Aus dem Fachbereich Medizin

der Johann Wolfgang Goethe-Universität

Frankfurt am Main

Zentrum der Hygiene

Institut für Medizinische Virologie

Direktor: Prof. Dr.med.H.W.Doerr

Characterization of The Longitudinal HIV-1 Quasispecies Evolution in HIV-1 Infected Individuals Co-infected with *Mycobacterium tuberculosis*

Dissertation

zur Erlangung des Doktorgrades der theoretischen Medizin des Fachbereichs

Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main

vorgelegt von

Tsigereda Biru Leulebirhan

aus

Addis Ababa, Äthiopien

Frankfurt am Main 2010

Dekan	Prof. Dr. J. Pfeilschifter
Referent	Prof. Dr. med. H.W. Doerr
Koreferent	Prof. Dr. med. L.G. Gürtler
Tag der mündlichen Prüfung	06. September 2010

Dedicated to my parents:

Nigatuwa Bekele

and Biru Leulebirhan

Table of Content

1. Introduction 1

1.1 Brief introduction to HIV and AIDS 1

1.1.1. Discovery of AIDS and its causative agent 1

1.1.2. Human Immunodeficiency Virus (HIV) 1

1.2. Epidemiology of HIV/AIDS 2

1.3. Entry and replication of HIV 3

1.4. HIV co-receptors and biological phenotypes 4

1.5. Genomic organization of HIV-1 5

1.5.1. HIV-1 gp120 7

1.5.2. The HIV-1 V3 loop 8

1.6. Genetic diversity of HIV 9

1.6.1. Genetic subtypes and groups 10

1.6.2. Genetic evolution of HIV-1 within individuals and concept of quasispecies 11

1.7. Tuberculosis 12

1.7.1. The tubercle bacillus 12

1.7.2. Stages of TB infection 13

1.7.3. Global burden of TB 14

1.8. HIV and TB Interaction 15

1.9. Aim of the study 17

2. Patients, materials and methods 18

2.1 Patients, data and plasma 18

2.1.1. Frankfurt HIV cohort 18

2.1.2. EPIDEM database 18

2.1.3. HIVCENTER plasma bank 19

2.1.4. Sample selection criteria 19

2.2. Materials 20
2.2.1. Laboratory equipments 20
2.2.2. Chemicals 21
2.2.3. Commercial kits, reagents, buffers, enzymes, primers and bacteria 22
2.2.4. Software and databases 27
2.3. Methods 28
2.3.1. HIV-1 RNA isolation from plasma 28
2.3.2. HIV-1 C2V5 *env* reverse transcription and PCR amplification 28
2.3.3. HIV-1 C2V5 *env* semi nested PCR 29
2.3.3. Molecular cloning 29
2.3.4. Sequencing 32
2.3.5. Sequence analysis 32
2.3.6. Statistical analysis 36
3. Results 37
3.1. Subjects and specimen collection 37
3.2. Cloning and sequencing 40
3.3. Phylogenetic tree 42
3.4. Phenotype prediction 43
3.5. Quasispecies diversity 44
3.6. Intra-patient quasispecies diversification 46
3.7. Quasispecies divergence for HIV-1/TB 46
3.9. Synonymous and Non synonymous nucleotide substitutions 50
3.10. Positive selection using data monkey programme 50
3.11. Positional variation along the C2V5 amino acid sequence 50
3.12. Potential N-linked glycosylation sites (PNGs) 51
3.13. Correlation between HIV-1 viral load, CD4 cell count, intra-patient diversity and divergence 52

4. Discussion 54
4.1. Active TB sustains HIV-1 quasispecies diversity for longer period 56
4.2. Active TB increases rate of HIV-1 divergence 58
4.3. TB might lead to slow evolution of X4 variants 59
4.4. Other findings 60
4.5. Limitations of the study 61
Summary 63
Zusammenfassung 65
Literature 67
Appendix 81
Abbreviations 81
Acknowledgement 84
Lebenslauf 86

1. Introduction

1.1 Brief introduction to HIV and AIDS

1.1.1. Discovery of AIDS and its causative agent

In the early 1980s, a new clinical syndrome was discovered among homosexual men in the United States and in 1981 the first article related to AIDS was published [1].This article reported that there was a random increase in *pneumocystis carinii* pneumonia (PCP), a rare lung infection. The infection was commonly associated with immunodeficiency, and many of the patients had very low numbers of CD4+ T-cells [1]. In 1982, the new disease was called acquired immunodeficiency syndrome (AIDS) and was properly defined by the center for disease control (CDC).

The first indication that AIDS could be caused by a retrovirus came in 1983 when scientists from the Pasteur Institute of Paris isolated a new virus containing RT activity from a patient with lymphoadenopathy [2].This virus was called lymphadenopathy associated virus (LAV). Shortly after, a similar virus was isolated from an AIDS patient by an American group [3] and it was evident that the new virus was the causative agent of AIDS and was called HTLV-III (human T-cell leukemia virus type III). At the same time Levy and coworkers [4] reported the identification of retroviruses named AIDS-associated retroviruses (ARVs) from AIDS patients with known risk groups as well as from symptomatic and some healthy people. In 1986 the International Committee on Taxonomy of Viruses (ICTV) recommended giving the AIDS virus its present name, Human Immunodeficiency virus (HIV) [5].

1.1.2. Human Immunodeficiency Virus (HIV)

According to ICTV classification system [6], HIV is grouped in the family, *Retroviridae*; subfamily *Orthoretrovirinae* and genus *Lentivirus*. *Retroviridae* comprises groups of RNA viruses which are enveloped and replicate in a host cell via the enzyme reverse transcriptase to produce DNA from their RNA genome [7]. This DNA is transported to the nucleus and integrated in to the cellular chromosome. The genus Lentivirus (lenti = slow) consists of groups of viruses which have long incubation period until manifestation of disease. Presently five serogroups are recognized designated with the associated type of vertebrae host (primates, sheep

and goats, horses, cats, and cattle) [6]. HIV belongs to the primate lentiviruses. Two species of HIV known to exist: HIV-1 and HIV-2. HIV-1 is the virus that was initially discovered and termed LAV; it is more virulent [8], and is the cause of the majority of HIV infections globally. HIV-2 has a lower pathogenicity and lower transmission capacity compared to HIV-1 and is largely confined to West Africa and countries with past socio-economical links with Portugal, including southwest India [9].

Morphologically HIV-1 and HIV-2 have the characteristics of a lentivirus, with a cone shaped core and a diameter of about 100 to 200 nm [7]. Projections of envelope make the surface appear rough. Tiny spikes made of two envelope glycoproteins, gp120 (external surface envelope protein) and gp41 (transmembrane protein) (about 8 nm) may be dispersed evenly over the surface [7]. The matrix protein just below the viral membrane forms the inner shell. Beneath is the capsid protein which is concentric and rod-shaped, or shaped like a truncated cone which encloses the viral RNA. Deep inside the isometric core, nucleocapsid, two strands of RNA molecules of approximately 9.6 kb nucleotide bases, integrase, a protease, ribonuclease, and two other proteins, known as p6 and p7 are found [7].

1.2. Epidemiology of HIV/AIDS

Since AIDS was first recognized in 1981, it has led to the deaths of more than 25 million people, making it one of the most destructive diseases in recorded history [10]. Globally, the number of people living with HIV is rising due to new infections and the beneficial impacts of anti retroviral therapy (Fig. 1). In the year 2008 an estimate of 33.4 million [31.1 million–35.8 million] people were living with the virus [10].

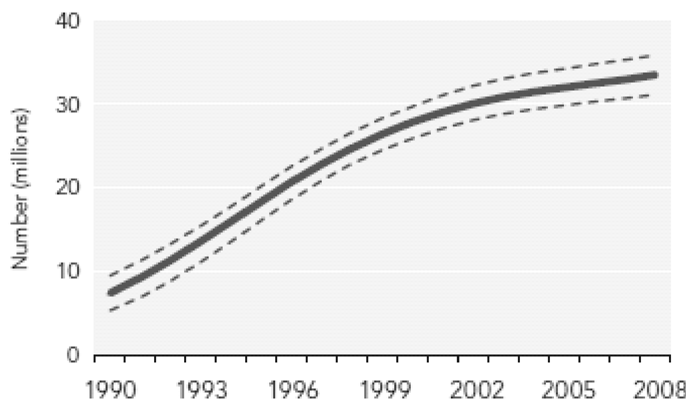


Figure 1 Global estimates of the number of people living with HIV from 1990–2008 (UNAIDS 2009) [10].

There is an extreme geographical variation in HIV prevalence within and between countries. In general sub-Saharan Africa is the most heavily affected region, accounting for 71% of all new HIV infections in 2008 followed by south and south-east Asia (Fig. 2).

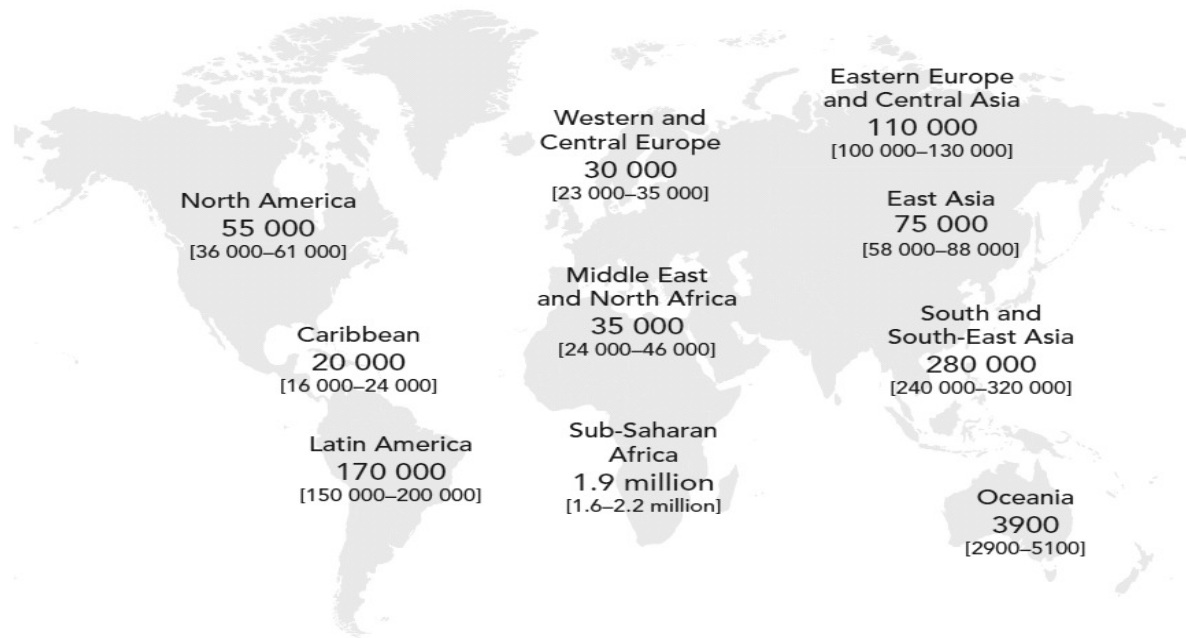


Figure 2 Estimated number of adults and children newly infected with HIV, 2008 (UNAIDS, 2009) [10].

1.3. Entry and replication of HIV

The main cellular receptor for HIV is the CD4 molecule [11]. CD4 is a 55-kd surface glycoprotein composed of highly charged cytoplasmic domain, a single hydrophobic membrane spanning domain and four distinct extracellular domains, D1 to D4 [12]. Its physiological function is to stabilize the interaction between the T-cell receptor on the surface of T lymphocytes and class II major histocompatibility complex (MHC-II) molecules on antigen-presenting cells. CD4 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 co-receptors, CXCR4 and CCR5, adjacent to the CD4 molecule in the cell membrane [13]. Co-receptor 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 [13].

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 [14]. 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 intergrase, in the context of a pre-integration complex, and transported to the nucleus by a process that requires ATP [15].

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 [14]. 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.

1.4. HIV co-receptors and biological phenotypes

After the identification of CD4 as the major HIV receptor, it was recognized that the CD4 receptor alone was neither sufficient nor the sole means of viral entry [7]. Some human cells expressing high levels of the CD4 protein, including undifferentiated CD4+ monocytes, are not susceptible to HIV infection [16-18]. Some animal cells, derived through molecular or somatic cell hybrid techniques to express human CD4 on the cell surface, could not be infected [18]. For more than a decade, the identity of the potential HIV-1 co-receptor remained elusive [19].

Later, it was demonstrated that members of the G protein-coupled receptor superfamily of seven-transmembrane domain proteins provided the long-sought co-receptor function [20-23]. The two major co-receptors for HIV infection are CXCR4 and CCR5. In recognition of the importance of CXCR4 and CCR5 in determining HIV tropism, a nomenclature scheme was devised based on co-receptor usage [19]. Isolates that used CCR5 were denoted R5 isolates; strains that preferentially used CXCR4 were named X4 viruses; and dual-tropic stains that used both CCR5 and CXCR4 were denoted R5/X4 [19].

Before the discovery of co-receptor usage as a classification system, strains of HIV-1 were classified on their different biological phenotypes [24] i.e., based on cell tropism, replication rate in peripheral blood mononuclear cells (PBMCs), or the cytopathology in MT-2 cells [25]. Isolates of HIV which 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 has been called rapid/high (R/H) syncytia inducing (SI) or MT-2 positive [26]. Other isolates which have a slower growth rate and do not induce syncytia (NSI) in PBMC and do not infect cell lines has been called slow/low (S/L) non-syncytia inducing (NSI) or MT-2 negative [26].Viruses expressing the first, rapid phenotype are often X4 viruses whereas the second, slower phenotypes belong to R5 viruses [27].

1.5. Genomic organization of HIV-1

The basic genome organization of the HIV-1 provirus is similar to all other retroviruses with respect to the three major open reading frames (ORFs) encoding the structural proteins *gag*, *pol*, *env* (Fig. 3). *Gag* and *pol* are produced from the unspliced transcript, whereas *env* is produced from an mRNA in which an intron is spliced out [28]. HIV-1 contains 6 additional genes termed *tat*, *rev*, *vif*, *vpr*, *vpx*, and *nef* that are produced from alternative splicing [29]. In total, five 5'-splice sites and eleven 3'-splice sites have been identified which give rise to more than 40 different mRNAs grouped into three different classes, namely: the unspliced primary transcript (~9 kb), a class of singly spliced RNAs (~4 kb), and a class of two or multiple spliced RNAs (~2 kb) [28-33]. A brief summary of gene and gene products of HIV is given in table 1[7].

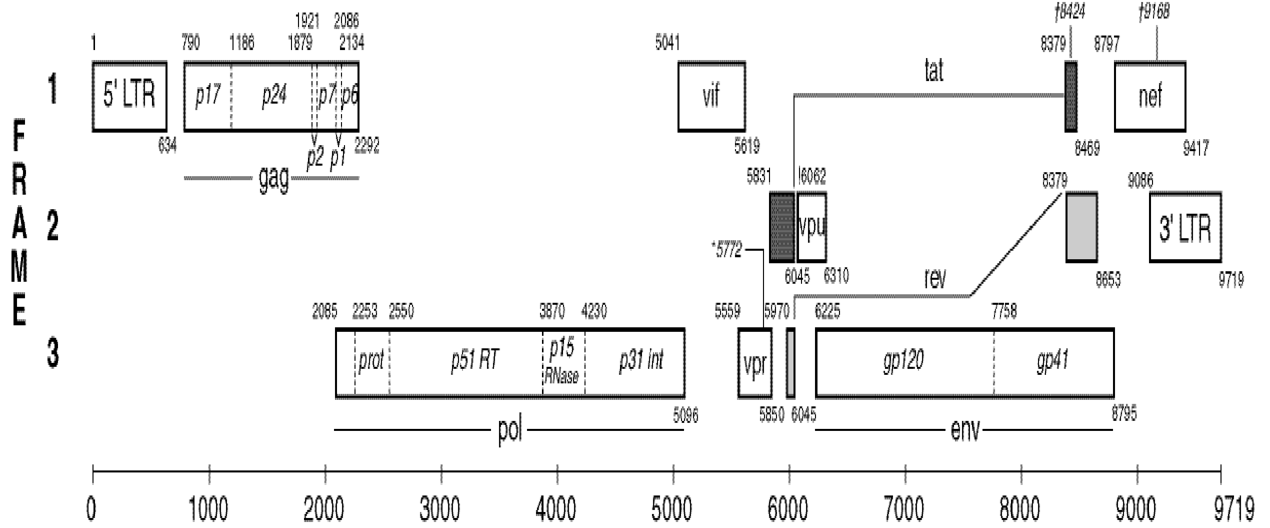


Figure 3 Landmarks of the HIV-1 genome, HXB2 strain (from Los Alamos HIV database; http://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html).

Table 1. HIV proteins and their function (Adapted from Ref. no. 7)

Proteins/ genomic structural elements	Designation(s) and size(kDa)	Function
LTR		Contains important regulatory regions, especially those for transcription initiation and polyadenylation.
Gag	p24	Capsid(CA),structural protein
	p17	Matrix(MA) protein, myristoylated
	p7	Nucleocapsid(NC) protein; helps in reverse transcription
	p6	Role in budding (L domain)
Polymerase(Pol)	p66,p51	Reverse transcriptase(RT): RNase H-inside core
Protease(PR)	p10	Posttranslational processing of viral protein
Integrase(IN)	p32	Viral cDNA integration
Envelop(Env)	Gp120	Envelope surface(SU) protein
	Gp41	Envelope transmembrane (TM) protein
Tat	p14	Transactivation
Rev	p19	Regulation of viral mRNA expression
Nef	p27	Pleiotropic can increase or decrease virus replication
Vif	p23	Increases virus infectivity and cell-to-cell transmission; helps in proviral DNA synthesis and/or in virion assembly
Vpr	p15	Helps in virus replication; transactivation
Vpu	p16	Helps in virus release; disrupts gp160:CD4 complexes
Vpx	p15	Helps in entry and infectivity
Tev	p26	Tat/rev activities

1.5.1. HIV-1 gp120

The gp120 (Fig. 4) is a glycoprotein that is located on the surface of HIV. It contains the binding site(s) for cellular receptors and the major antibody-neutralizing domains. Gp120 gene is around 1.5 kb long and codes for around 500 amino acids [34]. Three gp120s, bound as heterodimers to a transmembrane glycoprotein, gp41, are thought to combine in a trimer to form the envelope spike [35].

By comparing the genetic sequences of the envelope gp120 from HIV-1 virus isolates, five conserved (C) and five divergent or variable (V) regions have been identified [36-38]. Intra-molecular disulfide bonds in the gp120 glycoprotein result in

the incorporation of the first four variable regions into large, loop like structures [36]. Antibody binding studies and deletion mutagenesis have indicated that the major variable loops are well exposed on the surface of the gp120 glycoprotein [39, 40]. The more conserved regions fold into a gp120 core where CD4 binding determinants in *env* map to the C3 and C4 domains of gp 120 [41]. V1/V2, C2 and V3 domains on the other hand help to stabilize the association [43].

