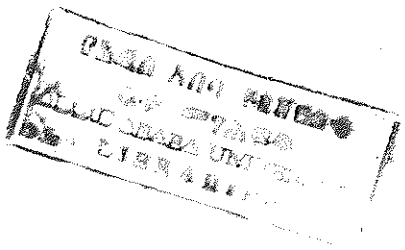
Addis Ababa University
School of Graduate Studies
Department of Biology

VIROLOGICAL AND IMMUNOLOGICAL FEATURES OF HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1) PRE AND POST TREATMENT OF TUBERCULOSIS IN COINFECTED PATIENTS



BY

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MARCH 2005

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TSIGEREDA BIRU LEULEBIRHAN

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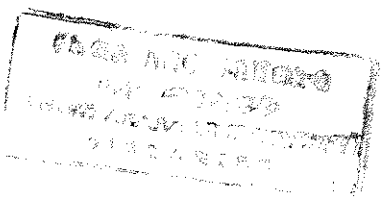


ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
bp	base pair
CCR5	CC Chemokine Receptor 5
CRF	Circulating Recombinant Form
CXCR4	CXC chemokine Receptor 4
dn	the relative proportion of non-synonymous substitution
DNA	Deoxyribonucleic acid
ds	the relative proportion of synonymous substitution
Env	envelop
EPTB.	Extra pulmonary tuberculosis
Gag	Group specific antigen
gp	Glycoprotein
HIV	Human Immunodeficiency virus
IFNγ	Interferon γ
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-12	Interleukin 12
kb	kilobases
LTR	Long Terminal Repeats
MTB	Mycobacterium tuberculosis
Nef	Negative factor
NFκB	Nuclear factor Kappa B
NSI	Non-syncytium Inducing
NJ	Neighbour joining
PBMC	Peripheral Blood Mononuclear cells
PCR	Polymerase chain reaction
Pol	polymerase
PTB	Pulmonary tuberculosis
Rev	Regulator of virion protein
R/H	Rapid/High
RNA	Ribonucleic acid
RT	Reverse transcriptase
SI	Syncytium Inducing
S/L	Slow/low
SIV	Simian Immunodeficiency Virus
Tat	transactivator of transcription
TB	Tuberculosis
TNFα	Tumor necrosis factor α
Vif	Virion infectivity factor
Vpr	Viral protein R
Vpu	Viral proteinU

Abstract

The association between HIV and Tuberculosis (TB) is complex and bi- directional. Recent studies demonstrated that TB accelerates the course of AIDS. In the present study we sought for virological and immunological features of HIV in TB co-infected individuals. The study subjects are selected from Ethio-Netherlands AIDS research project (ENARP) cohort. 8 HIV/TB co-infected individuals prior to diagnosis of TB, during and after completion of successful TB chemotherapy and 7 CD4 matched HIV-1 infected patients without the diagnosis of TB and with one year follow-up were involved. As routine laboratory analysis viral load and CD4+cell count were done at the time of blood sample collection. For this study, RNA was isolated from plasma and the C2V3 region of HIV-1 envelope gene was amplified by nested polymerase chain reaction (PCR).Direct sequencing was performed for all the PCR positive products. The HIV/TB co-infected individuals showed a significantly elevated plasma HIV-1 viral load ($P<0.05$) during treatment of TB with steeper decline in CD4+ cell counts ($P<0.05$). However, The ds (synonymous nucleotide substitution) and dns (non synonymous nucleotide substitution) comparison between the study groups and the controls at intake (before start of treatment) and ≈12 months later (after completion of TB treatment) didn't show a significant difference between the two groups. The ratio of ds and dns between the two time points for both groups was approximated to 1, which shows a positive but weak selection of evolution of the virus. We conclude therefore that plasma HIV-1 viral load is elevated during and after treatment of active tuberculosis, but this may not be due to the presence of highly replicative HIV viruses. We recommend future research should focus on isolating the different HIV quasispecies so that one can see if there is a difference in HIV genetic diversity, diversification and diversification rate between the HIV/TB and HIV only study population.

1. INTRODUCTION

1.1 HIV and AIDS

In the early 1980s, a new clinical syndrome was discovered among homosexual men in the United States. The infection was commonly associated with immunodeficiency, and many of the patients had very low numbers of CD4+ T-cells (Gottlieb, *et al.,* 1981). The new disease was called acquired immunodeficiency syndrome (AIDS), and scientists all over the world began to search for the cause of the disease. In 1983, scientists from the Pasteur Institute of Paris isolated a new virus from a patient with lymphoadenopathy (Barre-Sinoussi, *et al.,* 1983). Shortly after, the same virus was isolated from an AIDS patient by an American group (Gallo, *et al.,* 1984) and it was evident that the new virus was the causative agent of AIDS. Three years later it was named human immunodeficiency virus (HIV). In 1986, a second, closely related virus was discovered that is now called HIV-2 (Clavel, *et al.,* 1986), while the first virus is referred to as HIV-1.

At the time of the discovery of HIV and AIDS, it was difficult to imagine the proportions that the epidemic would grow to. By the year 2004 the epidemic has reached its highest level ever attained: an estimated 39.4 million people are living with the virus (UNAIDS-WHO report, 2004, *www.unaids.org*).

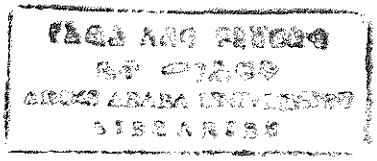
Ethiopia is one of the countries strongly hit by the virus. Although the epidemic started relatively late (in the mid 1980s), by the end of 2003, 1.5 million adults and children are living with the virus. The virus prevalence rate in the adult population is 4.4% (range 0.9%-7.3%) (UNAIDS, 2004).

1.2 HIV genome and proteins

HIV is a member of the lentivirus subfamily that belongs to the Retroviridae family (Dimmock & Primrose, 1987). The virus is roughly spherical, about one ten-thousandth of a millimeter across. Its outer envelope or coat is composed of a double layer of lipid envelope that bears numerous spikes. Each spike is composed of four molecules of gp120 and the same number of gp41 embedded in the membrane. Beneath the envelope is a layer of matrix protein that surrounds the core (capsid).The capsid has a hollow, truncated cone shape and is composed of a protein, p24 that contains the genetic material of the virus. Two strands of RNA molecules of approximately 9.6 KB nucleotide bases, integrase, a protease, ribonuclease, and two other proteins, p6 and p7, fit inside the viral core (Hutchinson, 2001).

Like other retroviruses, HIV genome consists of three genes *gag, pol, and env*, encoding the structural proteins and enzymes used in the replication cycle (Dimmock & Primrose, 1987; Fauci, 1988; Green, 1993; Stin, 2000). HIV and SIV are different from other retroviruses in having more genes with complex interactions. The common and assumed ancestral genetic structure for primate lentiviruses is *LTR-gag-pol -vif- vpr- tat-rev-env-nef-LTR* (Beer *et al.,* 1999; Stine, 2000). The function of each gene is not fully understood, but the *gag* gene codes for the manufacture of the dense cylindrical core proteins, the viral nucleocapsid. The *pol* gene codes for reverse transcriptase, protease, ribonuclease, and integrase (Dimmock & Primrose, 1987; Greene, 1993). The *env* gene codes for the two envelop proteins gp120 and gp41, the transmembrane that binds gp120 with the exterior of HIV(Dimmock & Primrose,1987; Stine,2000).The *tat* gene produces a regulatory protein that increase transcription of the HIV provirus whereas *Nef* may modify the host cell, enabling it to produce HIV virions later (Hutchinson,2001). *Rev* appears to be responsible

for switching the process of viral RNA transcripts to the pattern that dominates after the cell has been infected for over 24 hours (when two new size classes of RNA are created) (Greene,1993;Rosen,1991). The long terminal repeats are not part of the 9749 bases of the HIV genome but contain sequences that help other regulatory genes control *gag-pol- env* gene expression (Hutchinson, 2001).



1.3 The viral replication cycle

The main cellular receptor for HIV is the CD4 molecule (Dalgleish *et al.,* 1984). CD4 is a surface glycoprotein whose physiological function is to bind class II major histocompatiblity (MHC) molecules on the surface of antigen-presenting cells. It is present on a variety of hematopoeitic cells, including T lymphocytes, macrophages, dendritic cells and microglial cells in the brain. HIV-1 typically enters host cells through the interaction of the viral envelope protein, gp120, with CD4. This binding induces a conformational change in gp120 so that other regions are exposed that can bind to coreceptors adjacent to the CD4 molecule in the cell membrane (Doms and Trono, 2000). Coreceptor binding further induces a conformational change in the transmembrane part of gp41 so that a 'fusion peptide' is exposed and inserted into the cell membrane and this triggers the fusion of the viral envelope to the cell membrane.

Subsequent to internalization and uncoating process, the genome-containing core is exposed to the cytoplasm in a form suitable for reverse transcription to be completed (Hahn, 1994). Here the genomic RNA is reverse transcribed into complementary DNA by a virally coded enzyme, reverse transcriptase. Viral nucleic acid remains associated with the reverse transcriptase and the intergrase, in the context of a preintegration complex, is transported to the nucleus by a process that requires ATP but is independent of cell division (Bukrinsky *et al.,* 1992).

Once in the nucleus, the viral DNA genome integrates into the host cell genome via the action of the viral integrase, leading to the formation of a provirus. Provirus integration sites are random but may be influenced by chromatin structure (Hahn, 1994). Because the HIV provirus is covalently integrated into the host cell chromosome, it represents a stable component that is replicated and transmitted to target cells in synchrony with cellular DNA. The proviral DNA also serves as a template for the production of viral RNAs that include both genomic RNA molecules for incorporation into progeny virions as well as mRNAs for viral protein synthesis. Proviral gene expression is controlled by combination of viral and host cellular proteins that interact with viral DNA and RNA regulatory elements (Hahn, 1994).

Retroviral gene expression is regulated by terminally redundant long terminal repeats (LTR) elements within the HIV genome (Hahn, 1994). These LTR elements are generated during the reverse transcription process and are thus present in their entirety only in the DNA copy of the viral genome (provirus). Transcriptional activation of the HIV genome requires host transcription factors as well as virally encoded transactivators, both of which bind to specific recognition sequences in the LTR (Gaynor, 1992). The architecture of HIV enhancer and promoter elements has been studied most extensively and numerous binding sites for cellular transcription factors have been experimentally determined (Hahn, 1994). A number of these regulatory sequences resemble those present in human cellular genes and are able to specifically bind numerous host cell transcription factors. In this way, HIV-1 is able to harness the cellular transcriptional machinery in order to replicate, and these results in the coordination of viral transcription and cellular activation.

1.4. Co receptors and biological phenotypes

Isolates of human immunodeficiency virus type 1 (HIV-1) are classified according to their use of different cellular receptors, or coreceptors, in conjunction with CD4 for virus binding and entry (Hoffman *et al.,* 2002). Viruses using the seven transmembrane chemokine receptor CCR5, CXCR4, or both are termed R5, X4, and R5X4, respectively (Berger *et al.,*1998).Other receptors have been implicated in virus entry in vitro, but their relevance in vivo has not been well documented, and all viruses at a minimum use CCR5 or CXCR4 (Hoffman *et al.,*2002).

Strains of HIV-1 are also classified based on their different biological phenotypes (Schuitemaker *et al.,* 1992). Some isolates grow rapidly to high titers in cell culture and induce the formation of multinucleated giant cells (syncytia) in PBMC and certain cell lines such as the MT-2 cell lines. This phenotype has been called rapid/high (R/H) syncytia inducing (SI) or MT-2 positive. Other isolates have a slower growth rate, do not induce syncytia (NSI) in PBMC and do not infect cell lines. This phenotype has been called slow/low (S/L) non-syncytia inducing (NSI) or MT-2 negative. Viruses expressing the first, rapid phenotype are often X4 viruses and are isolated from patients at late AIDS stages; whereas the second, slower phenotypes belong to R5 viruses and usually dominate in more recently infected, asymptomatic patients (Bjorondal *et al.,* 1997).

