, and increased natural killer cell activity (Edelman et al, 1989). Several pieces of evidence implicate the involvement of an HIV superantigen (Torres et al, 2001). One such super antigen could be the viral protein Nef. Nef, is a regulatory protein expressed early in the infection of CD4+ T cells, it has been shown to induce Vß-specific T-cell proliferation in the absence of processing (Tanabe et al, 1997). A model on the hypothetical role of Nef in the pathogenesis of HIV is presented in Figure. 1-4. Nef is thought to be released in a soluble form as the result of lysis of infected cells. Interaction of T cells with Nef activates T cells to proliferate and produce cytokines such as IFN7 and IL-2. An outcome of Nef stimulation is the establishment of cellular reservoirs of activated CD4+ T cells for virus production and infection, with eventual depletion of T cells via virus replication, anergy and/or apoptosis. Nef activation of T-cells also promotes B-cell differentiation as a result of activated CD4+ cells providing help and producing cytokines that aid in B-cell maturation. This in turn may result in the polyclonal activation of B-cells, suggested by increased immunoglobulin levels seen

throughout the course of HIV infection (Edelman et al, 1989). The outcome of Nef-induced immune activation thus includes increased viral yield with T-cell anergy/apoptosis and polyclonal B-cell activation, the latter resulting in hypergammaglobulinemia and possible autoimmune-like sequelae (Torres et al, 2001).

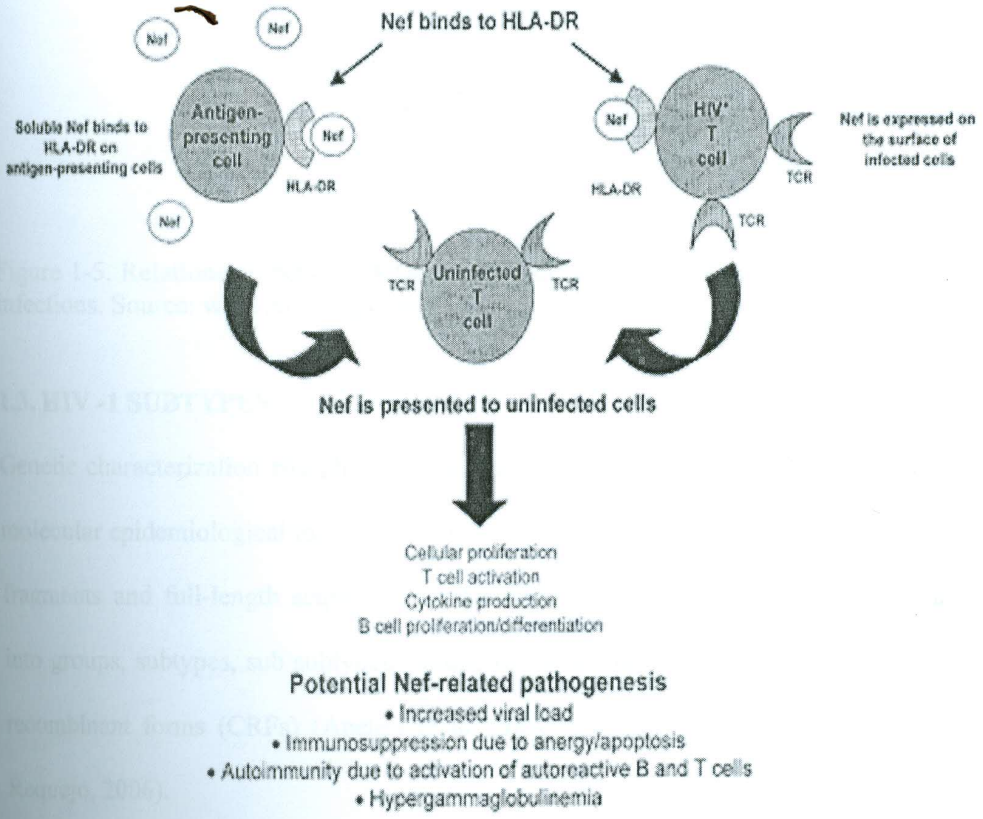


Figure 1-4. Model for the role of Nef in the pathogenesis of HIV. Source; Torres et al; Experimental Biology and Medicine 2001; 226:164-176

Once the CD4 cell count drops below about 400; infected individual may present with constitutional symptoms, such as fever, weight loss, fatigue, night sweats, diarrhoea, and persistent generalized lymphadenopathy. This is then followed by infection with opportunistic pathogens. As the CD4 cell count continues to fall below 200, other opportunistic infections (Pneumocystis carinii pneumonia, Kaposi's sarcoma, candidiasis, coccidioidomycosis, cryptosporidiosis, cytomegaloviral infections, toxoplasmosis of the brain, HIV encephalopathy, etc.) ravage the body until one or more of them cause death (Figure 1-5).

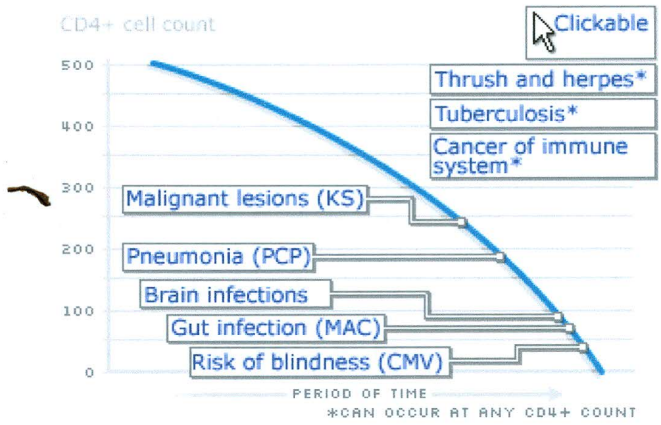


Figure 1-5. Relationship between CD4 count decline and development of opportunistic infections. Source; www.medscape.com

1.3. HIV -1 SUBTYPES

Genetic characterization and phylogenetic analysis of the isolated HIV-1 strains, using molecular epidemiological tools such as HMA, HTA, partial sequencing of env and gag fragments and full-length sequencing, have facilitated the division of HIV-1 isolates into groups, subtypes, sub subtypes, unique recombinant forms (URFs) and circulating recombinant forms (CRFs) (Apeteri et al, 2004, Lal et al, 2005, McCutchan, 2006, Requejo, 2006).

HIV-1 subtypes are defined by significant clustering across the genome in phylogenetic analyses. At least three epidemiologically unlinked sequences are needed to define a subtype (Robertson et al, 2000). Most HIV-1 isolates identified to date associated with the AIDS pandemic belong to a group designated M, for major. There are at least two other HIV-1 groups namely O (outlier) and N (novel/new), but these are largely confined to a restricted geographical area in West Central Africa. Group M is the only group with evidence of significant geographic spread within and outside Africa (Peteers et al, 1997, Simon et al, 1998, Peeters, 2000, Panathanasopoulos et al, 2003).

Full genomic sequencing of group M HIV-1 strains has revealed that there are at least 9 identifiable subtypes of M, designated A (1-4) B, C, D, F1,F2, G, H, J and K (McCutchan, 2006, Requejo, 2006 Taylor et al, 2008).

The amino acid distances in the env gene between the subtypes in the major group have reached 25-35 %, while in the gag gene it is about 15 % (McCutchan, 2000, Peeters et al, 2000). Within subtypes A and F, there are separate subclusters that can be distinguished, designated subclades or sub-subtypes A1, A2, F1 and F2 respectively (Brodine et al, 1995). HIV groups O and N, are genetically very divergent from group M, and represent less than 5 % of the worldwide infections and have almost exclusively been detected in West Central Africa (Simon et al, 1998, Robertson et al, 2000, Stebbing et al, 2003). Group O viruses have been identified in people who have links to Cameroon (Rayfeild et al, 1996) while Group N infections have only been identified in Cameroon so far (Bodelle et al, 2004, Roques et al, 2004).

1.3.1 HIV-1 Intersubtype Recombinants

Following the designation of group M subtypes, it was realized that certain isolates clustered with different regions of their genomes when phylogenetic analyses were performed. These intersubtype recombinants of HIV-1 strains are also a major component of the global epidemic, along with genetic subtypes of M. These recombinant forms have been identified in infected individuals especially in geographic regions where the epidemic has existed for a long time (Peeters et al, 2000, McCutchan, 2006). Some of these recombinants have become genetically stable and are associated with distinct out breaks in certain parts of the world. These stable recombinant forms are referred to as Circulating Recombinant Forms or "CRFs". There are currently over 43 recognized CRFs (Table. 1-1) (McCutchan, 2006, Taylor et al , 2008). To date, CRFs have been from all over the world where epidemics exist with two or more subtypes co circulating and may account for over 10 % of new HIV infections (Peeters et al, 2000, McCutchan 2006, http://www.hiv.lanl.gov/content/hiv-db/CRFs)

TABLE I. HIV Strains in the Global Epidemic

HIV-1[a]	Sub-classification[b]	Description	Main geographic concentrations	Global prevalence[c]	Compete genome sequences[d]
Group M					
Subtype	A	Includes sub-subtypes A1, A2, and A3	East and West Africa, former Soviet Republics	High	62
	B	B prime (B') variant in Southeast Asia	America, Western Europe, Australia	High	129
	C	50% of all HIV-1infections worldwide	Southern Africa, East Africa, India	High	209
	D	East and West African variants	East and West Africa	High	47
	F	F1 and F2 sub-subtypes	West Central Africa	Very low	10
	G		West Central Africa	Very low	8
	H		West Central Africa	Very low	3
	J		West Central Africa	Very low	2
	K		West Central Africa	Very low	2
CRF	CRF01_AE	Former "E"; subtypes A, E	Southeast Asia	High	52
	CRF02_AG	Subtypes A, G	West Africa	High	38
	CRF03_AB	Subtypes A, B	Former Soviet Republics	Very low	3
	CRF04_cpx	Former "I"; subtypes A,G,H,K		Very low	3
	CRF05_DF	Subtypes D, F	Central Africa	Very low	3
	CRF06_cpx	Subtypes A, G, J, K	West Africa	Very low	5
	CRF07_BC	Subtypes B', C	China	Very low	3
	CRF08_BC	Subtypes B', C; related to CRF07_BC	China	Very low	4
	CRF09_cpx	Subtypes A, F, G, related to CRF02_AG	West Africa	Very low	3
	CRF10_CD	Subtypes C, D	Tanzania	Very low	3
	CRF11_cpx	Subtypes G, J	West Central Africa	Very low	10
	CRF12_BF	Subtypes B, F	Argentina	Very low	7
	CRF13_cpx	Subtypes A, G, J, and CRF01_AE	West Central Africa	Very low	3
	CRF14_BG	Subtypes B, G	Spain, Portugal	Very low	6
	CRF15_01E	CRF01_AE, subtype B	Thailand	Very low	4
	CRF16_A2D	Sub-subtype A2, subtype D	Kenya	Very low	3
URF	AC, AD, CD, ACD	Estimated 30% of strains in East Africa	Kenya, Rwanda, Tanzania, Uganda	Moderate	44
	Complex	Common in West/West Central Africa	Nigeria, Cameroon	Moderate	33
	BF	Abundant in South America	Brazil, Argentina	Moderate	31
	CRF01_AE/B	Emerging in Southeast Asia	Thailand, Myanmar,	Very low	13
Group O		West Central Africa	Cameroon, Senegal	Very low	18
Group N		West Central Africa	Cameroon	Very low	3
HIV-2	Subtypes A and B		West Africa	Moderate	22

[a]HIV Type 1 and HIV Type 2; HIV-1 strains are classified into groups M, N, and O, respectively. Group M is subdivided into subtypes, circulating recombinant forms (CRF), and unique recombinant forms (URF).

[b]Subtypes are assigned letters in order of their discovery; CRF are numbered sequentially in order of discovery with the component subtypes after the underscore. "cpx" (complex) indicates that more than three subtypes are present. Some CRF also contain unclassified regions. URF are grouped according to their geographic origin for this analysis.

[c]Estimated from degree of geographic spread, prevalence of HIV in countries and regions, and proportion of total HIV infections in the country or region. Dark grey fill indicates the six most globally prevalent strains; light grey fill indicated strains of moderate abundance.

[d]Based on the HIV sequence database at http://hiv-web.lanl.gov. Because of recombination, only complete or virtually complete genomes of HIV provide unequivocal classification. Partial genome sequences and multiple sequences from a single individual, were excluded. CRF16_A2D includes two complete genomes from our unpublished data. At least five additional CRF have been described but sequences are not yet publicly available.

1.3.1.1. HIV Intergroup Recombinants

Recombination between highly divergent groups such as M and O, have been reported from Cameroon (Peteers et al, 1999, Takehisa et al, 1999). These intergroup recombinant strains from distant lineages may in future contribute substantially to new infections; especially if the intergroup recombinant achieves greater viral fitness than the original parental strain. The presence of these intergroup recombinants threatens the current available methods, for diagnosis, subtyping and treatment especially when it has already been shown that group O viruses have different antiretroviral susceptibilities (Descamps et al, 1997).

1.3.2 Global HIV-1 Subtype Distribution

The HIV-1 genetic subtypes are unevenly distributed in different geographical locations (Table. 1). The predominant viral forms in the global epidemic are subtypes A and C, followed by subtype B and the recombinants CRF01-AE and CRF02-AG (McCutchan, 2006, Requejo, 2006). Subtype B viruses are more prevalent in North America, Latin America and the Caribbean, Europe, Japan and Australia. Almost every subtype is present in sub-Saharan Africa, with subtypes A and D predominating in West, Central and Eastern Africa, and subtype C in Southern Africa. Subtype C is also prevalent in India and in Tanzania (Morison et al, 2001, Hoelscher et al, 2002, Lal et al, 2005, Gerhardt et al 2005, Herbringer et al 2006, McCutchan 2006). Subtype F has been described in Latin America, Central Africa and West Africa (Triques et al 1994, Hemelaar et al, 2006). The most recent subtypes namely G and H can be found in Central and West Africa (Hemelaar et al, 2006 McCutchan, 2006).

In 2004 subtype C accounted for 50% of all infections worldwide. Subtypes A, B, D and G were responsible for 6% of the infections, respectively. Subtypes F, H, J and K together caused 0.94% of infections. The two major CRFs, CRF01_AE and CRF02_AG, were each responsible for 5% of infections, whereas CRF03_AB was responsible for only 0.1% globally. Other recombinants accounted for the remaining 8%

of infections. All recombinant forms taken together were responsible for 18% of infections worldwide (Hemelaar et al, 2006).

1.3.2.1 Molecular Epidemiology of HIV-1 in Tanzania

The first AIDS case in Tanzania was reported in the Kagera region in the northwestern part of the country in 1983 (USAID Brief, 2002). In 1985 eight of the twenty one regions of Tanzania had reported 404 AIDS cases to the Ministry of Health (MOH). The number reached 1,525 by 1986 and all the regions in the country had reported AIDS cases (NACP- MOH report, 2005). In 1988, the Ministry of Health established the National AIDS Control Programme (NACP) to coordinate the AIDS control activities and monitor the HIV/AIDS epidemic in Tanzania. Twenty-two years after the first case was reported (82-84) NACP estimates that there were over 1,532,222 people living with HIV in Tanzania. It is estimated that in that year the HIV prevalence was 6.8% in adults aged between 15-24 years, 9.9% in adults aged between 25-34 years and 8.1% in adults aged between 35 years and above, with heterosexual HIV transmission accounting for 77.2% of all HIV transmissions. With the current trend of HIV infections in Tanzania the NACP estimates that national HIV prevalence rates will increase from the current 7.4% to 7.5 % by the year 2010 (figure 1-6).

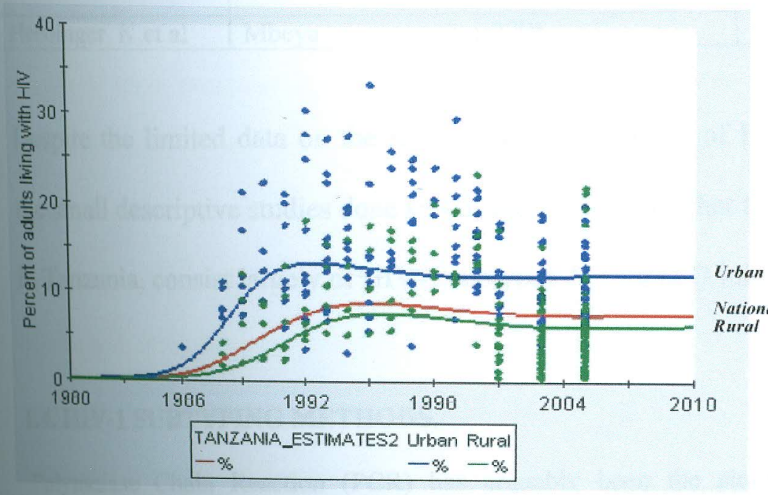


Figure 1-6. Depicts the estimated and projections of HIV prevalence for Tanzania for the period 1980 to 2010. Source; NACP-MOH Tanzania National AIDS Control Programme HIV/AIDS/STD surveillance report 2005: report No:19

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It is clear from the graph above that the number of HIV/AIDS cases in Tanzania is on the rise. Increased infections result in early mortality, loss of human resources and an increase in the number of orphans, a major impact on the economic and social stability of Tanzania. Effective preventive methods that are inexpensive, accessible and effective are therefore urgently needed in response to the current HIV/AIDS situation. An effective HIV preventive vaccine would be the most logical solution. However, this option is limited by the fact that there is limited HIV molecular epidemiological data for Tanzania and limited resources for developing and testing HIV vaccine candidates (Table 1-2).

Table 1-2. Summary of molecular epidemiology studies of HIV-1 in Tanzania

Author	Place of Study	Year of Publication	HIV-1 subtype identified
Hoelscher M et al	Mbeya	1989	A,C, D and G
Lyamuya E et al	Dar es Salaam	2000	A,C and D
Hoelscher M et al	Mbeya	2001	A,C,D and URF
Blackard JT et al	Dar es Salaam	2001	A,C, and D
Koulinska IN et al	Dar es Salaam	2002	A,D and URF
Kiwelu I E et al	Moshi	2003	A,C, D and URF
Renjifo B et al	Dar es Salaam	2003	A,C, and D
Arroyo M et al	Mbeya	2004	A,C,D and URF
Gerhardt M et al	Mbeya	2005	A,C, AC (triple infection)
McCutchan F et al	Mbeya	2005	ACD, AC (super-infection)
Herbinger K et al	Mbeya	2006	A,C,D and URF

Despite the limited data on the molecular epidemiology of HIV-1 in Tanzania the small descriptive studies done so far, clearly indicate that the AIDS epidemic in Tanzania, consist largely of HIV-1 subtypes A, C, and D infections.

1.4. HIV-1 SUBTYPING METHODS.

Polymerase Chain Reaction (PCR) has arguably been the single most important development in the past three decades with regard to the ability to characterize and

compare the genomes of viruses. It has not only been at the centre of numerous assays that have aimed at the detection of minute levels of virus but has also allowed detection of viruses that had previously been very difficult to detect, enabling the diagnosis of cases that would otherwise have gone undetected. In addition to sensitivity and specificity, another major advantage that PCR provides is the ability to amplify a relatively small portion of the viral genome (a few hundred to a few thousand base pairs) that may have been subjected to evolutionary pressures and to enter that portion into a comparison or subtype analysis while excluding the remainder of the genome. Thus, relatively small variable regions that actually define the subtype can be compared without the background clutter of the much larger genome. It is this ability that has made PCR based techniques invaluable in the molecular characterization of the HIV into subtypes, clades and recombinant forms.

To date the molecular characterization of the HIV employs numerous methods, however the most established and commonly used methods include; V3 Peptide - binding Immunoassays (PEIA), Partial/Full-Length Sequencing, Heteroduplex Mobility and Heteroduplex Tracking Assays (HMA/HTA), and the Multiregion Hybridisation Assay (MHA).

1.4.1. Peptide-Binding Enzyme Immunoassay (PEIA).

Peptide-binding enzyme immunoassays enable the detection of subtype-specific antibodies using an enzyme substrate reaction. Standard enzyme linked immunosorbant assay plates are coated with specific peptides, representing different HIV-1 subtypes. Subtype-specific antibodies in the serum samples bind to the respective peptides. An anti-human immunoglobin enzyme conjugate is added followed by the appropriate substrate. The result of the reaction is a coloured product that can be measured spectrophotometrically (figure 1-7).

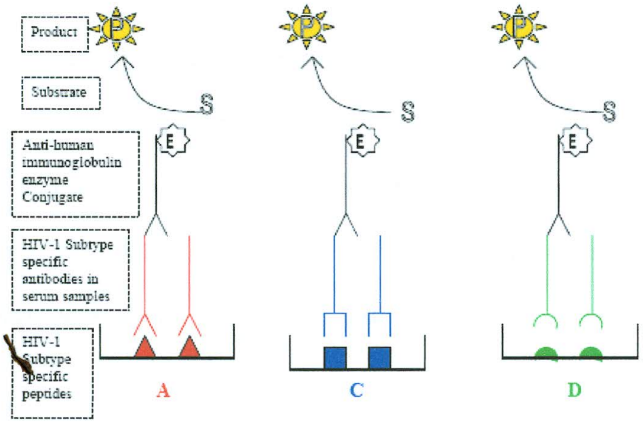


Figure 1-7. Schematic presentation of HIV -1 PEIA

HIV-1 V3 serotyping has been successfully used in several studies in Thailand and Uganda where subtypes E and B and subtypes A and D are predominant, respectively (Pau et al, 1993, Cheingsong-Popov et al, 1994, Luo et al, 1996, Subbarao et al, 1996 Kaleebu et al, 2000). In areas where subtypes A, C and D are predominant serotyping has however been difficult. This is due to lack of peptide specificity and frequent cross-reactions are observed. Generally subtype A cross-reacts with C and, to a lesser extent, subtype B with D. Although the development of the antigen limiting dilution assay that detects high avidity antibodies has improved the specificity of the V3 PEIA; its reduced specificity still limits this tool for use in countries where multiple HIV subtypes co-circulate as in the case of Tanzania (Janssens et al, 1997, Nkengasong et al, 1998, Hoelscher et al, 1998, Kaleebu et al, 2000).