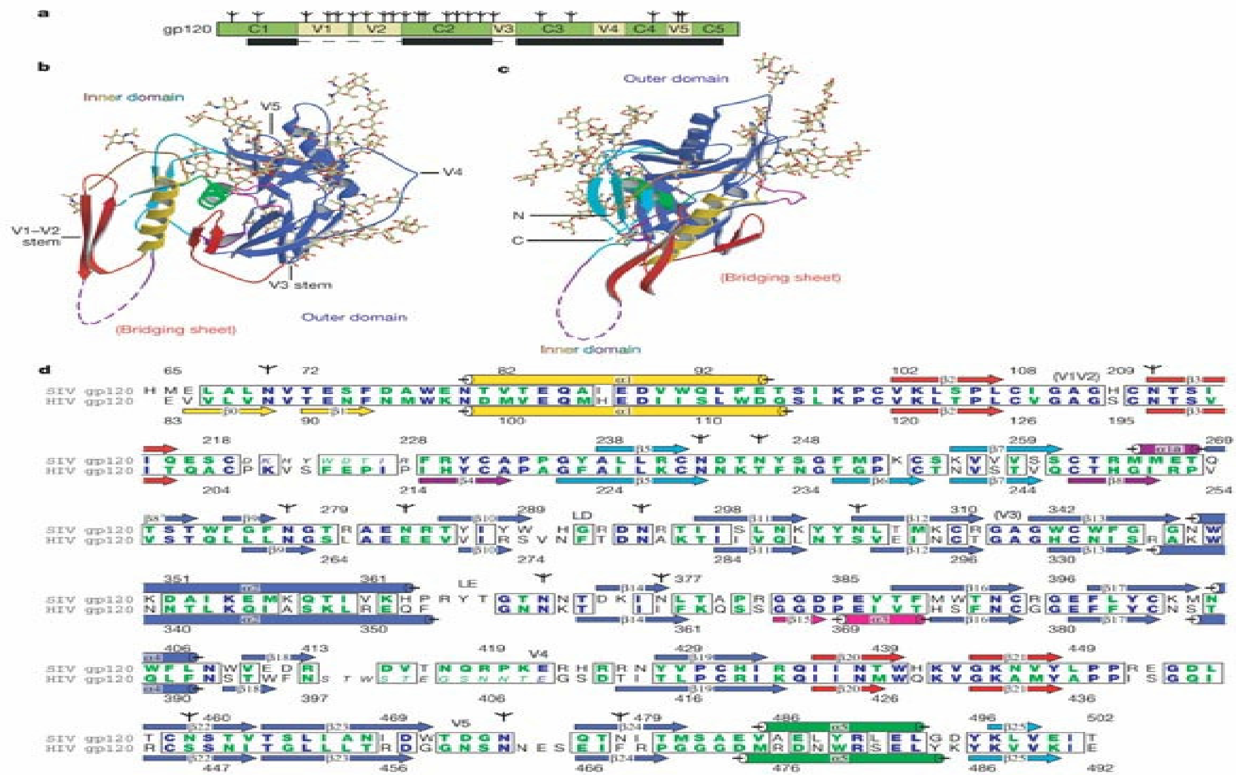


Figure 4 Structure of an unliganded simian immunodeficiency virus gp120 core (Adapted from Ref. no. 42).

1.5.2. The HIV-1 V3 loop

The third variable loop or V3 loop is a part or region occupying the gp120 positions 296 to 331 relative to HXB2. It is typically 34 to 36 residues in length and encodes a surface accessible loop formed by a disulfide bridge between two invariant cysteines. It contains an important immunodominant neutralizing domain also called principal neutralizing domains (PND) [43]. V3 loop-derived peptides have been found to be structurally similar to distinct chemokines, the natural ligands of CCR5 and CXC4 [44] which suggested that alternative V3 conformations are responsible for selective interactions with the co-receptors.

There is a marked variability in the amino acid sequence of the V3 loop among different isolates, especially in the regions flanking the highly conserved glycine-proline-glycine (GPG) central part [45]. The GPG crown (or crest) is situated in the center of the neutralizing domain [46] and forms a beta turn, with the flanking regions as the two strands of an anti-parallel beta sheet [47]. Sequence changes close to the GPG motif can alter the stability of the beta sheet and/or alter the surface accessibility, thereby influencing co-receptor usage [46].

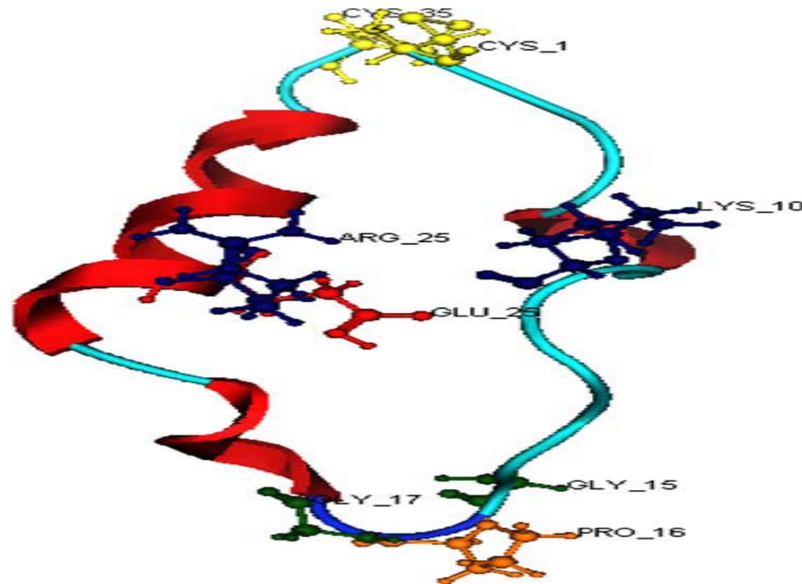


Figure 5 3D structure of the V3 loop obtained from NMR studies. The GPG (Gly15-Pro16-Gly17) sequence is shown at the crown of the loop in green, orange, and green, respectively. The disulfide bridge is represented by the two Cys residues in yellow (Cys1-Cys35). Position 25 is represented by both Arg (positively charged) and Glu (negatively charged) residues in red blue and red, respectively. Position 10 is represented by Lys in blue (positively charged).(Adapt from ref. no.48).

1.6. Genetic diversity of HIV

One of the earliest and most striking observations made about HIV is the extensive genetic variation that the virus has within individual hosts [49]. This variation makes HIV one of the fastest evolving of all organisms. Rapid evolution of HIV is the result of combination of factors. First, the virus experiences a high rate of mutation, with reverse transcriptase making 1.1 mutations (range, 0 to 3) in each viral genome during one infection cycle [50, 51]. Second, HIV has remarkable replicatory dynamics: with a viral generation time of ~2.5 days and production of ~10^{10}–10^{12} new virions each day [52]. Since the HIV genome is an estimated 10^4 base pairs in length and the baseline rate of viral production is approximately 10^{10} virions per day, millions of viral variants are produced within any infected person in a single day [52].

Finally, HIV-1 recombination can lead to further viral diversity and occurs when one person is co-infected with two separate strains of the virus that are multiplying in the same cell. Frequent recombination and natural selection further elevate its rate of evolutionary change.

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. There is only a 55% similarity in amino acid sequence among HIV isolates for the *env* gene while it is 82% for *gag*; 75% for *pol*; 83% for *vif*; 87% for *vpr*; 74% for *rev*; 67% for *tat*; and 77% for *nef* [7].

The clinical implications of high genetic variability are extensive. It allows the virus to escape the host immune system, develop drug resistance and escape the immunity induced by candidate vaccines. Therefore, knowledge about genetic variation of HIV-1 is important for the design of drugs and vaccines and for improving combination therapy.

1.6.1. Genetic subtypes and groups

Over the past decades, advances in sequencing technology and expanded disease surveillance have allowed researchers to characterize the variation in HIV-1 within individual patients and around the world. Phylogenetic analysis of HIV-1 and related viruses from nonhuman primates suggest at least four independent transmission events in the 20th century spawned four HIV-1 groups: major (M), outlier (O), non major and non-outlier (N) and P [53,54,55].

Group M is by far the most prevalent and contains the vast majority of genetic variants. It has been divided into subtypes, denoted with letters, and sub-subtypes, denoted with numerals [reviewed in ref. no. 56]. Currently nine subtypes (A-D, F-H, J, and K), six sub-subtypes (A1–A4, and F1–F2) of the major group M are recognized [reviewed in ref. no. 56].Over the past decade, advances in full-genome sequencing of HIV also have led to the identification of 48 circulating recombinant forms (CRFs) [57] and a number of unique recombinant forms (URFs). These are the results of recombination between subtypes within a dually infected person, from whom the recombinant forms are then passed to other people. The recombinant progeny are classified as circulating recombinant forms if they are identified in three or more

people with no direct epidemiologic linkage; otherwise they are described as unique recombinant forms [58].

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 travelling and immigration [59]. In South America, subtype B is the most common but subtypes F, C and recombinant CRF12_BF are also found [56]. In Asia subtypes B and C are the most common non-recombinant subtypes [56]. However the highest degree of genetic diversity is seen in Africa. In sub-Saharan Africa, all known HIV-1 subtypes are found to circulate in different regions, with different relative frequencies. In Central and West Africa subtype A seem to be the predominant form, but all other subtypes have been found in this region together with many recombinant forms [56]. In east African countries, such as 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 [56]. Fig.6 summarizes the subtypes and recombinant forms circulating throughout the world.

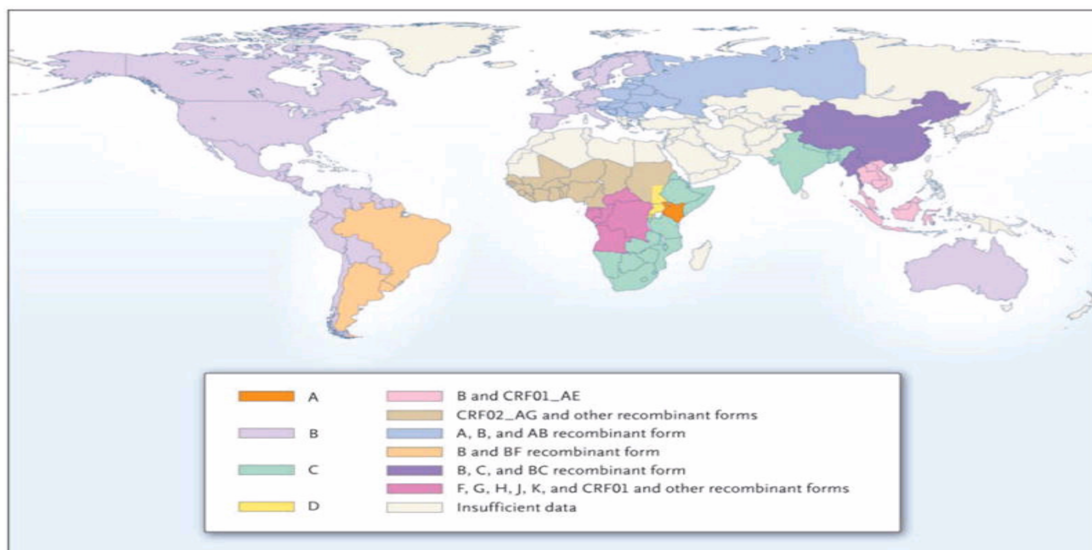


Figure 6 Current Global Distribution of HIV-1 Subtypes and Recombinant Forms (Adapted from Ref. no. 56).

1.6.2. Genetic evolution of HIV-1 within individuals and concept of quasispecies

HIV-1 evolves over the course of an individual's infection. Upon infection, the individual has a homogenous viral population [60-62]. 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 capacity, would be preferentially increased [63]. 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 [64]. As a consequence, the infected individual harbours 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 of closely related genomes are called quasispecies [65] .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 [66].

Viral sequence heterogeneity also exits in different body compartments within HIV-1 infected individuals. As HIV infects different cells and tissues, rare mutants escape the immune response and increase in frequency [62]. Tissue compartmentalization is evident in the lung [67, 68] genital tract [69, 70] and lymph node [69] but is most notable in the brain [71]. Some patients have distinct HIV-1 quasispecies phylogenies between the brain and blood, while others have quasispecies that migrate readily between these compartments [72].

1.7. Tuberculosis

1.7.1. The tubercle bacillus

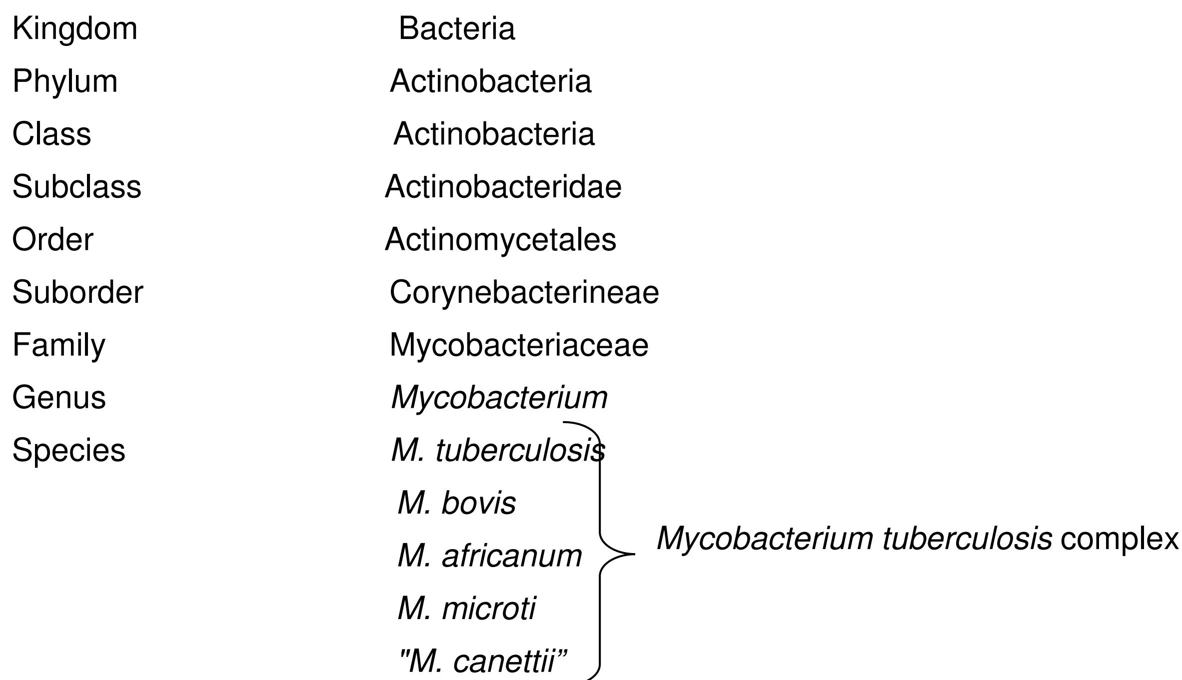
Tuberculosis or tubercle forming disease is an acute or chronic bacterial infection that primarily attacks the lungs, but which may also affect the kidneys, bones, lymph nodes, and brain. The disease is caused by *Mycobacterium tuberculosis* (MTB) first discovered by Robert Koch.

Bacteria of the genus *Mycobacterium* are acid fast, non-motile and non-sporulated rods. They have unusual high content (61-71%) of guanine plus cytosine (G+C) in the genomic DNA, and a high lipid content in the wall called mycolic acid. They are structurally more closely related to Gram-positive bacteria. However, mycobacteria do not fit into the Gram-positive category as the molecules attached to the cell wall are lipids rather than proteins or polysaccharides. Frequently, they do not retain the

crystal violet and appear as “ghosts” after Gram staining. Unlike Gram-negative bacteria, mycobacteria do not have an additional membrane in the outer layers of the cell wall. *M. tuberculosis* divides every 12 to 24 hours. This pace is extremely slow compared to that of most cultivable bacteria, which duplicate at regular intervals ranging from about 15 minutes to one hour.

Phylogenetically, *Mycobacterium* is grouped in the suprageneric rank of actinomycetes with other closely related genera like *Corynebacterium, Gordona, Tsukamurella, Nocardia, Rhodococcus* and *Dietzia* [73]. Only a few mycobacteria became successful pathogens of higher vertebrates while the rest of them live and replicate freely in natural ecosystems. Host-dependent mycobacteria that cannot replicate in the environment are *Mycobacterium leprae, Mycobacterium lepraemurium, Mycobacterium avium* subsp*. Paratuberculosis,* and members of the *Mycobacterium tuberculosis* complex [73].

Tubercle bacillus is the generic name for *Mycobacterium tuberculosis* (MTB) *complex*. The various etiologic agents of tuberculosis (TB) have distinct hosts, zoonotic potential and reservoirs. Lineage of the agents of TB is shown below [73]:



Kingdom	Bacteria	
Phylum	Actinobacteria	
Class	Actinobacteria	
Subclass	Actinobacteridae	
Order	Actinomycetales	
Suborder	Corynebacterineae	
Family	Mycobacteriaceae	
Genus	*Mycobacterium*	
Species	*M. tuberculosis*	*Mycobacterium tuberculosis* complex
	M. bovis	
	M. africanum	
	M. microti	
	"M. canettii”	

1.7.2. Stages of TB infection

MTB is transmitted from person to person through inhalation of bacteria-carrying air droplets. From those infected with the bacteria only 5 to 10% actually develop TB symptoms [74]. The disease is manifested in two stages: primary and secondary. In

primary TB, a person becomes infected with the TB bacteria but often experience no noticeable symptoms. At this stage the patient is not contagious. Macrophages, ingest the TB bacteria and transport them to the lymph nodes where they may be inhibited, destroyed, or continue multiplication.

If the bacteria multiply, active primary tuberculosis will develop. If the bacteria are inhibited, rather than destroyed, the immune cells and the bacteria form a mass known as a granuloma or tubercle. On a cellular basis, the immune cells form a wall around live and inactive bacteria. As long as the immune system remains strong, the bacteria remain encapsulated and inactive for many years. If the immune system becomes weakened, the tubercle opens; releasing the bacteria and the infection may reactivate to secondary TB. The formerly dormant bacteria multiply and destroy tissue in the lungs. They also may spread to the rest of the body via the blood stream.

1.7.3. Global burden of TB

Worldwide, one person out of three is infected with *Mycobacterium tuberculosis* – two billion people in total. TB currently holds the seventh place in the global ranking of causes of death [73]. In 2008, there were an estimated 9.4 (range, 8.9–9.9 million) million incident cases (equivalent to 139 cases per 100 000 population) of TB globally [75]. Most of the estimated number of cases in 2008 occurred in Asia (55%) and Africa (30%), with small proportions of cases in the Eastern Mediterranean Region (7%), the European Region (5%) and the Region of the Americas (3%) [75]. The 22 high-burden countries (HBCs), defined as the countries that rank first to 22nd in terms of absolute numbers of cases and which have received particular attention at the global level since 2000, account for 80% of all estimated cases worldwide [73,75].