The surface subunit of the HIV-1 *env* glycoprotein, gp120, controls entry related phenotypes. The determinants of co-receptor usage and MT-2 cell tropism lie largely with in the 35 amino acids of V3 (Hoffman *et al.,* 2002).However, changes in this region alone are not always necessary or sufficient to confer a particular phenotype in viruses (Hoffman *et al.,* 2002).

1.5. Genetic diversity of HIV

HIV-1 is characterized by high rates of mutation. This is the result of one or more error – prone steps in the virus life cycle. An important contributor to the high rate of HIV-1 mutation is the HIV-1 reverse transcriptase (RT) which does not possess 3' to 5' exonucleolytic proof reading activity. Fidelity studies using purified HIV-1 RT in cell-free systems indicated that nucleotide misincorporation frequencies are quite high ($2.5X10^{-4}$ to $5.8 X 10^{-4}$) (O'Neil *et al.,* 2002).Deletion, insertions and duplications are also frequently introduced, as well as recombination events. The overall rate of nucleotide substitution is approximately one million times higher than that of human genes (Li *et al.,* 1988).

There are large differences in genetic variation between different genetic regions of HIV. The *env* gene, which is divided into 5 variable regions (V1-V5) and 5 more constant regions (C1-C5), is particularly variable. The main explanation for this is that the protruding *Env* protein is a major target for the immune system and extensive variation in these domains mediates immune escape, which is beneficial for the virus. The principal neutralizing domain is the V3 loop (Goudsmit *et al.,* 1988; Palker *et al.,* 1988; Rusche, *et al.,* 1988). The V3 region also contains sites that determine coreceptor usage and infectivity (Chesebro *et al.,* 1992; Ivanoff, *et al.,* 1992). In contrast, the *pol* gene shows considerably lower variability, since it encodes important enzymes that have to maintain their functions and thus cannot afford much variation.

The clinical implications of the high genetic variability are extensive. It allows the virus to escape the host immune system, develop drug resistance and escape candidate vaccines. Therefore, knowledge about genetic variation of HIV-1 is important for the design of drugs and vaccines and for improving combination therapy. It can also help us understand more about the natural history and pathogenesis of the virus.

1.6. Genetic subtypes and groups

The extensive genetic variation of HIV-1 together with founder effects have resulted in several genetically divergent lineages that can be classified into groups and subtypes based on their phylogentic relationships. Three distinctive groups have been identified: M ('main'), O ("outlier") and N ("novel" or "non-M, non-O"). Group M is by far the largest and contains the vast majority of genetic variants. It is subdivided into nine genetic subtypes named A, B, C, D, F, H, J and K (Louwagie, *et al.,* 1995; Robertson *et al.,* 1999). The subtypes differ by up to 30% amino acids in the *env* gene and by up to 15% in *gag*. In order to define a new subtype the following criteria should be fulfilled (Robertson *et al.,* 1999)

- at least three representative strains should be identified in at least three individuals with no direct epidemiological linkage.
- Three full-length genomic sequences are preferred but two complete genomes in conjunction with partial sequences of a third strain are sufficient.
- The new subtype should be roughly equidistant from all previously characterized subtypes in all genomic regions as analyzed by phylogenetic and distance analysis.

There are clear differences in the geographic distribution of HIV-1 subtypes. In Europe, North America and Australia, subtype B was the first to be discovered and is still the predominant variant, although other subtypes have also been introduced through traveling and immigration (Thomson and Najera, 2001). In South America, subtype B is the most common but subtypes F and C are also found (Russell *et al.,* 2000). In Asia subtypes B and C are the most common non-recombinant subtypes (http://hiv-web.lanl.gov/content/hiv-db/mainpage.html). However the highest degree of genetic

diversity is seen in Africa. In sub-Saharan Africa, all know HIV-1 subtypes are found to circulate in different regions, with different relative frequencies. In Central and West Africa subtypes A and C seem to be the predominant forms, but all other subtypes have been found in this region together with many recombinant forms (Janssens *et al.,* 1997; Peeters, 2000). In east African countries, such Uganda, Rwanda, Kenya and Tanzania, the epidemic has involved mainly subtypes A and D. Subtype C predominates in Southern African countries like Zimbabwe, Zambia , Malawi, Botswana and South Africa (Janssens *et al.,* 1997).

In Ethiopia, according to the studies done by Abebe *et al.,* (1997; 2000) in Addis Ababa (the Capital city) and other urban areas of the country, subtype C is the predominant cause of the HIV epidemics. More importantly the circulating subtype C virus showed a significant amino acid differences when compared to all database sequences of subtype C. It is separated phylogenetically in two sub-groups which are designated main group C and subcluster C′. The C and C′ groups were equally distributed regardless of geographic location, time of sample collection or risk group(s) in the country. The prevalence of non-C HIV subtypes is extremely low. So far subtypes A and D have been isolated (Hussein *et al.,* 2000; Melaku, 2003).

1.7. Recombination in HIV-1

As in all other retroviruses, HIV-1 also recombines (Hu and Temin, 1990). By this means the virus is provided with far more adaptive potentials than is available from nucleotide substitution alone. Recombination occurs when the reverse transcriptase jumps back and forth between the two RNA templates during the reverse transcription of the viral genome. It has been estimated that HIV-1 undergoes approximately two to three recombination events per replication cycle (Jetzt *et al.,* 2000). Recombination is thus a natural step in the

replication cycle of retroviruses, and any newly synthesized viral genome will be recombinant between the two parental strands. However, recombination is only obvious when it occurs between parental strands with large genetic difference, such as different subtypes of HIV-1.

Inter-subtype recombination requires the simultaneous infection of a cell with two viruses of different subtypes, allowing the encapsdation of one RNA transcript from each provirus into a heterozygous virion. During subsequent infection of a new cell, the strand jumping polymerase will generate a mosaic provirus that is recombinant between the two parental subtypes (Wilbe, 2004). Recombination has also been detected between different groups of HIV-1 (Peeters *et al.,* 1999; Takehsa *et al.,* 1999) and between viruses of the same subtype(Diaz *et al.,*1995).The efficient spread of recombinant viruses has generated several so called circulating recombinant forms (CRFs). A CRFs is described as a lineage of recombinant viruses that plays an important role in the HIV-1 pandemics (Robertson *et al.,* 1999).

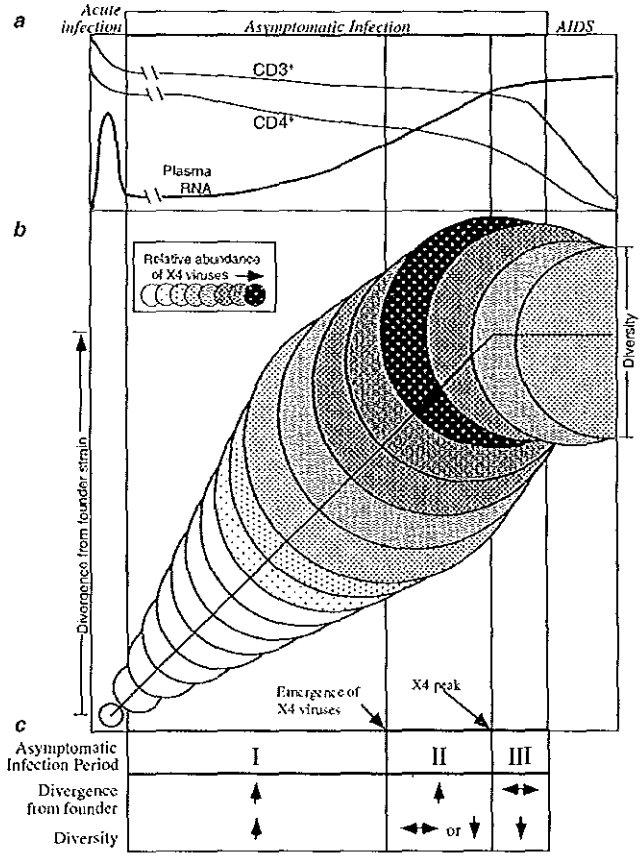
1.8. Genetic evolution of HIV within and among patients

HIV evolves over the course of an individual's infection (Fig 1). Upon infection, the individual has a homogenous viral population (Bonhoeffer *et al.*, 1995; Nowak, 1995; Wolinsky *et al.,*1996). Stable viral population equilibrium is found when the initial virus is relatively fit and replicating in a relatively constant environment. In this environment a particular genetic variant, regardless of its pathogenic ability, would be preferentially increased (Hutchinson, 2001). Early in the infection the immune response reacts quickly and strongly against common viral variants. An HIV-1 –specific immune response may lead to selection of HIV-1 escape variants, whereas the release of cytokines and

chemokines, due to a more general immune activation, appears to stimulate HIV-1 replication and increase virus levels (Collins *et al.,* 2002). This provides strong selection pressure for HIV viral diversification (Hutchinson, 2001). As a consequence, the infected individual harbors a population of genetically related but non-identical viruses that are under constant change and ready to adapt to changes in their environment. These genetically heterogeneous populations are termed "swarms" or "quasispecies" (O'Neil *et al.,* 2002).The viral population diversifies until genomic sequences differ as much as 10-15% in the V3 region. At late AIDS stage, the genetic diversity diminishes again, probably as a result of immune system failure (Shankarappa *et al.,* 1999).

Within HIV-1 infected individuals, viral sequence heterogeneity exits in different body compartments. As HIV infects different cells and tissues, rare mutants escape the immune response and increase in frequency (Wolinsky *et al.,* 1996). Tissue compartmentalization is evident in the lung (Itescu *et al.,* 1994; Nakata *et al.,* 1995) genital tract (Gupta *et al.,* 2000; Zhu, *et al.,* 1996) and lymph node (Wong *et al.,* 1997) but is most notable in the brain (Korber *et al.,* 1994). Some patients have distinct HIV-1 quasispecies phylogenies between the brain and blood, while others have quasispecies that migrate readily between these compartments (Korber *et al.,* 1994).

Figure 1. *Schematic illustration of proposed consistent patterns in development of HIV disease in moderate progressors.* **(Adapted from Shankarappa *et al.*, (1999).**



(a) Clinical phases of HIV infection as well as typical patterns of CD4+ and CD3+ T cells and plasma viral RNA loads. (b) Viral sequence evolution. Circle diameters represent the mean viral population diversities and vertical displacement of the circles represents the extent of viral population divergence from the founder strain. Shading represents the proportion of the viral population comprised of viruses with an X4 genotype. (c) Characteristic changes in viral evolution in the three periods of the asymptomatic phase identified in this study (↑, increasing; ↓, decreasing; ⇆, stable).

1.9. Ways of studying HIV evolution

A widely used means to distinguish between the various mechanism that may contribute to the fixation of mutations such as positive and negative selection as well as stochastic effects like bottlenecking and the massive destruction of virus and virus infected cells by intense immune response, is the analysis of non-synonymous (n) and synonymous (s) nucleotide substitutions (Kils-Hutten *et al.*,2001). Synonymous nucleotide substitutions (s) do not change the amino acid whereas non-synonymous substitutions (n) do change the amino acid. The *ds* describes the amount of synonymous substitutions that have occurred in proportion to all possible synonymous substitutions that can occur within the genetic region that is analyzed. A higher *ds* compared to *dn* is considered indicative of negative or purifying selection, which means that the gene is striving to be conserved. If *dn* is greater than the *ds* the gene is under positive or diversifying selection, a *dn* is probably driven by

industrialized nations by the year 2010. However, by the mid-1980s, the number of TB cases began to increase worldwide and by the year 2000, the TB bacteria had infected one third of the world's population (Encarta, 2003).