1.4.2. Heteroduplex Mobility and Heteroduplex Tracking Assays (HMA/HTA).

1.4.2.1. Heteroduplex Mobility Assay.

Delwart developed the heteroduplex mobility assay (HMA) to evaluate HIV-1 viral heterogeneity (Arnes, 1999). Heteroduplexes are formed when two non-identical but closely related single-stranded DNA fragments anneal. They form structurally distorted

DNA molecules at mismatched base pairs and at unpaired bases where an insertion or a deletion in the nucleotide sequence has occurred. These structural distortions cause the heteroduplex to migrate more slowly than the homoduplex during acrylamide gel electrophoresis and thus can be detected (Figure 1-8). The HMA method has been used efficiently to analyze the relatedness of various HIV strains (quasispecies) from a single individual and from populations around the world (Delwart et al, 1993, Renjifo et al, 2003,)

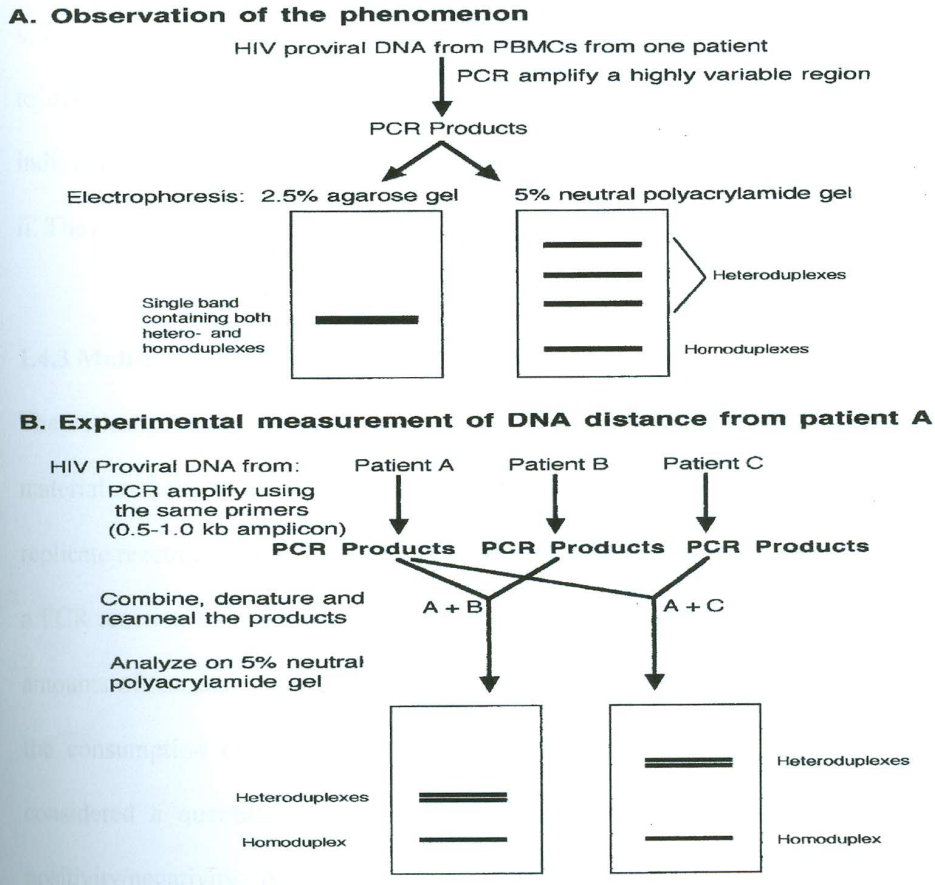


Figure 1-8.Diagramatic depiction of the Heteroduplex mobility assay. Source; Arnes M. Clinical Microbiology Rev. 1999; 12(4):612-26.

1.4.2.2. Heteroduplex Tracking Assay

A variation of the HMA methodology is to amplify by reverse transcriptase PCR (RT-PCR), products that represent an entire pool of quasispecies from an individual. This pool of PCR products is then hybridized to a radioactively labelled probe (made from a cloned region of HIV DNA), and the resulting heteroduplexes are detected by electro-

phoresis and autoradiography. This is called the heteroduplex tracking assay (HTA). Since the heteroduplexes displayed in the gel represent the relatedness of many of the quasi-species, HTA may provide the best overall analysis of genetic distances between quasispecies generated within a single individual. The HMA and HTA methods have had a huge impact on the molecular characterization of HIV subtypes worldwide. However these methods are not effective tools for screening large population samples due to the fact that;

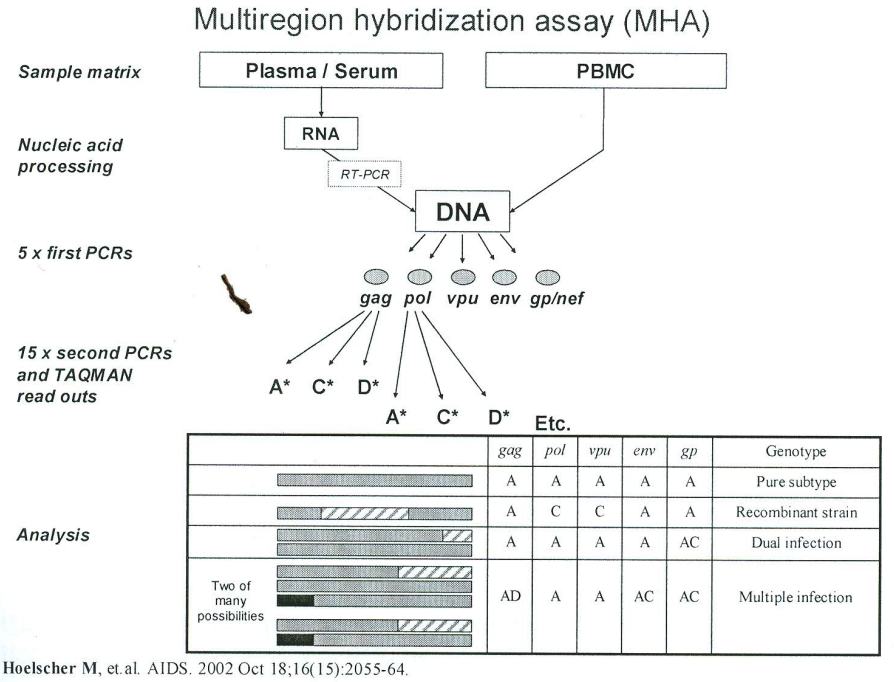
i. They only screen within two regions of the HIV genome. As a result they are unable to detect inter-subtype recombinants, intra-subtype recombinants or multiply infected individuals outside these regions.

ii. They are labour intensive and have relatively low through-put.

1.4.3 Multiregion Hybridisation Assay (MHA)

Theoretically, there is a quantitative relationship between the amount of starting material and the PCR product at any cycle. However, it is a common experience for replicate reactions to yield different amounts of PCR product. Furthermore, at the end of a PCR reaction in which the number of cycles is empirically pre-determined, different amounts of starting material can yield similar amounts of amplification product due to the consumption of the reagents. As a consequence, conventional PCR cannot be considered a quantitative assay and the results are usually expressed in terms of positivity/negativity. A semi-quantitative evaluation is possible, on the basis of the appearance of the electrophoretic band as weak or strong positive. This approach however, is highly subjective and the sensitivity is very low. To address the issue of PCR product quantification, methods such as quantitative real-time PCR (qr-PCR), have been developed (Mackay et al, 2002). Real time – PCR allows a highly sensitive quantification of transcriptional levels of the gene of interest in a few hours with minimal handling of the samples. The "Real time" PCR system utilizes the intercalator SYBER green or ethidium bromide that binds to the increasing amounts of amplified

double stranded DNA, resulting in an increase of fluorescence. An improved form of Real time PCR involving the use gene specific fluorescent probes in the PCR reaction also known as the TaqMan assay is commonly in use. The TaqMan methodology is based on the 5' nuclease assay first described by Holland et al, in 2002 which uses the 5'-3' exonuclease activity of Taq DNA polymerase to cleave a dual-labelled probe annealed to a target sequence during PCR amplification. DNA is added to a PCR reaction mixture containing standard PCR components plus a probe that anneals to the template between the two primers. This probe contains both a fluorescent reporter dye at the 5'-end (6-carboxyfluorescein, 6-FAM; emission λmax = 518 nm) and a quencher dye at the 3'-end (6-carboxytetramethylrhodamine, TAMRA; emission λmax = 582 nm). The quencher can only quench the reporter fluorescence when the two dyes are close to each other. In other words, if the probe sequence fails to bind to its target, as a result non amplification of target DNA. In event of the probe binding as a result of successful amplification of target DNA during the 2nd round PCR; the homologue probes bound to the amplicon is then cleaved by the endonuclease activity of the Taq-polymerase. This cleavage causes a disruption of the fluorescent marker and its quencher, which results in an emission of light. The intensity of this signal is directly proportional to the amount of PCR product and can be detected by an ABI 7700 TaqMan-system, Roche Light Cycler or Biorad I-Cycler (Figure 1-9).



Hoelscher M, et.al. AIDS. 2002 Oct 18;16(15):2055-64.

Figure1-10. Schematic presentation of the Multiregion Hybridization Assay (MHAacd).

1.4.4. Partial /Full-Length Sequencing

The complete sequence of the HIV-1 genome was published in 1985 using full length sequencing (Muessing et al, 1985). Sequencing (figure 1-11) basically involves extracting the target nucleic acid from cells or plasma and converting the target nucleic acid into DNA if necessary. This is then followed by amplification of the DNA, purification of the PCR products from unincorporated nucleotides and primers, re-amplification of the sequencing template that was "amplified" in another PCR mixture containing the sequencing primers, a mixture of deoxy-and dideoxynucleotides, and modified Taq polymerase. To facilitate the nucleotides sequences that make the target DNA, the dideoxynucleotides are coupled to fluorescent dyes. A different colour for each of the four bases is used; these act as terminators of the growing oligonucleotides. The large arrays of oligonucleotides are then separated in a polyacrylamide gel which is continuously scanned by a laser during the run, and the colours are detected as they pass the scan point. Each oligonucleotide in the array, as they move through the gel, is only one base shorter than the oligonucleotide above it in the gel. The detected information is

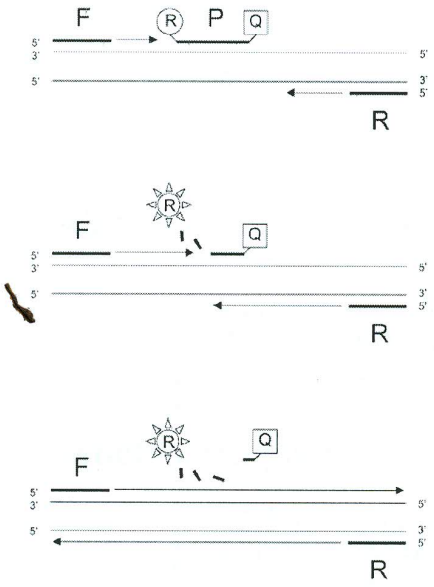


Figure 1-9. Schematic presentation of real time PCR. Source; Arnes M. Clinical Microbiology Rev. 1999; 12(4):612-26.

Hoelscher et al in 2002, using the basic principles of the TaqMan assay developed a real time PCR method, the Multiregion Hybridization Assay (MHAacd), to characterize HIV subtypes, inter-subtype recombinants and to detect HIV dual infections (Figure 1-10). This assay uses fluorescent-labelled hybridisation probes that are able to discriminate between HIV-1 subtypes A, C and D in 5 genome regions (gag, pol, vpu, gp41/MSD and intracellular gp41). Hybridisation sites were chosen in regions where HIV-1 subtype specific sequence signatures are conserved within one subtype but differ between subtypes. A difference of at least three mismatches between each subtype combination (A/C, C/D, A/D) is necessary to ensure subtype specificity. For each of the genome regions a first round PCR amplification is followed by a nested PCR, using four universal primers. To avoid cross-reactivity caused by overlapping emission spectra of different dyes, each probe is tested in a separate PCR reaction.

then fed directly into a computer for analysis and construction of the four-color electropherogram, which is then translated into the linear sequence by commercially available software.



1.5. STUDY RATIONALE

In 1984, the United States Secretary of the Department of Health and Human Services, Margaret Heckler announced to a jubilant audience "we hope to have a vaccine against AIDS ready for testing in about two years." Lifted by the general euphoria caused by the isolation of the virus that caused AIDS, Heckler concluded, "yet another terrible disease is about to yield to patience, persistence and outright genius" (Heckler, 1984). However more than twenty years later, a vaccine for HIV is still not available despite the best efforts of the international scientific community. Why then after 23 years of intensive research we still do not have a vaccine despite having developed and tested 95 candidate vaccines to date according to the International AIDS Vaccine Initiative (IAVI) report of 2007. We simply do not know enough about the factors that foster HIV viral diversity, nor do we understand the impact of viral diversity in terms of eliciting protective correlates of immunity in the host. The few studies to date worldwide that have tackled the issue of the impact of viral diversity and its role in the pathogenesis of AIDS have so far yielded no conclusive answers.

1.5.1 Impact of HIV Variability on Diagnosis

HIV-1 serologic assays are based on detection of antibodies to structural proteins, p24gag and gp41env, which are conserved among most HIV-1 isolates (Turner et al, 1999). Studies so far have shown that individuals infected with HIV-1 group O viruses, due to gp41 variations, fail to be diagnosed accurately by some of the serologic tests. This problem has to some extent been overcome by addition of group O lysate/peptides in the standard antigen mixture (Parry et al, 2003). Most of current enzyme immunoassays (EIA) and rapid tests are sensitive and specific for diagnosing persons with chronically established infections with HIV-1 group M subtypes, however some difficulties still remain in diagnosing persons with recent infections with non-B subtypes (Koch et al, 2001, Rouet et al, 2004). Pilcher et al in 2004 highlighted this when studying 1360 men attending STD clinic in Lilongwe Malawi. He demonstrated

that 24 men who had previously tested seronegative by conventional HIV antibody tests had in fact unrecognized acute HIV infections that only could be detected by viral load assay. These findings indicate that there is a need for the routine evaluation of the available commercial assays on the market to ascertain their ability to detect the majority of the newly emerging HIV variants.

HIV molecular diagnostic tests are even more challenged by the effects of viral diversity than serological tests. The generic primers in current use do not only facilitate the detection of infection with divergent viruses but also allow subsequent phylogenetic analysis of subtypes and quasi species for molecular epidemiologic studies. The primers in use however require regular updating because of the increasing number of HIV viral variants world wide because of recombination and spontaneous mutations (Peters, 2000, Lal et al, 2005).

Molecular detection of HIV plasma viremia has increased considerably in clinical settings, especially for monitoring patients on antiretroviral (ARV) therapy. Four commercial viral-load assays are available; Amplicor HIV-1 Monitor v1·5 (Roche Molecular Diagnostics), Nucleic-acid-sequence-based amplification (NASBA, Organon Teknika), HIV-1 Quantiplex branched DNA (bDNA, Chiron Diagnostics), and the LCx HIV-RNA quantitative assay (LCx, Abbott). The ability of these assays to quantify viral load from non-B subtype infections has been examined in several studies including Tanzania (Yang et al, 1999, Elbeik et al, 2000, Elbeik et al, 2002). Although, much progress has been made to improve the sensitivity of nucleic acid-based assays to quantify viral load across all subtypes of HIV-1, some RNA viral load assays still produce erroneous results (Amendola et al, 2002, Gottesman et al, 2004). Thus as with the case of serological assays more efforts have to be implemented to ensure that HIV diagnosis can be done accurately and in the early stages on infection. Early and accurate diagnosis of subjects with HIV infection may have major implications on the opportunities for HIV surveillance to change the natural history of the disease as well as provide opportunities for prevention of HIV transmission.

1.5.2. Impact of HIV Diversity on HIV Biological Characterization, Transmissibility, and Pathogenicity

HIV viral sequence variability is thought to dictate biological differences among HIV strains and partially explain the reasons for the different epidemic patterns seen in different regions of the world (Lal et al, 2005). Indeed, several reports have documented that HIV-1 subtypes may differ with respect to viral load (Hu et al, 2001), chemokine co- receptor usage (Tscherning et al, 1998), transcriptional activation levels (Jeeninga et al, 2000) and antiretroviral drug susceptibility (Wainberg, 2004, Kantor et al, 2004).

The differences in co-receptor usage and syncytia-inducing capacity displayed by HIV clades may have an impact on disease progression. The cytopathic property of B strains can be either syncytium inducing (SI) or non-SI (NSI), the former being consistent with a virus that infects T cells and replicates swiftly, whereas the latter is characteristic of a virus that infects macrophages and grows more slowly. The chief co-receptor of SI viruses is the ß chemokine receptor CXCR4, whereas that of NSI viruses is the ß chemokine receptor CCR5. Most HIV clades cause disease by assuming the CCR5+/NSI phenotype during early disease and the CXCR4+/SI phenotype during the end stages of disease (Ltyhgo, 2004, Stevenson, 2003, Thomson et al, 2002). However, this correlation does not hold true for clade A, C or D. Clade A viruses tend to favour CCR5 even at later stages, a pattern that is seen to a more extreme extent in C strains, which rarely become CXCR4+/SI even in moribund patients. Subtype D displays simultaneous tropism for CCR5 and CXCR4 throughout the course of disease (Tscherning et al, 1998, Xiao et al, 1998, Johnston et al, 2003, Cilliers et al, 2003, Pollakis et al, 2004). These findings clearly indicate that more research is needed to determine the effect HIV subtype diversity has on host tissue tropism.

Even though the exact functional consequences of viral pathogenesis of subtype diversity is not that clearly understood, there is some conflicting evidence that such differences may result in altered rates of disease progression and mother-to-child

transmission (MTCT) rates. A study done in Tanzania suggested that subtypes A, C and recombinants of A and C were more likely to be perinatally transmitted than subtype D viruses (Renjifo et al, 2001). While a large cohort study in Uganda reported no difference in rates of perinatal transmission for subtypes A and D, a large perinatal transmission study from Kenya provided a strong evidence that women infected with subtype D were more likely to transmit virus to their infants than those infected with subtype A (Yang et al, 2004).

HIV-1 subtype-specific differences in disease progression have also been studied in several natural history cohorts. For instance, a study in Senegal with limited number of patients reported that women infected with non-A subtypes were 8 times more likely to develop AIDS than those infected with subtype A (Kanki et al, 1999). A large cohort study in Uganda based on more than 1000 patients demonstrated that subtype D was associated with faster progression to death and with a lower CD4 cell count during follow-up, compared with subtype A (Kaleebu et al, 2002). However, no difference in disease progression have been found among patients infected with subtypes A, B, C and D in Sweden (Alaeus et al, 1999),or subtypes B and CRF01_AE in Thailand (Amornkul et al, 1999)

The conflicting results about the impact of viral diversity clearly indicate that additional longitudinal studies with various HIV subtypes are needed. These studies may aid in increasing our understanding of the virological characteristics of each subtype. They may help researchers to innovate superior strategies for disease control, especially when one considers that the effect of viral diversity on viral pathogenesis may be complicated by host, social and virological factors that are not yet fully understood. Furthermore, it may allow tracking of the spread of the epidemic between different populations, countries, and continents. Such information will be required for the design of HIV candidate vaccines, and may be essential for planning strategies for the utilization of future HIV/AIDS vaccines. It may also provide important information about factors, which drive the genesis of HIV recombinants. As more and more HIV- 1 variants,

inevitably intermix in different parts of the world, the likelihood of generating new recombinant viruses will increase. Continued monitoring of the HIV epidemic in Tanzania and globally is therefore necessary. The proper selection of a genetic subtyping tool that is specific, rapid and has a high through put will greatly facilitate this exercise. The MHAacd is such a tool. This tool is technically undemanding, and was specifically designed, to survey and screen populations to assess the presence of HIV-1 subtypes. Apart from its ability to detect recombinants, by virtue of the fact that the tool screens within five regions of the HIV genome instead of two regions, it has the potential ability to detect potential dual infections and super-infections. Furthermore, it is an assay that can be performed with a high through-put capacity at relatively modest costs as compared to sequencing. It is for these reasons that it was decided to use the MHAacd method to characterize the HIV-1 subtypes circulating in Mbeya region, Tanzania for this study.