Of the 9.4 million incident cases in 2008, an estimated 1.2–1.6 million (13–16%) were HIV-positive, with a best estimate of 1.4 million (15%) [75]. From these HIV-positive cases, 78% were in the African Region and 13% were in the South-East Asia Region [75]. There were an estimated 0.5 million deaths among incident TB cases who were HIV-positive [75].

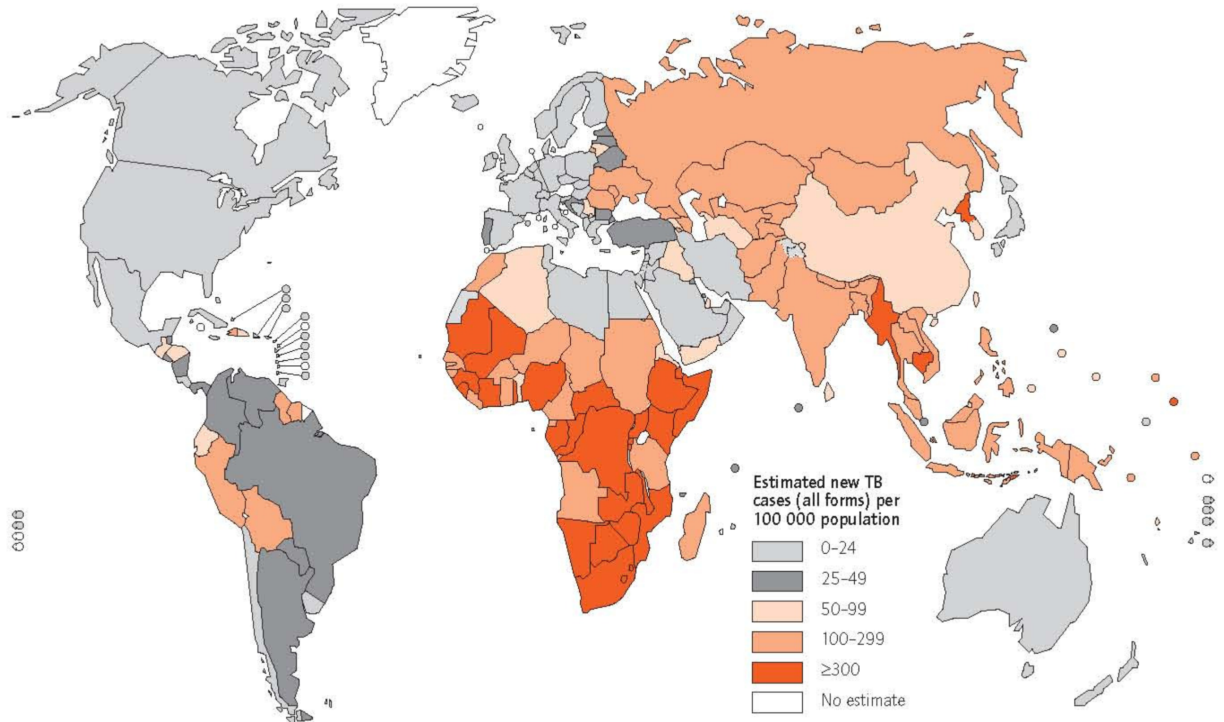


Figure 7 Global estimates of TB incidence rate in 2008 (Adapted from ref. no. 73).

1.8. HIV and TB Interaction

The discovery of modern antibiotic treatment in the middle of 1940's had significantly decreased the prevalence of TB at least in the developed world. Following the decline, most medical experts expected that the disease would be completely eliminated in industrialized nations by the year 2010. Paradoxically, the disease re-emerged in the late '80s fueled by the HIV/AIDS pandemic [76].

A complex biological interplay occurs between *M. tuberculosis* and HIV-1 in the co-infected host that results in the worsening of both pathologies. HIV promotes progression of *M. tuberculosis* either by endogenous reactivation or exogenous re infection. In HIV-infected hosts active TB develops at a yearly rate of 8 % [77, 78]. Clinical and epidemiological observations have demonstrated that HIV infected individuals have a 113 times higher risk of developing active TB upon exposure to MTB and 170 times higher risk during AIDS compared with uninfected persons [79].

On the other hand, the course of HIV-1 infection is also 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 [80].

In HIV/TB co-infected individuals, there are several signs of generalized immune activation in the peripheral blood [82] which are quantitatively more pronounced compared to HIV negative TB patients. In addition, active TB has been shown to increase HIV replication [82-84]. An investigation done by Nakata *et al.,* [83] 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 suggested a significant correlation between tumour necrosis factor α (TNFα) concentrations and viral load [85]. Apart from lung segments, plasma RNA in HIV-infected persons showed a 5-160 fold increase in viral load during acute phase MTB [82].

1.9. Aim of the study

- To analyze the C2V5 *env* quasispecies diversity in HIV-1 infected individuals with TB (HIV-1/TB) during active TB and after two months of intensive TB chemotherapy, in comparison to baseline CD4+ matched HIV-1 infected individuals without tuberculosis (HIV-1/non TB) with an effort to include individuals:
    a. who came from various geographical regions and were living in Germany
    b. who are natives in Europe
- To analyze the C2V5 *env* quasispecies divergence in HIV-1/TB within two time points (i.e. during active TB (TP-1) and after two months of intensive TB chemotherapy (TP-2)).
- To analyze HIV-1 co-receptor usage profile in quasispecies of HIV-1/TB (at TP-1 and TP-2) and HIV-1/ non TB.
- To analyze if there is any positive selective pressure in HIV-1/TB (TP-1 and TP-2) and HIV-1/non TB by calculating synonymous and non synonymous nucleotide substitutions.
- To analyze if there is any specific C2V5 *env* region that is highly variable in HIV-1/TB as compared to HIV-1/non TB.
- To analyze whether there is a difference in the number of N-linked glycosylation sites along the C2V5 *env* HIV-1/TB (at TP-1 and TP-2) in comparison to HIV-1/non TB.

2. Patients, materials and methods

2.1 Patients, data and plasma

2.1.1. Frankfurt HIV cohort

For this study plasma samples were retrospectively selected from participants in Frankfurt HIV cohort. Frankfurt HIV cohort is one of the largest HIV cohorts and treatment center in Europe with over 3000 regular patient visits (Fig. 8). It is part of the Hospital of Johann Wolfgang Goethe University, Frankfurt am Main, HIV Treatment and Research Unit (HIVCENTR).

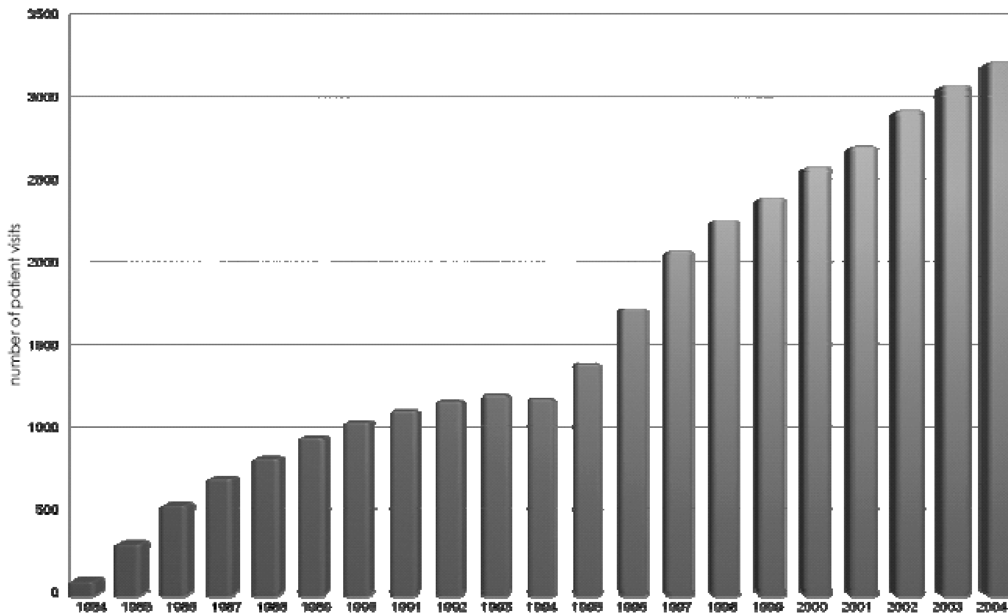


Figure 8 Patient visits at the HIV Treatment & Research Unit 1984 – 2004 (Adapted from ref no. 86).

2.1.2. EPIDEM database

The Frankfurt HIV Cohort stores data related to its patients in Microsoft Access based database system called EPIDEM. Data related to patients in EPIDEM database include a unique EPIDEM ID for each patient, age, sex, country of origin (citizenship), HIV history, HIV risk factors, anti retroviral therapy (ART) history, any infection diagnosed, CD4 and CD8 cell counts, HIV viral load. Data storage has been made by authorized data manager. For this study a small query was written by the authorized data personnel to select patient who fulfilled the selection criteria (see below).

2.1.3. HIVCENTER plasma bank

Since the year 1996, HIVCENTER plasma bank has stored plasma samples drawn from Frankfurt HIVCENTER cohort patients' in-70^{0}C deep freezer. All data pertaining storage has been saved in computerized system.

2.1.4. Sample selection criteria

Using EPIDEM and HIVCENTER plasma bank, plasma samples were selected from patients who fulfilled the following selection criteria.

2.1.4.1. Study subjects

HIV-1 infected patients with microbiologically and clinically confirmed active tuberculosis:-

- who had blood plasma samples collected and stored around the time of diagnosis for active TB and after 2 months of TB chemotherapy before ART initiation
- who had clinical and laboratory data in EPIDEM
- who took standard tuberculosis treatment after active TB was diagnosed and did not take ART at least in the first two months of intensive TB treatment

2.1.4.2. Control subjects

HIV-1 positive patients whose CD4 cell count had been matched to those of the study subjects(see 2.1.4.1) and had stored plasma samples at that particular time point

- who had no obvious active co-infections
- who were ART naïve by the time of sampling
- who had clinical and laboratory data in EPIDEM

2.2. Materials

2.2.1. Laboratory equipments

Table 2. List of laboratory equipments used during the study.

Product	Manufacturer
0.2 ml, 8-strip PCR tubes	Biozym Scientific GmbH, Oldendorf, Germany
0.2ml, 96-strip PCR tubes	Biozym Scientific GmbH, Oldendorf, Germany
8-cap strips	Biozym Scientific GmbH, Oldendorf, Germany
96-cap strips	Biozym Scientific GmbH, Oldendorf, Germany
1.5 ml Safe-Lock Micro centrifuge	Eppendorf, Hamburg, Germany
96-well plate caps	Applied Biosystems, Darmstadt, Germany
ABI Prism 3100 Avant sequencer	Applied Biosystems, Darmstadt, Germany
ABI Prism 3100 AVANT capillary-Array 50cm	Applied Biosystems, Darmstadt, Germany
Lab-Centrifuge	Hettich Zentrifugen, Tuttlingen, Germany
Empty petri dish Greiner (94x16mm)	Sigma-Aldrich, Steinheim, Germany
Electrophoresis accessories	München, Germany
GREINER 15ml polypropylene centrifuge tubes	Greiner bio one, Germany
Incubator	Heraeus, Thermoscientific
Magnetic stirer	GLW Gesellschaft für Laborbedarf GmbH, Wuerzburg, Germany
MicroAmp Optical 96-Well Plates	Applied Biosystems
Table centrifuge	Eppendorf, Hamburg, Germany
Thermomixer	Eppendorf, Hamburg, Germany

Thermocycler (TP Basic Gradient)	Biometra, Goettingen, Germany
Transluminator Biometra T1 2	Biometra, Goettingen, Germany
Shaker incubator	Infors AG, *Bottmingen*, Germany
Disposable gloves	Paul Hartmann, Heidenheim, Germany
UV Gel Documentation system for *electrophoresis* gels	PeQlab (Biotechnologia GmbH)
Vortexer	IKA works Inc Wilmington, NC

2.2.2. Chemicals

Table 3. List of chemicals used during the study.

Product	Manufacturer
Absolute Ethanol	Sigma-Aldrich, Steinheim, Germany ; J.T.Baker, Stuttgart, Germany
Ampicillin sodium salt	Sigma-Aldrich, Steinheim, Germany
ABI Prism 3100 POP-6 Polymer	Applied Biosystems
Boric acid crystals	Merck, Darmstadt, Germany
Bromophenol blue	Sigma-Aldrich, Steinheim, Germany
Difco^{TM} Lauria Bertani, miller Broth	Becton Dickinson (BD), Heidelberg Germany
Difco^{TM} Lauria Bertani Agar	Becton Dickinson (BD) Heidelberg Germany
DNA Mass Ladder	Fermentas Life Sciences, Leon-Rot, Germany
Ethidiumbromid	Sigma-Aldrich, Steinheim, Germany
Ethylenediaminetetraacetic acid	Merck Darmstadt, Germany
Glycerol	Roth, Karlsruhe, Germany

HPLC-Water RNAse and DNAse free	Eppendorf, Hamburg, Germany
Hi-Di Formamide	Applied Biosystems
Incidin plus	Ecolab,Duesseldorf,Germany
LE GP Agarose	Biozym Scientific GmbH, Germany
TRIS	SAFC, Andover, UK

2.2.3. Commercial kits, reagents, buffers, enzymes, primers and bacteria

2.2.3.1. QIAamp Viral RNA mini kit (Cat No 217004)……………… QIAGEN GmbH, Hilden, Germany

Contents:

QIAamp Spin Columns
Collection Tubes (2-ml)
Buffer AVL
Buffer AW1
Buffer AW2
Buffer AVE
Carrier RNA (poly A)
Handbook
2.2.3.2. QIAGEN one step RT-PCR kit (Cat No 210212)…………… QIAGEN GmbH, Hilden, Germany

Contents:

QIAGEN OneStep RT-PCR Enzyme Mix (Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq DNA polymerase)

QIAGEN OneStep RT-PCR Buffer,

Q-Solution

dNTP Mix

RNase-free water

Handbook

2.2.3.3. Taq PCR core kit (Cat No 203645)…………………………… QIAGEN GmbH, Hilden, Germany

Contents:

Taq DNA Polymerase

QIAGEN PCR Buffer

Q-Solution

MgCl2

dNTP Mix

Handbook

2.2.3.4. CloneJET^{TM} PCR cloning kit (Cat No K1232)…….Fermentas Life Sciences, Leon-Rot, Germany

Contents

PJET1.2/blunt Cloning Vector

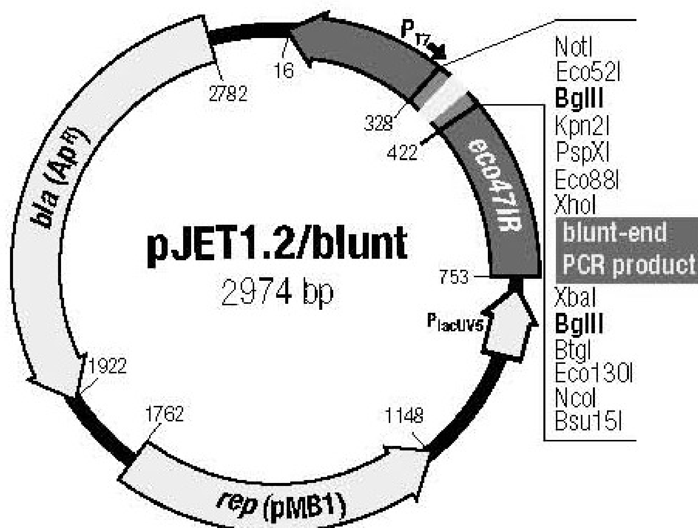


Figure 9 Genomic organization of PJET1.2/blunt cloning Vector.

2x Reaction Buffer

T4 DNA Ligase

DNA Blunting Enzyme

PJET1.2 Forward Sequencing Primer [5'-CGACTCACTATAGGGAGAGCGGC-3']

PJET1.2 Reverse Sequencing Primer [5'-AAGAACATCGATTTTCCATGGCAG-3']

Control PCR Product

Water, nuclease free

2.2.3.5. Transformation Aid Bacterial Transformation kit (Cat No K2711) ……Fermentas Life Sciences, Leon-Rot, Germany

Contents

C-Medium

T-Solution (A)

T-Solution (B)

2.2.3.6. Gene Jet Plasmid Miniprep kit (Cat No 1222)………………Fermentas Life Sciences, Leon-Rot, Germany

Contents

GeneJET™ Spin Columns assembled with Collection Tubes

Resuspension Solution

Lysis Solution

Neutralization Solution

Wash Solution (concentrated)

RNase A Solution

Elution Buffer

Handbook

2.2.3.7. FastDigest® XbaI (Cat No FD0684) …………………………Fermentas Life Sciences, Leon-Rot, Germany

Cutting site

5'...T^C T A G A...3'

3'...A G A T C^T...5'

2.2.3.8. FastDigest® XhoI (Cat No FD0694)………………………Fermentas Life Sciences, Leon-Rot, Germany

Cutting site

5'...C^T C G A G...3'

3'...G A G C T^C...5'

2.2.3.9. ABIprism 3100 Avant Sequencing kit …………….. Applied Biosystems

Contents

Ready Reaction Mix

pGEM®-3Zf(+) double-stranded DNA Control Template

–21 M13 Control Primers (forward)

BigDye Terminator v1.1/3.1 Sequencing Buffer (5X)

2.2.3.10. DyeEx 96 Kit (Cat No 63206; Cat No 63183)………………QIAGEN GmbH, Hilden, Germany

Contents
DyeEx 96 Plates
Waste Collection Plates
Handbook
2.2.3.11. PCR primers

Forward primers:

C2V5out5 (5′- AAGACGTTCAATGGRACAGG 3′) corresponding to nucleotide (nt) 6915-6934 in HXB2

C2V5in5 (5′-GCA CAG TACA ATGYACACATGG-3′) corresponding to 6952-6973 nt in HXB2 and the same reverse primer (C2V5_3)

Reverse primer:

C2V5_3 (5′ CAA TTG TCC CTC ATA TYT CCT CC-3′) corresponding to 7660-7638 nt in HXB2

2.2.3.12. TBE-buffer (0.5x working solution)

5X –TBE stock solution
108 g Tris
55g Borate
40ml, 0.5M EDTA
1 lt H_2O
pH 8.0
2.2.3.13. 1.5% Agarose gel

Agarose gel was made by mixing 1.5g agarose with 100ml TBE buffer in 250 ml Erlenmeyer flask and boiling in a microwave oven until all of the small translucent Agarose particles were dissolved. After cooling to 60^{0}C, the gel was poured on to an assembled gel cassette with combs. When the agarose gel was solidified, it was transferred in to fixed gel caster electrophoresis chamber which contained 0.5% TBE buffer. A mixture of 5-10μl of the PCR product or RE digest product (depending on the type of DNA) in 5μl of loading dye (Bromophenol blue) was loaded on the gel. 100bp λDNA fragment was used as a molecular weight marker. Electrophoresis run at 100 milliamperes (180V) for 1 hour. It was then examined under UV light and photographed. Positive results were the presence of right size (measured in base pair) band or bands.

2.2.3.14. Luria-Bertani (LB) agar plate

1 L of purified water

40 g Difco TM Lauria Bertani Agar

Autoclaved 121℃ for 15 minutes

Poured to empty Greiner petri dish.