Multiple factors are attributed to the global increase in TB. The single greatest risk however, is infection with HIV. Clinical and epidemiological observations have demonstrated that HIV infected individuals have a 113 times higher risk of developing active tuberculosis upon exposure to MTB and 170 times higher risk during AIDS compared with uninfected persons (Villarino *et al.*,1992). The increase of TB incidence is highest in Africa and Asia, areas with the highest number of people infected with HIV (Corbett *et al.*,2003).

Infection with MTB is characterized by strong immune response. Studies in animal models and in humans have demonstrated the wide range of immune components involved in the effective response against MTB. These components include T cells (both CD4+ and CD8+), cytokines, including IFN-γ, IL-12, TNFα and IL-6, and macrophages. The precise role and functions of these cells and molecules (and others) is still being defined and may differ in acute and chronic infection. These immune responses are directed towards containing or eliminating the tubercle bacillus within the tissues of the host (Flynn, 2004).

In HIV/TB co-infected individuals, several signs of generalized immune activation in the peripheral blood has been reported by Vanham *et al* (1996).The changes are qualitatively similar in HIV negative and HIV positive TB patients, but quantitatively more pronounced in the latter.

1.10.1. Effect of TB on HIV replication and diversification

Due to the association of TB with chronic immune stimulation, active TB has been shown to increase HIV replication (Goletti *et al.* 1996; Nakata *et al.*, 1997; Garrait *et al.* 1997)

and diversification (Nakata *et al.,* 1997; Collins *et al.,* 2000).An investigation done by Nakata *et al.,* (1997) using bronchoalveolar lavage (BAL) showed that HIV replication in diseased lung segments has a ten fold increase compared to unaffected segments or in patients without lung infection. The results suggest a significant correlation between tumour necrosis factor α (TNFα) concentrations and viral load. Besides lung segments, plasma RNA in HIV-infected persons showed a 5-160 fold increase in viral load during acute phase MTB with reductions in levels after successful treatment Goletti *et al.* (1996).

The role of MTB in modulation of HIV replication in monocytic cells has at least two mechanisms. First, alveolar macrophages could be co-infected at sites of disease where there are large numbers of bacilli. Second, macrophages can be stimulated by MTB to release cytokines that enhance HIV-1 replication. Since HIV-1 replication is closely regulated by the host cell transcriptional machinery, it comes under the influence of a complex network of proinflammatory and immunoregulatory cytokines (Lawan *et al.,* 2001). TNFα induces HIV-1 transcription in both macrophages and T lymphocytes via the NFκB pathway (Matsuyama *et al.,* 1991). Other proinflammatory cytokines (IL-1,IL-2,and IL-6) also induce HIV-1 replication (Lawan *et al.,*2001). IL-6 synergizes with TNFα to enhance HIV-1 replication at transcriptional and post transcriptional levels in monocytic cells but not lymphocytes (Poli *et al.,*1990). IL-1 increases HIV-1 replication in promonocytic cell lines by enhancing TNFα-mediated induction of NFκB (Granowitz *et al.,*1995). The potential for TB to increase the HIV-1 load in vivo may be greater than that of other common opportunistic infections because of the chronic clinical course of active TB.The critical role that TNFα plays in the host response to mycobacterial disease, and the

marked systemic immune activation that accompanies *M.tuberculosis* /HIV-1 co-infection is the basis for enhancement of HIV replication (Lawan *et al.*,2001).

It is demonstrated by Collins *et al.*, (2000) that, HIV-1 patients with pulmonary TB harbor a more heterogeneous HIV-1 population in the blood than individuals with HIV-1 alone. Quasispecies from HIV-1 infected participants without TB tended to have phylogenies with few phylogenetic clusters and relatively short branch lengths. In contrast, quasispecies from HIV/TB individuals had phylogenies with longer intrapatient branches and more genetically divergent populations. In their study the mutation frequency of HIV-1 quasispecies in HIV-1 infected adults with TB was at least two to three folds greater than in HIV-1 patients without TB. The authors speculated that divergent evolution of HIV quasispecies in the blood is due to "spill over" that introduced new quasispecies from the lung into the blood (Collins *et al.*, 2002).These authors concluded that the divergent evolution of HIV during active TB is independent of the effect of TB on viral replication; it is rather due to an HIV-1 directed stimulation and activation of both cellular and humoral immune responses and the increase in HIV-1 heterogeneity may be necessary for immune avoidance.

1.10.2. The effect of treatment of TB on HIV

TB is treated with a combination of several antibiotics for at least six months. When effective therapy is given, symptoms typically improve within four weeks and sputum cultures become negative with in three months. This pattern of clinical improvement and sputum conversion is seen in over 90 percent patients who are HIV positive (Johnson and Gold,1993).

TB is treated with a combination of several antibiotics for at least six months. When effective therapy is given, symptoms typically improve within four weeks and sputum cultures become negative with in three months. This pattern of clinical improvement and sputum conversion is seen in over 90 percent patients who are HIV positive (Johnson and Gold,1993).The standard treatment of drug-sensitive TB in HIV-positive individuals includes the following : Isoniazid (INH) (300mg/day),Rifampin (450 to 600 mg/day), Pyrazinamide (PZA) (20 to 30 mg/kg/day for nine months), Ethambutol (15 to 25 mg/Kg/day) or Streptomycin (15mg/Kg/day) (Johnson and Gold,1993).

Regardless of the time of occurrence and the form of disease, pulmonary TB in HIV-1 infected subjects appears to remain responsive to anti-tuberculosis chemotherapy to the same extent that in HIV uninfected subjects, except in patients with advanced HIV-1 disease, in whom extrapulmonary and /or disseminated TB are often present concomitantly (Toossi,2003) .

However the course of HIV-1 infection is accelerated subsequent to the development of TB. Both relative risk of death and rates of development of new opportunistic infections are increased in HIV-1/TB co-infection compared with CD4 cell-matched HIV-1 infected control subjects (Toossi, 2003). According to Goletti *et al* (1996), the change in plasma viral load in HIV-infected patients during treatment for tuberculosis has shown a significant decrease in four western subjects after as little as 1 month of successful anti-tuberculosis treatment and no reduction in two patients whose anti-tuberculosis treatment failed. In contrast, studies in sub-Saharan Africa documented no reduction in viral load during anti-tuberculosis treatment (Lawn *et al.*,1999; Wolday *et al.*,2003).

After treatment, most immune activation markers have been observed to decrease except TNFα in West African subjects (Lawn *et al,* 1999). In Ethiopia, Wolday *et al.,* (2005) indicated that immune activation did not decrease after successful TB treatment. If chronic immune activation remains persistent, it can be expected that a heterogeneous HIV population may continue to exist in order to evade the host immune response. Therefore, in the present study we hypothesized that TB induced immune activation enhances systemic HIV-1 heterogeneity leading to persistently elevated HIV-1 viral burden after treatment of active tuberculosis.

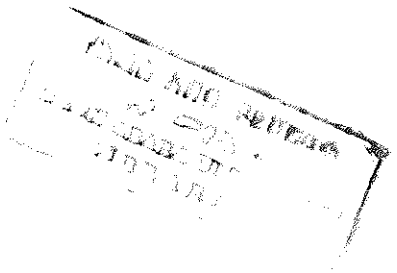
2. OBJECTIVE

2.1. General Objective

To determine the impact of Tuberculosis and its treatment on HIV-1 virological and immunological characteristics.

2.2. Specific Objective

1. To compare the genetic diversity of HIV-1 isolates from TB/HIV patients with those from HIV only.
2. To determine the impact of effective TB treatment on the magnitude of HIV-1 replication and diversity.
3. To determine the impact of HIV-1 diversity on CD4 cell count and plasma HIV-1 viral load in TB/HIV co-infected patients.



3. MATERIALS AND METHOD

3.1 Study participants

The study participants were selected among the HIV natural history study cohort population at Ethio-Netherlands AIDS Research Project (ENARP). ENARP has two HIV-1 cohort sites: Akaki and Wonji that enrolled factory workers. Enrollment started on February 26, 1997 in Akaki, and October 7,1997 in Wonji. In addition, information was available for some study subjects who are also participated in pilot studies one to two years before at Akaki (between Dec 20, 1995 and April 2, 1996) and Wonji (between Jume 20, 1996 and October 21, 1996). HIV status of participants of the pilot study was linked to the actual cohort data, in order to document a possible sero-conversion. At present, the cohort has enrolled 1641 factory worker in the two sites (801 Akaki and 840 Wonji). Cohort participants were enrolled only after signing an informed consent form. Withdrawal from the study can be at any time, without obligation to give reasons. As an incentive for their participation, study subjects and their immediate families were offered medical care to the standards of the country for the duration of the study. All participants attended an individual HIV-1 pre-test counseling session and knowledge of HIV status is voluntary. Blood samples were drawn every six months and other clinical and behavioral data are recorded during these visits. Ethical clearance for the study was given by the National and Institutional, Ethiopian Health and Nutrition Research Institute (EHNRI) Ethical clearance committee. Participants were prospectively recruited and followed up in out-patient ENARP clinics, where they were evaluated by primary clinicians at entry and at 6-months follow-up visits using a standard questionnaire and thorough clinical evaluation.

The cohort study has prospectively collected blood samples for each participant every 6 months. A complete blood cell count, HIV screening, CD4 T-cell count from whole blood and HIV-viral load from plasma were all done for each blood sample collected, and then the isolated plasma samples stored at -80°C.

Study participants were also evaluated by primary clinicians for any clinical symptoms including TB. If TB is suspected, chest radiograph and sputum smear for acid –fast bacilli are performed as described by Wolday *et al.* (2003). Standard short term chemotherapy was also given after positive TB diagnosis Wolday *et al.* (*2003*).

This study was a case control retrospective study. The study subjects were all HIV-1 infected patients with active pulmonary TB (HIV/TB) from April 1996 till the end of 2000 and had at least a one year follow up after development of active TB and were successfully treated. Within this time interval seventeen tuberculosis cases have occurred in the HIV positive individuals in this cohort. Of which four individuals died with out finishing the TB treatment. Five co-infected individuals didn't come in their regular follow up visit. Therefore we based our study on the rest eight individuals. The controls were CD4 matched HIV positive individuals with out active tuberculosis. Every attempt was made to include only those with out other opportunistic infections.

From the blood samples collected in the routine follow up every six months, we selected the plasma samples before the start of treatment, during treatment and after treatment of TB. Usually the time of diagnosis of TB and the time of blood collection do not coincide; therefore the very close date of blood collection to the diagnosis of TB is taken as the first base line (represent "before treatment of TB"). The CD4 count at this time point was used to select the controls.

The study population included eight study subjects and seven controls. Study subject 3 (individual 3) developed active tuberculosis two times and were included in both conditions. For the eight study subjects 26 plasma samples were selected. For patient number 1 four time points, for patient number 3 five time points, for patients' number 2,4,5,6 and 8 three time points for patient number 7 two time points were taken. For the seven controls two time points with one year difference was taken. A total of 40 samples were selected.

As a routine clinical and laboratory work, tuberculosis diagnosis, HIV screening, enumeration of lymphocyte subsets and viral load were determined as follows.