1.5.3. Impact of HIV Diversity on Response to Antiretroviral Therapy and the Emergence of Antiretroviral Resistance.

The question of the influence of HIV subtype diversity on response to antiretroviral treatment is an urgent one. The most studied HIV-1 is subtype B which accounts for only 12% of the global HIV infections in contrast to clade C viruses that account for 47% of all new HIV-1 infections (Hemelar et al, 2004). The lack in the availability of clinical data for non-B subtypes with regard to response to therapy is mainly because, until recently, antiretroviral treatment has been largely unavailable in many countries with non-B subtypes of HIV-1 infections. Two concerns arise from the analysis of the available data from treatment cohorts in Africa. First, is the issue of the probability of certain subtype of HIV-1 to spread and progress more rapidly than others, thus making treatment decisions more challenging (Kaleebu et al, 2002). Secondly, the applicability of baseline data on antiretroviral susceptibility derived from studies of subtype B in non-B subtype infections (Snoeck et al, 2006). There are in fact ,many potential

problems when comparing responses to therapy among persons infected with group M, non-B–subtype strains who often live in settings of limited resources with those infected with subtype B that often live in affluent settings. Assured availability of antiretrovirals may be just one of the factors that may affect response to therapy. The available data however , is thus far encouraging. A French cohort study of 416 patients, 24% of whom carried non-B subtypes of HIV-1, showed that at 3, 6, and 12 months after initiation of therapy, that HIV-1 subtype did not affect clinical progression, CD4+ count, or viral load in response to treatment (Bocket et al, 2005). Similarily in the Paediatric European Network for Treatment of AIDS (PENTA) 5 trial there was no significant difference according to HIV-1 subtype in the virologic response to treatment or in the frequency of development of resistance among children (Pillay et al, 2002). Overall, it appears that HIV-1 subtypes do not contribute major differences in the response to antiretroviral therapy. What has emerged however is a growing body of evidence that polymorphisms found in various subtypes before antiretroviral therapy is begun may affect genetic pathways of resistance.

HIV resistance to antiretroviral therapy can be divided into two categories: primary resistance, which reflects acquisition of a drug-resistant strain of HIV by a newly infected person; and secondary, or acquired resistance, which develops after a period of HIV treatment. Studies of non-B subtypes in patients who have never received antiretroviral therapy reveal that protease and reverse transcriptase sequences vary from those of the subtype B reference strains (Kantor et al, 2004). For example, polymorphisms in the reverse transcriptase gene do not typically occur in known sites of resistance to nucleoside reverse-transcriptase inhibitors (Deroo et al, 2002, Bellocchi et al, 2005). Data from several studies indicate that protease sequences from non-B subtypes in patients who were antiretroviral therapy naive contained amino acid substitutions associated with mutations in subtype B known to contribute to secondary

resistance. These, included mutations K20→R, M36→I, and H69→K/Q (Grossman et al, 2001, Holguin et al, 2004). These genotypic changes however, do not confer consistently decreased susceptibility by themselves when viral strains are subjected to phenotypic testing. Recent data on in vitro resistance against maraviroc, the recently approved entry inhibitor and CCR5 antagonist, do not suggest that there are differences among subtypes. However, the differences in co receptor tropism among subtypes noted above suggest that intersubtype variation in response to therapy could exist in vivo (Westby et al, 2007). Studies of resistance patterns that have emerge in non-B subtypes in patients receiving antiretroviral therapy indicate that polymorphisms present in these subtypes before therapy may provide a background for the emergence of subtype-specific pathways to secondary resistance (Taylor et al, 2008). Mutations leading to resistance appear to be similar among subtypes, but certain mutations seem to occur more frequently in non-B subtypes in particular. In a study of patients in Botswana infected with subtype C, in those without a response to a didanosine- or stavudine-based regimens, virus containing K65→R mutations developed within 8 months, more rapidly either D30→N or L90→M nelfinavir-resistance mutations (Soares et al, 2003, Grossman et al, 2004). Overall, than is seen in patients infected with subtype B (Frater et al, 2001). Subtype C viruses have been shown to develop resistance against nonnucleoside reverse-transcriptase inhibitors through either the K103→N or V106→M mutations, whereas subtype B viruses rarely develop V106→M mutations (Grossman et al, 2004). Nelfinavir resistance appears to occur primarily through L90→M mutations in subtypes G and C and other non-B subtypes, whereas subtype B acquires it appears that most antiretroviral resistance mutations in non-B subtypes are accounted for within the current available HIV resistance databases; however, further studies of treated cohorts

1.6. STUDY OBJECTIVES

1.6.1. Broad Objective

To conduct the molecular characterization of HIV-1 subtypes circulating in the Mbeya Region Tanzania.

1.6.2. Specific Objectives

1. To characterize and monitor the distribution of HIV-1 subtypes in Mbeya.
2. To characterize and monitor the emergence and distribution of HIV-1 recombinants.
3. To monitor the emergence and prevalence of HIV-1 dual/multiple infections.
4. To determine the prevalence of genotypic antiretroviral drug resistance mutations in drug naïve HIV infected individuals.
5. To determine the prevalence and persistence of HIV drug resistance mutations in drug naïve HIV infected pregnant women receiving single dose of nevirapine prophylaxis for the prevention of mother to child transmission of HIV.

CHAPTER 2. HIV-1 MOLECULAR SUBTYPE CHARACTERIZATION OF A FEMALE HIGH RISK COHORT IN MBEYA TANZANIA

2.1. INTRODUCTION

The HIV-1 genome in nature is characterized by its rapid evolution, which permits the virus to escape immune surveillance and to develop drug resistant variants as well as making it difficult to produce an effective vaccine. The evolution of HIV-1 diversity is driven by two major mechanisms, recombination and a high mutation rate. The extensive genetic variability of HIV-1 is primarily due to the high error rates of the viral reverse transcriptase, which results in approximately 10 genomic base changes per replication cycle, in addition to substitutions, deletions and insertions which may also occur (Keulen et al, 1997, Drosopoulos et al, 1998, Hahn et al, 2000). With a mutation rate of 2.5 to 5.8 per 10,000 nucleotides, the AIDS virus is thus able to maintain a constant pool of viral quasispecies which are on average 3-5% divergent (Peeters, 2000). Accumulations of these mutations overtime may lead in a shift of the viral sequence composition, increasing the diversity of the existing viral quasi species. The effects of mutations are attributable to the differential geographic distribution of the multiple genetic subtypes of HIV-1. Within a single geographic region, the range of genetic variability in the env gene is estimated to be 6-19%, although differences higher than 30% have been documented (Lal et al, 2005, Stebbing J et al, 2003, Peteers et al, 2000). The extent of genetic variability in a given geographical location increases over time after the introduction of a particular subtype in a population. If immune protection is dependent on variable HIV sequences, then HIV vaccine development will become a moving target, which may require the continuous adaptation of specific vaccines, as is the case today with influenza vaccines

The issue of HIV diversity is further complicated by the fact that HIV has to its disposal, an additional mechanism of generating diversity, namely recombination. The

effects of recombination are much more visible than those of mutation alone. However the prerequisite for recombination is the simultaneous co-infection of a single cell with at least two different provirus strains. Co-infection of a cell with at least two proviral strains, results in the generation of recombinant viruses within two weeks following co-infection (Levy et al, 2004). Infections with more than one proviral strains have been reported with HIV-1 and HIV -2 infections (Peeters, 2000); with different subtypes of HIV-1 group M strains and divergent strains of the same subtype (Hoelscher et al, 2002, Gerhardt et al, 2005, McCutchan et al, 2005). These forms of multiple HIV infections by two distinct viruses can occur simultaneously or near the same time or one after another. Recombination of the two distinct viruses in the host cells, results in radically different progeny genomic combinations that may stable as in the case of HIV-1 CRFs.

2.1.1 Viral Super-Infection and Its Implications

The worldwide prevalence of multiple strains in HIV infected individuals is an area of HIV research that has until of recently been uncharted waters. The discovery of large numbers of recombinant viruses clearly implies that co infection with divergent HIV-1 strains is not as rare as once thought. Indeed, during the past few years, a number of reports have described HIV-1 superinfection in human subjects (McCutchan et al, 2005, Jung et al, 2002, Koelsh et al, 2003). These reports have challenged the assumption that HIV-1–specific immune responses generated during primary infection are protective against subsequent infection and have raised concern, not only with respect to HIV-1–positive individuals engaging in unsafe sex but also from the standpoint of developing effective vaccines.

There are two basic mechanisms by which multiple HIV-1 infections are thought to occur. These include the dual HIV infection mechanism, which is a result of either co infection with two viral variants infecting at or near the same time before seroconversion, or the superinfection mechanism in which an individual acquires a second HIV strain some time after seroconversion from a primary HIV infection. The

frequency of HIV superinfections and pathogenic consequences of HIV superinfections are largely unknown, although there are several studies that have clearly proved that dual infection does occur (Jung et al, 2002, Papathanasopoulos et al, 2003). Studies on the pathogenic consequences of dual infections have indicated that dual infection may be associated with rapid disease progression to AIDS (Gottlieb et al, 2004). Determining the prevalence of super/dual infection may assist in answering basic future questions such as; is it likely that virus superinfection and recombination will become increasingly common as more people become infected with HIV but live longer as a result of more effective treatments. Our understanding of the mechanics behind multiple infections in terms of the; (1) the effectiveness of the immune response induced by initial HIV infection in conferring protection against subsequent superinfection; (2) the biological consequences of superinfection with regard to development of recombinants, viral fitness, viral drug resistance, virus transmissibility, and the natural history of disease and its progression ; (3) how to monitor for viral diversity and the development of more-sophisticated techniques for assessing superinfection; and (4) how to assess the relevance of superinfection and recombination in future vaccine designs, the development of diagnostic tests, antiretroviral drug susceptibility testing, and virus load measurement are key to controlling the global HIV and AIDS epidemic.

2.2. STUDY DESIGN

2.2.1. Study Site

The study site was Mbeya region (figure 2-1), located in South-western Tanzania. Mbeya region is located along the Trans African highway, which interlinks Kenya, Uganda and the northern parts of Tanzania with Zambia, Malawi, Zimbabwe and South Africa. Twenty sites, including fourteen villages that serve as trading areas along this highway, several border towns, and other areas that have busy markets, were included in the study

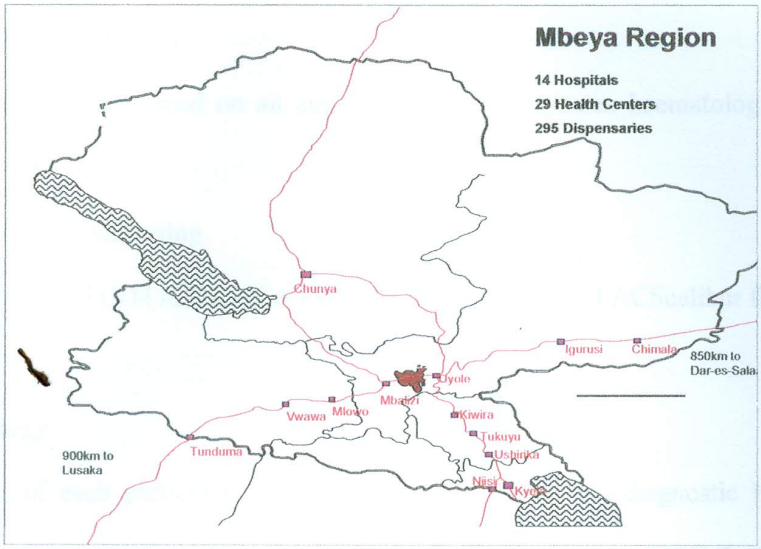


Figure 2-1 Mbeya Region Tanzania . Source www.mmrp.org

2.2.2 Study Population

Six hundred women from a high-risk population (bar and local brew shop sellers) in Mbeya region, Tanzania, were enrolled between September and November 2000 into a prospective HIV Superinfection Study (HISIS) within the Mbeya Medical Research Programme. The women were aged 18-35 years and worked in bars along the Trans-African highway and were suspected to be highly exposed to HIV-1 subtypes A, C, and D, the three predominant HIV-1 subtypes circulating in East Africa. At each visit an information/education campaign was conducted to promote condom utilization and safe sex practices. The participants were also provided with free health care, and prompt treatment of sexually transmitted infections (STI's) and other diseases throughout the study period of 36 months. At the time of this study antiretroviral treatment was not yet available as standard treatment for HIV and AIDS in the country.

2.2.3 Laboratory Processing of Samples

After providing informed consent, the following specimens were collected every 3 months for 3 years from each of the study participants.

2.2.3.1 Haematological analysis

Full blood counts were performed on an automated KX-21N sysmex haematological analyser.

2.2.3.2 CD4 Immunophenotyping

CD4 and CD8 counts and CD4 /CD8 ratios were determined by a BD FACScalibur flow cytometer.

2.2.3.3 HIV serology

The HIV-1 status of each participant was determined by using two diagnostic HIV antibody assays, enzyme-linked immunoassay test (Enzygnost Anti HIV1/2 Plus (DADE Behring Germany) and Determine HIV 1/2 rapid (Abbott UK) (Gerhardt et al, 2005). Samples reactive on both tests were considered HIV positive. Discordant results were resolved by Western blotting as per manufacture's instructions (Genelabs Diagnostics USA).

2.2.3.4 HIV viral load assay

HIV viral load testing was performed using a ROCHE Amplicor HIV monitor version 1.5.

2.3 MHA (acd) HIV-1 MOLECULAR SUBTYPING

Cyropreserved peripheral blood mononuclear cells (PMBC) isolated from the citrated blood Ficoll-hiscopaque centrifugation; in aliquots of 5-10 million that had been stored in liquid nitrogen were used for HIV-1 subtyping.

2.3.1. HIV DNA Extraction

HIV DNA was extracted from 200µls of thawed cyropreserved PBMCs from all prevalent HIV cases from the HISIS cohort; using the Roche High Pure Viral Nucleic Acid Kit (Cat. No. 1 858 874) according to the manufactures instructions. The extracted DNA was then stored at -20^0 C for further processing.

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2.3.2. HIV -1 Genetic Characterization by the Multiregion Hybridization Assay (MHAacd)

The MHAacd real time PCR method was performed as described by Hoelscher et al in 2002 see Table 2-1.

Table 2-1. MHA (acd) Primers and Probes

REGION	OUTER PRIMERS	INNER PRIMERS	SUBTYPE SPECIFIC PROBES
GAG HXB2-pos. 1195 1212	MHGAG1 : AGT ATG GGC AAG CAG GGA	MHGAG3: GGT CAG YCA AAA TTA YCC TAT AG	SUBTYPE A:GAGsAp; CAA AAT GCA CAA GGG CAA SUBTYPE C: GAGsCp CAG AAT CTC CAA GGG CAA SUBTYPE D:GAGsDp CAA AAC CTA CAG GGR CAA ATG
	MHGAG2: CTG CAG CYT CNT CAT TGA TGG	MHGAG4: GGC TCC TTC TGA TAA TGC TG	
POL /PROTEASE HXB2-pos 2168 2193	MHPOL1 : TTC AAC TGT GGC AAR GAA GG	MHPOL3: TTT AGG GAA AAT TTG GCC TTC	SUBTYPE A:POLsAp; CCC YCA TCC CAA AGA TCT CTG SUBTYPE C: POLsCp TGT CTC CTC GAA CCT GAA GC SUBTYPE D:POLsDp TCC TCC CCA AAC CCG AA
	MHPOL2: ACT GTA TCA TCT GCT CCT GT	MHPOL4: TGC CAA AGA GTG ATT TGA GG	
VPU (Beginning of envelop) HXB2-pos.8582-8589/ 8631-8657	MHVPU1 : CCT ATG GCA GGA AGA AGC GG	MHVPU3: GCA GAA GAY AGT GGC AAT GAG	SUBTYPE A:VPUsA1p; TC CCC AAG ATC ATA GTT CCC SUBTYPE A: VPUsA2p TT CCC AGG ATT ATA GTT CCC C SUBTYPE C:VPUsCp ATC CAA AAG CCT AAG ATG CC SUBTYPE D:VPUsDp CCA AGG AGC ATG ATG CC
	MHVPU2: CTT GTG GGT TGG GGT CTG	MHVPU4: GGT ACA CAG GCA TGT GTR GCC C	
ENV REGION HXB2-pos.8111-8134 / 8199-8222	MHENV1 : ACG CTG ACG GTA CAG GCC AG	MHENV3: GGA TCA ACA GCT CCT AGG RAT TTG G	SUBTYPE A:ENVsAp; CAY TTG TCC AAT GCC AAT AAG TC SUBTYPE C:ENVsCp CCA ACT GTC CAA TGC TAR TAA ATC SUBTYPE D:ENVsDp
	MHENV2: CTT GCT CTC CAC CTT CTT CTT C	MHENVU4: GGT GAR TAT CCC TGC CTA ACT C TA	

			TTG TCA ATT TCT YTT TCC CAC TCC
End of GP41 REGION HXB2-pos.8111-8134 / 8199-8222	MHGP1: GAA GAA GAA GGT GGA GAG C	MHGP3: CCT GTG CCT CTT CTG CTA CC	SUBTYPE A:GPsAp; CCA GTC TCA ACC CCT TGA GAC
	MHGP2: TTC NAA GCC CTG TCT KAT TC	MHGP4.1: CAG CCA GCT ACT RCT ATT GCT MHGP4.2: CCY TCA GCT ACT RCT ATT GCT	SUBTYPE C:GPsCp CTG TAG TCC CCT GAG ACT GCT G SUBTYPE D:GPsDp AGC ACT ATT CTT TAG TTC CTG AAT CCA

2.3.2.1 MHA First Round PCR

Five first round MHAacd PCR's were set up representing the five regions of the HIV-1 genome to be amplified, namely gag, pol, vpu, env and gp. For each PCR reaction, five separate master mixes (Table 2-2) were prepared using the appropriate primers.

Table 2-2. MHAacd first round PCR master mix

PCR Components	Volume per sample	Reagents Source
Aqua dest H_2O	26.5 µl	House aqua dest
dNTPs (each 2.5mM)	10µl	Q-BIOgene (Germany) Cat no. NTACG100
10x PCR Buffer (100mM Tris-HCL and 500mM KCL)	5.0 µl	Applied Biossytems (USA) Cat no. 4311820
MgCl2 (25mM)	3.0µl	Applied Biossytems (USA) Cat no. 4311820
Primer 1 (e.g. MHAGAG 1) (20µl)	2.5µl	Thermoelectron Corporation (Germany)
Primer 2 (e.g. MHAGAG 2) (20µl)	2.5µl	Thermoelectron Corporation (Germany)
AMPi Taq Gold polymerase (5u/µL)	0.5 µl	Applied Biossytems (USA) Cat no. 4311820
DNA Template	4 µl	
TOTAL VOLUME	54 µl	

The 50µl master mix was used per sample. One template was prepared for each of the five HIV-1 regions to be amplified; for the negative controls (NC) 4µl of H_2O was added instead of DNA.

The thermocycler protocol for the first round MHA cycle was as follows: 95°C for 10 min; 36 cycles of 95°C for 15 sec, 52°C annealing for 45 sec, and 72°C for 2 min; followed by one cycle of 72°C for 10 min and a final cycle of 20°C for 30sec. The first round PCR product was stored at -20°C in between PCR runs

2.3.2.2 MHA Second Round PCR / Taqman Run

For each first round PCR product three separate second round MHA PCR runs or Taqman runs were run. These runs correspond to three subtype probes required for subtype recognition. 25µl of the second round master mix per sample was used. To each well, except for the negative controls 1µl of first round PCR product was added see Table 2-3.

Table 2-3. MHAacd Taqman run master mix

PCR Components	Volume per sample	Reagents Source
Aqua dest H2O	10.5 µl	House aqua dest
Taqman Universal Master mix	12.5 µl	Applied Biossytems (USA) Cat no. 4324020
Primer 4 (e.g. MHAGAG 3) (480nM)	0.5µl	Thermoelectron Corporation (Germany)
Primer 2 (e.g. MHAGAG 4) (480nM)	0.5µl	Thermoelectron Corporation (Germany)
Probe (e.g. GAGspA) (220nM)	0.5 µl	Applied Biossytems (USA)
1st round PCR product	1.0 µl	
TOTAL VOLUME	25.5 µl	

The 96 well PCR plates covered with special Applied Biosystems (ABI) optical caps were placed in an ABI TAQMAN 7770 for thermocycling. The thermocycler protocol

for second round (MHA Taqman) run was as follows: 95°C for 10 min; 40 cycles of 95°C for 15 sec, 55°C annealing for 45 sec, and 72°C for 90 sec; followed by one cycle of 72°C for 10 min and a final cycle of 20°C for 30sec.

2.3.2.3 Confirmation MHA Taqman PCR Results

To confirm the MHA Taqman results, and to exclude PCR contamination or subtype probe binding failure all the second round PCR products were subjected to Agarose gel electrophoresis. A 2% agarose Gel (2gm in 100ml 1xTBE buffer + 3µl ethidium bromide) was prepared. 10µl of second round PCR product mixed with 4µl Bromophenol blue dye was loaded onto the gel, mixed together with 2µl of a 100bp molecular weight ladder. Electrophoresis followed immersion of the loaded gel immersed in 1xTBE buffer and subjected to 80-100 volts for 1-1.5 hours. After band separation, the gel was examined under UV light (302 nm), photographed and stored using the computer program GRABBIT. PCR contamination was excluded if the negative controls were blank. The Taqman results were accepted if the gel bands conformed to the expected PCR product size.

2.3.3. Cloning

The samples that gave a dual probe signal in the Taqman assay and had the correct agarose gel band were considered as potential dually infected. DNA from samples of potentially dually infected study subjects, were re-extracted and subjected to a nested PCR using a multiple primer combination approach. The multiple primer approach was used to increase the sensitivity of recovering viral quasispecies. Amplicons of 1.1 to 1.4 kb from regions 1 (gag/pol), 2 (vpu/gp120), and 3 (gp41/nef) were generated. Region 1 stretches from the p17 protein gene in gag to the beginning of the pol gene; region 2 starts in the middle of vpu and covers the entire gp120 gene, and genome region 3 contains the gp41 gene and half of the nef coding region. Two outer primer pairs and two inner primer pairs were chosen and used for each of the three regions. Four

different combinations of the primers were used in four separate PCR reactions (figure 2-2).