Petridish kept at room temperature until solidified and stored at 2-8^{0}C refrigerator until use.

2.2.3.15. LB-Ampicillin agar plate

1 L of purified water

40 g Difco TM Lauria Bertani Agar

Autoclaved 121℃ for 15 minutes

the solution was allowed to cool down to 55℃ ; 2ml stock solution (100mg/ml) of Ampicillin to final conc. 100 μg/ml was added and poured on to empty Greiner Petri dish; Petridish kept at room temperature until solidified, and stored at 2-8^{0}C refrigerator until use.

2.2.3.16. Luria-Bertani (LB) broth

1 L of purified water

25g DifcoTM Lauria Bertani, miller Broth

Autoclaved 121℃ for 15 minutes

Stored at room temperature until use

2.2.3.17. *Escherichia coli* JM107

A vial of powder of freeze dried *E.coli* JM107 culture was received free of charge from Fermentas Life Sciences, Leon-Rot, Germany. 0.5ml LB Medium was added aseptically and mixed. The mixture was streaked on an LB agar plate and allowed to grow over night. A single colony was sub cultured in to 5ml LB media and grown at 37^{0}C over night. The next day 25% (i.e., 0.125ml to 0.5 ml) glycerol was added to the turbid culture, thoroughly mixed and aliquoted in small vials; kept for 1hr at room temperature and stored at -80^{0}C in a deep freezer until use.

2.2.4. Software and databases

Table 4. List of software and databases used to analyze and present the data in this study.

Bioedit (for windows) version 7.0.9.0	See ref. no.87
Consensus Maker	*www.hiv.lanl.gov*
Datamonkey	*www.datamonkey.org*
Editor	Microsoft Windows XP
FinchTV	Geospiza; http://www.geospiza.com/Products/finchtv.shtml
Genebank, NCBI	http://www.ncbi.nlm.nih.gov/genbank/
Geno2pheno	http://www.geno2pheno.org/
Genecutter	HIV sequence database Los Alamos, http://www.hiv.lanl.gov/content/sequence/GENE_CUTTER/cutter.html
GraphPad Prism version 5.00 for Windows	www.graphpad.com Graph Pad Software, San Diego, California USA
Los Alamos HIV-1 database	*www.hiv.lanl.gov*
Microsoft excel 2003	Microsoft, Seattel, Washington
Microsoft power point 2003	Microsoft
Microsoft word 2003	Microsoft
Mega 4	See reference no.98
NCBI Blast	http://blast.ncbi.nlm.nih.gov/Blast.cgi
N-glycoSite	*www.hiv.lanl.gov*
Shannon Entropy	*www.hiv.lanl.gov*
SNAP	*www.hiv.lanl.gov*
zt partial Mantel test tool	See reference no.105

2.3. Methods

2.3.1. HIV-1 RNA isolation from plasma

HIV-1 RNA was isolated from plasma using QIAamp® Viral RNA Mini Kit. QIAamp® Viral RNA Mini Kit uses the principle of selective binding properties of a silica-gel–based membrane to bind and isolate RNA through optimum buffering condition.

For this purpose, plasma samples that were stored in -70^{0}C deep freezer were thawed to room temperature. One milliliter of thawed plasma was ultra-centrifuged at maximum speed (20,000g) for one hour. After removing the supernatant, 560μl AVL buffer was added on to the pellet, mixed by pulse-vortexing and incubated at room temperature for 10min. After the incubation period, 560μl absolute ethanol was added and mixed very well. From the total mix, each time 630μl was carefully pipetted and applied on to QIAamp Mini spin column, centrifuged at 6000g for 1 min so that all intact RNA could bind on to the spin column. The filtrate containing collection tube was discarded and replaced by a new collection tube. When this step was completed, the mini spin column was washed two times with 500μl buffer AW1 and buffer AW2 and centrifuged at 6000g. To completely remove traces of liquid a final centrifugation at 20,000g for 3 min was done. 60μl of elution buffer (Buffer AVE) was carefully added at the center of the spin column and centrifuged at 6000g for 3min. The RNA containing tube was kept at -70^{0}C until further experiments were performed.

2.3.2. HIV-1 C2V5 *env* reverse transcription and PCR amplification

QIAGEN One-step RT PCR kit was used for reverse transcription of viral RNA to cDNA and first PCR in one tube. The QIAGEN OneStep RT-PCR kit contains specially formulated enzymes for both reverse transcription and PCR amplification. For this purpose 20μl of eluted RNA was taken and mixed with a master mix. The master mix was prepared from 4μl RNase-free water, 10μl 5xQIAGEN One step RT-PCR Buffer, 2.0μl dNTP mix, 10μl 5x Q-solution, 0.5μl each primers, C2V5out5 and C2V5_3, 2μl QIAGEN One Step RT-PCR Enzyme Mix and1μl RNase inhibitor. The PCR condition for reverse transcription and PCR amplification was, one cycle of reverse transcription at 50°C for 30 min, followed by initial PCR activation at 95°C for 15 min; 40 cycles of denaturation, annealing, and elongation at 95°C for 30 sec, 55°C

for 45 sec, and 72°C for 3 min respectively; followed by 1 cycle of extension at 72°C for 10min and a final holding temperature at 4 °C.

2.3.3. HIV-1 C2V5 *env* semi nested PCR

Semi nested PCR was performed using Qiagen Taq PCR core kit. PCR reaction was carried out in 50μl reaction mixture that contained: 10x QIAGEN PCR Buffer (5μl), 5x Q-solution (10μl), dNTPs mix (10 mM each) (1μl), Taq polymerase (0.25μl), C2V5in5 (0.5μl), C2V5_3 (0.5μl) and 27.75μl distilled water with 5μl of the first PCR product. The following amplification condition was used: 1 cycle of denaturation at 94°C for 3 min, 40 cycles of denaturation, annealing, and elongation at 95°C for 30sec, 55°C for 45 sec, and 72°C for 3min respectively followed by 1 cycle of final elongation at 72°C for 10 min and holding it at 4°C till the next step.

Presence of right sized amplified DNA was confirmed by electrophoresis in 1.5% agarose gel.

2.3.3. Molecular cloning

2.3.3.1. Ligation experiment and Competent cell preparation

Molecular cloning was performed using FERMENTAS GeneJet PCR cloning and Bacterial Transformation Kits. The procedure was as follows: On the first day, frozen *E.coli* JM107 was streaked on LB agar plate and incubated at 37°C for approximately 16hrs. On the second day, a colony from the first day culture plate was taken and streaked on an LB agar plate and incubated in a similar condition as above. On the third day, one or two colonies were picked from the second day culture and mixed with a pre-warmed 1.5ml Transform AID C-medium in a 2ml centrifuge tube. The tubes were then incubated on a Thermomixer for 2hrs at 37°C at 1000 rpm. Ligation was performed in the mean time under the following condition: A blunting reaction was prepared by mixing 10μl 2X Reaction buffer, 2μl PCR product, 5μl nuclease-free water and 1μl DNA Blunting enzyme. The reaction mix was vortexed briefly and incubated at 70°C for 5min followed by chilling on ice for several seconds. Then a ligation reaction was made by adding 1μl PJET1.2 blunt Cloning Vector (50ng/μl), 1μl T4 DNA Ligase (5U/μl) on to the blunting reaction mixture. The ligation reaction was vortexed briefly, spin centrifuged and incubated for 30 min to 1hr.

2.3.3.2. Transformation

Ampicillin containing LB agar plates were prewarmed at 37^0C for at least 20min. T-solutions A and B were thawed and vortexed vigorously. TransformAid™ T-solution was prepared by mixing equal volumes of T-solution (A) and T-solution (B) (500 for each 2 transformations) and kept on ice. Freshly prepared bacterial culture in C-medium (above mentioned) was centrifuged at 10,000g for 1min, supernatant was removed and immediately kept on ice and resuspended in 300μl TransformAid™ T-Solution. After 5 min incubation on ice, the suspension was centrifuged in a similar way as above and the supernatant was discarded and the pelleted cells were resuspended in 120μl TransformAid™ T-Solution and incubated on ice for 5min.

Five μl of the incubated ligation mixture (mentioned above) was dispensed into a new microcentrifuge tubes and kept on ice for 2min. After 2 min, 50μl of the resuspended cells were added and incubated on ice for 5min. The mix was then plated on prewarmed ampicillin containing LB agar plates (mentioned above) and incubated at 37^0C overnight.

2.3.3.3. Selection and growth of colonies

After 18 hours of incubation, the LB agar was checked for any colony growth. As PJET1.2/blunt is a positive cloning vector, only bacterial cells with recombinant plasmids are able to grow. Therefore each single colony that was grown was picked and immersed in an ampicillin containing LB media in 15ml Greiner centrifuge tube and incubated overnight at 37^0C, on a 200 rpm shaker. On the next day, the bacterial pellet was harvested by centrifugation at 5000g for 5min.

2.3.3.4. Plasmid miniprep

Miniprep is a process used in molecular biology to analyze bacterial clones. It is used for small scale isolation of plamid DNA from bacteria. The isolation method is based on alkaline lysis. For this purpose; we used Gene Jet Plasmid Miniprep kit (Fermentas). First the bacterial pellet (see above) was resuspended in 250μl Resuspension Solution by vortexing and pipetting up and down. The mix was then transferred in to a 1.5ml centrifuge tube and 250μl of Lysis Solution was added and mixed thoroughly by inverting the tube up and down until the solution turned to be viscous. Immediately, 350μl of Neutralizing Solution was added and mixed thoroughly and centrifuged at 14,000g for 5min. The supernatant was then

transferred to GeneJET^{TM} spin column by careful pipetting not to disturb the white precipitates (contain cell debris and chromosomal DNA). The spin column was centrifuged for 1min and the filtrate was discarded; washed two times by adding 500μl Wash buffer and centrifuging at 10,000g for one minute and a final centrifugation for 3minutes to remove residual solution. Finally, plasmid DNA was eluted by adding 50μl elution buffer at the center of the spin column membrane and centrifuging at 10,000g for 3min. The plasmid DNA (clone) was then stored in a

-20^{0}C freezer

2.3.3.5. Restriction digest and analysis of DNA

Double digest restriction enzymes (or restriction endonucleases), XhoI and XbaI were used to confirm the presence of the right size insert of interest in the PJET1.2 vector. For this purpose, 20μl of reaction mix was prepared by mixing 2μl purified plasmid DNA, 2μl 10X Fast digestion^{TM} buffer ,1μl XbaI ,1μl xhoI and 16μl of nuclease-free water. After gentle mixing and spinning, the mix was incubated overnight on a Thermomixer at 37^{0}C and 300rpm. By agarose gel (1.5%) the presence of two bands with sizes around 650 bp and 2900 bp for insert and plasmid DNA respectively was verified.

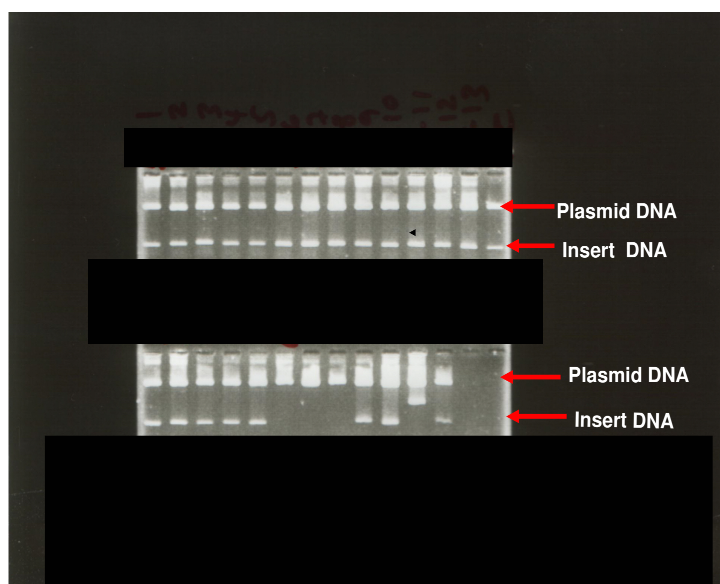


Figure 10 A typical restriction digest plasmid fragments to verify presence of right size insert after cloning.

2.3.4. Sequencing

All clones that contained right size inserts were sequenced using ABI prism 3100 Avant genetic analyzer. The ABI PRISM 3100-Avant is a four capillary genetic analyzer that uses a single argon-ion laser. The light from the array is collected by a spectrograph system with a CCD camera detector that simultaneously provides low noise, full-spectrum data from all four capillaries. To sequence the cloned DNA, first, a sequence reaction mix was prepared by mixing 9,4μl deionized water, 4μl v1.1 big dye Terminator Ready Reaction Mix, 4μl 5X sequencing buffer,2μl plasmid DNA (containing insert of interest) and 0,6μl PJET1.2 (forward or backward) primers. This mix was subjected to by 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 30 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 Qiagen DyeEx^{TM} Kit to remove unincorporated dyes. For this purpose, DyeEx 96 plate was taken out of the bag and placed on the top of the provided collection plate and centrifuged for 3 min at 750g. The flow through was discarded and 300μl aquadist was added to each well and centrifuged for 3min and the flow through was discarded. 20μl of sequencing samples were carefully applied on to the gel bed of each well and centrifuged as above. All the flow-through was eluted in MicroAmp Optical 96 Well plate and 10μl Hi-Di Formamide was added on to it. 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 50 cm with performance optimized polymer 6(POP6 ^{TM}), an Ultra seq- POP-6^{TM} default module with dye set E mobility file. The result was read using the data extraction software.

2.3.5. Sequence analysis

The obtained DNA sequence chromatogram was viewed using FinchTV Version 1.4 and edited manually. FinchTV is a popular, freely downloadable chromatogram viewer which can be used in operating systems; Linux, Mac OSX, Windows, and Solaris. It not only used to view chromatogram but also can be used to edit and reverse complement sequences and save as a new file.

The identity of the sequences was confirmed using NCBI BLAST. BLAST is a sequence similarity searching program or algorithm. BLAST compares query sequences such as nucleotide sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. NCBI (the National Center for Biotechnology Information) is responsible for making available the GenBank DNA sequence database.

After confirming the identity of the sequences, multiple sequence alignment was performed using Bio edit software package which has an integrated ClustalW. Any errors in alignment and confirmation of the proper alignment were made using Los Alamos freely available online tool, Genecutter. A brief description of the softwares and databases that were used to analyze our data are described below:

2.3.5.1. Los Alamos HIV database

Los Alamos HIV database [88] is one of the largest and freely available online HIV database under Los Alamos National Laboratory funded by Division of AIDS of the National Institute of Allergy and Infectious Diseases. The database contains data on HIV genetic sequences, immunological epitopes, drug resistance-associated mutations, and vaccine trials. It also gives access to a large number of online bioinformatics tools (software). From this database we retrieved reference sequences of all HIV-1 subtypes. Besides, the following online tools were used [72; 89-94]:

Gene cutter: is a sequence alignment and protein extraction tool. It uses Hmmer, an implementation of profile hidden Markov models (profile HMMs) for biological sequence analysis [95-97] with a training set of the full-length HIV genome alignment. Using this online tool, multiple sequence alignments were done and coding regions extracted.

HIV Sequence Locator: is a tool to find the start and end coordinates of HIV input sequence(s) relative to the reference HIV strain HXB2. It shows graphically which HIV genes or proteins a sequence covers relative to the reference sequence and displays both the nucleotide sequence and protein translation. For this study, this tool was used to see if the generated sequences were in the right orientation and if there was any stop codon. Sequences that contained premature stop codons were excluded from any further analysis.

Consensus Maker: this tool was used to generate a consensus sequence for clones derived from a patient at a single time point.

Entropy: this tool was used to measure variation in protein sequence alignments.

N-glycosyte: was used to highlight and tally N-Linked Glycosylation site (Nx [ST]) patterns, where x can be any amino acid) in amino acid alignments that were generated.

SNAP: was used to calculate synonymous and non-synonymous substitution rates.

2.3.5.2. Molecular evolutionary genetics analysis (MEGA) package

MEGA:- is a very well known and publicly available standalone software package (integrated tool) [98]. Its applications include sequence alignment, inferring phylogenetic trees, mining web-based databases, estimating rates of molecular evolution, and testing evolutionary hypotheses. For this particular study, MEGA version 4 was installed and the following analyses were made:-

Pairwise distance between sequences:- evolutionary distances in units of substitutions per site were calculated using Kumura-2 parameter method [99] by considering the rate variation among sites had a gamma distribution (shape parameter = 0.5).

Phylogenetic tree:- all phylogenetic trees were constructed using the neighbour-joining method [99]. This approach was selected because other methods like parsimony or maximum likelihood could not handle such a huge number of sequences that were generated for this study.

Bootstrap:- was used to test the percentage of replicate trees in which the associated taxa clustered together in 1000 replicates [98].

Intra-patient viral diversity:- was calculated by averaging distances within all clone sequences of each sample as implemented by “within group average calculation” option of MEGA.

Viral divergence:- defined as the genetic distance difference between the newly produced viruses compared to founder viruses. It was calculated by estimating the net average distances between the two time point sequence samples. Analyses were conducted using the Maximum Composite Likelihood method in MEGA4 [98, 101]. The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.5). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option).

Viral diversification:- defined as the change in intra-patient diversity or the difference between earlier and later time point intra-patient diversity was analyzed by calculating pairwise distance of each clone sequence to a consensus sequence generated.

2.3.5.3. Phenetic analyses using mantel's test

Mantel's test is a generalized regression permutation procedure which compares two distance matrices [102]. This method is an established method [102-104] to determine if sequences from any compartment (or time point) shared more genetic identity with each other than with sequences from other compartments (or time points). In the present study, we used Mantel's test to evaluate if clone sequences derived from a patient at a time point shared more genetic identity with each other than sample from the other time point. For this purpose, two distance matrices were generated. The first matrix consisted of pairwise evolutionary distances generated from all sequences obtained at both time points, and the second distance matrix consisted of an ideal matrix (M_d) of the same dimension as the first, such that:

$$M_d(i,j) = \begin{cases} 0 \text{ if sequence } i \text{ is from the same time point as sequence j} \\ 1 \text{ otherwise} \end{cases}$$

The test was implemented using the zt partial Mantel test tool [105] using 10,000 to 100,000 randomization. The hypothesis that there is no genetic identity between two time points was rejected if the associated p-value exceeded 0.05.

2.3.5.4. Datamonkey

DataMonkey is a webserver of the HyPhy package used for estimating the rates of nonsynonymous and synonymous changes at each site in a sequence alignment in order to identify sites under positive or negative selection [105]. Data monkey has the alternatives of three different codon-based maximum likelihood methods, (Single Likelihood Ancestor Counting (SLAC) [106], fixed effects likelihood (FEL) [107] and random effects likelihood (REL) [107]. For this study SLAC was used to detect positive selection sites. Multiple sequence alignment of the coding region of the clone sequences for each sample was used as a raw data.

2.3.5.5. Web pssm and geno2pheno

WebPSSM [108] and geno2pheno [109] are bioinformatic tools for predicting HIV-1 co-receptor usage from the amino acid sequence of the third variable loop (V3) of the envelope gene.