3.2 Diagnosis & Treatment of Tuberculosis

The diagnosis of TB was made on the presence of the following: 1) sputum smear 2) histological evidence and 3) radiographs consistent with pulmonary TB as assessed by a radiologist and a clinician. From the 17 TB cases described above, 12 cases were confirmed using chest radiography and clinical examination. One case was confirmed using histological examination and another one using sputum smear and clinical examination. For the rest 3 cases the data about the TB diagnosis was missed. After TB diagnosis, all patients received directly –observed therapy (DOT) with isoniazid 5 mg/Kg body weight (300 mg maximum), rifapicin 10 mg/Kg (600mg maximum),pyrazineamide 15-30mg/Kg (2 gm maximum) and ethambutol 15-25 mg/Kg. DOT was provided by well trained nursing staff throughout the entire course of follow up.

3.3. HIV screening

HIV screening was performed by HIV-SPOT (Gene labs Diagnostics, Singapore) and ELISA (Vironstika®- HIV Uni-Form II, Organon Teknika, The Netherlands).In addition,

samples screened as reactive/positive by the above assays and in the case the two reading contradict a confirmed were done by Western –bolt (HIV Blot 2.2,Genelabs Diagnostics, Singapore).

3.4. Enumeration of Lymphocyte Subsets

Absolute number of leukocytes per micro liter of whole blood was obtained using counter T540 (Coulter Electronics, Florida, U.S.A).Lymphocyte subsets were enumerated based on three colors staining in whole blood, using a FACScan flow cytometer (Becton Dickinson, USA),according to the manufacturer's recommendations. The percentage of lymphocyte and the absolute count of leukocyte were used to calculate absolute count of the corresponding cell populations (CD4+,CD8+,B cells and NK cells) using the Multiset Analysis software. However, only the CD4 count was used in this analysis.

3.5. Viral Load Determination

Plasma RNA viral load was determined using the modified form of a nucleic acid sequence based amplification assay NASBA (Organon Teknika, the Netherlands) on 200µl plasma according to the manufacturer's recommendations. Briefly, RNA was extracted using an established guanidinium isothiocyanate-based method, and then was isothermally amplified in the presence of 3 RNA standards (Qa, Qb, Qc) of known high, medium and low concentrations respectively. These RNAs serve as internal calibrators each differing from the HIV-1wild type (WT) RNA by only a small sequence. Detection of amplified RNA was performed on NucliSens reader (Organon Teknika, The Nether lands), using the RNA standards for calibration and calculation of viral loads where the lower detection limit was 80copies/ml. Results were also presented as $\log_{10}$ RNA copies/ml plasma.

For this study we did nucleic acid isolation, Reverse transcription, first and second rounds of PCR and Sequencing as follows.

3.5 RNA isolation, cDNA synthesis, PCR and sequencing

3.5.1. Nucleic Acid Isolation and Purification

HIV-1 RNA was isolated from the collected plasma according to guanidinium isothiocyanate (GuSCN) silica bead RNA extraction method of Boom *et al.* (1990). For this purpose, 100μl of plasma was added to 900μl lysis buffer (L_6)[1] containing GuSCN. 50μl silica was added to the plasma lysis buffer mixture and mixed. After incubating for 10min. at room temperature the mix was centrifuged at 14000 rpm for 40 seconds. After discarding the supernatant the pellet was washed with washing buffer (L_2)[2], and ethanol two times each and finally with a one time with acetone. After washing, the pellet was dried at 56°C for 10min and RNA eluted with 50 μl Low TE^{-4} (Louwagie Tris-EDTA) buffer for 15 min. by shaking.

3.5.2. Reverse transcription

10μl of eluted RNA was taken and reverse transcribed in to cDNA by using avian myeoloblastosis virus reverse transcriptase (AMV). Each RT reaction tube contain 20μl of reaction mix,: RT buffer (2.0μl of 10X), $MgCl_2$ (0.9μl of 100 mM), dNTP's (0.8μl of mM), RNAsin (0.3μl), 3'OUTpgV3rev primer, (5'TTC TCC TCT ACA ATT AAA GCT ATG TGT T), (1.0μl of 100ng/*ul*), AMV-RT (5.0µl of 1U/*ul*) and RNA (10μl). The RT tubes were then kept at 42°C for 45 min for cDNA synthesis. After 45 min. the product of the reverse transcription reaction was kept at 95°C for 5min to inactivate the AMV.

3.5.3 Amplification of the C2V3 Region by Polymerase Chain Reaction (PCR)

The cDNA was subjected to two rounds of amplification by PCR in a thermal cycler (Applied biosystems 2700).

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[1] L_6 is prepared from 120g GuSCN to 100ml L_1 buffer , 8.8ml of 0.5M EDTA and 2.4ml (=2.6g) Triton X-100 at PH=8.0 and temperature of 60°C.

[2] L_2 is prepared by mixing 120 g Guandium isothiocyanate to 100ml of L_1

L_1 buffer is prepared by mixing 12.1 g Tris to 900ml distilled H_2O with 42ml 37% HCl PH + 8.0 up to the total volume of 1L.

3.5.3.1.First round PCR

A DNA fragment that contain the C2V3 region of the virus was amplified using Taq polymerase and 5OUTgpV3dir (5'TGTCAGCACAGTACAATGTACACA) primer. Each 1st PCR tube was filled with 100μl of the reaction mixture; $MgCl_2$ (2.4μl of 100mM), PCR buffer (8.0μl of 10X),Taq polymerase (Ampli Taq,ABI) (0.4μl of 5U/ul), 5OUTgpV3dir (1.0μl of 100ng),dH_2O (68.2 μl),and cDNA (20μl). The c DNA was amplified in the following condition: 1 cycle of denaturation at 95°C for 4 min, 35 cycles of cycles of denaturation, annealing, and elongation at 95°C for 1min, 55°C for 1min, and 72°C for 2 min respectively, followed by 1 cycle of extension at 72°C for 10min and a final holding temperature at 4 °C.

3.5.3.2. Second round PCR

A nested PCR amplification of the C2V3 region of the gp120 was performed by using a pair of primers 5INNgpCV3-dir (5'CGC CAG GGT TTT CCC AGT CAC GAC TTA AAT GGT AGT MTA GCA GAA) and 3INNpgV3C-rev (5'CGA CGT TGT AAA ACG ACG GCC AGT GTT GTA ATT TCT AGG TCC CCT CCT GA). The nested PCR reaction was carried out in 50μl reaction mixture that contain: PCR buffer (5μl),$MgCl_2$ (7.2μl), Taq polymerase (0.4μl), dNTPs (0.4 μl), 5INNgpCV3-dir (0.5μl), 3INNpgV3C-rev (0.5μl) and 31 μl distilled water with 5μl of the first PCR product. The following amplification condition was used: 1 cycle of denaturation at 94°C for 4 min, 25 cycles of denaturation, annealing, and elongation at 95°C for 1min,55°C for 1 min, and 72°C for 1min respectively followed by 1 cycle of final elongation at 72°C for 10 min and holding it at 4°C till the next step.

3.5.4 Agarose Gel Electrophoresis

The second PCR product was subjected to run on 1 % agarose gel stained with 0.5μg/ml Ethidium bromide (Et) to confirm the presence of the amplified product. A mixture of 10μl of the PCR product, and 5μl of loading dye was loaded on the gel.100bp λDNA fragment was used as a molecular weight marker. The gel was covered with 1500ml 1xTAE (Tris Acetate EDTA) buffer and run at 100millamper (120V) for 1 hour. It was then observed under UV light where PCR positive specimens fluoresced. Finally Polaroid pictures were taken and 300 bp bands checked.

3.5.5 DNA sequencing

All the PCR positive samples were directly cycle sequenced using ABI prism 3100 (Applied biosystems, Applera Corporation, U.S.A) genetic analyzer with the primers used in the second PCR. First, the PCR products were diluted 1:10 (5ul of DNA in 45ul dH_2O). The 5X sequencing buffer was also diluted using dH_2O to 1X sequence buffer which is used to dilute (1:3) v1.1. Big dye terminator ready reaction mix. A mixture was then formed using 8 μl diluted big dye terminator ready reaction mix, 1ul (50ng/ul) nested PCR primer (5INNgpCV3-dir) and 11 μl of the diluted PCR product. These mixes run to PCR using the following PCR condition to give the sequence product (extension product): 1 cycle of denaturation at 96°C for 1 min, 25 cycles of denaturation, annealing, and elongation at 96°C for 10 sec,50°C for 5 sec, and 60°C for 4min. respectively and a final hold to 4°C.

The extension product (sequence product) was then purified using ethanol-EDTA-sodium acetate (NaAc) or Ethanol-sodium acetate (NaAc) purification procedure. First 50 μl of 100% Ethanol was added to each tube which contain the extension product. Then a mix of 2 μl of EDTA and 2 μl of 3M NaAc was added. After vortexing, the mix was put at room

temperature for 20 min. exactly after 20min. it was centrifuged for 20 min at maximum speed (14000g). The supernatant was removed completely and the pellet washed 2X with 70% ethanol with maximum centrifugation for 5 min. at each wash. Then the pellet was dried completely and 10 μl formamide was added. After 30 min. the sequence product was transferred to 96 well plates and denatured at 95°C for 5min. Finally the plate was loaded to the sequencer with the following running module: A capillary length of 36 cm with performance optimized polymer 4(POP4 ^{TM}), an Ultra seq- POP-4^{TM} default module with dye set E mobility file. The result was read using the data extraction software within 2 hours.

3.5.6. Sequence analysis

The obtained sequences were viewed using Chromas (version 2) software and edited manually. The sequences were then aligned with previously obtained HIV-1 *env* C2V3 reference sequences of Ethiopian strains and with the consensus HIV-1 sequences for different subtypes found in the Los Almos HIV database. DNADIST, NEIGHBOR, DRAWTREE, SEQBOOT and CONSENSE programs of the PHYLIP package were used for phylogenetic analysis of sequence with the Kimura two – parameter model. MEGA (Molecular Evolutionary Genetics Analysis) software was used for synonymous and non synonymous genetic distance calculation with modified Nei-Gojobori method and with the Jukes Cantor correction. DNA sequences were translated into amino acid sequences using the MEGA software.

3.6. Statistical Analysis

The statistical analysis was done using the statistical package STATA (Intercooled Stata version 7.0 Stata Corporation, College Station, TX,USA). Differences between any two

groups was compared using non-parametric statistical tests-Wilcoxon rank test and Mann-Whitney U test of independence.

Ethical Consideration

The ENARP/EHNRI Natural HIV Disease Progression Cohort study have been reviewed and approved by Institutional and National Ethical Clearance Committee's. There was no requirement of collecting new materials and all the experiments mentioned in this study were performed on samples already available at ENARP laboratory plasma bank. For this particular study ethical clearance was obtained from the research ethical clearance committee (RECC)/EHNRI and IRB/AAU-Biology Department.

4. RESULT

4.1. Follow-up and Tuberculosis associated features of the HIV/TB co-infected patients

The different follow-ups associated with tuberculosis for HIV/TB co-infected patients (n=8) are presented in table 1. The table shows the first date the patients started enrollment at ENARP cohort (beginning of enrollment) and the time active tuberculosis was diagnosed. These patients are survivors for at least one year after TB diagnosis.