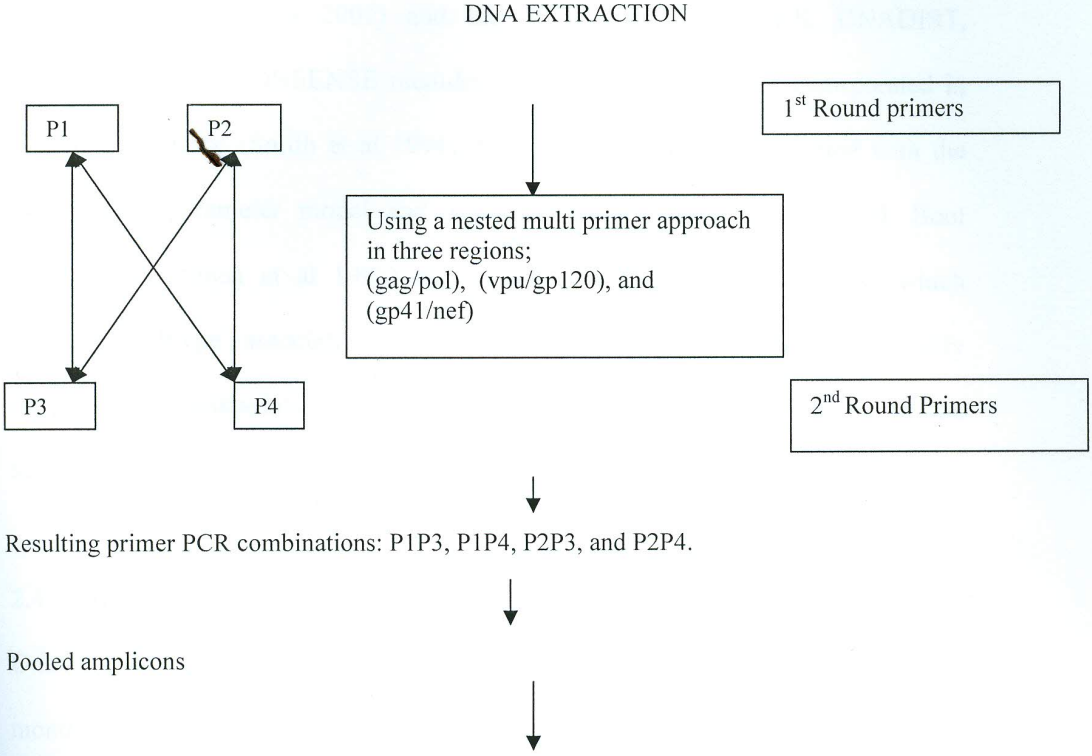


Figure 2-2. Diagrammatic representation nested PCR using a multiplex primer approach.

The PCR mixtures contained a 200 µM concentration of each deoxynucleotide triphosphate, 1.5 mM MgCl2, 15 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.2 µM (each) primers, and 3.75 U of AmpliTaq Gold . Five microliters (for genome regions 1 and 2) or 10 µl (for genome region 3) of extracted DNA was added to the first-round PCRs. One microliter (for regions 1 and 3) or three microliters (For region 2) of the first-round products were used for the second round of amplification. The thermocycler protocol was as follows: 95°C for 10 min; 35 cycles of 95°C for 10 sec, 52 ^{0}C annealing for 30 sec, 72°C for 2 min for amplification; and one cycle of 72°C for 10 min. Table 2-4 shows the primers and annealing temperatures used.

2.3.3.2. Phylogenetic Analysis

The sequences were assembled with Sequencher, aligned with reference sequences of all HIV-1 subtypes, and phylogenetically analyzed with the MEGA, version 3.1, program (Kumar et al 2001) and the SEQBOOT, DNAPARS, DNADIST, NEIGHBOR, and CONSENSE modules of the PHYLIP package incorporated in the GDE interface (Smith et al 1994). Distance analysis was performed with the Kimura two-parameter model and a transition/transversion ratio of 2.0. Boot scanning (Salminen et al 1995) and informative site analysis software, which tabulates subtype associations in 10-bp increments, were used to identify recombinant breakpoints, which were confirmed by visual inspections of the sequence alignment and/or by subregion trees.

2.4. MHA RESULTS

One hundred twenty one (121) prevalent HIV infected women each with at least 21 months follow-up samples and 2 samples of deceased HIV positive women with at least 12 months of follow-up were subtyped using the MHAacd method.

2.4.1. HIV Prevalence and Incidence

The overall HIV sero-prevalence for the 600 HISIS participants was 68.7% at enrolment, while the cumulative HIV incidence over 3 years follow up (36 months) was 17%.

2.4.2. MHAacd Assay Performance

A total 1653 Taqman runs were conducted on the 123 extracted samples. Out of the123 samples, 98 (80 %) could be subtyped in at least one region. Samples were accorded a subtype when probe signal could be detected in at least three regions. The amplification and probe detection efficacy of the assay was best in the gag region whereby 85.8 % of the samples resulted in a detectable result, in contrast, the amplification and PCR detection rate for the env region was only 48.2% (figure 2-3).

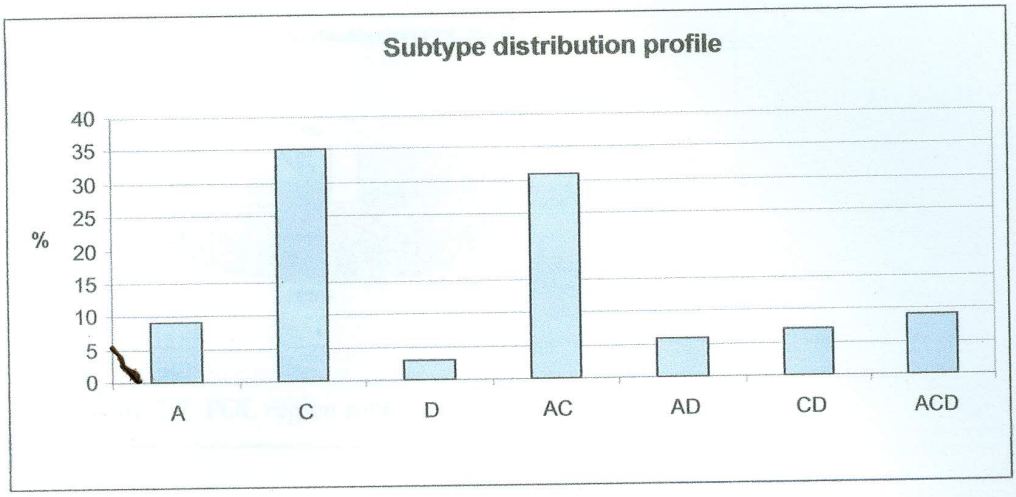


Figure 2-5. Graph depicting MHAacd Subtype distribution profile

2.4.3.1 Region Specific MHAacd Subtype Distribution Profile

The majority of samples in the gag and pol regions subtyped as clade C, 52% and 65% respectively. In the case of vpu, env and gp regions the majority of the samples subtyped as clade A, 78%, 44% and 50% respectively. 15.4% of all the samples gave a dual probe signal, with the gp region having the highest number of dual probe binding signals at 13% see Figures 2-6 to 2-10.

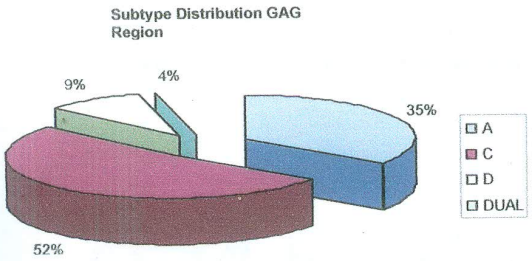


Figure 2-6. GAG Region subtype distribution chart

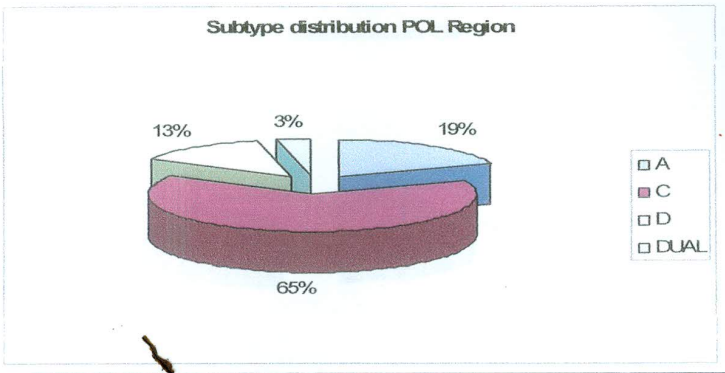


Figure 2-7. POL region subtype distribution chart

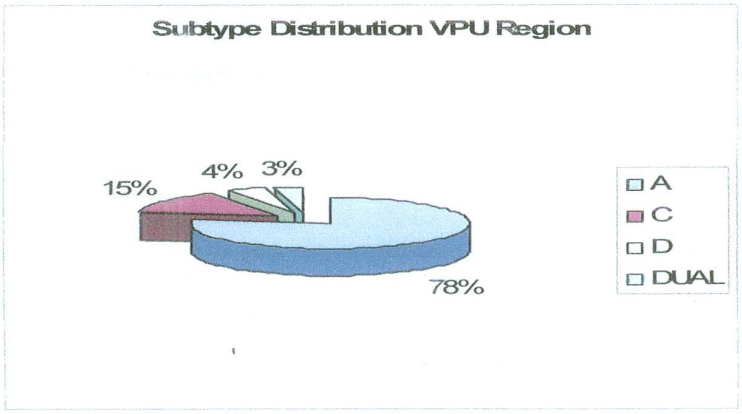


Figure 2-8. VPU region subtype distribution chart

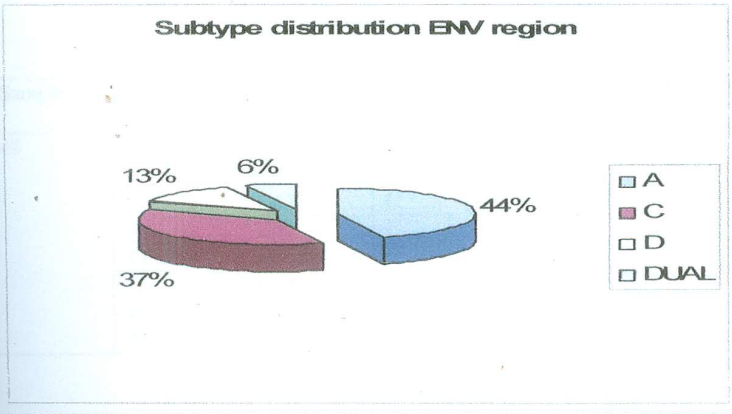


Figure 2-9. ENV region subtype distribution chart

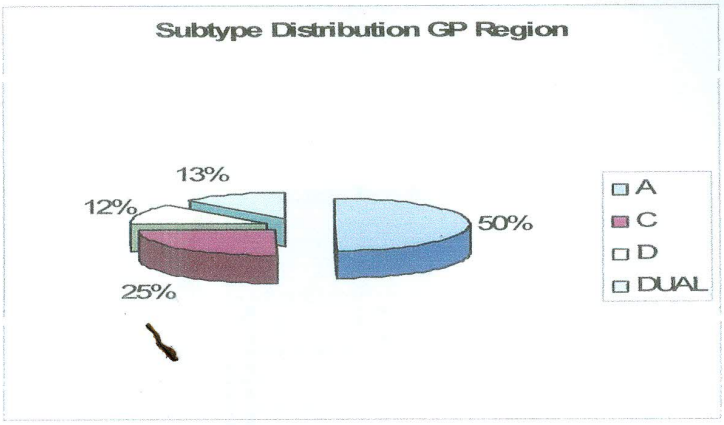


Figure 2-10. GP region subtype distribution chart

2.4.3.2 Confirmation of Taqman Multiply Infected Samples by Cloning and Sequencing

DNA from patient study number 507, a deceased 22 year old bar worker and patient study number 48; a 31 year old bar worker considered to be potentially dually infected (Table 2-5 and 2-6), were re-extracted and subjected to a nested PCR using a multiple primer combination approach. Three amplicons of 1.1 to 1.4 kb from regions 1 (gag/pol), 2 (vpu/gp120), and 3 (gp41/nef) were generated. The amplicons were cloned into an E.coli TOPO cloning vector according to the manufactures instructions (see figure 2-11).

Table 2-5. Taqman Results for Sample 48

Sample	Month of follow up	Gag	Pol	Vpu	Env	Gp
48	0	C	C	C/A	PCR-VE	C
	3	C/D	C	C	PCR-VE	C
	6	C/D	C	C/A	PCR-VE	C
	9	C/D	C	C/A	PCR-VE	C
	12	C	C	C/A	A	C
	15	C/D	C	C/A	A	C
	18	C	C	C/A	A	C
	21	C/D	C	A	PCR-VE	PCR +VE

Table 2-6. Taqman Results for Sample 507

Sample	Month of follow up	Gag	Pol	Vpu	Env	Gp
507	0	A	A	C/A	A	A
	3	A	A	C	A	A
	6	A	A	C/A	A	A
	9	A	A	A	A	A
	12	A	A	C/A	A	A

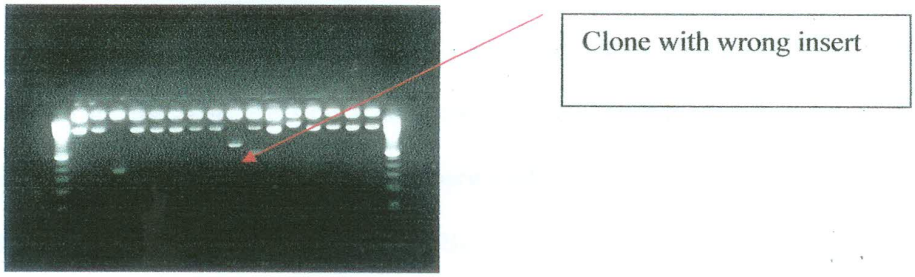


Figure 2-11. TOPO Cloning Vector with Correct and Incorrect Inserts For Sample 48

2.4.3.3. HIV Multiple Infections

The sequences from the clones of samples 507 and 48 were assembled with Sequencher, aligned with reference sequences of all HIV-1 subtypes, and phylogenetically analyzed with the MEGA, version 3.1, program and the SEQBOOT,DNAPARS, DNADIST, NEIGHBOR, and CONSENSE modules of the PHYLIP package incorporated in the GDE interface. Distance analysis was performed with the Kimura two-parameter model and a transition/transversion ratio of 2.0. Boot scanning and informative site analysis software, which tabulates subtype associations in 10-bp increments, were used to identify recombinant breakpoints, which were confirmed by visual inspections of the sequence alignment and/or by subregion trees. In depth phylogenetic analysis of the sequences recovered from sample 507 suggested that the patient may have been triply infected (Gerhardt et al, 2005). Figure 2-12 depicts the phylogenetic tree for region 1 (GAG/POL), of sequences from sample 507. Three distinct molecular forms could be deduced upon analysis of the GAG/POL region (region I); they included a pure A viral molecular form (I), and two AC viral molecular

forms with different breakpoints (form II and III), suggesting that the sample was multiply infected with at least two distinct HIV strains. Fig 2-13 represents the phylogenetic tree for region II (VPU/GP120) of sample 507. In this, figure eight (8) distinct molecular forms were identified (forms I-VIII). Forms I indicates an infection with a clade C viral strain, Form II a clade A1 viral strain, form III a clade A2 viral strain, forms IV-VII various forms of AC recombinant viral strains and form VIII various molecular forms of A1A2 intrasubtype viral recombinant forms. The findings from this region are highly indicative of a triple HIV-1 infection. Similarly, with regard to region III (GP41/NEF), the distribution of recovered viral molecular forms as presented in Fig 2-14, are similar to those recovered in region I. In region III, four molecular forms could be deduced, one pure clade A viral form (form I) and three A/C recombinant viral forms (forms II-IV). Based on the findings of the in depth analysis of the three regions of sample 507, it would be fair to conclude that participant 507 was multiply infected.

In the case of sample 48 it was however, only possible to recover the C clade virus sequences from the clones picked from regions GAG/POL and VPU despite repeated attempts, thus multiple infection could not be confirmed.

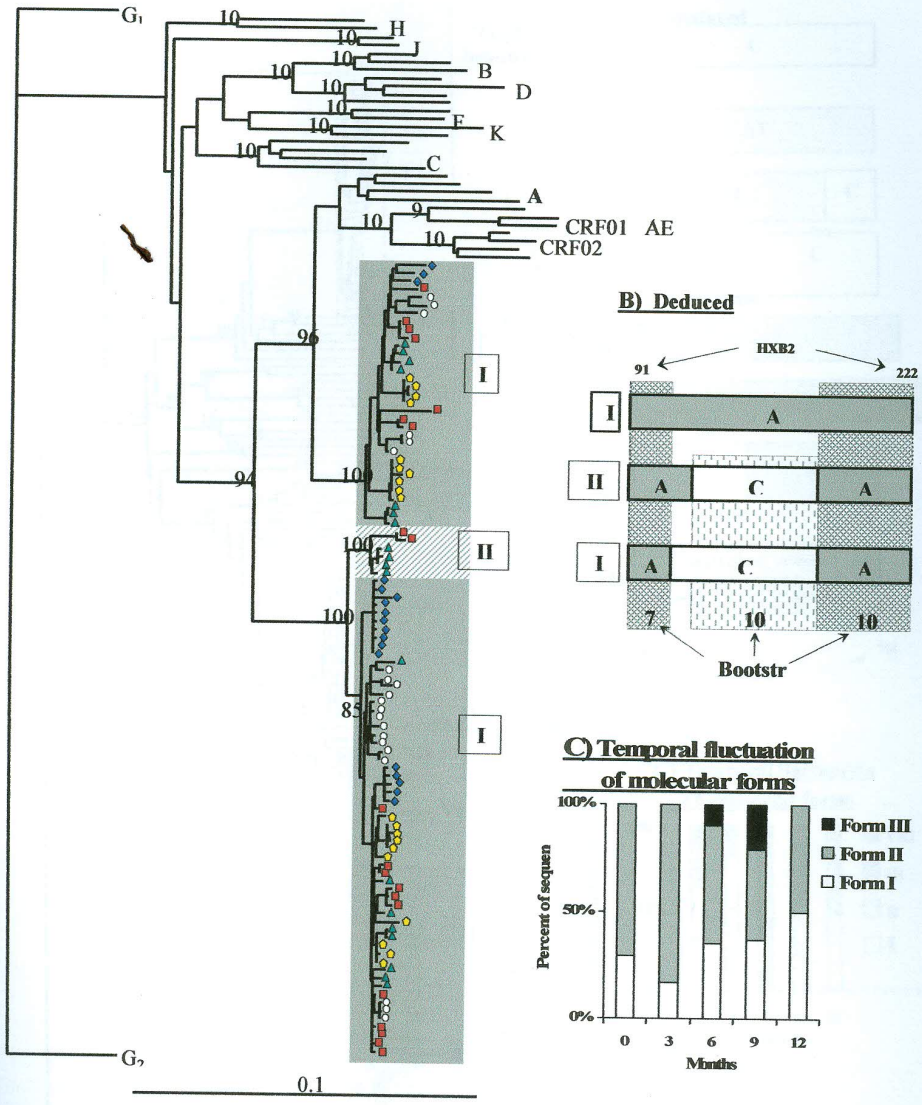


Figure 2-12. Phylogenetic Tree for Region 1 (GAG/POL).