2.3.5.6. Nucleotide sequence accession number

Sequin [110] is a stand-alone software tool developed by the NCBI for submitting and updating entries to the GenBank, EMBL, or DDBJ sequence databases. Sequin.win.exe version 10 was downloaded and sequences were annotated and have been deposited in Genebank.

2.3.6. Statistical analysis

Student's t-test, Man-Whitney U test, Wilcoxon rank sum test and linear correlations were calculated using Graph Pad Prism version 5.00 for Windows.

3. Results

3.1. Subjects and specimen collection

Using EPIDEM, all microbiologically confirmed active TB diagnosed patients from the year 1996 to 2006 who had follow-up and properly stored plasma samples were selected. Only eight TB patients had fulfilled the criteria of selection, [HIV-1/TB, n = 8] (subject ID: TB-1 to TB-8) (Table 5). Five of the HIV-1/TB had pulmonary TB, 2 extra pulmonary and 1 both pulmonary and extra pulmonary.

Seven HIV-1/TB patients were ART naïve when they were diagnosed for active TB. One patient (TB-5) was diagnosed with active TB 103 days after ART initiation; thus ART treatment was interrupted for TB treatment of this patient for approximately 17 weeks. Second time point samples were obtained for six of the eight patients (TB-1, TB-2, TB-3, TB-5, TB-6, and TB-7) at least after 2 months of antituberculous chemotherapy and before initiation of ART. Currently all patients are taking ART.

As controls, an equal number of HIV-1 infected, baseline CD4 cell count and CDC HIV staging matched patients without history of active TB from the same cohort [HIV-1/non TB, n = 8] (subject ID: NTB-1 to NTB-8) were selected (Table 5). Efforts were made to exclude patients with co-infections. While we excluded patients who had history of TB based on the results of ELISPOT TB, it was difficult to find patients free of all possible co-infections. Five of HIV-1/non TB did not have any recorded co-infections; the residual three, as analyzed only later on had minor infections like oral candidiasis and *Toxocara canis* after the time of sampling. All control patients were ART naïve at the first time point of sampling. However, second time point sampling of HIV-1/non TB was not possible because of the immediate start of HAART which lowered HIV viral load to very low level.

The country of origin for four of the HIV-1/TB patients was from European Union (EU) and the rest four immigrated from East and North Africa. Four of HIV-1/non TB were from EU, three from East Africa and one from Latin America. For all patients the exact date of HIV-1 infection is not known. From the records it is suspected that all patients had more than two years of HIV-1 infection history.

The mean age for HIV-1/TB was 42 years (range 29 to 58), mean CD4 cell count was 112 cells/µl (range 12 to 266), and mean HIV-1 viral load was 864,750 copies/ml

(range 361,000 to 2,200,000). For HIV-1/non TB group the mean age was 36 years (range 26 to 59), mean CD4 cell count was 134 cells/µl (range 24 to 266), and mean HIV-1 viral load was 124,405 copies/ml (range 3,560 to 675,000). There was a significantly elevated HIV-1 viral load (unpaired t-test, $p < 0.05$) in HIV-1/TB as compared to HIV-1/non TB (Fig. 11). Elevated viral load for HIV-1/TB did not decline in the second time point follow-up (Wilcoxon rank sum test, $p = 0.2$) Most of HIV-1/TB patients didn't show a decline in CD4 cell count during treatment for active TB (Fig. 12). Patients of African origin (n=4) had a significantly higher CD4 cell count when they developed active tuberculosis as compared to European origin patients (n=4) (unpaired t-test, $p < 0.05$).

Table 5. Clinical and routine laboratory data of the study participants.

Patient ID	Age	Sex	region of origin	Diagnosis	HIV time of infection (Y)[a]	Sampling time in weeks[b]	CD4 count (cells/µl)	Viral load (copies/ml)	ART[c]
TB-1	29	M	EU	Pulmonary and extra pulmonary TB	≥ 9	0	12	384000	naive
"						+12	11	750000	naive
TB-2	47	M	EU	TB septicemia	≥2	0	104	498000	naive
"						+ 8	89	137000	naive
TB-3	35	M	EU	Pulmonary TB	≥11	0	55	798000	naive
"						+25	94	194200	naive
TB-4	48	M	East Africa	Pulmonary TB	not known	0	62	484000	naive
TB-5	35	M	East Africa	Pulmonary TB (IRS)[e]	not known	-16	143	1500000	naive
"						+20	208	600000	STI[d]
TB-6	29	M	East Africa	Pulmonary TB	not known	0	226	2200000	naive
"						+7		288000	naive
TB-7	46	M	North Africa	Pulmonary TB	~8	-19	266	693000	naive
"						+24	38	750000	naive
TB-8	58	M	EU	Miliary TB, oral candidiasis , HBV	not known	0	26	361000	naive
NTB-1	59	M	Turkey	oral candidiasis	not known	0	24	675000	naive
NTB-2	35	M	EU	__	not known	0	143	36100	naive
NTB-3	28	M	East Africa	Toxocariasis	not known	0	140	242000	naive
NTB-4	38	F	East Africa	oral candidiasis	not known	0	71	8500	naive
NTB-5	34	F	East Africa	__	not known	0	170	5800	naive
NTB-6	41	M	EU	__	not known	0	266	3560	naive
NTB-7	30.5	F	EU	__	not known	0	179	8879	naive
NTB-8	26	F	Latin America	__	not known	0	82	15400	naive

[a]years are described if HIV diagnosis was done before TB diagnosis and unknown if the diagnosis of TB coincides with HIV diagnosis
[b] 0: sampling during TB diagnosis; - : sampling before TB diagnosis, +: sampling after TB diagnosis
[c]ART = Antiretroviral therapy
[d] STI = Structured treatment interruption
[e]IRS = Immune reconstitution syndrome

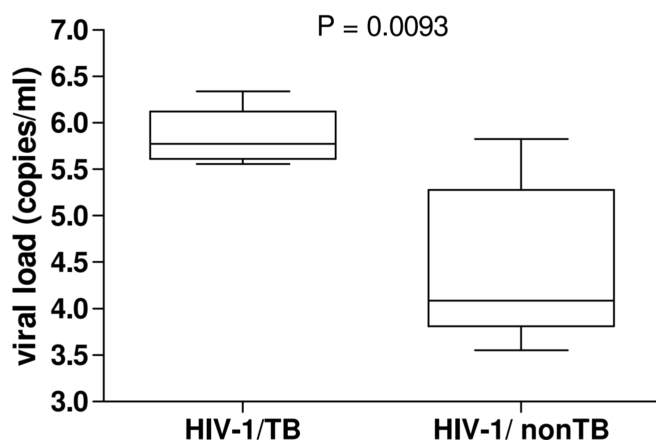


Figure 11 Box and whisker plot, showing statistically significant difference in HIV-1 viral load (copies/ml) for HIV-1/TB compared to HIV-1/ non TB.

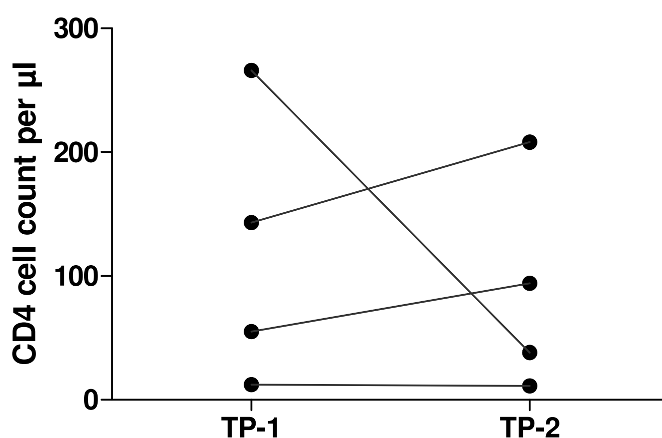


Figure 12 Line graph showing CD4 cell count of HIV-1/TB patients during diagnosis of active TB (time point-1, TP- 1) and after intensive TB chemotherapy (time point-2, TP-2).

3.2. Cloning and sequencing

A total of 360 clones (Table 6) encompassing 684bp C2V5 *env* (Fig.13) were sequenced. Sequences have been deposited in Genebank NCBI database with accession number GQ390435 to GQ390793.

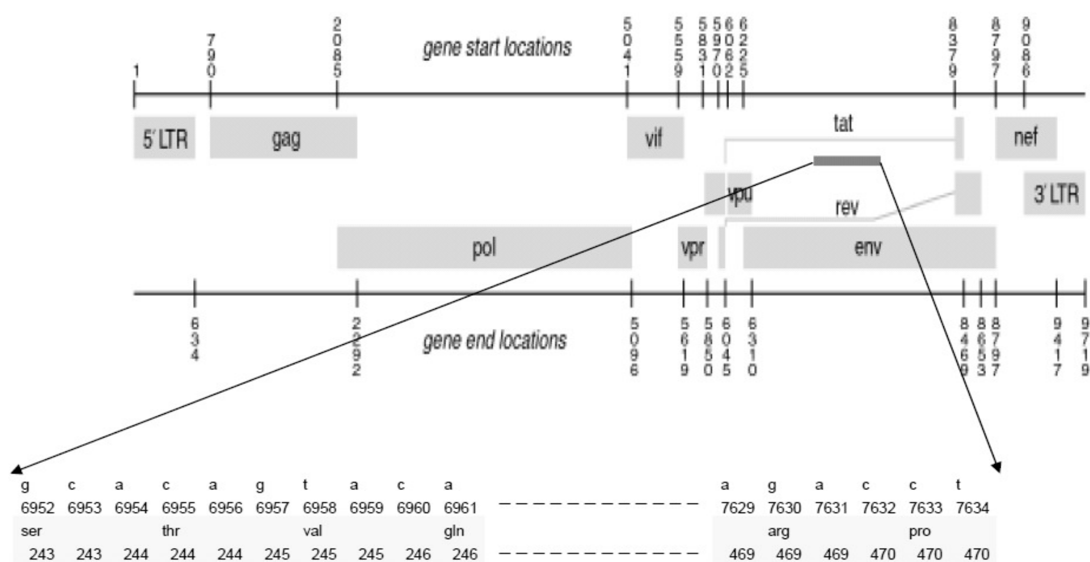


Figure 13 Genomic map of HIV-1 C2V5 *env* region (HXB-2 numbering) that was sequenced and analyzed in the study.

Table 6. Number of C2V5 *env* clones generated and sequenced, from HIV-1/TB and HIV-1 non TB.

Patient ID	Number of clone sequences
TB-1(TP_1)	13
TB-1(TP_2)	26
TB-2(TP_1)	23
TB-2(TP_2)	18
TB-3(TP_1)	9
TB-3(TP_2)	16
TB-4(TP_1)	17
TB-5(TP_1)	21
TB-5(TP_2)	10
TB-6(TP_1)	19
TB-6(TP_2)	22
TB-7(TP_1)	28
TB-7(TP_2)	15
TB-8(TP_1)	9
NTB-1	14
NTB-2	11
NTB-3	17
NTB-4	20
NTB-5	14
NTB-6	8
NTB-7	20
NTB-8	10

TP_1 - time point 1 (initial time point of sampling that corresponds to active tuberculosis diagnosis)
TP_2 - time point 2 (final time point of sampling that corresponds to more than 7 weeks of anti tuberculosis chemotherapy)

3.3. Phylogenetic tree

Neighbor-Joining trees were constructed to verify clustering pattern of clonal sequences and to determine HIV-1 subtypes (Fig.14). In the HIV-1/TB, three patients were infected with HIV-1 subtype C and the rest carried HIV-1 subtype B. In the HIV-1/non TB group, three patients were infected with HIV-1 subtype C, a further three with HIV-1 subtype B, and two patients with HIV-1 subtype A virus. Based on the country of origin, all HIV-1 subtype C patients were from East Africa.

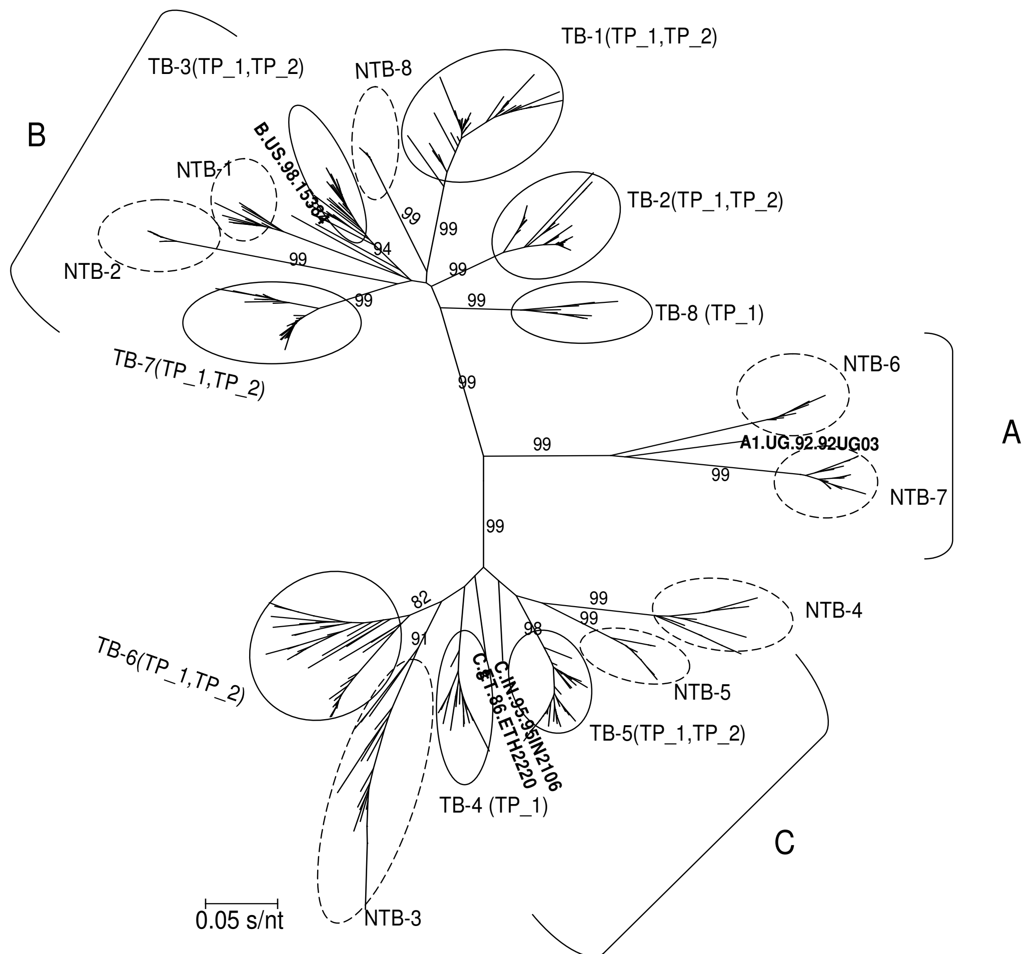


Figure 14 Neighbor-Joining tree showing the subtype and monophyletic clustering of all quasispecies for HIV-1/TB at two time points of follow-up (solid lines) and CD4 matched HIV-1/non TB (broken lines). Trees were drawn based on the Kimura-2 parameter method. Reference subtypes from Los Alamos HIV database were included for subtype determination. Bootstrap values in percent are indicated near the branches. The scale bar for the branch length represents 0.05 substitutions per nucleotide site. TP_1 = time point 1 and TP_2 = time point 2.

3.4. Phenotype prediction

The co-receptor usage of each HIV-1 quasisipecies was predicted using WEB PSSM and verified by Geno2pheno. 77 out of 360 clonal sequences (21.3%) were X4 tropic (Table 7). Two HIV-1/TB (TB-1 and TB-5) had a dominance of X4 viruses at one or both time points and three HIV-1/non TB had more than 20% of quasispecies X4 tropic (Table 7). In terms of subtypes, 17% of all subtype B and 51% of subtype C were X4.

Table 7. Co-receptor predictions of HIV-1/TB and HIV-1/non TB groups.

Patient ID	Number of clone sequences	Number of clones, predicted CXCR4 tropic	Predicted CXCR4 tropic viral population (%)	Subtype
TB-1 (TP_1) TB-1 (TP_2)	13 26	7 8	53.84 30.76	B
TB-2 (TP_1) TB-2 (TP_2)	23 18	0 1	0 5.5	B
TB-3 (TP-1) TB-3 (TP-2)	9 16	0 0	0 0	B
TB-4 (TP-1)	17	0	0	C
TB-5 (TP-1) TB-5 (TP-2)	21 10	20 10	95.2 100	C
TB-6 (TP-1) TB-6 (TP-2)	19 22	1 1	5.3 4.5	C
TB-7 (TP-1) TB-7 (TP-2)	28 15	1 0	3.8 0	B
TB-8 (TP-1)	9	0	0	C
NTB-1	14	3	21.40	B
NTB-2	11	11	100	B
NTB-3	17	11	64.7	C
NTB-4	20	2	10	C
NTB-5	14	1	7.1	C
NTB-6	8	0	0	A
NTB-7	20	0	0	A
NTB-8	10	0	0	B

3.5. Quasispecies diversity

Mean intra-patient HIV-1 diversity was calculated (Table 8) by averaging distances within all the clone sequences of one individual at a single time point. For HIV-1/TB ranged from 1.3 to 10.3% (mean 6.37%) at the initial time point and 2.7 to 10.1% (mean 6%) at the final time point (Table 8). For HIV-1/non TB, it ranged from 0.45 to 13.5% (mean 4.4%). All except two HIV-1/TB showed a very high diversity at the initial time point of sampling which was not observed in the HIV-1/non TB. An exceptionally high diversity was observed in only one HIV-1/non TB (NTB-3) who had been diagnosed for *Toxocara canis* after the time of sampling. When NTB-3 was excluded, a statistically significant two folds greater diversity was observed in the HIV-1/TB compared to HIV-1/non TB (unpaired t-test, p = 0.03) (Fig. 15). The increased diversity in HIV-1/TB didn't show significant reduction in the second time point sampling.

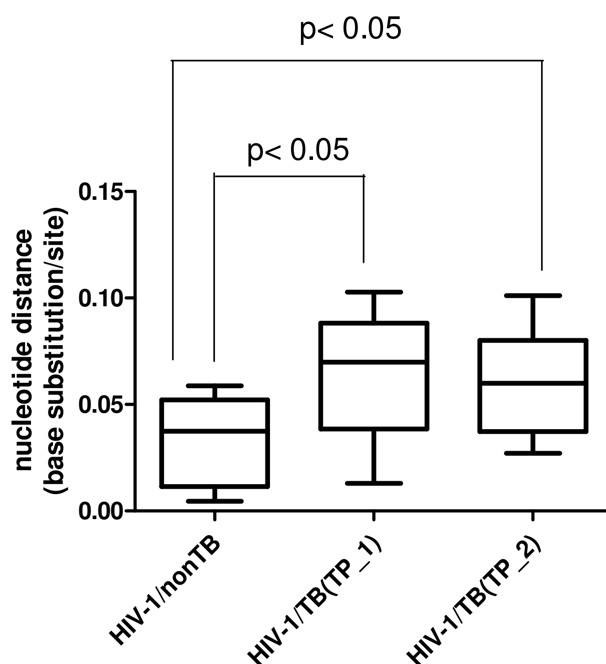


Figure 15 Intra-patient HIV-1 diversity of HIV-1/TB at the first time point (n = 8) and the second time point (n=6) compared to HIV-1/non TB at entry (single time point) (n=7) showing a significant difference (p<0.05). Evolutionary distances were shown as the number of base substitutions per site using the Maximum Composite Likelihood method. The y-axis indicates the nucleotide distance (base substitutions per site) and the x-axis the group names. TP_1 = time point 1, TP_2 = time point 2.