Table 1. Follow-up and tuberculosis associated features of the HIV/TB co-infected patients

Subject ID	Beginning of enrollment	enrollment continued till	date of TB diagnosis	Type of TB	out come	TB diagnosis	months of TB follow up
Patient 1	24/03/1997	19/05/03	8/3/1999	EPTB	BIOPSY	BIOPSY	45.2
Patient 2	11/6/1997	1/4/2002	19/05/98	PTB		CXR+CLINICAL	42.63
Patient 3	22/08/1998	18/03/02	18/04/00	PTB	PTB	CXR+CLINICAL	data missed
Patient 4	25/01/2000	19/05/03	28/08/00	PTB		CXR+CLINICAL	data missed
Patient 5	9/6/1997	11/12/2000	18/01/00	PTB		SPUTUM& CLINICAL	42.7
Patient 6	4/6/1997	1/12/1999	29/06/98	PTB	DEATH	CXR+CLINICAL	42.87
Patient 7	29/04/1997	22/05/00	1/11/1999	PTB		CXR+CLINICAL	44
Patient 8	2/11/1998	9/8/2001	5/8/2000	PTB		CXR+CLINICAL	data missed

CXR- chest radiography
Clinical- clinical examination
PTB- pulmonary tuberculosis
EPTB- extra pulmonary tuberculosis

4.2. Virological, Immunological and other profiles of the study subject

The clinical, virological and immunological data on the study population is shown in Table 1. The median age for the study subjects at baseline was 35 years (range 30- 43) and for the controls 32 years (range23-39). The median CD4 count for the study subjects before the start of treatment was 218 /ul (range 93-352) and for the control at baseline was 306/ul (range 92-351). The median viral load before the start of treatment for the study subjects and at corresponding time for the controls was 11,000 copies/ml (range 930 to 380,000) and 9,900 copies/ml (range 135 to 59,000) respectively. During treatment the median viral load was 152,500copies/ml (range 3,300 to 910,000) for the study subjects. The median viral load after treatment of TB or with one year follow up for the study subjects and the control was 29,000 copies/ml (range 270 to 345,000) and 11,000 copies/ml (range 80 to 160,000), respectively.

Table 2. Clinical, Virological and immunological data of the HIV and HIV/TB study population

Patient	Time point (In month)	AGE	SEX	CD4 cells/ mm^3	Plasma Viral load Copies/ml	Type of TB	PATIENT STATUS	Other Infections/Clinical signs	PCR amplification	HIV subtype
1	0	38	M	310	81000	EPTB	before TB diagnosis	None	+	C'
	7			295	290000		during TB treatment	None	+	
	13			208	2900		after TB treatment	None	+	
	23			220	160000		after TB treatment	None	+	
2	0	35	M	352	88000	PTB	before TB diagnosis	Oral candidiasis	+	C
	8			446	152500		during TB treatment	None	+	
	14			302	345000		after TB treatment	None	+	
3	0	30	M	175	9400	PTB	before TB diagnosis	None	+	C'
	12			128	29000		after TB treatment	Severe/recurrent pneumonia	+	
	18			137	11000	PTB	before TB diagnosis	None	+	
	26			170	20000		during TB treatment	None	+	
	32			185	15000		after TB treatment	None	+	
4	0	32	M	327	8000	PTB	before TB diagnosis	None	+	C'
	6			301	71000		during TB treatment	Recurrent upper respiratory tract infections	+	
	12			321	170000		after TB treatment	None	+	
5	0	30	M	304	5000	PTB	before TB diagnosis	Weight loss < 10% of body weight	+	CC'
	9			207	29000		during TB treatment	None	+	
	16			111	47000		after TB treatment	None	+	
6	0	40	F	218	300000	PTB	TB diagnosis	Vulvovaginal candidiasis > 1 month	+	C'
	5			207	180000		during TB treatment	None	+	
	11			175	170000		after TB treatment	None	+	
7	0	35	M	93	380000	PTB	before TB diagnosis	None	+	C'
	12			51	910000		during TB treatment	Minor mucocutaneous lesions, Weight loss < 10% of body weight	+	

+ indicates DNA is amplified by PCR
_ indicates DNA not amplified

EPTB – Extra pulmonary tuberculosis
PTB --- Pulmonary tuberculosis

8	0 11 18	43	M	212 147 178	930 3300 270	PTB	before TB diagnosis during TB treatment after TB treatment	Chronic diarrhea > 1 month None None	+ + +	
9	0 12	33	M	336 461	59,000 160,000	No TB	CONTROL	Recurrent upper respiratory tract infections None	+ +	C'
10	0 12	28	M	179 185	135 80	No TB	CONTROL	None None	+ –	C'
11	0 12	39	M	310 158	15000 24000	No TB	CONTROL	None None	+ +	C
12	0 14	32	M	306 389	9900 11000	No TB	CONTROL	None None	+ +	C'
13	0 6	29	M	92 58	50000 140000	No TB	CONTROL	None None	+ +	C'
14	0 12	39	M	224 275	8300 7000	No TB	CONTROL	Persistent generalized lymphadenopathy, Oral candidiasis,	+ +	CC'
15	0 15	23	F	351 414	730 710	No TB	CONTROL	Severe/recurrent pneumonia Minor mucocutaneous lesions	+ +	

For this study some of the study participants that were mentioned in the report of Wolday *et al.* (2003) were involved. But here individuals who survived for more than one year were the ones that are included. A significantly increased viral load ($P<0.05$) in the HIV/TB co-infected individuals was observed after the start of TB treatment compared to pre diagnosis of TB (Fig 2 A&B). The steeper increase in viral load was still persisted in most of the individuals after completion of TB treatment (Fig 3A). For the controls (HIV only) the viral load observed between 0 months and 12 months doesn't show a significant difference (Fig 3B).

A significantly decreased absolute CD4 cell count ($P<0.05$) was also observed in HIV/TB co-infected subjects after completion of TB treatment compared to the prediagnosis of TB(Fig 4 A&B).Such a decrease in CD4 count is not observed in the control subjects between 0 and 12 months time interval (Fig 5).

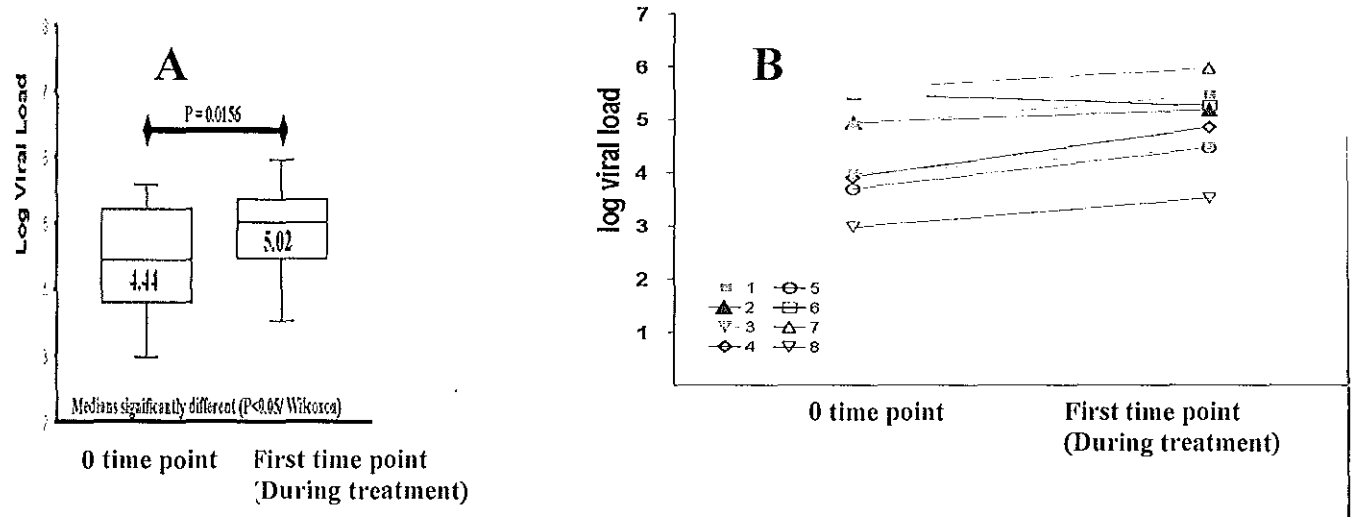


Figure 2*(A&B)Plasma Viral Load Variation in HIV/TB coinfected patientls (study subjects) before diagnosis (0 time point) and during treatment (n=8). Fig (A). The line in the middle of the box represents the median and the box extends from the 25th percentile (X[25] to the 75th percentile X[75] which is defined as the interquartile range(IQR). The whisker extend to the upper and lower adjacent values. Fig (B) The line extends from the 0 time point to the first time point (during treatment).*

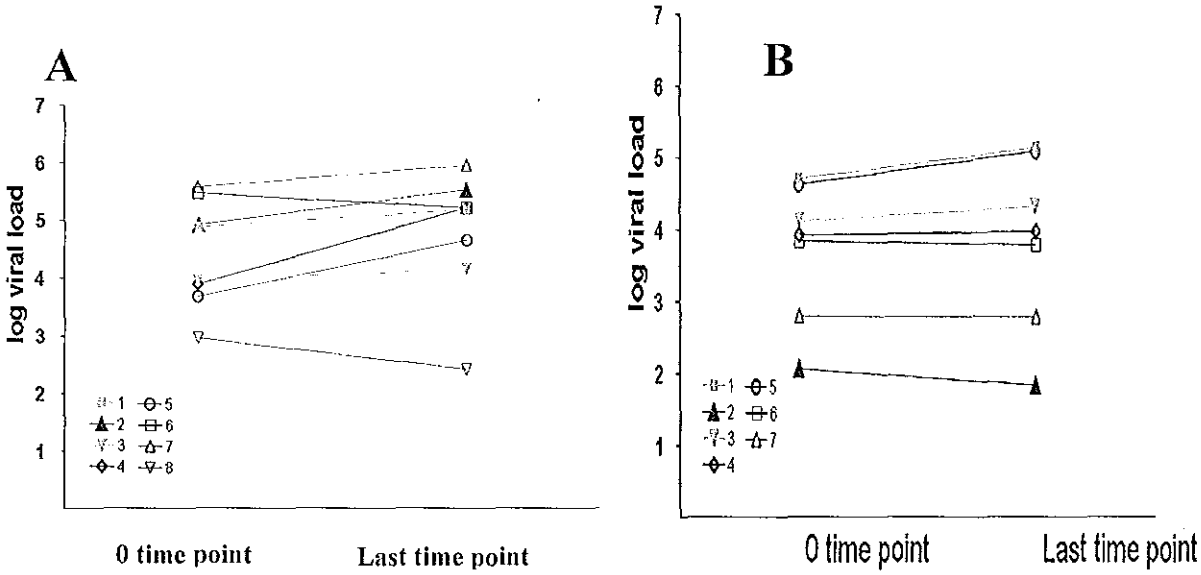


Figure 3 *(A) Plasma Viral Load Variation in HIV/TB coinfected patients (study subjects) before diagnosis (0 time point) and after treatment (last time point) (n=8). Fig 3 (B) Plasma Viral Load Variation in CD4matched HIV only patients (controls) at 0 time point and last time point (n=7).The line extends from 0 time point to the last time point.*