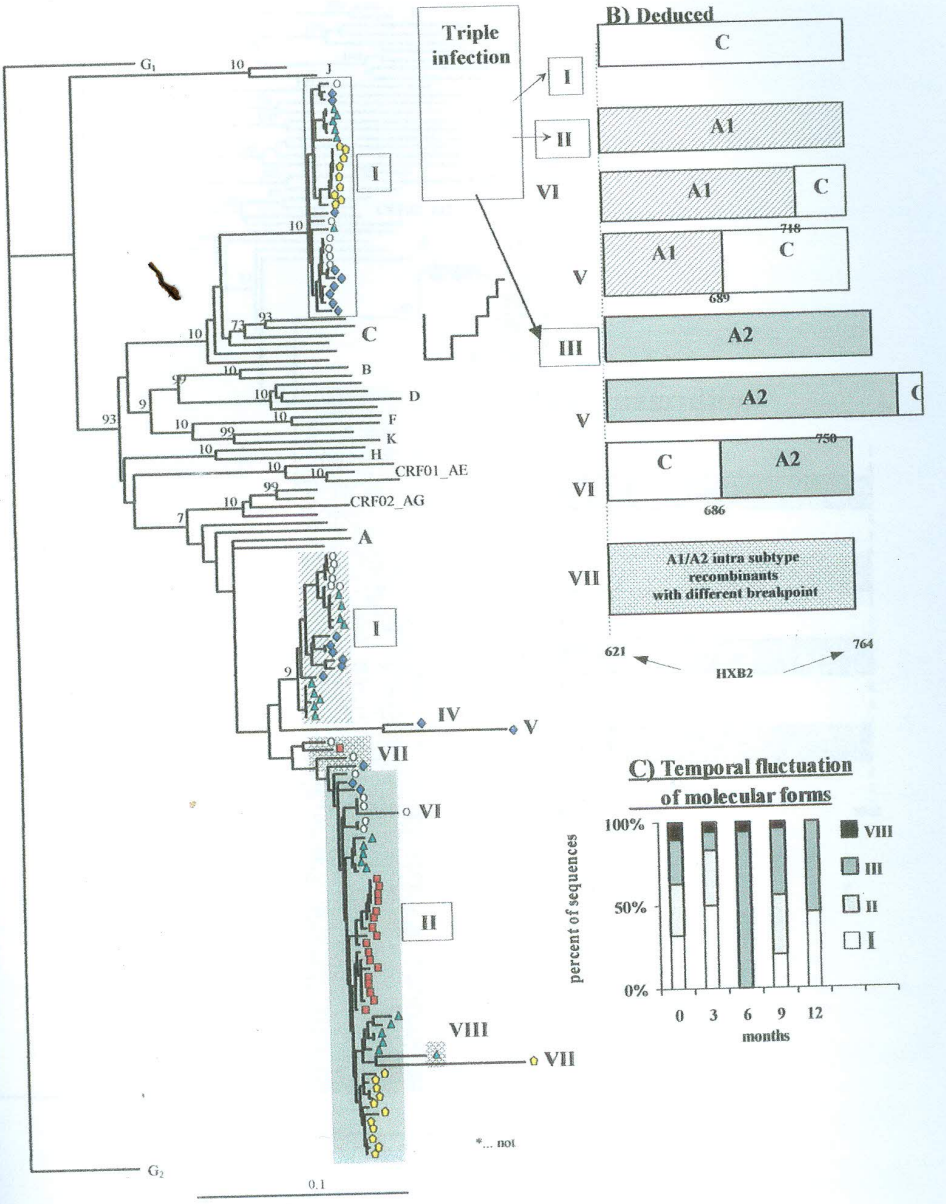


Figure 2-13. Phylogenetic Tree for Region 2 (VPU/GP120)

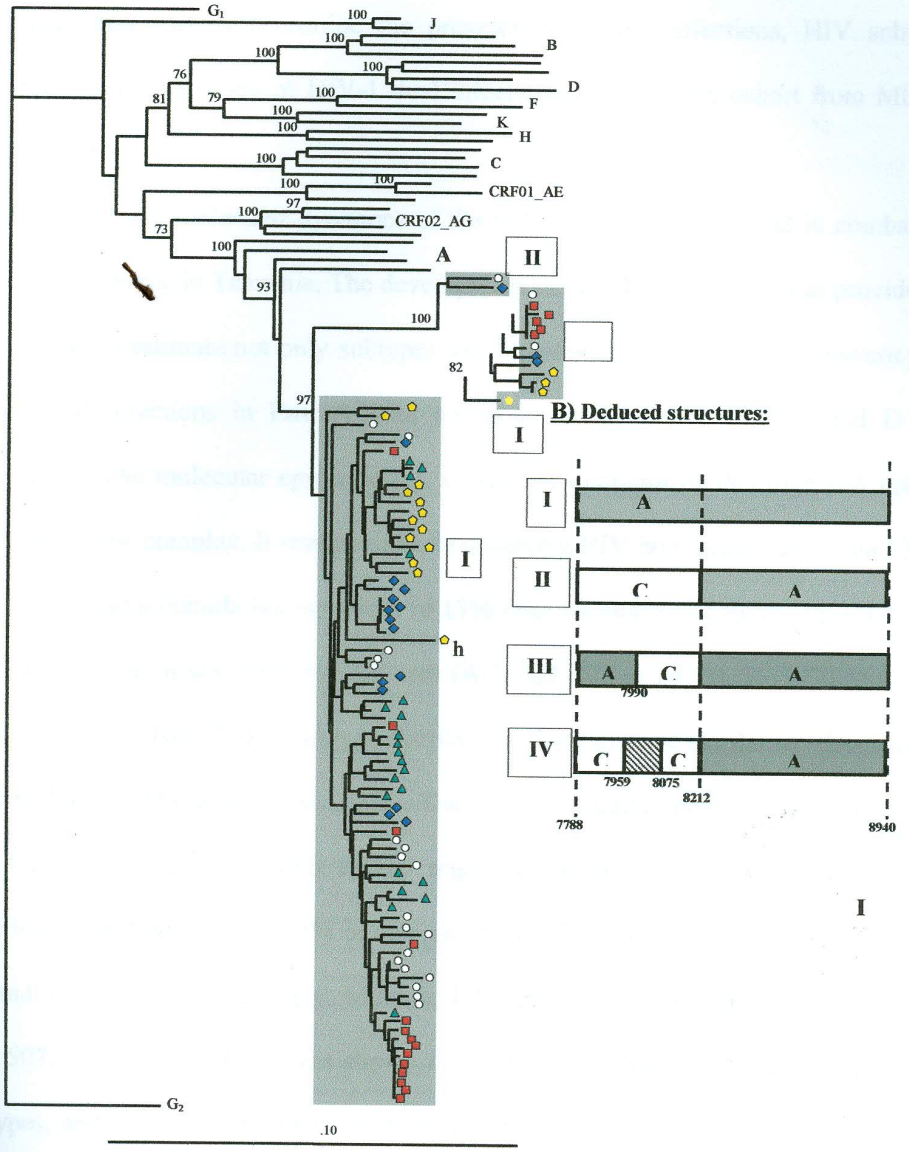


Figure 2-14. Phylogenetic Tree for Region 3 (GP41/NEF)

2.5. DISCUSSION

The study aim was to determine the prevalence of HIV infections, HIV subtype distribution and frequency of HIV-1 dual infection in a high-risk cohort from Mbeya region, Tanzania.

Understanding the molecular dynamics of the HIV-1 epidemic is crucial in combating the AIDS epidemic in Tanzania. The development of the MHAacd assay has provided a new avenue to estimate not only subtypes and recombinants, but also the frequency of HIV-1 dual infections in East African countries where subtypes A, C and D are circulating. The molecular epidemiology of HIV -1 subtypes of this high risk cohort was found to be complex. It was possible to observe a HIV prevalence rate of 68.7% at recruitment and a cumulative incidence of 17% over 36 months of follow up. The HIV subtype distribution was 54% recombinant (AC, AD, CD or ACD), 35% Clade C, 9% clade A and 3% clade D. The subtype distribution findings were similar to other studies done in Mbeya (Hoelscher et al, 2002). The general putative dual infection frequency was found to be 15.4 %, with the gp region harbouring the majority of the dual infections with frequency of 13% in comparison to 6%, 4%, 3% and 3% for env, gag, pol and vpu regions respectively. Multiple infection was established for study subject case 507. This study subject was shown to have been infected by at least 3 different subtypes, and it was possible to observe that there was temporal fluctuation of HIV-1 molecular forms in the multiply infected individual over time (Herbinger et al, 2006, Gerhardt et al, 2005).

The MHAacd assay with its 80%-90% ability to subtype HIV-1 samples seems an efficient tool for screening large population cohorts for HIV-1 subtype distribution. This tool's unique ability to detect dual infection, is however daunted by a number of short comings, the major one being primer mismatching (Herbinger et al, 2006, Gerhardt et al 2005, and Arroyo et al, 2005). There is a clear indication from the study, that regions that are generally conserved such as gag, have a high sample amplification rate (85.8%), whereas regions known to have high mutation rates such as pol or env have reduced

PCR efficiency of 60.8% and 48.2% respectively. The MHAacd primer mismatching observed seems to inadvertently lead to a regional bias when it comes to determining the HIV-1 subtype distribution patterns in populations. As a result, more conserved regions of the HIV genome will most likely give a PCR result while the reverse is true for hyper variable regions. One may then question whether the proportions of subtypes and recombinants determined by MHAacd is sufficiently accurate to support differences between population sites, as primer bias seems to influence the recovery of viral strains. It is thus imperative that in order to ensure a more accurate reproduction of the distribution patterns of the circulating quasispecies in populations in future, a multiple primer approach be employed in MHAacd subtyping assays.

MHA probe cross reactions and probe binding failures are the other drawback in terms of efficacy of the MHAacd assay. Probe cross reactions and probe binding failures may inadvertently lead to an overestimation or underestimation of the real frequency of HIV-1 dual infections in populations. The effect of probe binding failure has been demonstrated in the study of HISIS participant 507(Gerhardt et al, 2005). The MHAacd results for patient 507, suggested that the subject may have been dually infected with a clade A and recombinant A/C recombinant. In depth analysis of HIV PCR products from this subject using a multiple primer approach and partial sequencing, revealed that 507, was triply infected with two distinct clade A viruses , numerous clade A/C recombinant forms as well as several clade A intrasubtype recombinants. Case 507 clearly indicates that MHAacd assay underestimated the number of subtypes in a sample as it failed to differentiate between:

(i) intersubtype recombinants that had breakpoints outside the subtype specific probe region.

(ii) intrasubtype recombinants even if they had break points within the subtype specific region.

As a result, there was gross underestimation of the number of HIV molecular forms circulating in an individual if one were to use the MHA data alone.

On the other hand probe cross reactions may lead to an over estimation of dual infections as is with the case of HISIS study subject 48. The MHA data for this individual suggested a C/D dual infection in the gag region. However unlike in the case of 507 we could only obtain gag sequences corresponding to a clade C strain after cloning and sequencing. It thus can only be speculated that the MHA dual signal in the gag region was a result of probe cross reactions or that it was true signal that failed to amplify due to lack of appropriate primers for that particular viral clade D strain.

To date five versions of the MHA assay have been developed worldwide, MHAacd, MHAbf, MHAcrf02, MHAacd version 2.0 and MHAbce version 2.0 (Hoelscher et al, 2002, Hierholzer et al, 2002, Kijak et al, 2004 , Arroyo et al, 2005 and Kijak et al, 2007). These newer versions of the assay have tried to surmount some of deficiencies of original assays, but even these must be re-evaluated from time to time to ensure it will be possible to isolate the majority of the circulating HIV-1 strains worldwide to prove their efficacy.

The availability of MHA assays for practically all the major circulating HIV-1 subtypes worldwide gives the MHA a tremendous advantage over other subtyping tool, in addition to the fact that the MHA assays are non labour intensive, but still able to accurately with a high throughput characterize HIV-1 strains at an affordable cost. This makes them an attractive inexpensive alternative method as compared to full genome sequencing. All in all the HIV subtyping results of this study have opened interesting avenues for research in understanding HIV viral dynamics in relation to host immune response. It would be of interest to study the factors that control the temporal fluctuation of the viral molecular forms in multiply infected individuals that was observed in this study. The results of such studies may provide insights on viral factors that facilitate the persistence of some viral strains over others while evading the host immune system at the same time. An understanding of these factors including the global knowledge of HIV-1 subtype distribution profiles will expedite in depth studies on the effect HIV-1 subtype diversity on pathogenesis, drug resistance, transmissibility and the factors that

favour the genesis of recombinants. The target of an effective AIDS preventive or therapeutic vaccine for Tanzania or for the world may no longer be elusive.

2.6 CONCLUSION

In this work, it has been possible to determine that the HIV-1 molecular epidemiology of Mbeya region, Tanzania is complex, with a mixture of pure and recombinant HIV-1 forms are co-circulating and where multiple HIV subtype infections are common. The MHAacd assay used in this study has been shown to be a robust HIV-1 subtyping tool screening large population cohorts in Tanzania.

CHAPTER 3.0 GENOTYPIC RESISTANCE TESTING OF AN ANTIRETROVIRAL NAIVE HIV SEROPOSITIVE COHORT OF FEMALE BAR WORKERS OF MBEYA REGION

3.1. INTRODUCTION

The new generation of drugs targeting the virus has revolutionized the treatment of infected individuals with HIV and AIDS. There are currently 29 United States Food and Drug Agency (FDA) approved drugs belonging to four different classes including nucleoside analogue reverse transcriptase (RT) inhibitors (NRTIs), non-NRTIs (NNRTIs), protease inhibitors (PIs) and a fusion inhibitor which are currently used in various combinations to treat HIV-infected patients (FDA home page accessed April 2007). Highly active antiretroviral therapy (HAART) has dramatically altered the human immunodeficiency virus type 1 (HIV-1) pandemic in the developed world. Most patients treated with HAART will maintain clinically undetectable plasma virus loads with concomitant dramatic decreases in mortality and morbidity. Such therapy has sharply reduced HIV transmission, morbidity and mortality, but has also created the long-term problem of antiretroviral (ARV) drug resistance (Adje-Tourea et al, 2003). One of the most daunting challenges facing clinicians is the problem of treatment failure in their patients. There are several factors involved in the development of treatment failure in HIV-infected patients; these include pre-existing resistant viral variants, poor quality drugs, patient-specific factors such as adherence and pharmacologic genetic factors. Host immunologic status is also important because the more advanced the disease is, the less likely it is that a complete response to ARV therapy will occur.

Treatment failure due to the rise of drug-resistant viral strains remains the most serious problem confronting clinicians, pharmacists, clinical virologists and patients alike. Drug-resistant HIV variants are now becoming so widespread that an increasing number of patients are experiencing "primary" resistance, having been infected with drug-resistant and sometimes multidrug-resistant strains (Shafer, 2002, Adje-Tourea et al,

2003 , Kantor et al, 2003, Spira et al, 2003, Cheung et al, 2004, Weinstock et al, 2004, Konings et al, 2004, Grossman et al, 2005).

The genesis of HIV drug resistant strains in drug naive as a well in patients on HAART is a function of HIV's diversity as well as the infidelity of the viral RT enzyme (Oude E et al, 1997). It has been shown that the Y181C and Y181I mutations render group O and HIV-2 viruses resistant to all drugs within the entire NNRTI class, respectively (Quinones- Mateu et al, 1998). Clade F viral strains show some degree of resistance to the non-commercialized NNRTI, the TIBO compound, while remaining sensitive to other NNRTIs, such as nevirapine and delaviridin (DLV), as well as NRTIs and PIs (Apteri et al, 1998). Some clade C isolates from treatment-naive Zimbabweans have been reported to have inherent resistance against NNRTIs due to the presence of a G190A mutation (Loemba et al, 2002).

With the increasing frequency of non-subtype-B (non-B) isolates in the world and the recent introduction and scaling up of access to antiretroviral drugs in developing countries, there is a need to characterize the resistance mutations in non clade B HIV strains. The biological consequences and the impact of both viral and host genetic variation and polymorphism on the success of drug therapy in persons infected with HIV-1 non-B subtypes remain to be defined. Especially in view of the fact, that genotypic analyses of HIV viruses around the world have shown many nucleotide changes, polymorphisms and secondary mutations within the RT and protease regions, that may be associated with the emergence of resistance to drugs used for the treatment of HIV-1 (Valle-Bahena et al, 2006, Nkengasong et al, 2004, Adje-Toure et al, 2003,Vergne et al, 2003 Holguin et al, 2002 & 2006, Vergne et al, 2000).

The true role of clade diversity on antiretroviral therapy in HIV-1 infection has yet to be established. The few studies that have investigated this issue seem to indicate that clade diversity has an impact on the development of resistance to antiretrovirals. A recent study in the Ivory Coast, showed that HIV-1 infected patients with predominantly A/G recombinants rather than clade B viruses, had a higher prevalence of drug resistance

genotypic polymorphisms (57.4%) and were more likely to express drug-resistant strains after starting HAART (Adje et al, 2001). The Ugandan HIV network for prevention trials 012 study using a single-dose NVP to prevent mother-to child transmission of HIV, showed that selection of genotypic mutations associated with resistance to NVP occurred more frequently at 6-8 weeks postpartum in women infected with subtype D than in women infected with subtype A viruses (Eshleman et al, 2001).

3.2. STUDY DESIGN

One hundred and two (102) available -80^{0}C stored plasma samples of HIV -1 seropositive ART naive women part of the HISIS bar workers cohort, were retrospectively screened between June 2004 and October 2005 for genotypic antiviral drug resistance using an in House drug resistance genotyping method developed at the Max Von Pettenkoffer Institute, Munich, Germany.

3.3 MATERIALS AND METHODS

3.3.1. Extraction of HIV Nucleic Acid.

HIV nucleic acid was extracted from 400µls of stored plasma samples or 200 µls PBMCs stored in liquid nitrogen from 102 prevalent HIV cases of the HISIS cohort; using the Roche High Pure Viral Nucleic Acid Kit (Germany). The volume of Plasma and reagents used were double the volumes recommended by the manufacture in order to increase the efficiency of the extraction of samples with low viral loads (400-1000 copies/ml).

3.3.2. HIV -1 Genotypic Drug Resistance Testing

A modified nested PCR followed by sequencing method developed at the Pettenkoffer Institute Munich (Eberle et al, 1995) was used for this study. The first round PCR generated a 1.6Kbp fragment of the GAG/POL region, corresponding to HXB2 positions 2023–3645. The second round products generated three DNA fragments of

430bp, 442bp and 517bp in length respectively. These fragments corresponded to HIV 1 genome protease gene (PR) (codons 1-99), the left and middle gene fragments of the viral reverse transcriptase (RT) gene (1-255) as shown in figure 3-1 and table 3-1.

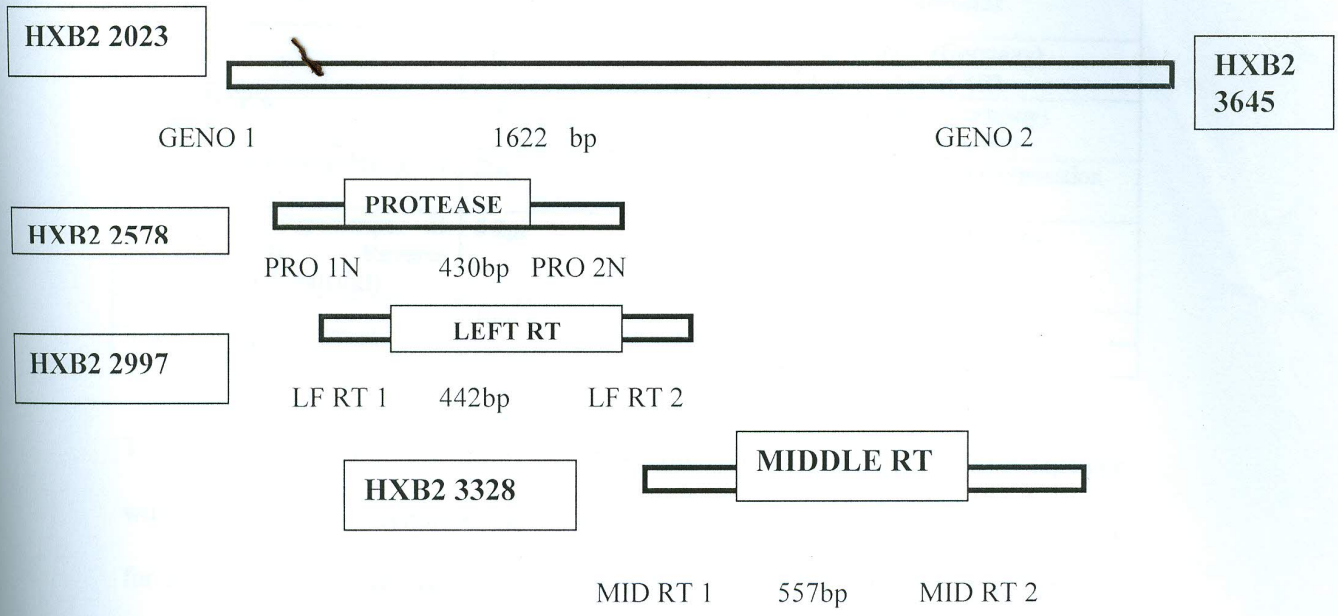


Figure 3-1. In House Resistance testing PCR Fragment Map.

Table 3-1. In House resistance testing PCR and Sequencing Primers

REGION	FORWARD PRIMER (5')	REVERSE PRIMER(3')
GAG/POL HXB2-pos. 2023 3645	Geno 1N: GGC TGT TGG AAA TGT GGR AAR GA	Geno 2N TTA CAT CAT TAG TGT GGG CA(G/C)
PROTEASE HXB2-pos. 2578 3010	PRO 1 AGA GCCC AAC AGC CCC RCC	PRO 2 ACT GGT ACA GTY TCA ATR GG
LEFT RT HXB2-pos. 2997 3440	LRT 5 TAG TCC TAT TGA RAC TGT RCC AGT	LRT 3 GAA GCA CAT TGT ACT GAT AYC TR
MIDDLE RT HXB2-pos. 3328 3890	MRT 5 TCT GGG ARG TIC ART TAG GIA TAC C	MRT 3 AAY TTC TGT ATR TCA TTG ACA GTC CA

3.3.3 cDNA synthesis (RT – PCR)

Extracted RNA was incubated at 65^0C for 10 minutes before transcription into cDNA to make it linear. The cDNA master mix was then prepared as shown in table 3-2.

Table 3-2. Resistance Genotyping cDNA master mix composition.

PCR Component	Per sample	Reagent Source
5x Buffer (SUPERSCRIPT BUFFER)	4µl	Invitrogen (Germany) Cat no. 18064-022
DTT	2µl	Invitrogen (Germany) Cat no. 18064-022
5mM dNTP's	2µl	Q-BIOgene (Germany) Cat no. NTACG100
Geno 2N (reverse primer)(10 µM	2µl	Thermoelectron Corporation (Germany)
Invitrogen Superscript II Rnase H Reverse Transcriptase (200U/µl)	0.2µl	Invitrogen (Germany) Cat no. 18064-022
Linear Viral RNA	10µl	
Total volume	20.2µl	

Tubes containing 10 µl heated (65^{0}C for 10 minutes) extracted RNA sample together with 10.2µl of cDNA synthesis master mix was placed in a 45^{0}C water bath for 1 hour for transcription to take place. The cDNA was stored at -20^{0}C till the first round PCR run.

3.3.4. First round genotypic drug resistance PCR

A single first round reaction was set up for each sample. For the first round PCR 10 µl of cDNA was inoculated into 40µl of first round PCR master mix (table 3-3).