Table 8. Mean Intra-patient HIV-1 diversity, mean synonymous (ds) and non synonymous (dns) nucleotide substitutions, co-receptor predictions of HIV-1/TB and HIV-1/nonTB.

Patient ID	Number of clone sequences analyzed	Intra-patient nucleotide sequence distance (mean ± SE) (10^{-2} substitutions/site)	Mean ds[a]	Mean dns[b]	Mean ds/dn[c]	Predicted CXCR4 tropic viral population (%)
TB-1(TP_1)	13	7.75 ± 0.8	0.069	0.059	1.43	53.8
TB-1(TP_2)	26	5.99 ± 0.61	0.084	0.044	2.80	30.8
TB-2(TP_1)	23	4.99 ± 0.57	0.047	0.040	1.89	0
TB-2(TP_2)	18	7.31 ± 0.86	0.055	0.053	1.46	5.5
TB-3(TP_1)	9	10.28 ± 1.07	0.098	0.078	1.42	0
TB-3(TP_2)	16	6 ± 0.66	0.069	0.045	1.88	0
TB-4(TP_1)	17	6.93 ± 0.77	0.049	0.060	0.87	0
TB-5(TP_1)	21	3.48 ± 0.42	0.038	0.029	1.47	95.2
TB-5(TP_2)	10	2.72 ± 0.41	0.039	0.021	2.40	100
TB-6(TP_1)	19	9.17 ± 1.03	0.082	0.070	1.87	5.3
TB-6(TP_2)	22	10.11 ± 0.93	0.087	0.075	1.20	4.5
TB-7(TP_1)	28	1.31 ± 0.15	0.027	0.023	1.59	3.8
TB-7(TP_2)	15	4.07 ± 0.49	0.037	0.019	2.47	0
TB-8(TP_1)	9	7.05 ± 0.84	0.064	0.055	2.16	0
NTB-1	14	5.22 ± 0.67	0.05	0.047	1.28	21.4
NTB-2	11	1.16 ± 0.24	0.025	0.009	3.67	100
NTB-3	17	13.48 ± 1.26	0.119	0.096	1.35	64.7
NTB-4	20	5.88 ± 0.64	0.067	0.048	1.66	10
NTB-5	14	1.59 ± 0.23	0.02	0.015	1.95	7.1
NTB-6	8	3.74 ± 0.48	0.03	0.027	1.19	0
NTB-7	20	3.83 ± 0.64	0.039	0.034	1.15	0
NTB-8	10	0.45 ± 0.14	0.014	0.003	5.17	0

TP_1 - time point 1 (initial time point of sampling that corresponds to active tuberculosis diagnosis)
TP_2 - time point 2 (final time point of sampling that corresponds to more than 7 weeks of anti tuberculosis chemotherapy)
[a] ds: The Jukes-Cantor correction for multiple hits of proportion of observed synonymous substitutions
[b] dn: The Jukes-Cantor correction for multiple hits of proportion of observed non-synonymous substitutions
[c] ds/dn: The ratio of synonymous to non-synonymous substitutions

3.6. Intra-patient quasispecies diversification

Diversification as defined in the method section was used to quantify the dispersion of the cloned sequences from a fixed single generated consensus sequence for each patient sample at each time point (Fig.16). Highest diversification was observed in patients NTB-3 and TB-6 (Fig.16). For two patients (TB-7 and TB-5) diversification in the two time points varied significantly. TB-7 showed higher diversification at the second time point compared to the first ($p < 0.05$), and TB-5 showed significantly lower diversification at the second time point compared to the first ($p < 0.05$). Although not significant ($p = 0.0774$), TB-3 showed a lower diversification at the second time point compared to the first. For the rest of the HIV-1/TB two time point pairs, no significant difference was found.

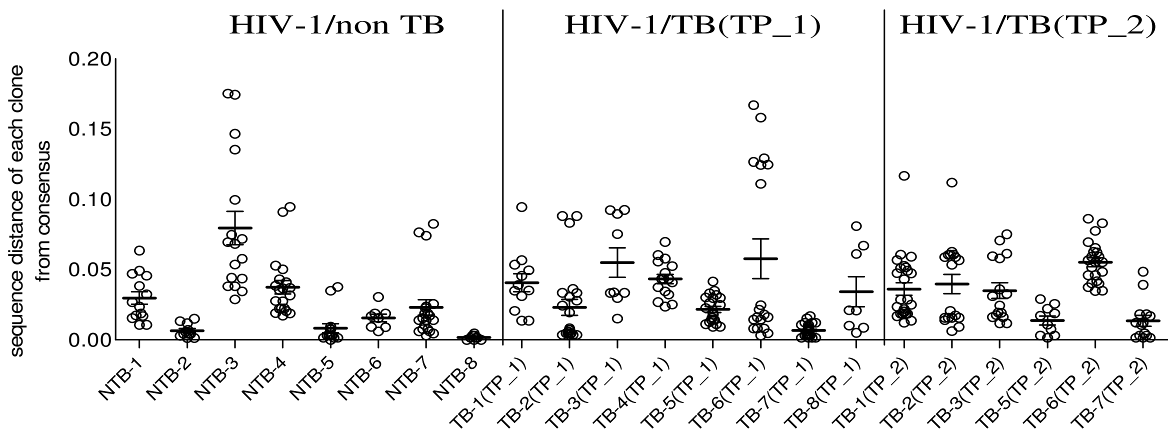


Figure 16 C2V5 *env* clone sequence diversification of HIV-1/TB and HIV-1/non TB. Each circular point indicates the pair wise comparison of the nucleotide distance of each clone sequence to a consensus sequence generated for each patient sample. The y-axis indicates the sequence distance of each clone from consensus in nucleotide substitution per nucleotide site. The x-axis indicates the ID for each patient sample. For HIV-1/TB, in the time points are indicated in brackets in addition to the patient ID. TP_1 = time point 1, TP_2 = time point 2.

3.7. Quasispecies divergence for HIV-1/TB

For HIV-1/TB, quasispecies divergence, i.e., evolution of founder viruses to later viruses within the first and second time point sampling was calculated (Table 9). In patients TB-2, TB-3, TB-4, and TB-6 quasispecies that were present at the

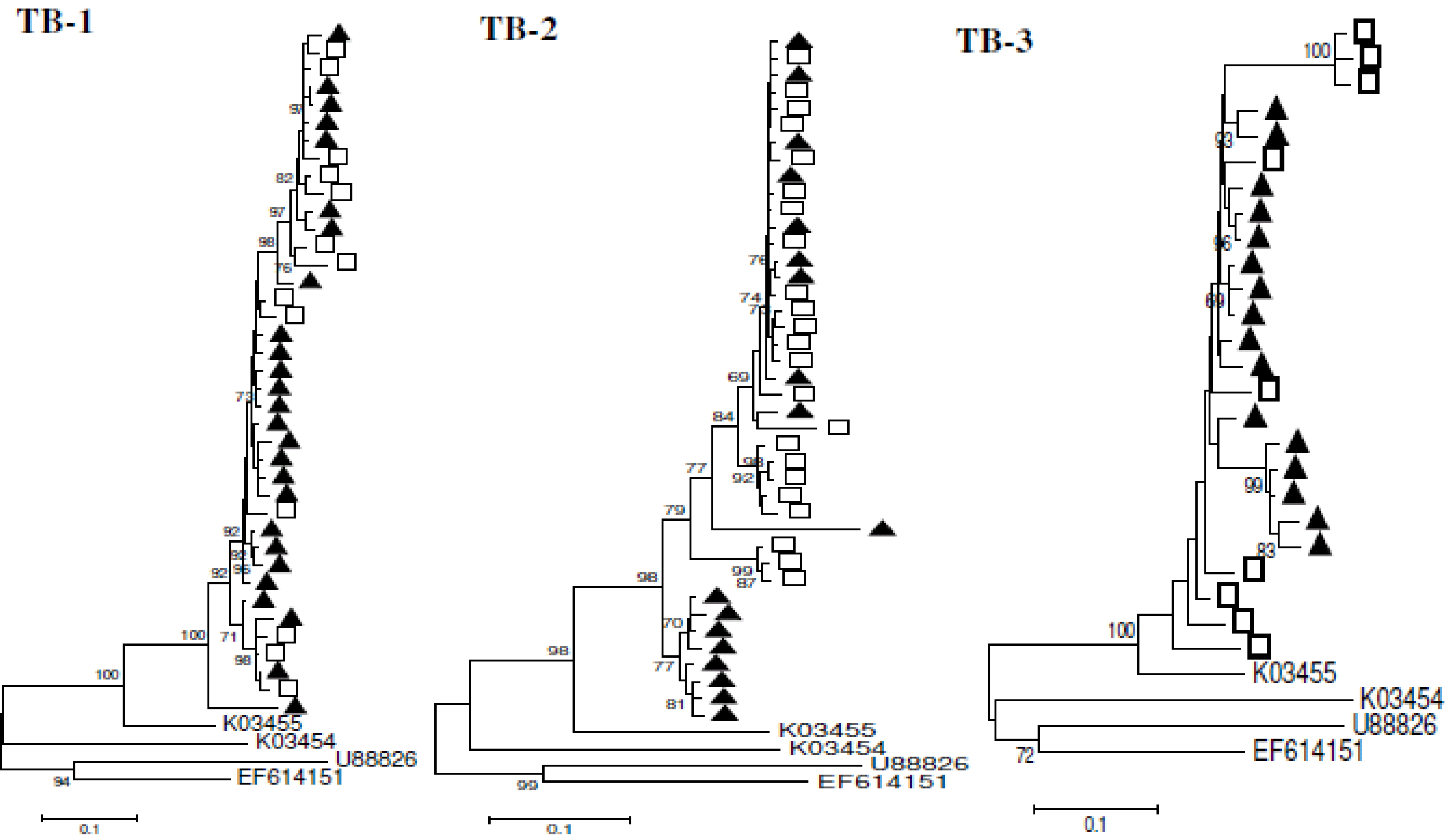
initial time point were replaced by distinct quasispecies at the second time point. This is quite strikingly observed in patients such as TB-7 (Fig. 17).

Mantel's test, which compared the identity of the viral populations at two time points, also confirmed this observation; in patients TB-1 and TB-5, the viral population at the two time points did not show a significant difference ($p > 0.05$). In the rest of the patients, the viral population belonging to the same time point showed more similarity to each other than to the viral population from the other time point ($p < 0.05$).

Table 9. Quasispecies divergence between two time points of sampling for HIV-1/TB.

Subject ID	Quasispecies divergence between TP-1 and TP-2 (substitutions/site)	Days interval between first and second time point
TB-1	0.00216 +/- 0.00059	84 days
TB-2	0.01310 +/- 0.00309	56 days
TB-3	0.01481 +/- 0.00244	175 days
TB-5	0.00113 +/- 0.00069	252 days
TB-6	0.03874 +/- 0.00578	49 days
TB-7	0.09198 +/- 0.01667	301 days

Rate of divergence was calculated by dividing the divergence to the time interval. A higher rate of divergence was observed in TB-2 (2.34×10^{-4} substitutions/site/day), TB-3 (8.46×10^{-5} substitutions/site/day), TB-6 (7.9×10^{-4} substitutions/site/day) and TB-7 (3.05×10^{-4} substitutions/site/day) as compared to TB-1 (2.57×10^{-5}substitutions/site/day) and TB-5 (4.48×10^{-6} substitutions/site/day).Interestingly these two patients who had a slow divergence rate had a dominance of X4 viruses at one or both time points.


TB-1
TB-2
TB-3
K03455
K03454
U88826
EF614151
0.1

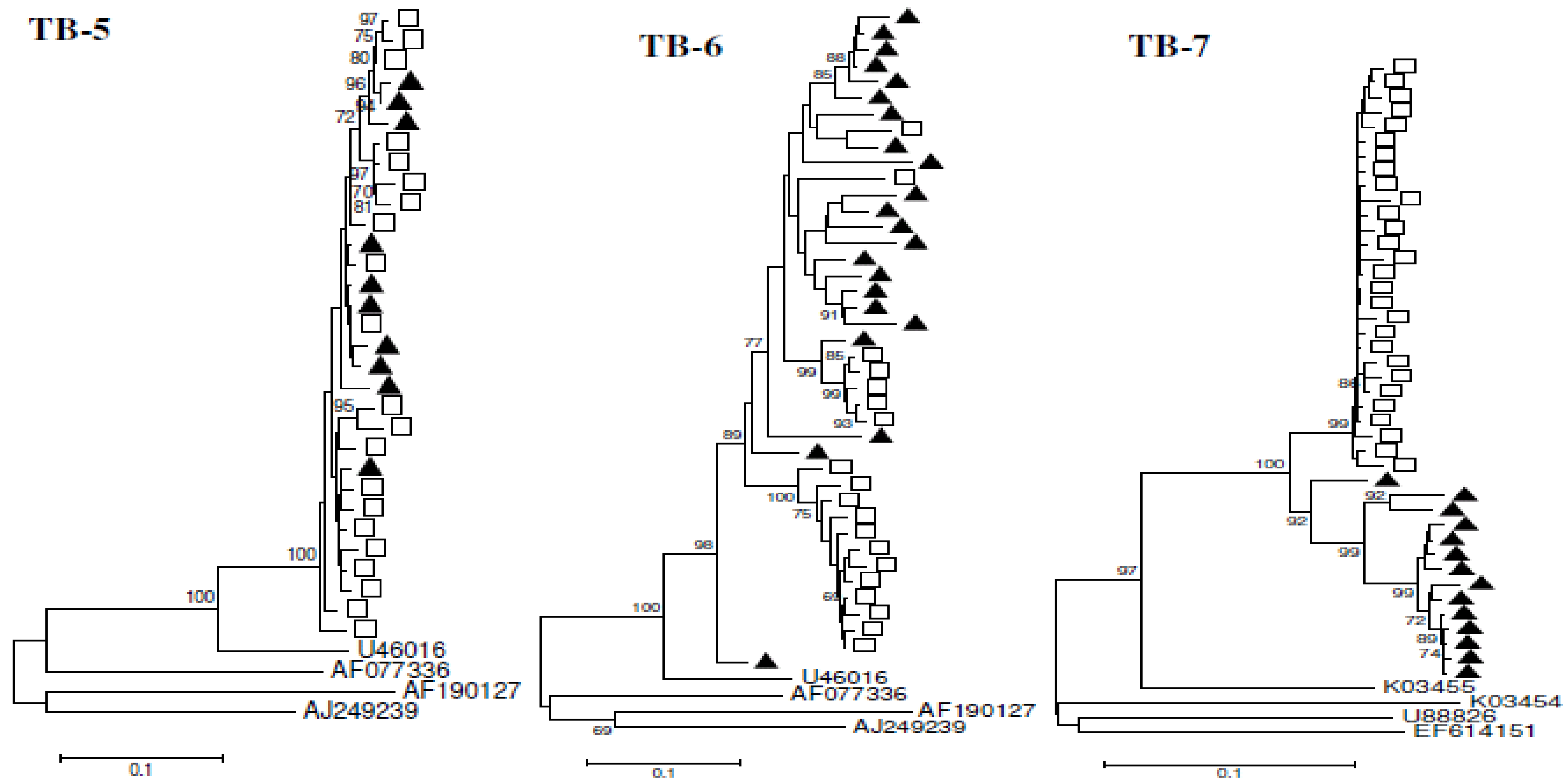


Figure 17 Phylogenetic analysis of C2 to V5 regions of all clones of HIV-1/TB at two time points of sampling for each patient. The trees show the clustering pattern of quasispecies over time. 9 to 25 clones for each time point were sequenced. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) and which have values greater than 70% are shown. The evolutionary distances were shown as number of base substitutions per site computed using the Maximum Composite Likelihood method. The scale bar represents 0.1 substitutions/nucleotide site. Reference sequences from Los Alamos HIV database were used as out groups. Squares (□) indicate clones derived during active TB diagnosis, while black filled triangles (▲) indicate clones derived from samples at least 7 weeks after the start of antituberculous chemotherapy.

3.9. Synonymous and Non synonymous nucleotide substitutions

Synonymous (amino acid not changing) and non synonymous (amino acid changing) nucleotide substitutions were calculated using SNAP. This analysis gives important information if there are positive, negative or neutral selection pressures on HIV-1 viral population. As shown in Table 8 all patients except TB-4, have greater synonymous (ds) to non synonymous (dn) substitutions and a ratio above one (ds/dn >1).

3.10. Positive selection using data monkey programme

Besides SNAP, data monkey was utilized to assess the presence of positively selected amino acids. Data monkey has the alternatives of three different codon-based maximum likelihood methods, SLAC, FEL and REL, to estimate the dN/dS (also known as Ka/Ks or ω) ratio at every codon in the alignment. The presence of positively selected amino acid sites using SLAC showed no positively selected amino acid sites for six of the HIV-1/TB and for all HIV-1/non TB. For the remaining two HIV-1/TB patients, HXB-2 referenced gp120 amino acid positions 466 (glutamic acid) and 467 (isoleucine) for TB-4, and 295 (asparagine) for TB-6 were under positive selection.

3.11. Positional variation along the C2V5 amino acid sequence

Using Shannon entropy as a measure of variation in DNA and protein sequence alignments we analyzed whether there is a difference in positional variation along the C2V5 *env* region of HIV-1/TB and HIV-1/non TB. There was a higher variability for HIV-1/TB along the V4 region independent of their subtypes compared to HIV-1/non TB (Fig. 18). The median intra-patient V4 diversity for HIV-1/TB was 0.184 nucleotide substitutions per site (range 0.01 to 2.59) while for HIV-1/non TB was 0.069 nucleotide substitutions per site (range 0.0034 to 0.2). On the other hand, the V3 diversity did not show a difference between HIV-

1/TB (median 0.0363, range 0.0056 to 0.0822) and HIV-1/non TB (median 0.032, range 0.04 to 0.0847).