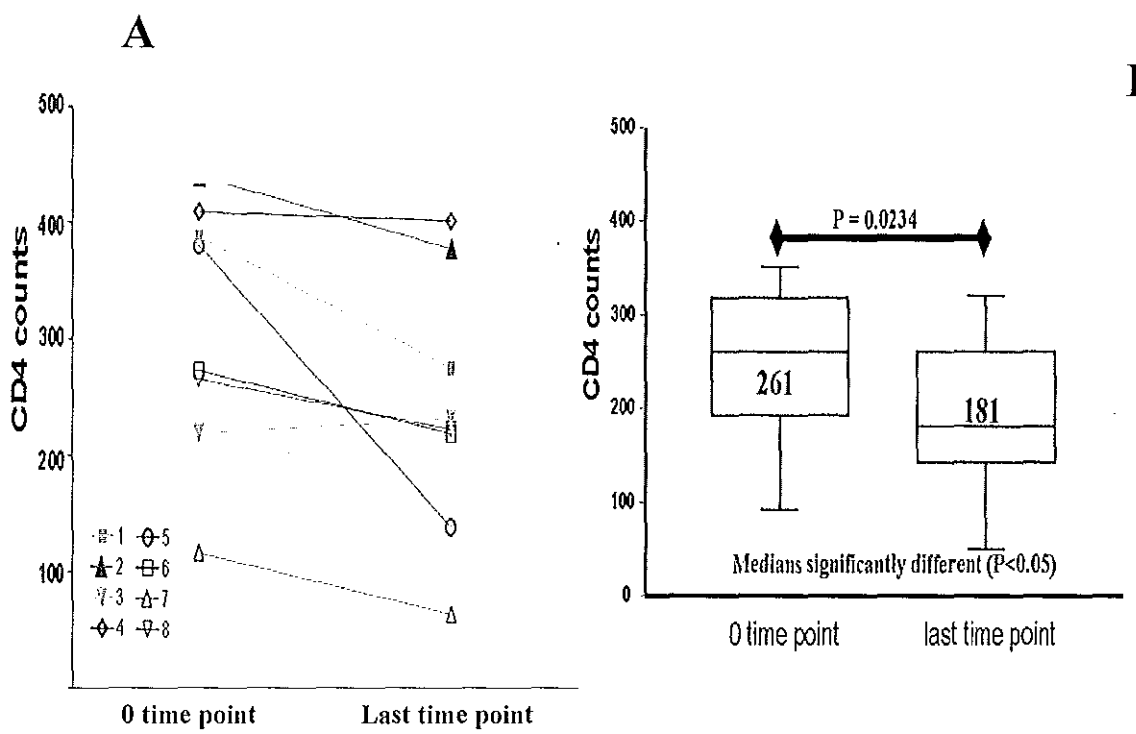


Figure 4*(A&B) CD4 count variation in HIV/TB coinfected patients (study subjects) before diagnosis (0 time point) and after treatment (n=8). Fig (A) The line extends from the 0 time point to the first time point (during treatment). Fig 4 (B) The line in the middle of the box represents the median and the box extends from the 25th percentile (X[25] to the 75th percentile X[75] which is defined as the interquartile range(IQR). The whisker extend to the upper and lower adjacent values.*

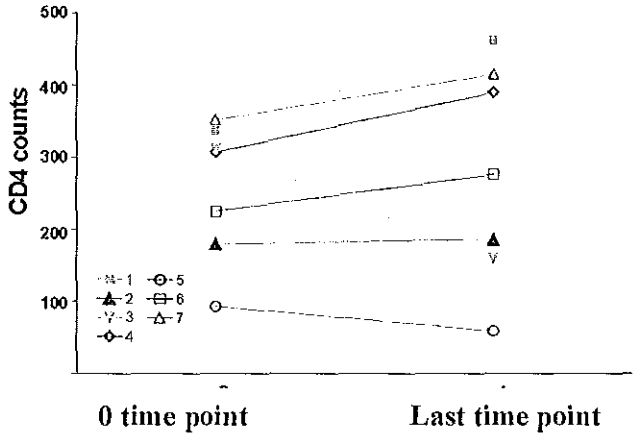


Figure 5. *CD4 count variation in CD4 matched HIV only patients (controls) at 0 time point and last time point (n=7).The line extends from 0 time point to the last time point.*

4.3. PCR and DNA sequencing

RNA was isolated from all the 40 plasma samples, of which 39 (97.5%) had amplifiable PCR product. One became negative (individual 10, after 12 months).From the 39 PCR positive specimens we were able to find an interpretable sequence for the 31 samples (79.5%).

4.4. Phylogenetic analysis

Based on 265 bp C2V3 region Neighbour Joining Tree was constructed to compare the phylogenetic relationship of all *env* sequences (Fig 6). Bootstrap values indicated that sequences from each patient formed a cluster with no aberrant or contaminating sequences. All the readable sequences obtained from 13 HIV and HIV/TB patients belonged to the Ethiopian subtype C sequence.

In this study, 9 out of 13 individuals (69.2%) belonged to subcluster C′ and 2 out of 13 (15.4%) sub-clustered to main group C viruses. The remaining two sequences showed a recombination between the two Ethiopian sub clusters C and C′ (Fig.7).

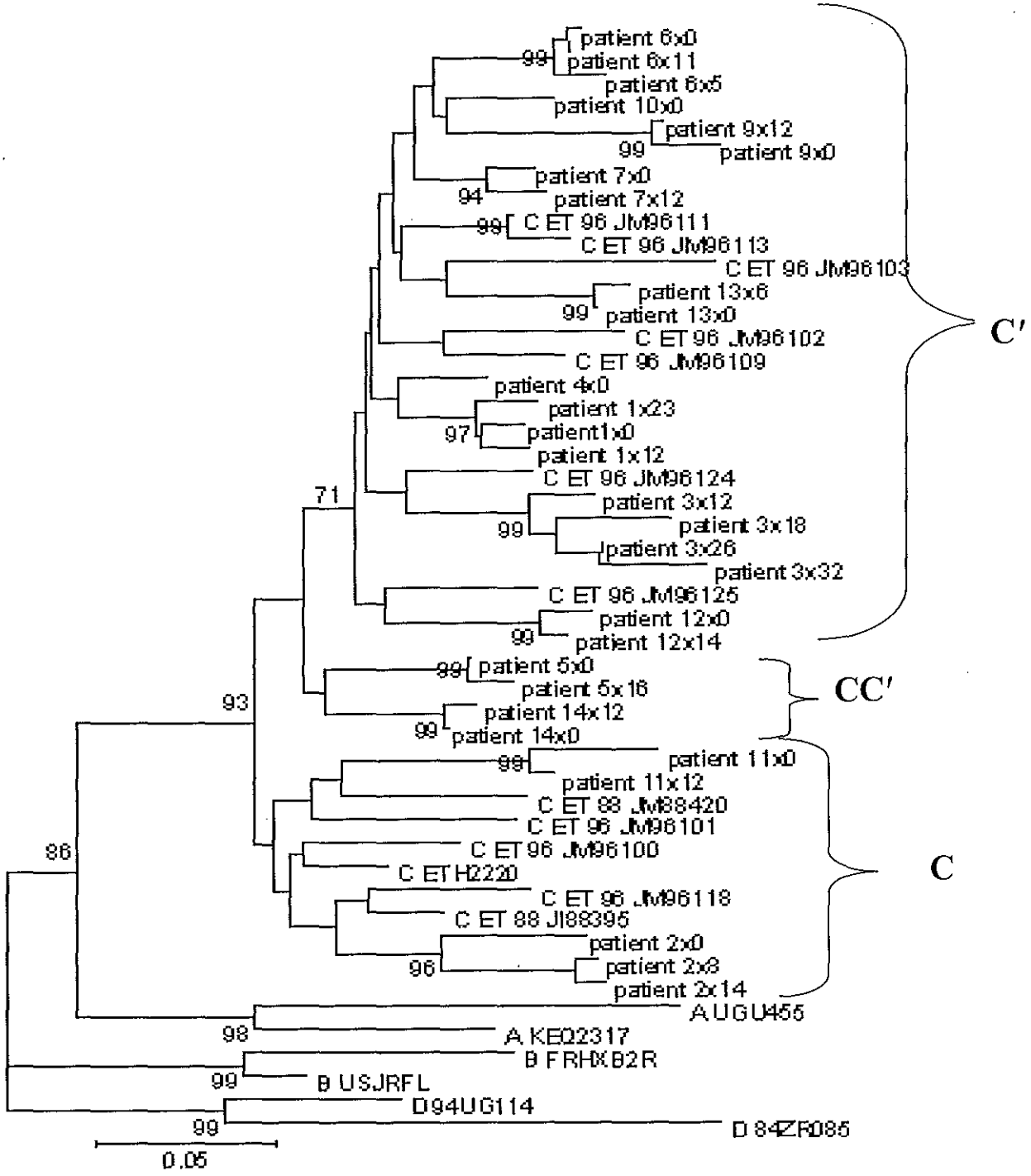


Figure 6. ***Neighbor- Joining tree of 265 bp C2V3 region of the HIV env showing HIV-1 subtypes of the HIV and HIV/TB study population.***

Reference sequences are Ethiopian subtype C sequences and other subtypes (A, B, and D) from Los Alamos HIV data base. Note In Ethiopia we see the formation of the C'cluster. Patients 5 and 14 are between the 2 clusters giving the indication that they are recombinants. The scale bar represents a genetic distance of 5 %.The numbers in each branching are the boot strap values which indicates biased estimates of accuracy.

The identification of the patient is like this:-Example: patient 6X11 means patient number 6 at the 11th months.

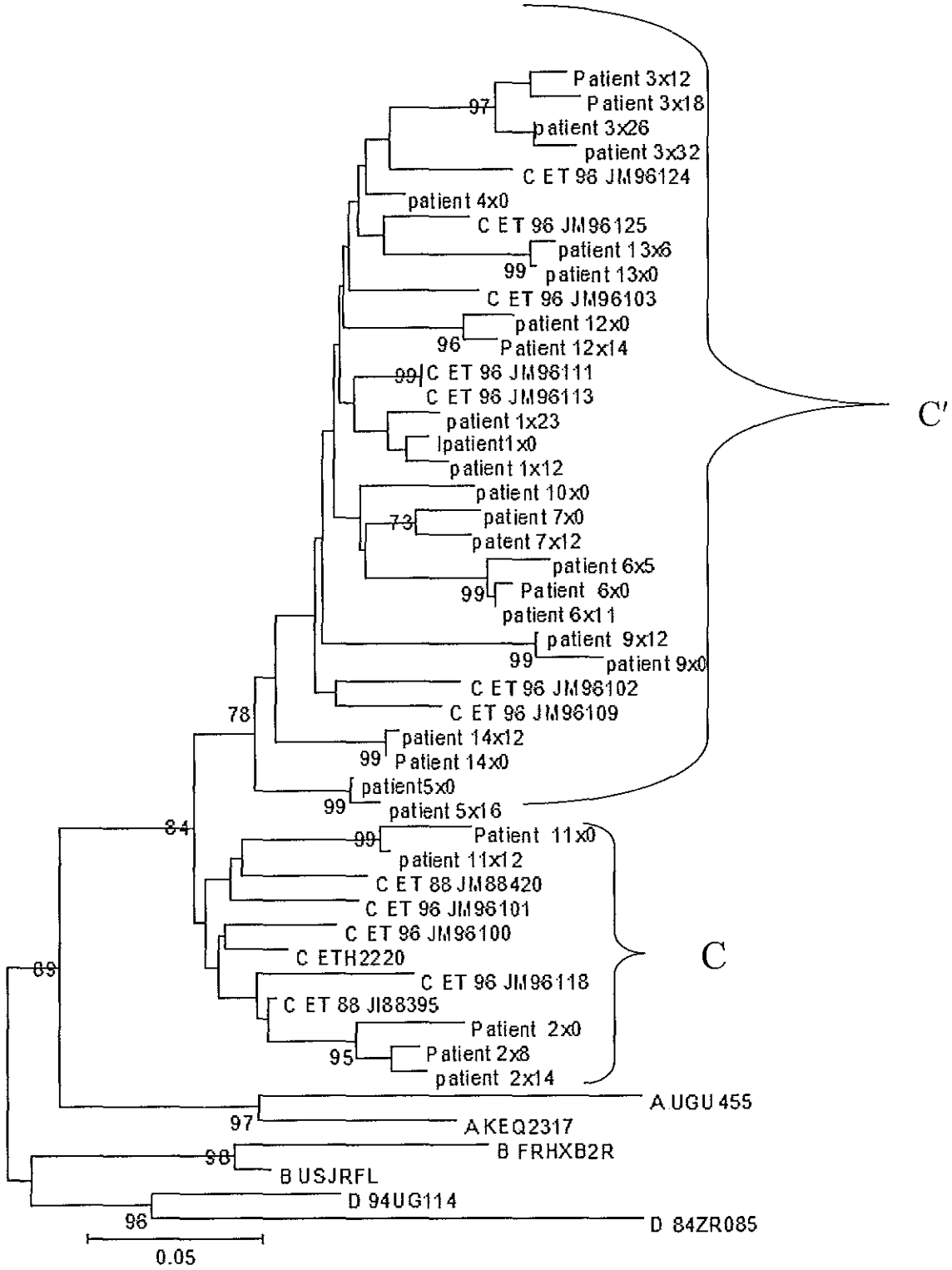
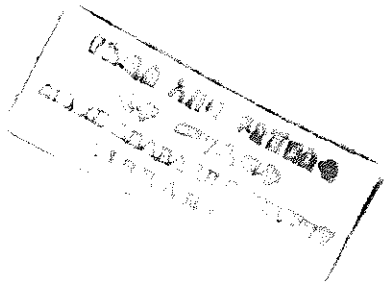


Figure 7. *A similar Neighbor-Joining tree depicted in fig.6. except the 3' end of the C2V3 region for patient 5 and 14 cut out. Here patient 5 and 14 become part of the C' cluster. The scale bar represents a genetic distance of 5 %.The numbers in each branching shows the boot strap value which indicates biased estimates of accuracy.*
The identification of the patient is like this:-Example: patient 6X11 means patient number 6 at the 11th months.