Table 3-3. Genotypic Drug Resistance Testing First Round PCR Master Mix

	Per sample	Reagent Source
H_2O	17µl	House aqua dest
1.25mM mix (62.5 of each) dNTP's	14µl	Q-BIOgene (Germany) Cat no. NTACG100
10 X Buffer No.1 with magnesium chloride	5µl	ROCHE (Germany) Cat no. 11681842001
Primer 1 (10µM) Geno 1N	2µl	Thermoelectron Corporation (Germany)
Primer 2 (10µM) Geno 2N	2µl	Thermoelectron Corporation (Germany)
Taq (Roche Expanded long Template PCR system (5U/µl)	0.75µl	ROCHE (Germany) Cat no. 11681842001
cDNA	10µl	
Total volume	50µl	

The thermocycler conditions for the first round was as follows: 92°C for 2 min; 30 cycles of 92°C for 10 sec, 52°C annealing for 30 sec, and 68°C for 120 sec; followed by one cycle of 68°C for 7 min.

3.3.5. Second Round In House Genotypic Drug Resistance PCR

Three sets of second PCR reactions namely PRO, LFRT and MRT were set up to correspond to the three regions of the HIV pol gene to be amplified. For each second round PCR reaction 1 µl of first round product was inoculated into 49µl of second round PCR master mix (table 3-4). The thermocycler conditions for second round was as follows: 92°C for 5 min; 30 cycles of 92°C for 1 min, 50°C for 30 sec, and 72°C for 90 sec; followed by one cycle of 72°C for 7 min.

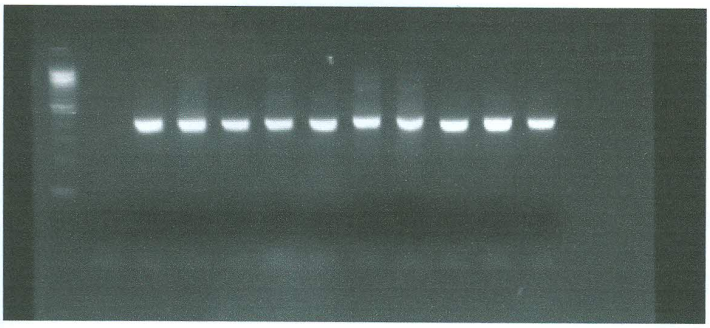
Table 3-4. Example of Genotypic Drug Resistance Testing Second Round PRO region PCR master mix.

	Per sample	Reagent Source
H_2O	26µl	House aqua dest
1.25mM dNTP's	8µl	Q-BIOgene (Germany) Cat no. NTACG100
10 X Buffer with magnesium chloride	5µl	GE Healthcare (USA) Cat no. 27-0799-05
e.g. Primer Protease 1 (10µM)	5µl	Thermoelectron Corporation (Germany)
e.g. Primer Protease 2 (10µM)	5µl	Thermoelectron Corporation (Germany)
Taq (Amersham Biosciences 5U/µl)	0.4µl	GE Healthcare (USA) Cat no. 27-0799-05
First round PCR product	1µl	
Total volume	50µl	

3.3.6. Confirmation of PCR Results

To confirm PCR results and exclude PCR contamination, all the second round PCR products were subjected to gel electrophoresis. A 2% Agarose gel (2gm in 100ml 1xTBE buffer + 3µl ethidium bromide) was prepared. 10µl of second round PCR products mixed with 4µl Bromophenol blue dye was loaded onto the gel mixed together with 2µl of a 100bp molecular weight ladder (Invitrogen Germany). Electrophoresis

followed in 1xTBE buffer at 90 volts for 1 hour. The resulting gel bands were examined under UV light (302 nm), photographed and stored using the computer program GRABBIT. PCR contamination was excluded if the negative controls were clearly negative. The PCR results were only accepted if the gel bands conformed to the expected PCR product size (figure 3-2).



100bp NC 1 2 3 4 5 6 7 8 9 10 11

Figure 3-2. Photograph of gel for 11-protease region (Pol) second round PCR products (517bp).

3.3.7 Sequencing

Second round PCR products of the correct size were sequenced according to the protocol described by Sanger et al in 1977.

Six sequencing reactions consisting of 1.5 µl of sequencing buffer, 0.5 µl 3.1 Big Dye, (Applied Biosytems), 1.5µl (pmol) or either forward or reverse primer (Thermo electron, Germany), and 2µl of second round PCR products were performed for each sample. The sequencing thermocycling conditions were as follows; 25 cycles of 96°C for 30 sec, 50°C for 15 sec, 60°C for 4 min.

The resulting sequencing reaction product was then cleaned with the Dye Ex 2.0 spin Kit (Qiagen, Germany), followed by a 99% and 70% ethanol precipitation clean up reaction to remove excess dideoxynucleotides and primers. Twelve microliters of ethanol precipitated sequencing product was dissolved in 13µl solution of HiDi

formamid (Applied biosytems, USA) before read being on a 96 well ABI 3730 sequencer.

3.4. RESULTS

3.4.1 PCR Efficacy.

One hundred and two (102) plasma samples were extracted and underwent nested RT-PCR. Of these 92 % of samples gave a positive PCR result. Two hundred and eighty eight (288) PCR products were sequenced. The DNASTAR analysis program was used to edit and create the sequence contigs, after which, 92 working protease contig sequences and 89 RT contig sequences were obtained for analysis and evaluation. The resulting sequences contigs were analyzed using the Stanford University HIVdb: Drug Resistance Algorithm (Beta Test) http://hivdb.stanford.edu/pages/algs/HIVdb.html. This database compares the sequences submitted to reference resistance sequences and scores the resistance mutations as shown in the example below.

HIVdb: Drug Resistance Algorithm (Beta Test) version 4.0

Home | Analyze a sequence | Analyze a mutation list | Release Notes

SeqID: B4PRO.fas_1 Contig_1 Date: 15-Mar-2005

Summary Data

Sequence includes PR: codons: 1 - 99

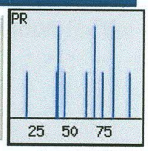
There are no insertions or deletions

Subtype and % similarity to closest reference isolate:

1. *PR: A (97.3%)*

Sequence Quality Assessment

Gene	QA Problem	Codons
PR	Stop Codons, Frame Shifts:	*None*
PR	B,D,H,V,N:	*None*
PR	Unusual Residues:	*None*



Blue lines indicate differences from consensus B; tall blue lines indicate sites associated with drug resistance. Red lines indicate QA problems.

Drug Resistance Interpretation

PI Major Resistance Mutations: None
PI Minor Resistance Mutations: **M36I, L63P, V77I**
PR Other Mutations: I13V, E35D, R41K, R57K, H69K, L89M

	Protease Inhibitors
APV	Susceptible
ATV	Susceptible
IDV	Susceptible
LPV	Susceptible
NFV	Susceptible
RTV	Susceptible
SQV	Susceptible

PR Comments

- M36I/V is associated with resistance to each of the PIs when present with other mutations. M36I occurs commonly in certain non-subtype B viruses. M36L is a rare variant of unknown significance.
- L63P is a common polymorphism that is associated with resistance to PIs when present with other mutations. The effects of other mutations at this position - which occur not too infrequently - have not been studied.

3.4.2 HIV subtype distribution of Protease and RT Sequences.

The majority of the sequences isolated for both the protease (69.9%) and RT region (67.5%) typed as clade C see figure 3-3 and 3-4.

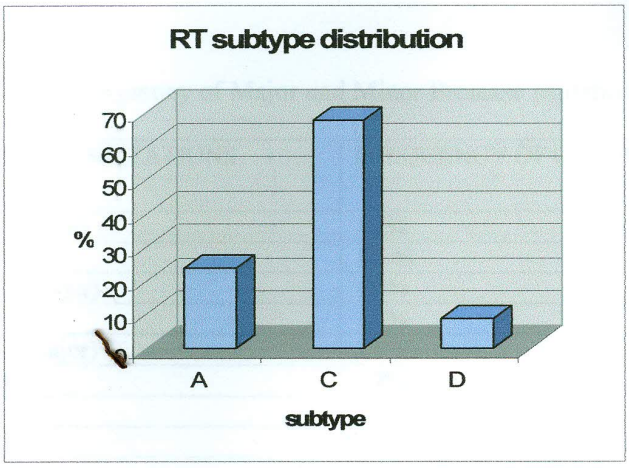


Figure 3-3. Graph depicting HIV subtype distribution for the RT region

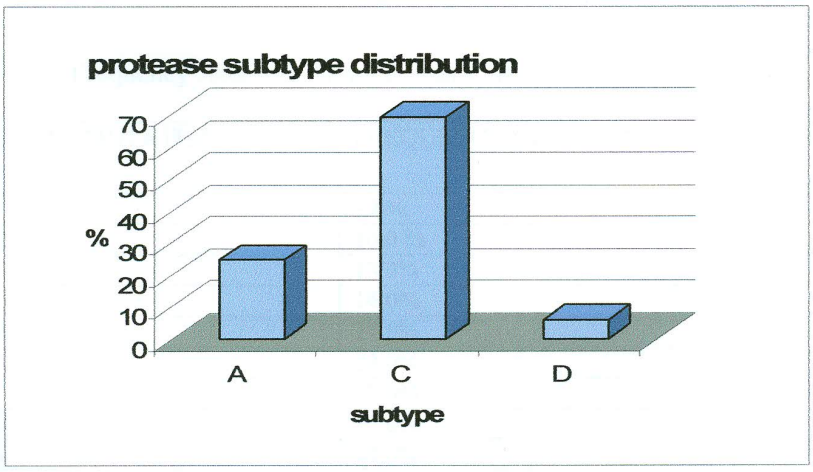


Figure 3-4. Graph depicting HIV subtype distribution for the POL region

3.4.3 Frequency of Protease and R T Region Mutations

Analysis of the recovered PR and RT sequences for HIV-1 drug resistance associated mutations, revealed only 3 primary mutations, namely M46V, L33F and L90M associated with the PR region and the Nucleoside Reverse Transcriptase associated mutation V118I (89 sequences)(see tables 3-5 and 3-6.)

Table 3-5. Frequency of Major and Minor Protease Mutations

PROTEASE MUTATIONS	FREQUENCY OF OCCURENCE (92 sequences)
L33F (major)	1.2%
A71T	1.2 %
M46V (major)	1.2%
I84V	1.2%
L90M (major)	3.5%
L10V	5.8%
D60E	5.8%
V77I	7.0%
V82I	7.0%
K20R/M	10.5 %
I93V	54.0 %
L36P	56.0 %
M36I/V	77.6 %

Table 3-6. Frequency Acessory Minor Protease Mutations

PROTEASE MUTATIONS	FREQUENCY OF OCCURENCE (92 sequences)
I64 V	8.0%
K45R	10.0 %
N37S	13.3%
G16E	14.0%
R57K	21.0%
K14R	23.0%
I13V	27.0%
E35D	35.0%
T12S	44.0%
I15V	50.0 %
L19I	67.0 %
L89M	80.0 %
H69K	90.0 %
R41K	90.0%

Table 3-7: Frequency of NRTIs mutations

NRTIs MUTATIONS	FREQUENCY OF OCCURRENCE (89 sequences)
K219E	1.0%
V118	1.3%

Table 3-8 Frequency of NNRTIs mutations

NNRTIs MUTATIONS	FREQUENCY OF OCCURRENCE (89 sequences
A98G	1.3%
K101 N	1.3%
K103R	1.3%

3.5 DISCUSSION

HIV resistance mutations are commonly labelled as either primary (or less commonly 'major') or secondary (or 'minor') Hirsch et al, 2000). Primary mutations reduce drug susceptibility by themselves whereas secondary mutations reduce drug susceptibility or improve the replicative fitness of isolates with a primary mutation (Shafer, 2002).

Antiretroviral drug resistance is the major obstacle to durable treatment of HIV-1. Our current understanding, of drug resistance is largely limited to HIV-1 epidemics in areas of the world where subtype B viruses dominate and where antiretroviral therapy is readily accessible. A number of studies of subtype B HIV-1 infections show that the presence of drug resistance before starting a new drug regimen is an independent predictor

of virologic response to that regimen (Hanna, 2001, D'Aquila, 2001,Haubrich, 2001, Demeter, 2001). In randomized clinical trials, subjects whose physicians have access to drug resistance data, particularly genotypic resistance data, respond better to therapy than control subjects, whose new regimens are crafted without access to the results of these assays (Meynard et al, 2002, Tural et al, 2002).

In this study we provide one of the first descriptions of the prevalence of resistance mutations to antiretroviral drugs in chronically HIV infected treatment-naive female patients from Southern highlands of Tanzania. All viral isolates subtyped belonged to HIV-1 group M subtypes, A, C and D. In depth analysis of the recovered PR and RT sequences for HIV-1 drug resistance associated mutations, revealed only 3 primary mutations, namely M46V, L33F and L90M associated with the PR region and the Nucleoside Reverse Transcriptase associated mutation V118I for the 89 sequences analysed (0.045%).

All PR region sequences isolated and analyzed had at least one secondary resistance associated mutation. Furthermore, all PR region sequences expressed at least one of the

three commonly detected secondary PR mutations, namely M36I, L36P and I93L. Ninety six percent (96 %) of all PR region sequences dually expressed these secondary associated mutations. All PR region sequences also expressed a number of accessory secondary mutations that have not as yet phenotypically been associated with primary ARV drug resistance (see table 3-7). Current knowledge of ARV drug resistance mutations has so far indicated that only primary ARV drug resistance mutations are important for development of drug resistance to PI's in clade B HIV-1 viral strains (Holguin et al, 2006). This may not be the case for Mbeya with its complex HIV-1 molecular epidemiology. The relevance of these accessory mutations such as L89M, R41K or H69K, that were observed in high frequency in the viral isolates from Mbeya, but which are not common in clade B virus sequences, is of interest. The impact of these mutational polymorphisms in terms of viral fitness, pathogencity, transmissibility and PI drug resistance development; especially in a region shown to have a high prevalence of multiple infections, is an area of research that needs to be explored (Gerhardt et al, 2005, McCutchan et al, 2005). Evidence is already mounting that some accessory mutations such as M36I may be associated with increased replicative fitness (Holguin et al, 2006). If this is the case, it is clear from the study findings that PI use must be optimal to avoid development of ARV drug resistance in Tanzania. It is imperative that measures to enhance drug adherence, such as the provision of adequate training and motivation to health care providers to facilitate proper management and monitoring of patients receiving antiretroviral therapy must be implemented. There may even be a need to establish local drug resistance interpretation algorithms to ensure optimal use of available drugs and to facilitate the understanding of factors that may promote PI drug resistance development in our settings.

3.6 CONCLUSION

The study results indicate that prevalence of major mutations conferring antiretroviral resistance in drug naïve chronically HIV-1 infected patients from Mbeya to be low

(0.045%). Thus the need to introduce genotypic drug resistance testing of chronically infected patients before initiation of therapy in Tanzania seems inappropriate. Additionally, this study has shown that drug naive HIV-1-infected women of Mbeya harbour viruses with single or dual secondary PI resistance-associated mutations. The impact of these secondary mutations detected in viral isolates from this region on the development of PI drug resistance needs to be investigated, to ensure the optimal use of the few available antiretrovirals.

CHAPTER 4. DETERMINATION OF THE PREVALENCE AND PERSISTENCE OF DRUG RESISTANT HIV-1 STRAINS AFTER NEVIRAPINE PROPHYLAXIS GIVEN TO DELIVERING MOTHERS TO PREVENT MOTHER TO CHILD HIV TRANSMISSION

4.1. INTRODUCTION

Mother-to-child transmission (MTCT) is the largest source of HIV infection in children below the age of 15 years. The WHO estimates showed that daily in 2006, 1,800 children were being infected with HIV worldwide, with the the vast majority through MTCT (UNAIDS report 2006, WHO PMTCT Guidelines, version 2, 2006). In Tanzania, approximately 12% of pregnant women attending antenatal clinics were living with HIV. Without intervention 30 – 40% of these HIV, infected pregnant women could have transmitted the virus to their newborns. In the absence of interventions, it is thus estimated that, 72,000 infants each year could become HIV infected via this route (Tanzania National Guidelines for Prevention of Mother-to-Child Transmission [PMTCT] of HIV Volume 2 March 2004, Tanzania MOH PMTCT Manual, September 2004).

MTCT of HIV can occur during pregnancy, during delivery, or at any point during breast-feeding. The risk of transmission through breastfeeding is cumulative; the longer the HIV-infected mother breastfeeds, the greater the risk for MTCT of HIV. A systematic review study done by De CocK et al in 2000 found that for mothers using replacement feeding there is obviously no transmission through breastfeeding. The study findings suggested that breastfeeding through 6 months leads to about 10% extra transmission (from 20% to 30%), while breastfeeding through 18-24 months leads to about 17.5% extra transmission (from 20% to 37.5%), compared to no breastfeeding. Furthermore, studies on mixed breastfeeding and MTCT transmission of HIV -1 have indicated that; the rate of MTCT of HIV -1 is increased through mixed breastfeeding rather than by exclusive breastfeeding alone (Nduati et al, 2000). As a result of these findings avoidance of breastfeeding, shortening the breastfeeding period and/or

encouraging exclusive breastfeeding are among the few current strategies employed to date for the PMTCT of HIV. Other strategies include the use ARV drugs during pregnancy, labour and in the early neonatal period or the advocacy of caesarean section delivery before the beginning of labour and membrane rupture.

The use of ARV prophylaxis during pregnancy, labour and in the early neonatal period is one of the most favoured methods for PMTCT of HIV. The first major breakthrough in the prevention of MTCT came in 1994 with the three-part Pediatric AIDS Clinical Trials Group (PACTG) 076 trial, which demonstrated that long-course

AZT prophylaxis given early in pregnancy and intravenously during delivery to the mother and for six weeks to the infant dramatically reduced the risk of vertical transmission from 25% to 8% (Connor et al, 1994). Although the PACTG 076 AZT regimen was rapidly introduced into practice in Europe and North America, it was costly and too complex to be used in many parts of the world with a high prevalence of HIV. The need and efficacy of shorter, simpler, and cost effective ARV regimens had to be evaluated in clinical trials to cater for the needs of low- and middle-income countries. Two such trials included the PETRA study; a multi-centre study that evaluated the efficacy of three short-course regimens of zidovudine and lamivudine in preventing early and late transmission of HIV-1 from mother to child in Tanzania, South Africa, and Uganda (PETRA study group 2002). The HIVNET- 012 trial in Uganda evaluated the use of single dose Nevirapine (SD-NVP) (Guay et al, 1999, Jackson et al, 2003).

The success of the HIVNET-012 study heralded the use of SD-NVP for PMTCT in low and middle-income countries. NVP is a non-nucleoside reverse transcriptase inhibitor (NNRTI), which binds to the HIV-1 reverse transcriptase directly, inhibits the synthesis of viral DNA and thus inhibits viral replication. It rapidly gets absorbed and crosses the placenta efficiently after a single oral dose of 200 mg to the mother. It has a very long elimination half-life of 40 hours. NVP for PMTCT is administered in a simple two-dose regimen (one dose each to mother and the newborn) to be taken by mouth (under supervision). It is inexpensive, does not require refrigeration and no significant side

effects have been noticed after a single dose administration. Due to its low cost and ease of use, this short-course regimen has been one of the most frequently used regimens for PMTCT in resource-poor settings. However the use of SD-NVP prophylaxis to prevent mother-to-child HIV transmission has been a subject of controversy. Discussions at the XVth International AIDS Conference 2004 were dominated by issues regarding the efficacy of NVP on PMTCT of HIV. Two studies presented at the conference reinforced long-standing concerns about the danger of resistance to NNRTIs caused by the use of SD-NVP prophylaxis. The first study, presented by Dr. Lynn Morris of South Africa, found that NVP resistant mutations persisted up to 6 months after delivery in 14% of mothers and 36% of infants who received SD-NVP prophylaxis. The second study, presented by Dr. T.R. Cressey of the Perinatal HIV Prevention Trial-2 (PHPT-2) trial in Thailand, found that providing SD-NVP to mothers receiving standard zidovudine (ZDV) prophylaxis could produce NVP concentrations in the mothers' bloodstream that were high enough to select for resistance 3 to 4 weeks following delivery. These studies, along with other recent findings from the PHPT-2 study in Thailand, led to calls from delegates to discontinue the use of SD-NVP for MTCT prophylaxis in favour of regimens of longer duration that contain 2 or 3 ARV drugs. This is regardless of the fact that SD-NVP given at the onset of labour, and a single dose administered to the neonate shortly after birth, to have been shown to reduce MTCT of HIV by almost 50%, and that the benefits of this regimen persist albeit at reduced levels, at a rate 41% for up to 18 months, despite continued breast-feeding (Jackson et al, 2003).

The cry to discontinue SD-NVP for PMTCT has been overwhelming; however so far there has been no conclusive evidence that SD-NVP adversely affects the clinical outcomes of women and children who are placed on ARV therapy after receiving prophylaxis. Current evidence suggests that resistant mutations in infants who are HIV infected usually arise "de novo" rather than being transmitted from the mother (Eshlman et al, 2004). Furthermore lack of alternative simpler, effective and affordable regimens

for HIV MTCT has delayed the displacement of SD-NVP in many resource poor countries. More studies are thus needed to confirm or disprove the available reports on the impact of HIV resistant strains that develop as a result of SD-NVP prophylaxis. Issues such as the effects of HIV clade differences, viral fitness, maternal viral load levels, maternal immune status with regard to development and persistence of NVP resistance mutations following SD-NVP exposure have to be investigated before one can conclusively advocate the discontinuation of SD- NVP for PMTCT.

In an attempt to tackle some of these issues we embarked on a study to determine the prevalence and persistence of NVP resistant strains among HIV-1infected pregnant women attending antenatal clinics in the Mbeya region of Tanzania, where a 42 % NVP prophylaxis coverage rate existed and where multiple HIV subtypes co-circulate (Kracher et al, 2006).