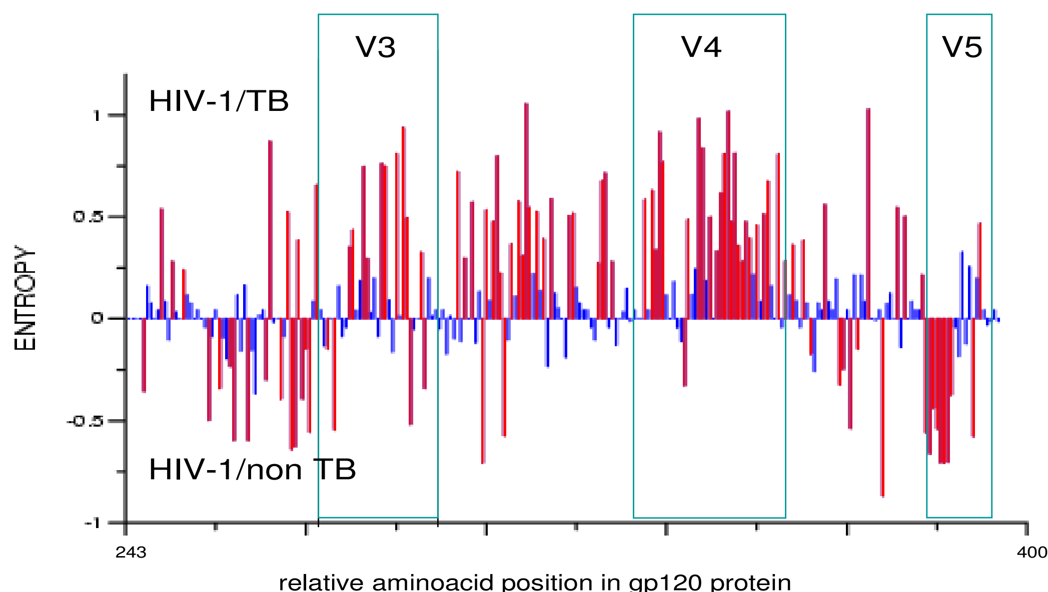


Figure 18 Entropy plot showing variation in the amino acid sequences of clones from HIV-1/TB and HIV-1/non TB. Entropy >0 indicates higher variability in HIV-1/TB compared to HIV-1/non TB. Entropy <0 indicates less variability in HIV-1/ TB as compared to HIV-1/non TB. Significant sites with p<=0.05 are shown in red.

3.12. Potential N-linked glycosylation sites (PNGs)

HIV *env* gp120 is among the most heavily glycosylated proteins in nature [111]. N linked glycosylation, sequon, has a pattern of NX [ST] (where X can be any amino acid). Here we analyzed the number of PNGs for C2V5 *env* of HIV-1/TB and HIV-1/non TB by averaging the number of PNGs for each quasispecies for a patient at a time point. As shown in Table 10, the mean number of PNGs was 14 (range 11 to 17) when both groups were included. No significant difference was found between HIV-1/TB and HIV-1/non TB. However, a significantly lower number of PNGS was observed in patients with predicted X4 dominating viral sequences as compared to R5 (p = 0.03).

Table 10. Average number of N-linked glycosylation sites for each patient obtained by averaging the PNGs obtained for each clone sequence.

Subject ID	Average number of N linked glycosylation sites
TB-1(TP-1)	13
TB-1(TP-2)	13
TB-2(TP-1)	16
TB-2(TP-2)	15
TB-3(TP-1)	14
TB-3(TP-2)	14
TB-4(TP-1)	14
TB-5(TP-1)	14
TB-5(TP-2)	13
TB-6(TP-1)	13
TB-6(TP-2)	14
TB-7(TP-1)	14
TB-7(TP-2)	13
TB-8	16
NTB-1	14
NTB-2	15
NTB-3	11
NTB-4	17
NTB-5	14
NTB-6	16
NTB-7	15
NTB-8	14

3.13. Correlation between HIV-1 viral load, CD4 cell count, intra-patient diversity and divergence

Viral divergence for HIV-1/TB showed a strong correlation with the initial time point CD4 cell count ($r^2 = 0.66$, $p < 0.05$, two tailed) (Fig. 19). No correlation was found between CD4 cell count and intra-patient nucleotide diversity for both HIV-1/non TB and HIV-1/TB at the two time points of sampling. Viral load was also not correlated with intra-patient viral diversity for both groups.

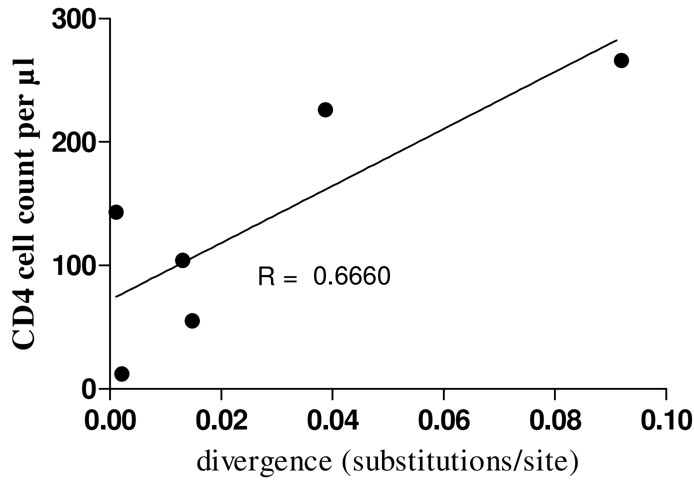


Figure 19 Two time point HIV-1/TB quasispecies divergence (nucleotide substitutions/site) plotted against CD4 cell count (cells/µl) at the initial time point showing strong correlation, $p<0.05$.

4. Discussion

HIV evolves at any time over the course of an individual's infection. Evolution of the virus is shaped by various selection pressures. Selection pressures can be exogenous in nature, such as the pressure exerted by antiretroviral drugs leading to the selection of drug resistance mutants; or can be endogenous, such as the pressure generated by the adaptive immune system, leading to selection of immune escape mutants. These two processes shape the virus in a fashion that may lead to individual-specific HIV-1 variants.

In the time course of HIV infection, which is divided in to primary, asymptomatic, symptomatic and AIDS (Fig. 20) HIV viral variant population dynamics varies. During transmission a dramatic evolutionary bottleneck occurs, with ~80% of heterosexual infections apparently initiated by a single variant [113]. After transmission, mutational escape and reversion rapidly shape HIV evolution [114,115]. Especially HIV-1–specific CD8 T cells which are detectable before seroconversion and long before neutralizing antibodies (NAbs) [116] have an important contribution in shaping HIV evolution and in the decline of acute phase viral viremia (viral set point) [117].

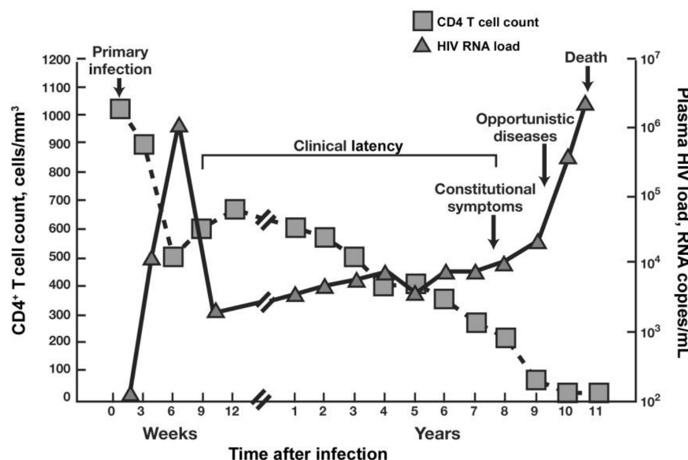


Figure 20 the typical course of HIV infection without intervention. Adapted from [112]

During the asymptomatic stage, evolution of HIV increases rapidly. A comprehensive analysis done by Shankrappa *et al* [66] divided HIV-1 evolution at this stage in to three phases quantified by diversity, divergence and emergence

of X4 viruses. According to Shankrappa *et al.* [66] in the first phase, both viral population divergence and diversity increased linearly and in the second phase the viral population continued to diverge from the founder strain at the same rate, while diversity levels off. In the third stage divergence also slows down or stabilizes, while diversity declines.

However such an evolutionary pattern can be perturbed when the immune system is activated due to infections or vaccination. Activation of the immune system is a normal response to antigenic stimulation which induces changes in the host characterized by proliferation of antigen-responsive cells, elaboration of cytokines, and subsequent proliferation of bystander cells [118-120]. Such a state of immune activation results in an increase in the number of available activated $CD4^+$ T cells that serve as susceptible targets for HIV. This leads to replication of HIV.

A number of researches have addressed the issue of HIV evolutionary pattern during immune activation. One of the early and most prominent studies was by Ostrowski *et al* [121] that characterized HIV quasispecies composition after a shot of Tetanus Toxoid vaccine. Patients who received the vaccine showed a dramatic shift in composition of plasma viral quasispecies.

Later, a study done by Collins *et al.* [122] was able to show influence of active TB on HIV quasispecies in a cross-sectional study design. In the present study, we were able to show effect of mycobacterial growth/TB on HIV-1 quasispecies diversity on a longitudinal basis.

The significance of our work can be viewed in the context of understanding the long term effect of active TB on HIV-1 in co-infected individuals, not only on quasispecies evolution but also on disease progression. Several reports from various parts of Africa showed that the increase in HIV-1 replication due to active TB does not decline after 2 months of intensive antituberculous chemotherapy [123-125]. The sustained increase in HIV-1 replication even after initiation of antituberculous chemotherapy unlike other opportunistic infections (OIs) [126, 127] is a puzzling phenomenon. The sustainability or reversal of the HIV-1

quasispecies heterogeneity after TB treatment is not known. Tetanus Toxoid vaccinated HIV-1 infected patients developed a transient increase in HIV-1 heterogeneity which was reversed after few weeks [121]. Emergence of a heterogeneous HIV-1 population within a patient may be one of the mechanisms to escape strong immune or drug pressure [65, 128]. The existence of better fitting and/or immune escape HIV-variants can lead to an increase in HIV-1 replication [129,130]. It might be that TB favourably selected HIV-1 variants which are sources for consistent HIV-1 replication.

Understanding the mechanisms underlying the impacts of TB on HIV-1 is essential for the development of effective measures to reduce TB related morbidity and mortality in HIV-1 infected individuals. There are on going researches to find out the reason that increased HIV-1 replication couldn’t decline even after MTB treatment by chemotherapy. In the present study we studied whether the increase in HIV-1 quasispecies diversity during active TB is reversed or preserved throughout the course of antituberculous chemotherapy. Our investigation might partly explain the reasons for sustained HIV-1 replication during or after active TB treatment in antiretroviral therapy (ART) naïve patients.

The main findings of the study are discussed here below:

4.1. Active TB sustains HIV-1 quasispecies diversity for longer period

In the present study, we compared:

1) HIV-1 quasispecies diversity during diagnosis and after 2 months of TB chemotherapy

2) Cloud of quasispecies during diagnosis of TB in HIV-1/TB with CD4 cell count matched HV-1/non TB.

The purpose of the latter comparison was to verify whether active TB can lead to high HIV-1 diversity in our patients.

As seen in Fig. 15 HIV-1 quasispecies diversity was significantly higher ($p<0.05$) during active TB and sustained up to two months of chemotherapy. This observation indicates that the impact of TB on HIV-1 diversity can even proceed after active TB treatment which is in line with other observations that showed HIV-1 replication remained elevated after TB resolution by chemotherapy [123-125]. Higher diversity is usually associated with faster HIV-1 disease progression to AIDS [65,131].

One way of explaining higher diversity during active TB might be generalized immune activation due to TB. Infection with MTB is characterized by strong immune response. Studies in animal models and in humans have demonstrated that a wide range of immune components are activated during TB. These include T cells (both CD4+ and CD8+), cytokines, including IFN-γ, IL-12, TNFα and IL-6, and macrophages [132]. In HIV/TB co-infected individuals several immune activation markers showed to increase including neopterin, TNF-α, β2-microglobulin [81,85,133], various chemokines such as MIP1-α, MIP1-β, RANTES and SDF-α and expression of chemokine receptors (CCR5 and CXCR4) [134].

Although most immune activation markers are reduced after start of TB chemotherapy, some might remain persistent which might lead to sustained HIV diversity. According to a study by Goletti *et al.,* [82] the level of immune activation markers after few weeks of successful TB treatment in co-infected individuals had shown a decline to baseline line level in western subjects. In other studies some but not all immune activation markers showed a decline [85,135]. Lawn and colleagues [85] found out that TNFα did not show a decline after 2 months of chemotherapy while other activation markers showed a decline. In a study done in Ethiopia, Wolday and colleagues [135] did not find a reduction in the increased chemokine and chemokine receptors after two months of anti tuberculosis chemotherapy. Kizza and colleagues [123] found two groups of patients, which showed a significant reduction and those which did not after 12 months of treatment follow up.

We also asked if a more specific HIV-1 directed immune response had an impact on the observed higher diversity. For this purpose we quantified selection pressure. A widely used means to measure selection pressure is the analysis of non-synonymous (n) and synonymous (s) nucleotide substitutions [60]. Synonymous nucleotide substitutions (s) do not change amino acid sequence although there is a change at the nucleotide level whereas non-synonymous substitutions (n) do change the amino acid sequence. 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 *ds* the gene is under positive or diversifying selection. A *dn* is probably driven by selective forces to change. A *ds/dn* ratio close to one indicates a neutralizing selection. Using this method, we obtained a *ds/dn* ratio approximately or above one for all patients which indicates that there was no strong positive selective pressure for all viral quasispecies isolated. This is in contrary to what was observed in the cross sectional study by Collins *et al* [122].

4.2. Active TB increases rate of HIV-1 divergence

When we quantified an average rate of C2V5 evolution in two time point follow-up of HIV-1/TB, we found an average rate of 2.4×10^{-4} substitutions/site/day. We compared this value with an average rate of *env* evolution from previous reports [136-140] .The average rate of genetic evolution of HIV-1 was estimated to be 2.3×10^{-3} to 6.7×10^{-3} substitutions/site/year at the *env* V3 region, while the rate for the whole *env* was 1.0×10^{-3} and 1.7×10^{-3} substitutions/site/year, which gives an approximation of 1.7×10^{-5} substitutions/site/day. Compared to these previous reports, our HIV-1/TB patients had 14 times higher mutation rate. However, we were unable to compare the rate with HIV-1/non TB group due to their immediate start of HAART before second time point sampling.

A higher viral divergence and divergence rate might indicate high replication and constant pressure for evolution. It is also clearly demonstrated in the phylogenetic trees reconstructed. For example in patient TB-3 (Fig. 17), the viral quasispecies composition at time point 2 is distinct from that of time point 1 within only 7 weeks. At longer time interval, approximately 43 weeks, in patient TB-7 a separation of two clusters of viral population was observed (Fig. 17).Especially, four of the six patients: TB-7, TB-2, TB-6, TB-3 had a higher evolutionary rate compared to the remaining two.

4.3. TB might lead to slow evolution of X4 variants

Using phyletic analysis by Mantel's test, we obtained a significant difference in genetic identity between quasispecies at TP-1 and TP-2 in four of the six HIV-1/TB.These patients namely: TB-7, TB-2, TB-6 and TB-3 had also a dominance of predicted R5 phenotype. In the residual two (TB-1 and TB-5), no significant difference in quasispecies identity between the two time points and a dominance of X4 viruses was observed.

In one in vitro study [134] it was observed that MTB increased CXCR4 surface expression on alveolar macrophages and allowed the entry and replication of X4 viruses while inhibiting R5 virus entry. Some earlier studies indicated that macrophages exposed to mycobacterial cell wall lipoarabinomannan become highly susceptible to HIV-1 [141]. It was further demonstrated that stimulated macrophages are highly susceptible to T-tropic HIV-1 (which otherwise poorly replicate in macrophages) and modestly susceptible to M-tropic HIV-1 [141]. At advanced stages of HIV disease the source of HIV replication are tissue macrophages [142].

The observation that slower divergence rate and similar viral population for a long period of time in the samples of HIV-1/TB with dominance of X4 viruses might be related to the direct selective pressure of MTB to HIV-1 to favorably select X4 viral populations. The proportion of X4 viruses, however, in all our HIV-

1/TB patients remained stable over the study period, which might be due to tuberculosis chemotherapy and needs further study.

4.4. Other findings

Besides the above mentioned findings, our study highlighted findings such as, the high variation of V4 loop in HIV-1/TB compared HIV-1/non TB. V4 as part of the env loop is likely to have an impact on *Env* conformation and glycan packing [143-145] although its role in neutralization resistance is not clear. In one study it was found that C3 and V4 regions combine to form important structural motifs and that epitopes in this region are major targets of the early autologous neutralizing response in HIV-1 subtype C infection [146].

In the present study, the numbers of N-linked glycosylation sites along the C2V5 were lower in X4 viruses compared to R5. This observation is in line with previous reports that demonstrated that decreased number of *N*-linked glycosylation sites (sequons) in gp120, especially within and around the V3 region, during evolution from the R5 to the X4 phenotype [147-149].

From our intra-patient diversity data (Table 8) and diversification plot (Fig. 16) one can see that single time point sequence diversification is variable. Even in HIV-1/TB there were individuals who showed extremely low intra-patient diversity like TB-7 and TB-5. As may be seen, this is independent of the duration of HIV-1 infection and HIV-1 subtypes. It might be that the initial time point sampling was a few months earlier than the diagnosis of active tuberculosis (approximately 5 and 4 months respectively). As MTB is a slow growing bacterium we assumed that these patients had TB infection even at that earlier time.

The observation that there was no difference in diversity and divergence between African origin patients living in Europe and native Europeans indicates that the country of origin and viral subtypes have no significant impact on the influence of TB on HIV-1 diversity. In our study, the HIV-1 viral load in HIV-1/TB patients after the start of antituberculous chemotherapy did not show a significant decline

compared to untreated TB. Similar observations in HIV-1/TB co-infected patients in Africa were frequently reported [123-125].

Although the sample size for the African participants in this study was small (n =4), we also observed the onset of active TB in African origin patients at a significantly higher CD4 cell count compared to European origin patients. This observation is in line with studies done in TB endemic countries, where the onset of TB occurs at higher CD4 cell counts [150,151] which might be explained by the reactivation of childhood tuberculosis in Africans.

4.5. Limitations of the study

Our study has a clear limitation to find control patients who fulfilled selection criteria such as matched CD4 cell count without opportunistic infections (OI) and with follow-up. Although we made efforts, it was difficult to find HIV-1 infected individuals with no other co-infection at CD4 cell count below 200 per µl. Control subjects such as NTB-1, NTB-3 and NTB-4 had documented co-infections like *Candida albican* and *T.canis*. Intra-patient viral diversity in these patients was higher than the residual 5 and was comparable to the HIV-1/TB subjects. This might suggest that OI can trigger HIV-1 sequence diversity. Besides an extremely high HIV-1 diversity was observed in one control subject (NTB-3). HIV-1 from this control subject showed the highest diversity of all the others. More than half of the quasispecies isolated from the sample of this patient harboured a predicted X4 phenotype. No other opportunistic infection has been documented except *T. canis*. Until to date, there are no reports which indicate the possible association of *T.canis* infection with HIV-1 genetic diversity. The possible association of this helminthic infection with HIV needs further investigation.

The strategy used in our study to isolate HIV-1 quasispecies is one of the few available methods used in many laboratories to date. It is however linked with method-specific limitations such as Taq induced recombination, nucleotide misincorportion, template resampling and cloning bias [152]. Primers used in the study were tested for different subtypes and their priming regions are at low

mutable or constant regions of HIV-1 C2C5 *env*. However, one cannot assure that all the quasispecies templates were amplified.