4.5. Predicted amino acid sequence

The deduced aminoacid sequence alignment for the V3 loop of all the sequences is presented in Fig 8. All V3 loops are 35 amino acids long and are flanked by the two cysteine residues. The characteristic C subtype pattern GPGQT (Glysine-proline-glysine-glutamine-threonine) motif at the apex is conserved in all the amino acid sequences. A high frequency of methionine (M) at the 12^{th} position is particularly observed in the C′ subcluster whereas the main group C doesn't possess it.

In this study, one individual had a higher overall positive charge (+5) but with the presence of N-linked glycosylation. Another individual has lost the N-glycosylation site but has a lower positive charge.



V3 LOOP											11													25										Charge
PATIENT 1x0	C	T	R	P	N	N	N	T	R	K	S	M	R	I	G	P	G	Q	T	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	+3
PATIENT 1x23	-	-	-	-	-	-	-	-	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 1x12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 2x0	-	-	-	-	-	-	-	R	-	E	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	L	-	Y	-	+2
PATIENT 2x8	-	-	-	-	-	-	-	-	-	E	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	+1
PATIENT 2x14	-	-	-	-	-	I	-	-	-	G	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+2
PATIENT 3x12	-	-	-	-	S	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	F	-	-	-	E	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 3x18	-	-	-	-	S	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	F	-	-	-	E	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 3x26	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	-	-	E	E	-	-	-	-	-	-	-	-	-	-	+2
PATIENT 3x32	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	-	-	E	E	-	-	-	-	-	-	-	-	L	-	+2
PATIENT 4x0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	+4
PATIENT 5x0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 5x16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 6x0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 6x5	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	K	-	-	-	-	+4
PATIENT 6x11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 7x0	-	-	-	-	-	-	-	-	-	T	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+2
PATIENT 7x12	-	-	-	-	-	-	-	-	-	T	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+2
PATIENT 12x0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	+4
PATIENT 9x12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	.	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 9x0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	.	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 12x14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	+4
PATIENT 11x0	-	P	-	-	-	-	-	-	-	E	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	+2
PATIENT 11x12	-	P	-	-	-	-	-	-	-	E	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+1
PATIENT 10x0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 13x6	-	-	-	-	S	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	F	-	-	-	A	-	-	-	N	-	-	-	-	-	-	+5
PATIENT 13x0	-	-	-	-	S	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	N	-	-	-	-	-	-	+5
PATIENT 14x12	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+3
PATIENT 14x0	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+3

Figure 8. ***Predicted amino acid sequence with its net charge of the V3 loop for the study population .Dashes (-) indicate concurrence with the amino acid sequence of PATIENT 1x0, dots(.)indicate gap, X indicates unknown amino acid. The GPGQT crown and N-linked glycosylation sites are shaded.***

The identification of the patient is like this:- Example: patient 6X11 means patient number 6 at the 11th months.

4.6. Genetic diversity difference between the study group and the control

4.6.1. Nucleotide divergence between 0 and 12 months viral sequences

A comparison in genetic divergence between the two groups is shown in Fig 9 and 10. Divergence can be defined as the distance from a founder sequence to a latter sequence (Mani *et al.*, 2002).

The *ds* (synonymous nucleotide substitution) and *dns* (non synonymous nucleotide substitution) comparison between the study groups and the controls is shown in Fig 9. The sequences obtained in both groups didn't show a significant difference.

ds comparison at 0 months (at intake) and ≈12 months later between the two groups is shown in fig10 (a). Here the median value for both groups at the two time points didn't show a significant difference. Fig10 (b) shows the *dns* (non synonymous nucleotide substitution) comparison between the two groups at 0 and 12 months time. Even though statistically not significant, the median *dns* value for the study groups has increased at 12 months when compared to 0 time point. For the control group *dns* value is almost identical at 0 and 12 months later.

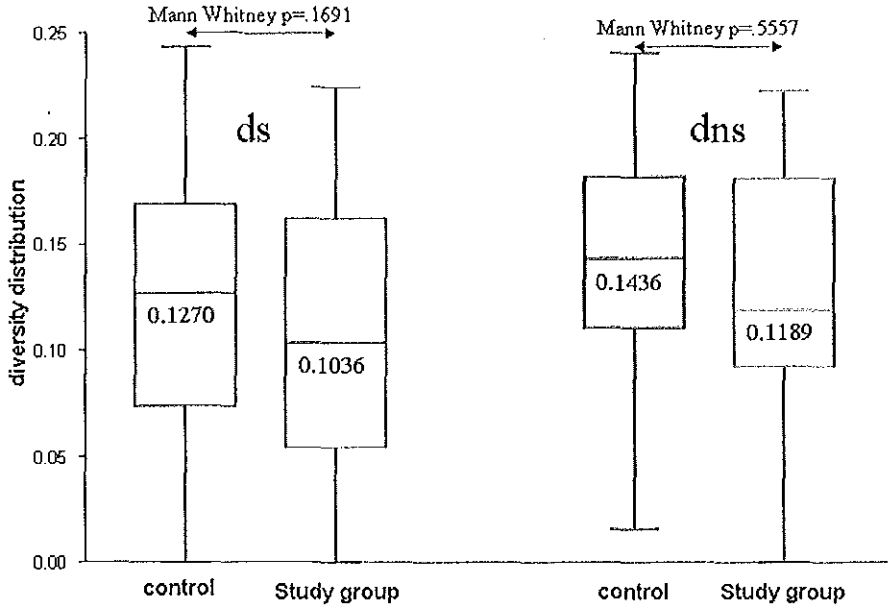


Figure 9.*Comparison of Synonymous and non synonymous distance distribution of 0 and 12 months between the control and study groups. The line in the middle of the box represents the median and the box extends from the 25th percentile (X[25] to the 75th percentile X[75] which is defined as the interquartile range(IQR). The whisker extend to the upper and lower adjacent values.*

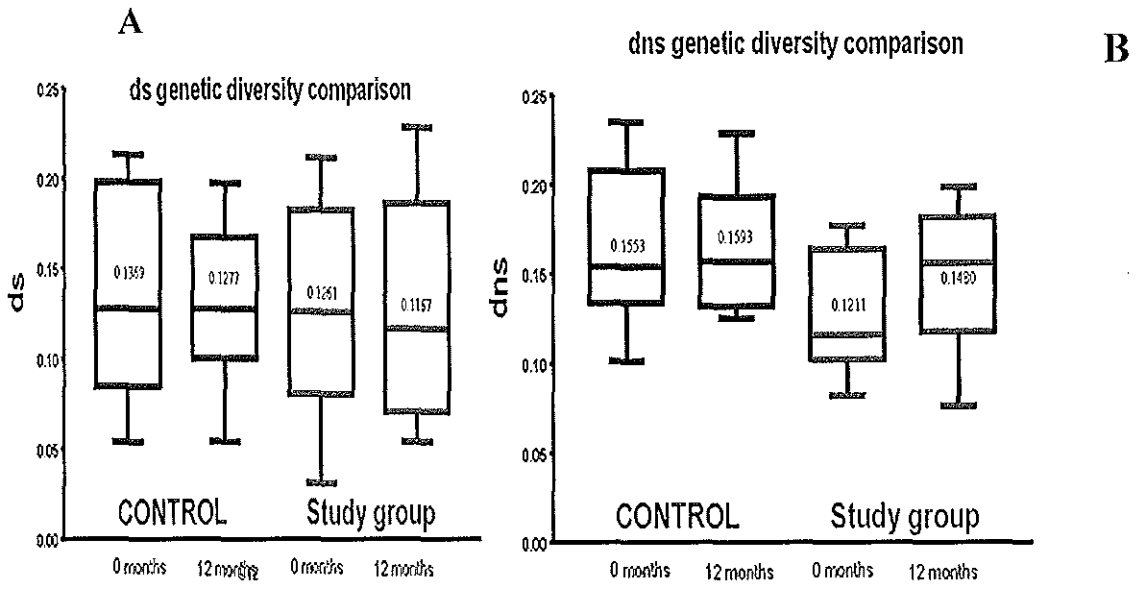


Figure 10. *(a and b). Box and Whisker plot for comparison of syonymous substitution (ds)(a) non synonymous substitution (dns) (b) of the study group and control at 0 month (initial) and approximately 12 months (latter). The line in the middle of the box represents the median and the box extends from the 25th percentile (X[25] to the 75th percentile X[75] which is defined as the interquartile range(IQR). The whisker extend to the upper and lower adjacent values.*

4.6.2. ds/dns ratio as a measure of direction of selection

The ratio of synonymous substitutions per synonymous site to non synonymous substitution per non synonymous site (ds/dns) is a measure of direction of selection, it indicates change in primary aminoacid sequence and is frequently used to describe viral evolution. A ds/dn ratio >1 suggest purifying selection whereas ratio lower than or approximately 1 are characterized by positive selection.

A comparison *ds* to *dns* ratio for the study groups and the controls is shown in Fig 11 (a) and (b).For the control groups the *ds/dns* value was slightly lower than 1 at 0 time point but after 12 months it almost becomes 1. For the study groups an identical *ds/dns* value to the controls was observed at 0 time point but 12 months latter the *ds/dns* ratio become lower than 1 which may indicate a positive selection although statistically not significant.