4.1.1. Study Site

The study site was Mbeya region located in south western Tanzania. Four government antenatal clinics within a radius of 20 km within Mbeya municipality were randomly selected. The selected antenatal clinics were Kiwanjampaka, Igawilo, Meta and Ruanda.

4.1.2. Study Population

Between March and August 2005, 121 (95% CI 0-40%) consenting HIV-1 seropositive women, with pregnancies of at least 28-36 weeks gestation attending at one of the selected 4 sites were recruited for the study. The recruited women were all residents of Mbeya municipality were aged between 18-38 years; with a median age of 25.7 years. The study participants HIV serostatus was reconfirmed at recruitment, and the participant's baseline viral load levels, CD4 + ve counts, and full blood counts were determined. Each NVP study participant was assigned to specific study nurse/midwife recruited specifically for study, to assist in delivery, questionnaire interviews and for provision of supervised administration of SD-NVP at onset of labour.

4.2. ETHICAL APPROVAL

Participation was voluntary and informed consent was obtained from all study participants. Ethical approval for the study was obtained from Muhimbili University of Health and Allied Sciences Research and Publications Committee and from the Mbeya Regional and Local Government ethical committee under the Regionals Medical Officer's Office. Approval was also obtained from the regional PMTCT of the HIV coordinator in Mbeya municipality.

4.3. STUDY SUBJECTS AND DATA COLLECTION

4.3.1 Interviews and Specimen Collection

The recruitment process began by informing the potential participants about the study and requesting them to fill out a semi- structured questionnaire consisting of close and open ended questions on their general HIV knowledge and on MTCT and PMTCT. The questionnaires were translated into Kiswahili and pre tested (see appendix 1A).

After providing informed consent 30 mls of whole blood was collected at each study round i.e. 0, 4, 6, 8,12,16,20, and 24 weeks after supervised SD-NVP intake from each of the 121 study participants.

4.3.2 Laboratory Processing of Samples

4.3.2.1 Haematological analysis

Full blood counts were performed on an automated KX-21N sysmex haematological analyser.

4.3.2.2 CD4 and CD8 Immunophenotyping

CD4 and CD8 counts and CD4 /CD8 ratios were determined by a BD FACScalibur flow cytometer.

4.3.2.3 HIV serology

The HIV-1 status of the participants was determined using an algorithm comprising of Enzygnost Anti HIV1/2 Plus (DADE Behring Germany) and Determine HIV 1/2 rapid (Abbott UK). Samples parallely reactive on both tests were considered HIV positive.

Discordant results were resolved by Western blotting as per manufactures instructions (Genelabs Diagnostics).

4.3.2.4 PBMC Isolation and HIV viral load assay

In each round: 4 x 2ml plasma, 3 x 3-7 million of peripheral blood mononuclear cells (PBMCs) were obtained by ficoll isopaque isolation (Sigma Diagnostics Germany). HIV viral load testing was performed using a ROCHE Amplicor HIV monitor version 1.5.

4.3.2.5 HIV ARV drug resistance genotyping

Plasma and isolated PBMCs to be used for genotypic drug resistance sequencing were shipped in liquid nitrogen to the Tropical Institute of the Ludwig Maximillian University in Munich, Germany, where DNA extraction, genotypic drug resistance PCR amplification and sequencing were performed.

4.4. RESULTS

4.4.1 Socio-demographic Characteristics

Table 4-1, shows the socio-demographic characteristics of the women attending antenatal clinics in Mbeya town. Five hundred sixty seven women were willing to be interviewed about their knowledge on HIV and AIDS from whom 171 were recruited into the NVP cohort. The mean age was 28.3 years for general population group and 26.5 years for the NVP group.

Table 4-1 Socio-demographic characteristics of the women attending antenatal clinics in Mbeya town (n =567)

Respondents N=567	Characteristics	Percentages (%)
Religion	Christian	93
	Muslim	6
	Other	1
Occupation	Petty Business women	36
	Farmer/ housewife/not working	37
	Civil servant	1
	Bar worker	8
	Domestic worker	17
Fertility	Number with living children	
	0	17
	1	25
	2	45
	3 or more	13

4.4.2 HIV and MTCT/PMTCT Knowledge of Women Attending Antenatal Clinics In Mbeya Municipality Tanzania.

4.4.2.1 AIDS awareness and HIV sexual behaviour

Of the 567 women interviewed 488 (86%) of the respondents were aware that there is currently no cure for HIV and AIDS. 159 (28%) of the respondents were not aware that HIV infected individuals could appear healthy. 244 (43%) of the respondents had their first sexual intercourse when they were younger than 18 years of age, while 15 (27%) of the respondents admitted that the last time they had sexual intercourse it was not with their regular sexual partner.

4.4.2.2 MTCT and PMTCT knowledge

Of the 567 women interviewed only 69 (12%) were not aware that breastfeeding could transmit HIV. While 209 (37%) of the participants were aware of the maximally recommended period of six months for breastfeeding for mothers who are HIV-1 positive. Only 206 (46%) of the women were willing to inform their partners or close relatives about their HIV serostatus. 505 (89%) women cited cost as the major factor that made them continue to breastfeeding albeit been aware of the risks associated with this practice. The benefits of the use of ARV in pregnancy as a means to reduce MTCT

were only known to 301 women (53%). Radio and television programs were cited as the major (82%) source for acquisition of information on MTCT/PMTCT knowledge by the participants. 357 (63%) of the participants indicated traditional beliefs and fear of HIV associated stigma as a reason to continue to breast feed for more than the recommended six months. 192 (34%) of the study participants admitted not to have a permanent sexual partner. Only 210 (37%) of the subjects claimed that they had never used a condom while having sexual intercourse.

4.4.3 Prevalence and Persistence of Drug Resistance Mutations

Out of the 121 women recruited for the study only 110 (92%) women completed at least five study rounds. Eleven women (7%) completed less than 3 study rounds. The cohort median baseline CD4 T cell counts was 418 cells/mm3 (range, 23–1232 cells/mm3) and the base line median plasma HIV-1 RNA level was 32,940 copies/ml. All viral isolates typed belonged to HIV-1 group M subtypes, A, C and D

4.4.3.1 Subtype Distribution of Protease and RT Sequences.

Ninety nine (99) sequences out 110 (90%) samples were successfully amplified. The majority of the sequences isolated for both the protease (74.9%) and RT region (78.9 %) were typed as clade C

4.4.3.2 Frequency of Protease and RT Region Mutations

Five major (5.0%) NVP associated drug resistance mutations could be detected in the 99 RT recovered sequences from plasma samples analyzed at weeks 6 (n=1) ,8(n=3), 12(n=1) weeks post supervised SD-NVP. Two (1.96%) major PI resistance mutations could be detected, from 102 recovered sequences analyzed. The primary PI resistance mutations detected were N88D and L76V. In accordance to the Stanford Genotypic Resistance Interpretation Algorithm, N88D causes intermediate resistance to Nelfinavir (NFV) (Grossman et al 2004) and possibly low-level resistance to Atazanavir (ATV)

and Saquinavir(SQV). L76V is a recently recognized non polymorphic mutation, that reduces susceptibility to Amprenavir (APV), Indinavir (IDV), and LPV (Lopinavir), while increasing susceptibility to SQV and ATV (see tables 4-2 and 4-3).

Table 4-2: NVP Study frequency of NNRTIs mutations

NRTIs MUTATIONS	FREQUENCY OF OCCURRENCE (89 sequences)
K103E	1.01%
V106M	1.01%
L210F	1.01%
K103N	3.03%

Table 4-3 Frequency of other RT mutations

OTHER RT MUTATIONS	FREQUENCY OF OCCURRENCE (89 sequences
V35T	10.6%
V179I	23.5%
D177E	74.4%
K122E	78.1%

Table 4-4: Frequency of protease mutations

PROTEASE MUTATIONS	FREQUENCY OF OCCURRENCE (102 sequences)
N88D (major)	1.0%
L76V(major)	1.0%
L90M (major)	1.0%
V11I (minor)	2.9%
K20R/M (minor)	10.5 %
L10V (minor)	11.8%
G16E (minor accessory mutations)	12.0%
L36P (minor)	30.0 %
I93L (minor accessory mutations)	50.0%
T12S (minor accessory mutations)	67.0%
H69K (minor accessory mutations)	85.0%
M36I/V (minor)	88.6 %

4.5. DISCUSSION

Mortality among children born to HIV-infected mothers in sub-Saharan Africa is substantially higher than among children born to HIV-uninfected mothers in the same environment. In a meta-analysis of data from African trials done by Newell in 2004, the mortality of nearly 3500 HIV positive children and of 700 of HIV-infected mothers was analyzed. The analysis estimated that 35% of HIV infected children would die by the age of 1 year, and 53% by age of 2 years, compared with 5% and 7.5% mortality rates in uninfected children (Newell et al., 2004). Reduction of this HIV linked infant morbidity and mortality is in line with the millennium goals of United Nations, 2000 Declaration of Commitment on HIV/AIDS. The declaration targets include a 25% reduction by 2010 in the percentage of 15—24-year-old pregnant women who are HIV infected and a 50% reduction by 2010 in infant HIV infection by encouraging practices that promote reduction or at best eradication of MTCT of HIV.

In the present study we report the findings of a cohort of 121 HIV seropositive pregnant women who were administered SD- NVP during labour for PMTCT. Analysis of the sequences recovered from the baseline PR region samples for HIV-1 drug resistance associated mutations, revealed 2 primary PR region mutations namely N88ND and N76V. N88D is a mutation associated with intermediate resistance to Nelfinavir (NFV), while the N76V mutation predisposes viral strains to reduced susceptibility to protease inhibitors APV, IDV and LPV (http://hivdb.stanford.edu/pages/algs/HIVdb.htm). RT region sequences from samples of week 6, 8 and 12 post SD-NVP intake contained primary mutations associated with resistance to NVP. The primary NVP resistance associated mutations detected; were K103N associated with a 50 fold resistance to NVP and (DLV) and 25 fold resistance to EFV, K101E associated with a 10 fold resistance to NVP and V106M, which is associated with a 50 fold resistance to NVP. Persistence of these mutations could only be established for a single sequence harbouring a K103N mutation 24 weeks post SD-NVP intake. The NVP mutations detected were equally distributed among clade A (n=2) and clade C (n=3) HIV viruses. No NVP associated

mutations was present in the clade D amplified virus sequences. One baseline clade D sequences was found to harbour a potential primary NRTI mutation. The NRTI mutation detected was L210F, an RT mutation of uncharacterized genotypical/ phenotypical significance but a probable polymorphism of related primary NRTI mutation of known significance L210W that contributes resistance to all NRTI's except 3TC. (http://hivdb.stanford.edu/pages/algs/HIVdb.htm).

The study results suggest that NVP resistance develops in some women when treated prophylactically with SD-NVP to prevent HIV-1 MTCT. These findings confirm the findings of the HIVNET-012 group of Uganda (Eshleman et al, 2001). However our findings differ slightly from previous SD- NVP resistance studies which have quoted high rates of NVP resistance mutations (40%) following the administration of a SD-NVP (Johnson et al, 2005, Flys et al, 2006). In this study it was only possible to establish a 5% prevalence rate of primary NVP associated mutations following SD-NVP intake. The low prevalence of primary NVP resistance mutations observed in this study, seems, however to correspond well to the results of other studies done in Tanzania on the prevalence of primary ART resistance in drug naïve pregnant women of Tanzania (Somi et al, 2007). The study's inability to detect more sequences containing primary NVP resistance mutations (15-40%) after a SD-NVP administration as reported by similar studies done in Uganda and South Africa is perplexing. However from results it cannot be conclusively concluded that the mutations observed were the only primary NVP mutations present in the pool of amplified sequences. What is much more likely; is that more sequences with primary NVP resistance mutations were present but failed to be detected by the genotyping tool at our disposal. It is probable that these undetected sequences containing primary NVP resistance mutations belonged to a pool of viral strains that were probably a minority population of the circulating HIV strains in the selected plasma samples. The inability to detect all viral strains carrying NVP associated resistance mutations is not unique. Johnson et al in 2005 demonstrated that one could increase the NVP primary drug resistance detection sensitivity from 40% to

65% by using of an ultra sensitive real-time resistance PCR assay for the mutations K103N and Y181C. (Johnson et al, 2005). The degree of specificity of the genotyping tool employed to detect NVP resistance mutations seems to be of the utmost importance. The more specific the tool the more resistance mutation one will detect. Taking into account that the genotypic method used in this study was primarily developed as an inexpensive method for HIV routine diagnostic resistance testing with a sensitivity level of 300 copies (Eberle et al, 1995). It probably failed as is the case with many established genotypic drug resistance methods to detect viral isolates that make up less than 20% of the sample strains (Hammer, 2005, Flys et al, 2006). However, this may not be the sole reason. It may be fair to alternatively argue that the low prevalence of primary NVP drug resistance mutations observed could be a result, of the complexity of the epidemiological diversity of circulating HIV-1 viral strains observed in Mbeya region. The fact the majority of the strains circulating in Mbeya have been shown to be recombinant (57%) and that a high prevalence of multiple infection has been documented for the region seems to suggest, that HIV-1 subtype diversity may have an effect on the detection of drug resistant viral strains (Gerhardt et al 2005. McCutchan et al 2005). The effects of HIV clade diversity and multiple infections is further complicated by the fact that there seems to be a temporal fluctuation of viral molecular forms in multiply infected individuals which may introduce sampling errors that may bias viral strain recovery (Gerhardt et al, 2005). The effects of HIV clade diversity and multiple infections on development of drug resistance needs to be studied in more detail before one can conclude that subtype has no effect on antiretroviral drug resistance development and detection. To date there is preliminarily evidence that subtype diversity may affect the accuracy of some HIV viral RNA quantification assays (Rouet et al, 2007). Whether or not plasma HIV viral RNA under quantification due to subtype diversity significantly affects the interpretation of ARV resistance data of the various HIV resistance genotyping methods used in practice, is an area that still needs to be thoroughly investigated.

What is clear without a doubt is that resistant viral isolates may develop after prophylaxis with SD-NVP. However whether these minorities' viral isolates are of clinical significance or have an impact on the patient's future exposure to ARV NNRTI regimens is an area that needs to be explored. Preliminary evidence so far indicates that these viral isolates could be of clinical significance. An observational study by Jourdain et al, in 2004 showed that women exposed to NVP who had detectable NNRTI-associated resistance mutations early during the postpartum period had an inferior virologic response to a subsequent NNRTI based treatment regimen at 24 weeks of therapy, compared with women not exposed to NVP. On the other hand women exposed to NVP who had no detectable resistance mutations early during the postpartum period had an intermediate-level virologic response, suggesting that NNRTI resistant mutants were likely present at low frequencies and were possibly contributory to a less-than-ideal response to treatment. These observations once more confirm that it may not always be possible to detect NVP resistant strain, but it does not mean that resistant strains are absent.

The issue then on hand for resource poor settings is, is it economically viable to invest in expensive drug resistant screening methods for their HIV PMTCT programs to detect these minority viral isolates? Or should they consider replacing single with dual drug regimens or triple drug regimens for HIV PMTCT to avoid the potential viral resistance problem? An alternative would be adopting the WHO 2006 PMTCT guidelines for use of SD-NVP prophylaxis, that recommends supplemented SD-NVP by the co administration of AZT and 3TC intrapartum and for seven days postpartum to prevent resistance to NVP. Unfortunately however there already reports in that this regime may fosters resistance to NVP and 3TC and thus compromise these women's future treatment with these drugs (WHO 2006 Guidelines on Antiretroviral Drugs for Treating Pregnant Women and Preventing HIV Infection In Infants).

It may not be easy to design a tailored PMTCT programs that will suffice the needs of individual countries. However, what is apparent is that one can no longer ignore the SD-

NVP resistance problem. Each country currently using SD-NVP prophylaxis for its PMTCT programs will have to implement changes in accordance to the resources available. It may be wise for countries initially to try and control the accessory factors that promote vertical transmission of HIV; such as maternal viral load, viral subtype, viral fitness, maternal CD4 counts and co presence of sexually transmitted infections in HIV pregnant women. (Newell, 2006). Implementation of measures that in early pregnancy reduce or eliminate factors that promote vertical HIV transmission may be the key to effective PMTCT. Furthermore, dismantling the barriers that affect the acceptability of local PMTCT programs is major factor that may affect the provision of effective care. Barriers such as;

(1) Low uptake of Voluntary Counselling and Testing (VCT) due to confidentially issues or due to inappropriateness of counselling. Only fifty three percent of our interviewees were aware that breastfeeding could transmit HIV and only twenty seven percent were aware that HIV positive mothers should not breastfeed for more than 6 months despite counselling.

(2) Failure to return for HIV test results or failure to return for follow up visits before starting antiretroviral therapy were additional challenges; three of our participants never returned to collect their results.

(3) HIV associated stigma. Nine of the study participants were lost to follow up due to fear that their positive serostatus would be revealed by continuing in the study. Sixty three percent of participants quoted HIV stigma a cause for them to continue to breastfeed for longer than the recommended period

(4) Lack of spouse /family cooperation. Four of the study participants were lost to follow up because they did not obtain their family or spouses consent before joining the study.

Tackling theses barriers may contribute to the overall reduction of vertical transmission of HIV-1.

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4.6. CONCLUSION

It can be concluded that NVP resistance develops after single dose administration. However the ability to detect NVP resistance mutations after single dose administration of NVP seems to be a function of the specificity of genotyping tool used. The more specific the genotyping tool the more likely one is able to detect these minority viral populations that have developed resistance after SD-NVP administration (Plamer et al, 2006). Conventional in house genotyping tools seem ineffective methods to monitor resistance development after SD-NVP administration in region with a complex HIV molecular epidemiology. The screening of populations for primary NVP resistance following SD-NVP administration in HIV regions that are epidemiologically complex is best undertaken by the use of sensitive and specific real-time PCR assays. These assays however, may probably be too expensive to be adopted by many resource poor countries whose budgets for health care are seriously constrained. Resource poor countries like Tanzania should thus concentrate on improving their existing PMTCT programmes by. adopting the WHO 2006 recommendations of supplementing SD-NVP with 3TC and or AZT/ZDV combination to avoid development of maternal NVP resistance after single dose administration (Shapiro et al, 2006). For those countries, which can afford it, PMTCT programmes using multiple drug regimens rather than a single drug would be the most appropriate choice. However in countries with limited health care budgets, continued use of NVP for PMTCT, should be considered with the establishment of a national register of women who have been previously exposed to NVP. The register would facilitate genotypic resistance screening of these women to determine if they harbour NVP primary resistance mutations that may compromise these women's treatment response to future HAART regimens that may contain NVP and form a basis for a national NVP drug resistance data base.

CHAPTER 5: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. GENERAL DISCUSSION

Understanding the molecular epidemiology of the HIV-1 strains circulating in Tanzania is crucial in the successful response the to AIDS crisis. Molecular biological methods are the most sensitive tools for the surveillance of the circulating genetic forms of HIV-1, and for the study of their origin, factors of propagation, factors for pathogenesis and characterization of resistance of different variants in various geographical areas and among different population groups with different risk practices.

In this study it has been possible to establish that the molecular epidemiology of HIV-1 in a high-risk cohort from Mbeya Tanzania to be complex where a variety of pure and recombinant HIV-1 viral forms co-circulate. The method used in this study MHAacd, has the advantage over other HIV-1 subtyping tools in that it provides a quite reasonable rapid estimate of the prevalence of recombinants and dual infections in populations infected with HIV-1 (Hoelscher et al, 2002). In this study it was possible to establish that there is high prevalence of HIV-1 recombinant viral forms (54%) over pure subtypes (46%) circulating in Mbeya; suggesting that multiple infections are likely to be more common than previously assumed. The general putative HIV-1 multiple infection frequency of 15.4 % observed in this study may be a gross underestimation of what actually occurs in vivo. As the subtyping tool used in this study could only differentiate between intersubtype recombinants but not among intrasubtype recombinant forms as was reflected in the in-depth analysis of a MHAacd sample 507 of a triply infected bar worker (Gerhardt et al, 2005). Future studies in Tanzania should focus on determining the prevalence of recombinants in various risk groups, the incidence of dual infections, and the factors that foster dual infection, resulting in the generation and transmission of recombinant forms of HIV-1. Such studies may provide information on how to limit the spread and complexity of circulating HIV-1 forms. A better understanding of the impact of multiple infections with regard to disease progression, viral transmissibility and

development of ARV drug resistance may result (Gottlieb et al, 2004). The future in controlling the AIDS epidemic may lie in the prevention of re-infection of those already infected with HIV-1. Acquiring insight on how to prevent re-infection and control the diversity of HIV-1, may improve our chances of finding effective HIV treatments and preventive or therapeutic vaccines.

Knowledge of the prevalence of the different HIV-1 variants is not only relevant in developing effective vaccines but it is also relevant for planning patient management in clinical practice, to ensure the validity of tests for diagnosis, reliability of laboratory markers of infection during follows up of patients and determining the effectiveness of treatment regimens. Our findings on the prevalence of drug resistance mutations in chronically infected drug naïve individuals in both a high and a low risk cohort was relatively low, 0.017% and 0.015% respectively. In depth, analysis of the protease region (PR) for both cohorts revealed a high degree of sequence polymorphisms. Over 90% of all PR sequences analyzed expressed mutations associated with secondary resistance to protease inhibitors, and over 50 % of the sequences expressed the accessory mutations I15V, L19I, R41K, H69K, and L89M. Despite that current knowledge on ARV drug resistance so far indicates that only primary mutations are important in developing drug resistance to PIs. The role of these accessory mutations in non-clade B viruses needs to be further investigated. The importance of investigating the role of these accessory mutations in non-clade B viruses becomes significant when one analyses the data of the SD-NVP study. In this study, it was possible to establish that a single dose of NVP could induce primary resistance to NVP in strains circulating in Mbeya. What will happen when PIs are introduced on large scale in a region like Mbeya, where the HIV viral strains circulating already harbor PI resistance associated accessory mutations? Will the up scaling in the provision of antiretrovirals currently taking place in Tanzania expedite the rate of PIs drug resistance development? How are we prepared to control PI resistant viral strains that develop, and sequester into the brain microgial cells for example? Does Tanzania have the resources to switch to newer and

better anti HIV therapy regimens once resistance to PIs develops? The answer to most of theses questions is that at the moment, Tanzania is probably not prepared to cater for needs of an extensive ARV drug resistance problem. The presence of these accessory mutations however, can be compared to the idiom "there is no smoke without fire". We have smoke and the fire may be just around the corner. What should then be done to prevent this imminent disaster with the current up scaling of ARV availability taking place in Tanzania? The simplest solution would be to strengthen the available strategies currently in place that may assist in minimizing the development of drug resistance (Landman et al, 2006). This would include strengthening of the available VCT services and HIV clinics. VCT centers and HIV clinics should have adequately trained staffs who are motivated. Well trained staff, are the backbone for monitoring and evaluating potential drug-drug, drug-food, and drug-herbal adverse reactions that may lead to patient non-compliance and consequently lead to the development of ARV resistance. Last but not least, having HIV clinics that are well equipped with an uninterrupted supply of drugs of assured quality is one of the cornerstones in building up patient's confidence with the health care system and ensuring patient adherence. Strategies to minimize HIV associated stigma at VCT centers and HIV clinics could be a starting point to promote patient adherence. Integrating the HIV clinics with the general medicine clinics may prevent the "finger pointing" that HIV and AIDS patients are commonly subjected to. We lost 9 of our NVP study participants as a result of the stigma associated with a specialized NVP HIV clinic that in their view drew attention to their HIV serostatus. Furthermore, boosting of VCT services at antenatal clinics to include facilities for partner counseling is of prime importance. Imparting to expecting mothers and their male partners the correct knowledge about HIV and the factors that promote MTCT such as STI co infections, maternal viral load and mixed feeding practices may convince partners to make the best-informed choices for methods of prevention of parent to child transmission (PPTCT) (Bajunirwe et al, 2005). Setting up male partners counseling services will reduce the barriers of lack of partner consent and

commitment, which seems to be a major obstacle to effective PMTCT, as reflected in the NVP study results where less than half the study subjects (46%) were willing to inform their partners about their serostatus.

Nonetheless what is urgently needed right away in Tanzania without doubt is the routine surveillance of resistance to ARV drugs; especially with looming NVP resistance problem that was highlighted in this study. The ability to detect primary ARV resistance early enough is the key to influencing policy makers to make changes in national drug policy guidelines to avoid spread of uncontrollable levels of resistance early. In the case of the resistance problem to NVP as a result of its use in single dose for PMTCT, a switch as possible to a multiple drug regimen for the Tanzanian PMTCT programs to start as soon as possible is advocated, especially for those with CD4 cell counts below 350 or 200. Adoption of the WHO 2006 recommendations of supplementing SD-NVP with 3TC and AZT could also be an alternative although even though this regimen has already been shown to induce resistance to NVP and 3TC (Highleyman, 2006). Whatever PMTCT regimen is as adopted partly as a result of these findings, continuous monitoring for development and spread of antiretroviral resistance of drug resistance with regard to HAART and PMTCT regimens goes without question. The earlier ARV drug resistance is monitored in Tanzania, the better the nation will be prepared to control and tackle ARV drug resistance. The W.H.O currently recommends surveillance of transmitted HIV drug resistance strains in resource-limited settings where ART access is being rapidly expanded. It is prudent therefore to consider setting up ARV drug resistance surveillance facilities within the few tertiary health research institutions in the country and empower them to act as centres of excellence where samples collected randomly and routinely nationwide are sent to be monitored for genotypic and phenotypic development of ARV drug resistance. Such an initiative would give rise to a national central data base that would provide policy makers, drug prescribes, drug dispensers, drug user's and researchers' with useful information on how best to utilize the few available ARVs effectively. This data base would not only be a repository for

national drug resistance profile data, but it could also be used to monitor the introduction and emergence of diverse HIV-1 subtypes within the country, an important issue in terms of HIV-1 vaccine development and monitoring changes in HIV-1molecular epidemiology.

The drug resistance data base however, will provide only information on when a particular resistance profile developed and on how frequent the resistance occurs. It will not provide answers as to why the resistance developed due to factors like patient non-compliance, inadequate dosing, injudicious prescription and spontaneous mutation. To minimize ARV drug resistance development and spread, other loopholes in the health care system that may foster resistance must be sought and controlled. This may include strengthening ARV drug pharmacovigilance. According to the 2006 WHO fact 257, 25% of medicines consumed in developing countries are counterfeit or substandard. Poor quality medications imported or manufactured locally in this country, may yet be another avenue for the development and spread of resistance to ARVs. The contribution of substandard drugs to the development of drug resistance is not a new phenomenon for many developing countries. Worldwide there have been numerous reports about fake antimalarials, which have contributed to the development of resistance to these drugs (Green, 2006; Atemnkeng_ et al, 2007). These products have been found to contain inappropriate amounts of active ingredients all in the name of so called "generic drugs" (Rimoy et al, 2002). The Tanzania Food and Drug Authority (TFDA), in collaboration with the Departments of Pharmaceutics and Therapeutics, Pharmaceutical Microbiology and Clinical Pharmacology of the Muhimbili University of Health and Allied Sciences (MUHAS) have the pivotal role of monitoring that all the ARV drugs used in Tanzania, are of the required quality as a means of preventing further emergence of resistance to ARVs.

One area of research that is yet unexplored in Tanzania, but which could have tremendous impact on the efficacious use of the few HIV antiretrovirals available is the role of Pharmacogenetics in the treatment of HIV/AIDS. A recent report on efavirenz

(EFZ), a commonly used ARV drug, reported higher plasma exposure levels and more frequent early side effects within individuals harbouring the homozygous variant of the hepatic cytochrome P450 enzyme CYP2B6 G516T, a polymorphism, which is more frequently found in African-American subjects rather than in Caucasian subjects (Cressey et al, 2007, Wang et al, 2006). What this study actually highlights is that the ARV dosing regimens designed for individuals of Caucasian origin may not necessarily be appropriate for individuals of African origin.

5.2. CONCLUSION

It is clear from the study findings that the use of molecular biological tools facilitates the unravelling of the molecular epidemiology of HIV-1 in the Mbeya region of Tanzania. It was possible to establish that the molecular epidemiology of HIV in Mbeya were complex with individuals in this region prone to multiple HIV-1 re-infections. It was also possible to show that HIV-1 strains circulating in Mbeya exhibited a high sequence polymorphism in the protease region. The complexity of the viral strains found in Mbeya; highlight how little the mechanisms of HIV pathogenesis are understood and the need of more studies to further understand the diversity of HIV in Tanzania. The findings of the NVP resistance following single use prophylactically as well as the detection of primary resistance to protease inhibitors, when this drug is not yet in wide circulation in the country, warrants a more extended country wide surveillance of the emergence and spread of ARV resistance.

5.3. RECOMMENDATIONS

From the study findings the following recommendations are proposed:

I. Future studies in Tanzania to concentrate on monitoring the prevalence of recombinants, the incidence of dual infections, and the factors that foster dual infection, resulting in the generation and transmission of recombinant forms of HIV-1

in various risk groups; as a means of improving our chances of finding effective HIV treatments and vaccines.

II. Setting up ARV resistance surveillance and monitoring facilities in selected academic research institutions, assigned with the task of establishing a national ARV drug resistance database.

III. Future studies on the impact of the sequence mutational polymorphisms detected in the protease region of the HIV viral isolates from Mbeya, with regard to development of resistance upon exposure to Protease Inhibitors.

IV. Consider adopting alternative cost effective mutiple drug regimens for the PMTCT of HIV instead of using SD-NVP as the sole drug for PMTCT.

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APPENDIX IA-NVP STUDY 2005/2006- SUBSTUDY 4
MTCT/ PMTCT KNOWLEDGE QUESTIONNAIRE 2005/2006 VERSION 4B (ENGLISH VERSION)

MA 1	Respondent ID	☐☐☐☐
MA2	Current date	☐☐ ☐☐ ☐☐ day month year
MA 3	Place of interview	
MA4	Sex of respondent	Female
MA4	Interviewer name	Int. Code ☐☐

Background characteristics

MA5	How old are you now?	Age in years ☐ refused 98 Unknown 99
MA6	Where have you lived most of your life?	Rural area 1 town 2
MA6	What is your religion?	Christian 1 Muslim 2 No religion 4
MA7	What kind of work were you doing most of your life?	Not working 1 Farmer/gardener 2 Unskilled labourer 3 Skilled labourer 4 Driver 5 Domestic employee 6 Civil servant 7 Military/police 8 Businessman/woman 9 Bar/pub/restaurant work 10 Other (specify) 11
MA7	Are you currently working?	Yes 1 No 2 Refused 98 Unknown 99

Fertility

MA8	How many children do you currently have altogether? (please remember the ones living with you, living away and those who have died).	number ☐ Refused 98 Unknown 99

MA9	Have you using any method or doing anything to delay or avoid having children?	Yes 1 No 2 Refused 8 Unknown 9
MA10	What method have you used? Indicate three methods only; start with the method which was mentioned first. Write 00 in boxes if no (more) method was mentioned	none 0 Pill 1 iucd 2 Injectables 3 norplant 4 sterilization 5 Partner sterilized 6 Diaphragm\foam 7 Male condom 8 Female condom 9 Withdrawal 10 Rhythm\calendar 11 Abstinence 12 Consuming herbs 13 Tying strings 14 Other 15 Refused 98 Unknown 99

Health behaviour

MA11	Have you ever had any of the following? 14.1 blood transfusion 14.2 unsterile needles 14.3 razor blade incision 14.4 tattooing	list them.
MA12	Have you undergone female genital mutilation?	Yes 1 No 2 Refused 98
MA13	when was the last time you used any form of medication?	Days 1 Weeks 2 Months 3 Years 4 Never took any medicine Number ☐
MA14	When become sick where do you get advice about the medication to use?	Doctor (hospital) 1 Pharmacy/drug store 2 Traditional healer 3 Friends, relatives or neighbors 4 Self medication 4
MA15	Are you in the habit of using traditional medicines together with western medicines to treat your ailments?	Yes 1 No 2

MA16	If yes when did you last visit a traditional healer?	Days 1 Weeks 2 Months 3 Years 4
MA17	When you last visited the traditional healer what were you suffering from?	List

Sexual risk behavior

MA18	At what age did you first have sexual intercourse?	Never had sex 0 Age in years ☐☐ Refused 98 Unknown 99
MA19	In your lifetime, have you ever used a condom?	Yes 1 No 2 Refused 8 Unknown 9
MA20	What kind of partner was the person you last had sex with?	Permanent 1 Knew before 2 Met first time 3 Refused 8 Unknown 9

Aids awareness and personal HIV history

MA21	Do you think it is possible that HIV infected people could look healthy?	Yes 1 No 2 Refused 8 Unknown 9
MA22	As far as you know, is there any way to cure aids?	Yes 1 No 2 Don't know 3
MA23	What is your present marital status?	Currently single 1 Not married, permanent partn. 2 Currently married one spouse 3 Currenty married two+ spouses 4 widower 5
MA24	How old is your spouse or permanent partner? (if two+ spouses refer to first spouse alive)	age in years ☐ refused 98 Unknown 99

PMTCT/ MTCT knowledge

MA25	Can a HIV positive mother transmit the HIV virus to her infant?	Yes 1

		No 2 Don't know 3
MA26	If yes how?	During pregnancy Through breastfeeding Other methods list 4.methods 1&2
MA27	Can breast milk transmit the HIV virus?	Yes 1 No 2 Don't know 3
MA28	Do you intend to breastfeed your infant after delivery?	Yes 1 no 2
MA29	If you could not what alternative feeding method do you intend to use instead of breast feeding?	 1cow milk 2 infant formula 3.other (state)
MA30	Is it recommended for HIV positive mothers to breastfeed their infants?	Yes 1 no 2
MA31	What would make you continue to breastfeed even if you knew it posed a risk for HIV transmission to your child?	1. Cost 2. Spouse/ family cooperation 3. Traditional beliefs 4. Other (please sate) 5. Factors 1& 2 6. Factors 1,2&3
MA32	Does the use of antiretroviral drugs in HIV positive mothers reduce the risk of MTCT?	Yes 1 No 2 Don't know 3
MA34	Is it advisable for expecting mothers to know their HIV serostatus?	Yes 1 No 2
MA35	Does your permanent partner know HIV serostatus?	Yes 1 No 2
MA36	Do know the HIV serostatus of your permanent partner?	Yes 1 No 2 Don't know
MA37	Did you receive pre and post HIV counselling?	Yes 1 No 2 Only pre counselling 3 Post counselling 4 Both pre and post counselling 5

MA38	Have you informed anybody other than your counsellor about your HIV serostatus?	Yes 1 No 2
MA39	If yes who?	Family member 1 Friend 2 Other state 3
MA40	Where do you get your HIV/AIDS information from?	Radio T.V. Flyers Medical professionals All the above Other

APPENDIX IB-NVP STUDY 2005/2006- SUBSTUDY 4
MTCT/ PMTCT KNOWLEDGE QUESTIONNAIRE 2005/2006 version 4b
(Swahili translation)

MA1	Nambari ya msalili	☐☐☐☐
MA2	Tarehe ya usalili	☐☐ ☐☐ ☐☐ tarehe mwezi mwaka
MA3	Mahali pa usalili	
MA4	Jinisia ya msalil	Mke
MA5	Jina la muuguzi	Int. Code ☐☐

Taarifa za jumla

	Una miaka mingapi?	Umri miaka ☐☐ Kakataa kujibu 98 Hakumbuki 99
MA6	Katika maisha yako umeishi zadi kijini au mjini?	Rural area 1 town 2
MA7	Nimuumini wa dhehebu gani?	Mkirsto 1 Muislam 2 Hana dini 4
MA8	Kwa kawaida unafanya kazi gani ?	Hafanyi kazi 1 Mkulima 2 Kazi isiyokuwa na ujuzi 3 Kazi yenye ujuzi 4 Dereva 5 Mfanyakazi wa serikali 6 Mfanyakazi kwa ndani 7 Mwanajeshi/polisi 8 Mfanyabiashara 9 Hudumu wa bar hoteli au kilabu 10 Kazi nyingine taja 11
MA9	Unafanya kazi kwa sasa?	Ndiyo 1 Hapana 2 Kakataa kujibu 98 Hakumbuki 99

Afya ya uzazi

MA10	Umesha zaa mara ngapi?	☐ Kakataa kujibu 98 Hakumbuki 99
MA11	Umesha wahi kutumia njia yoyote ya uzazi wa mpango?	Ndiyo 1 Hapana 2 Kakataa kujibu 98 Hakumbuki 99

	Ni njia ipi/ au zipi uliz0tumia? (nakali njia tatu za mwanzo alziotaja kama atataja zaidi ya njia moja)	Kidonge 1 ktanzi 2 Sidano 3 vijiti 4 kufunga kizazi 5 Kondom ya kiume 6 Kondom ya kike 7 Tarehe 8 Mitishamba 9 Nyingine 10 Kakataa kujibu 98 Hakumbuki 99

Mwenenedo wa afya

MA12	Umewahi kufanyiwa kimojawapo kati ya hivi: 14.1 kuongezewa damu kuchomwa sindano zisizo salama Kuchanjwa chale na wembe Kujichora na kitu chenye ncha kali	Kuongezewa damu 1 Kuchomwa sindano zisizo salama 2 kuchanjwa chale na wembe 3 Kujichora na kitu chenye ncha kali 4 Hajawahi kufanya 5
MA13	umekeketwa?	Ndiyo 1 Hapana 2 Kakataa kujibu 98
MA14	Ni lini umetumia dawa ya aina yoyote?	Siku 1 Wiki 2 Miezi 3 Miaka 4 Situmi dawa5 Namba ☐
MA15	Ukiwa unaumwa ushauri wa dawa za kujitibu unapata wapi?	Dakatari (hospitalini) 1 Duka la dawa 2 Mganga wa jadi Ndugu ;majirani au rafiki 3 Ujuzi wako wa dawa mwenyewe 4
MA16	Unamazoea ya kutumia dawa za mtishamba pamoja na za hospital kujitibu?	Ndiyo 1 Hapana 2
MA17	Kama ndiyo: ni lini ulikwenda kwa mganga wa jadi mara ya mwisho?	Siku 1 Wiki 2 Miezi 3 Miaka 4
MA18	Ulipokwenda kwa mganga wa jadi mara ya mwisho ulikuwa unasumbuliwa na ugonjwa gani?	.....taja

Mewnendo wa afya ya kujimiana

MA19	Ulikuwa na miaka mingapi ulipojamiiana kwa mara ya kwanza? At what age did you first have sexual intercourse?	Umri katika ☐☐ Kakataa kujibu 98 Hakumbuki 99

MA20	Umewahi kutumia kondomu maishani mwako?	Ndiyo 1 Hapana 2 Kakataa kujibu 98 Hakumbuki 99
MA21	Ni mpenzi wa aina gani ulijamiiana naye kwa mara ya mwisho?	Mpenzi wa kudumu 1 Mutu unaye mfahamu 2 Mtu uliye kutananaye kwa mara kwanza 3 Kakataa kujibu 98 Hakumbuki 99

Ufahamu kuhusu ukimwi

MA22	Je unafikiri mtu aliyeathirika na virusi vya ukimwi anaweza kuonekana mwenye afya njema?	Ndiyo 1 Hapana 2 Kakataa kujibu 98 Hakumbuki 99
MA23	Kwa ufahamu wako, kuna tiba yoyote kwa ukimwi?	Ndiyo 1 Hapana 2 Hajui 3
MA24	Hali yako ya ndoa kwa sasa (umeolewa/umetaliki/mjane)	Hajaolewa 1 Hajolewa, mpenzi kwa kudumu. 2 Ana ndoa ya mume moja 3 Ana ndoa ya waume wengi 4 Mjane 5
MA25	Mume wako au mpenzi wako wa kudumu ana miaka mingapi?	umri miaka Kakataa kujibu 98 Hakumbuki 99

MA26	Ufahamu kuhusu maambukizi ya ukimwi kutoka mama kwa mtoto	
	Kuna uwezekano wa mama mjamzito mwenye ukimwi kumuambukiza mtoto wake kabla au baada ya kujifungua?	Ndiyo 1 Hapana 2 Hajui 3
MA27	Kama ndiyo kwa njia ipi/zipi?	Akiwa tumboni 1 Kwa kumnoyesha 2. Njia namba 1 na 2 3 Njia nyingine taja 4
MA28	Kuna uwezekano mtoto kuambukizwa kwa kunyonya maziwa ya mama mwenye ukimwi?	Ndiyo 1 Hapana 2 Hajui 3
MA29	Baada ya kujifungua utamnyonyesha mtoto wako?	Ndiyo 1 Hapana 2 Hajui 3
MA30	Kama humnyonyeshi mtoto wako ni njia ipi utakayotumia kumpa mtoto wako maziwa?	Maziwa ya ngombe 1 Maziwa ya kopo 2 Njia Nyingine 3
MA31	Ni salama kwa wakina mama mwenye virusi vya ukimwi kumnyonyesha watoto wao?	Ndiyo 1 Hapana 2

	Ni nini kitakufanya unendelee kumnyonyesha mtoto hata kama unajua unahatarisha mtoto wako na maambukizi ya ukimwi?	Gaharma 1 Mapendekezo ya mume/ mzazi mwenzako 2 Mila na desturi 3 Mengineyo 4. Masawla namba 1 na 2 5. Masawla namba 1,2&3 6.
MA32	Je mama wenye ukimwi akitimuia dawa za ukimwi utapunguza uwezekano wa kuambuzika mtoto wake wakati wa ujauzito na wakati wakumonemyesha?	Ndiyo 1 Hapana 2 Hajui 3
MA34	Ni vema mama waja wazito wapimiwe ukimiwi kabala ya kujifungua?	Ndiyo 1 Hapana 2 Hajui 3
MA35	Je, mwenzi mume/mpenzi wa kudumu anafahamu hali yako ya kuwa na virus vya ukimwi?	Ndiyo 1 Hapana 2 Hajui 3
MA36	Je, unafahamu hali ya kuwa na virusi vya ukimwi vya mwenzi (mume/mpenzi wa kudumu)?	Ndiyo 1 Hapana 2 Hajui 3
MA37	Je, ulipata ushauri nasaha kabla na baada ya kupima?	Ndiyo 1 Hapana 2 Kabla ya kupima 3 Baada ya kupimia 4 Kabla na baada ya kupima5
MA39	Je , ulimfahamisha mtu yeyote zaidi ya mshauri nasaha kuhusu hali yako ya kuahirika na virusi vya ukimwi?	Ndiyo 1 Hapana 2 Hajui 3
MA40	Kama ndiyo nani?	Mwanafamilia 1 Rafiki 2 Mchungali/imama 3
MA40	Unapata kutoka wapi maelezo au elimu kuhusu ukimwi?	redio 1 Luniga 2 vipeperushi 3 Daktari au muuguzi 4 Zote hapo juu 5 Kwingine taja 6