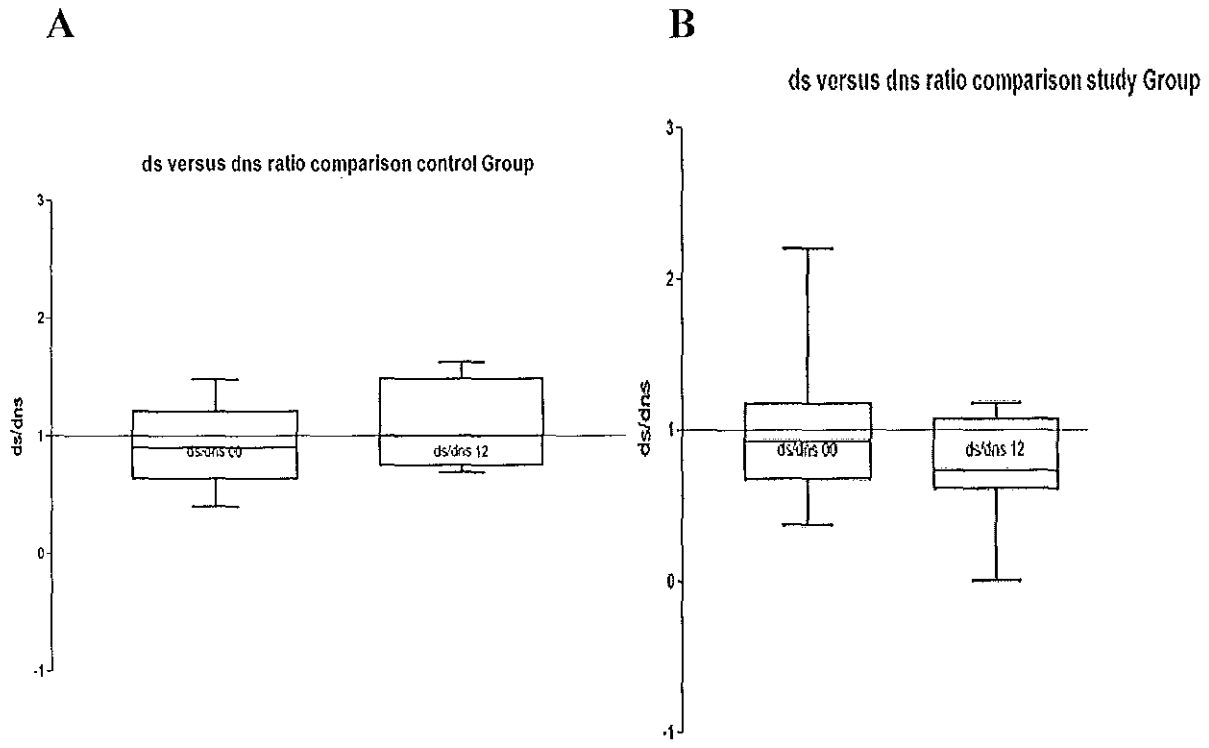


Figure 11. ***ds/dns comparison for the study and control groups at 0 (initial) and approximately 12 months (latter).***

ds/dns ratio >1 indicates purifying selection
ds/dns ratio<1 indicates positive selection
ds/dns ratio ≈1 indicates neutral selection

5. DISCUSSION

Few data exist worldwide on longitudinal CD4+ cell and HIV viral load measurement after the initiation of TB treatment during HIV/TB co-infection. Goletti *et al.*,(1996) and Nakata *et al.*,(1997) documented a significant decrease in HIV-1 viral load after the initiation of anti- tuberculosis treatment in western subjects. In contrast the present study and previous reports in Ethiopia(Wolday,2003;2005) and else where in sub Saharan Africa (Lawn *et al.*,1999) have shown a significant increase in plasma HIV-1 viral load after the start of TB treatment in co-infected individuals.

During the acute phase of MTB, both the western and sub-Saharan African patients show a significant increase in viral load. This is associated with the heightened systemic immune activation due to MTB (Goletti *et al.*, 1996; Vanham *et al.*, 1996). This systemic immune stimulation due to active tuberculosis decreased significantly during the first two months of anti-tuberculosis treatment in sub-Saharan African subjects except TNFα (Lawn *et al.*, 1999).And the raised plasma TNFα level has been suggested to be one of the causes for the elevated viral load during treatment for TB.

The study subjects in this study were also involved in anther study that sought a possible association between tuberculosis and immune activation (Wolday, *et al.*, 2005). In that study it is demonstrated that, there is persistently elevated chemokine and chemokine receptors (markers for immune activation) despite successful TB treatment in HIV/TB co-infected individuals.

This persistently activated immune system may partially explain the elevated viral load observed in TB co-infected HIV patients after successful TB treatment. The mutation

frequency of HIV-1 which was considerably higher during active TB (Nakata *et al.*,1997;Collins *et al.*,2000) may have contributed to highly replicative HIV-1 viruses that lead to persistent viral load elevation.

However, our result based on direct sequencing of PCR products obtained from HIV/TB patients prior to TB treatment and after approximately 12 months follow up (post treatment) when compared to CD4 matched HIV only patients (controls) at the corresponding time interval showed no significant difference in nucleotide genetic distance (divergence). Moreover the selection pressure (ds/dns) ratio approximated to 1, suggest positive, but weak selection on both groups (Kils-Hutten *et al.*,2001).

Therefore our result didn't support the hypothesis that HIV/TB co-infection would provide an advantage to certain variants of HIV viruses with a high replicative capability.

In HIV/TB co-infected individuals a heterogeneous HIV population and high mutation frequency in the blood was observed in the study done by Collins *et al.*,(2000). The explanation given by the authors is that, active TB may have caused an HIV-1 directed stimulation and activation of both cellular and humoral immune responses. In turn, an increase in HIV-1 heterogeneity may be necessary for immune avoidance (Collins *et al.*, 2000).

In our study participants, there is immune activation during HIV/TB coinfection even after successful TB treatment (Wolday *et al.*, 2005), but this immune activation may not be responsible for selection of specific viral variants (i,e may not be HIV specific). Therefore it may cause viruses in CD4+ and macrophages to replicate at the lung sites irrespective of the virus genotype they contain.

A similar explanation was given by Ostrowski *et al* (1998) in immunization by tetanus toxoid that resulted in transient burst in plasma viremia after immunization. The viral burst may reflect a non specific increase in viral replication leading to a generally reversible shift in composition of plasma viral quasispecies, and did not to any evidence on selection of specific viruses (Ostrowski *et al.,* 1998).

This puts the earlier explanation given by Collins *et al.,* (2000) in question. In the other study done by the same authors (Collins *et al.,*2002) found a genetically distinct viral population at the lung site in TB co-infected individuals and this resulted in dissemination to the blood and caused a genetically heterogeneous HIV population in the blood. Nickel *et al.*, (2003) suggested that this genetically heterogeneous HIV population in the pleural spaces is due to stimulation of HIV from a latent reservoir pool as opposed to Collins *et al.,* (2002) suggestion that the diversity is from new rounds of viral replication and selection.

The incidence of active TB in Ethiopian HIV patients occurs usually at a CD4+ cell count below 200 cells/mm^3 (Wolday *et al.,* 2003). The very low CD4 count that continued to show a significant decline even after successful TB treatment was similar to what has been reported to be characteristic of a stabilized or slowed HIV genetic divergence from the founder strain and an indicator of a decline in diversity (Shankarappa *et al.,*1999).

The V3 amino acid sequences gives information about the co-receptor usage of the virus. Specific variability in V3 that are associated with different co-receptors can be used to predict phenotype with reasonable success (Hoffman *et al.,* 2002). In particular studies had determined that the presence of at least one basic substitution at V3 position 11 or 25 (HXB gp 160, 306 and 322) is associated with X4, R5X4 and SI phenotypes (Hoffman *et*

al., 2002). More over, the R5-to-X4 phenotype switch of HIV-1 was shown to be associated with an increase in the overall positive charge of the V3 region and the lack of the *N*- linked glycosylation event within the V3 loop for the different subtypes of HIV- 1 (Nabatov, *et al.,* 2004).

However in the present study, analysis of the V3 amino acid sequences revealed none of the HIV and HIV/TB samples to possess a positively charged amino acid at the particular positions which indicates they belong to NSI/CCR5 tropic viral phenotypes.

Nakata *et al.,*(1997) demonstrated that co-infection with tuberculosis and HIV-1 not only markedly increased the amount of HIV-1 RNA produced in the lung but also induced more basic amino acid substitutions in the gp 120 V3. In the present study, however, none of the HIV/TB co-infection individuals possessed a basic amino acid at the 11^{th} and 25^{th} position of the V3 region.

Immune activation may also be explained by the type of tropisms of viral variants that the individual harbors. NSI viruses have an apparent replicative advantage over SI viruses in the setting of immune activation (Ostrowski *et al.,* 1998). Since all the HIV/TB and HIV subjects were shown to harbor NSI strains, in the present study, this is ample indication for existence of immune activation in all the study population.

Generally, we need more information in the interpretation of genetic analysis in subtype C sequences. Subtype C has some peculiar features when compared to other subtypes for which most genetic diversity analysis studies were carried out. First, total sequence variability among subtype C V3 sequences is low-observation that subtype C V3 sequences are more highly conserved than in other subtypes, and second, basic amino acid substitutions are rare among a large collection of V3 sequences from subtype C viruses.

Such substitutions are the hallmark of X4 variants among subtype B viruses. Third, there is a lack of correlation between the appearance of multiple V3 sequence variants and more extensive V3 sequence evolution as a function of decreasing $CD4^+$-T-helper-cell levels (Ping *et al.,* 1999).

As it is well documented (Abebe *et al.,* 2000) the Ethiopian subtype C sequence is sub-clustered into C′ and main C. The phylogenetic analysis done in this study confirmed that the C and C′ sequences cluster separately with 93% boot strap values. Sub-cluster C′ is the dominant subtype C cluster in the study population with no difference between the HIV and HIV/TB subjects. This is in line with other recent studies done in Ethiopian sequences (Measho, 2001; Melaku, 2003).Consistent with previous works done on Ethiopian HIV subtypes (Abebe *et al.*,2000) the predicted amino acid sequences of the V3 loop showed a high frequency of methionine at the 12^{th} position in sub-cluster C′ but not in the main C, the biological explanation for this observation still needs to be sought.

This study has also revealed a recombination of C/C′ at the *env* region for the first time (fig 6&7). Previously recombination on C/C′ was detected on the *gag* region where all the recombinants had a C′ envelop (Pollakis *et al.,* 2000). In general recombinant viruses may have advantages over the parental strain, including eventual modifications in tropism and replication efficiency (fitness). Several studies have found that, under the selective pressure imposed by antiretroviral drugs, recombination between strains with different drug sensitivities occurred, resulting in new HIV-1 variants with dual drug resistance (Moutouh *et al.*,1996).

Our result also showed the PCR efficiency (97.5%) which is the highest ever attained in previous works at this laboratory. Only one sample became PCR negative may be because

of the viral load which is very low (log 1.9 or 80 copies/ml). High PCR efficiency was obtained possibly because a better PCR machine (applied biosystem 2700) was used and the viral load for most of the samples was high.

But the sequence efficiency for this work (79.5%) is unexpectedly low. This is because of the lack of some facilities in the laboratory for the preparation of the sequence products by the time this work was done. First we didn't purify the PCR products because we didn't have a column purification kit. Second we did not use a plate centrifuge for purifying the extension product, which made us lose pellet during the various washing steps.

The limitation of this work is that our result is based solely on the direct PCR products of the dominant sequences which obscure the sequences of the various quasispecies. We were unable to observe intra patient diversity, diversification and diversification rate.

6. CONCLUSION AND RECOMMENDATION

The pathogenesis of persistently elevated plasma HIV viremia in patients co-infected with TB after completion of TB treatment in Africa remains undefined. No significant HIV genetic divergence between HIV/TB co-infected and HIV only patients after TB treatment /one year follow-up in this study. This suggests that, during or after TB co-infection in HIV infected individuals, the heightened immune activation does not lead to highly replicative HIV variants. This may be because the immune activation is not HIV specific but rather a more generalized one. The generalized immune activation during HIV/TB co-infection caused the virus to replicate persistently but did not cause selection of specific viral variants.

Future researches should focus on isolating the different HIV quasispecies so that one can see if there is a difference in HIV genetic diversity and the diversification rate between the HIV/TB and HIV only study populations.

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