Summary

One of the earliest and most striking observations made about HIV is the extensive genetic variation that the virus has within individual hosts, particularly in the hypervariable regions of the *env* gene which is divided into 5 variable regions (V1-V5) and 5 more constant (C1-C5) regions.

HIV evolves at any time over the course of an individual's infection and infected individuals harbours 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 of closely related genomes are called quasispecies [65].

Tuberculosis or tubercle forming disease is an acute and/or chronic bacterial infection that primarily attacks the lungs, but which may also affect the kidneys, bones, lymph nodes, and brain. The disease is caused by *Mycobacterium tuberculosis* (MTB), a slow growing rod-shaped, acid fast bacterium. It is transmitted from person to person through inhalation of bacteria-carrying air droplets.

Worldwide, one person out of three is infected with *Mycobacterium tuberculosis* – two billion people in total. TB currently holds the seventh place in the global ranking of causes of death [73]. In 2008, there were an estimated 9.4 (range, 8.9–9.9 million) million incident cases (equivalent to 139 cases per 100 000 population) of TB globally [75].

A complex biological interplay occurs between *M. tuberculosis* and HIV in co-infected host that results in the worsening of both pathologies. HIV promotes progression of *M. tuberculosis* either by endogenous reactivation or exogenous reinfection [77, 78] and, the course of HIV-1 infection is accelerated subsequent to the development of TB [80].

Active TB is associated with an increase in intra-patient HIV-1 diversity both systemically and at the infected lung sites [64,122]. The sustainability or reversal of the HIV-1 quasispecies heterogeneity after TB treatment is not known.

Tetanus toxoid vaccinated HIV-1 infected patients developed a transient increase in HIV-1 heterogeneity which was reversed after few weeks [121]. Emergence of a heterogeneous HIV-1 population within a patient may be one of the mechanisms to escape strong immune or drug pressure [65,128]. The existence of better fitting and/or immune escape HIV-variants can lead to an increase in HIV-1 replication [129,130]. It might be that TB favourably selected HIV-1 variants which are sources for consistent HIV-1 replication.

Understanding the mechanisms underlying the impacts of TB on HIV-1 is essential for the development of effective measures to reduce TB related morbidity and mortality in HIV-1 infected individuals. In the present study we studied whether the increase in HIV-1 quasispecies diversity during active TB is reversed or preserved throughout the course of antituberculous chemotherapy.

For this purpose Two time point HIV-1 quasispecies were evaluated by comparing HIV-1 infected patients with active tuberculosis (HIV-1/TB) and HIV-1 infected patients without tuberculosis (HIV-1/non TB). Plasma samples were obtained from the Frankfurt HIV cohort and HIV-1 RNA was isolated. *C2V5 env* was amplified by PCR and molecular cloning was performed. Eight to twenty five clones were sequenced from each patient. Various phylogenetic analyses were performed including tree inferences, intra-patient viral diversity and divergence, selective pressure, co-receptor usage prediction and two time point identity of quasispecies comparison using Mantel's test.

We found out from this study that:

1) Active TB sustains HIV-1 quasispecies diversity for longer period

2. Active TB increases the rate of HIV-1 divergence

3) TB might slow down evolution of X4 variants

And we concluded that active TB has an impact on HIV-1 viral diversity and divergence over time. The influence of active TB on longitudinal evolution of HIV-1 may be predominant for R5 viruses. The use of CCR5-coreceptor inhibitors for HIV-1/TB patients as therapeutic approach needs further investigation.

Zusammenfassung

Eine der ersten und überraschenden Beobachtungen, welche bei der Analyse des HI-Virus gemacht wurden ist seine ausgeprägte Genetische Variabilität besonders die hypervariable Region des *env* Genes betreffen. Dieses wird in 5 variable Regionen (V1-V5) sowie 5 stärker konservierte Regionen (C1-C5) unterteilt.

HIV wandelt sich zu jedem Zeitpunkt im Verlauf der Infektion und jedes infizierte Individuum ist Träger einer Population von genetisch verwandten jedoch nicht identischen Viren, welche sich kontinuierlich verändern und an die Erfordernisse innerhalb der Umgebung anpassen. Diese genetisch heterogenen, jedoch eng verwandten Populationen werden Quasispecies genannt.

Tuberkulose ist eine mykobakterielle Infektion, welche sowohl akute als auch chronische Verläufe zeigt. Neben den Lungen als primärem Manifestationsort können auch die Nieren, Knochen und andere Organe befallen sein. Eine von drei Personen weltweit ist mit *Mycobacterium tuberculosis* infiziert, insgesamt 2 Milliarden Menschen.

In HIV/TB Co-Inifzierten Menschen entsteht ein komplexes Zusammenspiel zwischen HIV und *M. tuberculosis,* welches zu einer Verschlechterung beider Krankheitsbilder führt. HIV führt durch endogene Rekativierung oder exogene Re-Infektion zu einer Progression der Tuberkulose, welche im weiteren Verlauf die Krankheitsprogression von HIV beschleunigt. Sowohl Morbidität als auch Mortalität sind in HIV-1/TB Co-Infizierten Menschen erhöht.

Aktive Lungentuberkulose und Miliartuberkulose gehen mit dem Anstieg der Diversifität der HIV Viren innerhalb eines Wirtes einher. Wie lange diese erhöhte Heterogenität der HIV Quasispecies nach der erfolgreichen Behandlung einer Tuberkulose bestehen bleibt ist bisher noch unklar.

Das Verständnis des dem Zusammenspiel von HIV und TB zugrundeliegenden Mechanismus ist essentiell für die Entwicklung von effektiven Massnahmen zur Senkung der Morbidität und Mortalität in HIV/TB Co-infizierten Menschen.

Die gegenwärtige Forschungsarbeit folgte daher der Frage, ob wärend einer aktiven TB Infektion eine Zunahme der Diversität der HIV-1 Quasispecies zu beobachten ist und ob diese Diversität während einer TB Therapie erhalten bleibt oder sich zurück bildet.

Hierfür wurden die HIV-1 Quasispecies zu zwei Zeitpunkten untersucht, wobei Proben von HIV-1 infizierten Patienten mit aktiver Tuberkulose (HIV-1/TB) und HIV infizierte Patienten ohne Tuberkulose (HIV-1/non TB) verglichen wurden. Aus Plasmaproben der Frankfurter HIV Cohorte wurde HIV-1 RNA isoliert. *C2V5 env* wurde durch PCR amplifiziert und molekular cloniert. Acht bis fünfundzwanzig Clone wurden für jeden Patienten sequenziert. Mehrere phylogenetische Analysen wurden durchgeführt, welche tree inferences, Intra-Patienten- und virale Diversität und Divergenz, Selektionsdruckanalysen, Vorhersage der Co-Rezeptornutzung sowie Zweipunktanalysen der Identität von Quasispecies mit Hilfe des Mantel's Test miteinschlossen.

Die Analysen ergaben die folgenden Ergebnisse:

1) Eine aktive TB erhält die Diversität von HIV-1 Quasispecies über einen längeren Zeitraum.

2. Eine aktive TB verstärkt die HIV -1 Divergenz

3) TB könnte zu einer langsameren Evolution von X4 Varianten führen.

Schlussfolgerung: eine aktive TB beeinflusst die Entwicklung der viralen Diversität und Divergenz von HIV-1 im Verlauf der Krankheit. Der Einfluss der aktiven TB auf die longitudinale Evolution von HIV-1 könnte insbesondere R5 Viren betreffen. Der Einsatz von CCR5-Corezeptor Inhibitoren in HIV-1/TB co-inifizerten Patienten sollte daher in Langzeitstudien untersucht werden.

Literature

[1] Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, and Saxon A: *Pneumocystis carnii* pneumonia and mucosal candidasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N Eng J Med 2005; 305:1425-31.

[2] Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L: Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).Science 1983;220:868-71.

[3] Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleske J, Safari B: Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 1984; 224: 500-3.

[4] Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro M, and Oshiro LS: Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science 1984; 225:840-842.

[5] Coffin J, Hasse A, Levy JA, Montagnier S. Oroszlan S, Teich N, Temin H, Toyoshima K, Varmus H, Vogt P, Weiss R: Human Immunodeficiency viruses. (Letter). Science 1986; 232:697.

[6] International Committee on Taxonomy of Viruses. Virology Division. http://www.ictvonline.org.

[7] Levy JA: HIV and the pathogenesis of AIDS. Third edition. ASM press, VA USA. 2007.

[8] Gilbert PB, McKeague IW, Eisen G, Mullins C, Guéye-NDiaye A, Mboup S, Kanki PJ: Comparison of HIV-1 and HIV-2 infectivity from a prospective cohort study in Senegal. Statistics in Medicine 2003; 22: 573–593.

[9] Reeves JD and Doms RW: Human immunodeficiency virus type 2. J Gen Virol 2002; 83: 1253–65.

[10] AIDS epidemic update. UNAIDS. World Health Organization December 2009.

[11] Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, and Weiss RA: The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 1984; 312: 763-7.

[12] Maddon PJ, Dalgleish AG, McDougal JS, Clapham PR, Weiss RA, Axel R: The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell 1986; 47:333-348.

[13] Doms RW, Trono D. The Plasma membrane as a combat zone in the HIV battlefield. Genes Dev 2000; 14: 2677-88.

[14] Hahn BH: Viral genes and their products; in Broder S, Merigan T, Bolognesi D (editors): Text book of AIDS Medicine. Williams & Wilkins, USA, 1994 pp.21-45.

[15] Bukrinsky MI, Sharova N, Dempsey MP, Stanwick TL, Bukrinskaya AG, Haggerty S, Stevenson M: Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc Natl Acad Sci USA 1992; 89:6580-6584.

[16] Chesebro B, Buller R, Portis J, Whrly K: Failure of human immunodeficiency virus entry and infection in CD4-positive human brain and skin cells. J Virol 1990; 64:215-221.

[17] Evans DJ, McHugh TM, Stites DP, and Levy, JA: Differential ability of human immunodeficiency virus isolates to productively infect human cells. J Immunol 1987; 138:3415-3418

[18] Kikukawa R, Koyanagi Y, Harada S, Kobayashi N, Hatanaka M, Yamamoto: Differential susceptibility to the acquired immunodeficiency syndrome retrovirus in cloned cells of human leukemic T cell line Molt-4. J Virol 1986; 57:1159-1162.

[19] Freed EO, Martin MA: HIVs and Their Replication. Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, and Straus SE (editors). 2107-2123. 2007. Philadelphia, Williams and Wilkins; A Wolters Kluwer Business. Fields Virology. Volume 2. 5th Edition. Ref. Type: serial (Book, Monograph).

[20] Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA: CKR5: A RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 1996; 272:1955-1958.

[21] Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, Sodroski J: The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 1996; 85: 1135-1148.

[22] Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR: Identification of a major coreceptor for primary isolates of HIV-1. Nature 1996; 381:661-666.

[23] Feng Y, Broder CC, Kennedy PE, Berger EA: HIV-1 entry cofactor: fuctional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 1996; 272:872-877.

[24] Schuitemaker H, Koot M, Kootstra NA, Dercksen MW, de Goede RE,van Steenwijk RP, Lange JM, Schattenkerk JK, Miedema F, and Tersmette, M: Biological phenotype of Human Immunodeficiency Virus type- 1 Clones at different stages of infection: Progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. J. Virol 1992; 66:1354–1360.

[25] Fenyo E, Schuitemaker H, Birgitta A, McKeating QS, and the EC concerted action HIV variability: The history of HIV-1 biological phenotypes; in Korber B, Hahn B, Foley B, Mellors J, Leitner T, Myers G, McCutchan F, Kuiken C (editors) : Human Retroviruses and AIDS 1997 Compendium. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, 1997, pp. 13.

[26] Karlsson A, Parsmyr K, Sandström E, Fenyö EM, Albert J: MT-2 cell tropism as prognostic marker for disease progression in human immunodeficiency virus type 1 infection. J Clin Microbiol 1994; 32(2):364-70.

[27] Bjorndal A, Deng H, Jansson M, Fiore JR, Colognesi C, Karlsson A, Albert J, Scarlatti G, Littman DR, and Fenyo EM: Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. J Virol 1997; 71: 7478–7487.

[28] Schwartz S, Felber BK, Fenyö EM, Pavlakis GN: Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. J Virol 1990; 64(11):5448-56.

[29] Lützelberger M, Reinert LS, Das AT, Berkhout B, Kjems J: A novel splice donor site in the gag-pol gene is required for HIV-1 RNA stability. J Biol Chem 2006: 7; 281(27)

[30] Purcell DF, Martin MA: Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. J Virol 1993; 67(11):6365-78.

[31] Furtado MR, Balachandran R, Gupta P, Wolinsky SM: Analysis of alternatively spliced human immunodeficiency virus type-1 mRNA species, one of which encodes a novel tat-env fusion protein. Virology 1991; 185:258-70.

[32] Muesing MA, Smith DH, Cabradilla CD, Benton CV, Lasky LA, Capon DJ: Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature 1985; 313:450-8.

[33] Robert-Guroff M, Popovic M, Gartner S, Markham P, Gallo RC, Reitz MS: Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. J Virol 1990; 64(7):3391-8.

[34] Kuiken C, Leitner T, Foley B, Marx P, Wolinsky S: HIV Sequence Compendium: 2008 Los Alamos National Laboratory.

[35] Zhu P, Winkler H, Chertova E, Taylor KA, Roux KH: Cryoelectron tomography of HIV-1 envelope spikes: further evidence for tripod-like legs. PLoS Pathogens 2008: 4 (11): e1000203. doi:10.137/journal.ppat.1000203. PMID 1900895.

[36] Leonard CK, Spellman MW, Riddle L, Harris J : Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombination human immunodeficiency virus envelop glycoprotein (gp120) expressed in Chinese hamster ovary cells. J Biol Chem 1990; 265:10373-10382.

[37] Starcich BR, Hahn BH, Shaw GM, McNeely RD, Modrow S, Wolf H, Parks ES, Parks WP, Josephs SF and Gallo RC: Identification and characterization of conserved and variable regions in the envelope gene of HTLVIII/LAV, the retrovirus of AIDS. Cell 1986; 45:637-648.

[38] Willey RL, Rutledge RA, Dias S, Folks T, Theodore T, Buckler CE, Martin MA: Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. Proc. Natl. Acad. Sci. USA 1986; 83:5038–42

[39] Moore JP, Sattentau QJ, and Wyatt R, Sodroski J: Probing the structure of the human immunodeficiency virus surface glycoprotein gp120 with a panel of monoclonal antibodies. J Virol 1994; 68(1):469-84.

[40] Wyatt R, Sullivan N, Thali M, Repke H, Ho D, Robinson J, Posner M, Sodroski J: Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions. J Virol 1993; 67(8):4557-65

[41] Wyatt R, Sodroski J: The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science 1998; 280(5371):1884-8.

[42] Chen B, Vogan EM, Gong H, Skehel JJ, Wiley DC, Harrison SC. Structure of an unliganded simian immunodeficiency virus gp120 core. Nature 2005; 433:834-41.

[43] Broliden PA, von Gregerfelt A, Clapham P, Rosen J, Fenyo EM, Wahren B, Broliden K: Identification of human neutralization-inducing regions of the human immunodeficiency virus type 1 envelope glycoprotein. Proc Natl Acad Sci USA 1992; 89:461-465

[44] Sharon M, Kessler N, Levy R, Zolla-Pazner S, Gorlach M, Anglister J: Alternative conformations of HIV-1 V3 loops mimic beta hairpins in chemokines, suggesting a mechanism for co-receptor selectivity. Structure (Camb) 2003; 11: 225-36.

[45] LaRosa GJ, Davide JP, Weinhold K, Waterbury JA, Profy AT, Lewis JA, Langlois AJ, Dreesman GR, Boswell RN, Shadduck P, Holley H, Karplus M, Bolognesi DP, Matthews TJ, Emini EA, Putney SD: Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. Science 1990; 249 (4971):932-5.

[46] Rusche JR, Javaherian K, McDanal C, Petro J, Lynn DL, Grimaila R, Langlois A, Gallo RC, Arthur LO, Fischinger PJ, Bolognesi DP, Putney SD and Matthews TJ: Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120.Proc Natl Acad Sci U S A 1988; 85(9):3198-202.

[47] Tugarinov V, Zvi A, Levy R, Hayek Y, Matsushita S, Anglister J: NMR structure of an anti-gp120 antibody complex with a V3 peptide reveals a surface important for co-receptor binding. Structure 2000; 8(4):385-95.

[48] Sirois S, Sing T, Chou KC: HIV-1 gp120 V3 loop for structure-based drug design. Curr Protein Pept Sci 2005; 6(5):413-22.

[49] Holmes EC, Zhang LQ, Simmonds P, Ludlam CA & Leigh Brown AJ: Convergent and divergent sequence evolution in the surface envelope glycoprotein of human immunodeficiency virus type 1 within a single infected patient. Proc Natl Acad Sci USA 1992; 89, 4835–4839

[50] Preston BD, Poiesz BJ & Loeb LA: Fidelity of HIV-1 reverse transcriptase. Science 1988; 242:1169–1171.

[51] Gao F, Chen Y, Levy DN, Conway JA, Kepler TB, Hui H. Unselected mutations in the human immunodeficiency virus type 1 genome are mostly nonsynonymous and often deleterious.J Virol. 2004; 78:2426-33.

[52] Perelson AS, Neumann AU, Markowitz M, Leonard JM & Ho DD. HIV-1 dynamics *in vivo*: virion clearance rate, infected cell life-span, and viral generation time. Science 1996; 271: 1582–1586.

[53] Keele BF, Van Heuverswyn F, Li Y, Bailes E, Takehisa J, Santiago ML, Bibollet-Ruche F, Chen Y, Wain LV, Liegeois F, Loul S, Ngole EM, Bienvenue Y, Delaporte E, Brookfield JF, Sharp PM, Shaw GM, Peeters M, Hahn BH: Chimpanzee reservoirs of pandemic and non pandemic HIV-1. Science 2006; 313:523–6.

[54] Korber B, Muldoon M, Theiler J, Gao F, Gupta R, Lapedes A, Hahn BH, Wolinsky S, Bhattacharya T: Timing the ancestor of the HIV-1 pandemic strains. Science 2000; 288:1789–96.

[55] Plantier JC, Leoz M, Dickerson JE, De Oliveira F, Cordonnier F, Lemée V, Damond F, Robertson DL, Simon F: A new human immunodeficiency virus derived from gorillas. Nat Med. 2009; 15:871-2.

[56] Taylor BS, Sobieszczyk ME, McCutchan FE, Hammer SM: The challenge of HIV-1 subtype diversity. N Engl J Med 2008; 358(15):1590-602.

[57] The Circulating Recombinant Forms (CRFs). http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html (June, 2010).

[58] HIV Sequence Compendium 2005. Leitner T, Foley B, Hahn B, Marx P, McCutchan F, Mellors J, Wolinsky S, and Korber B (editors). Published by Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, NM, LA-UR 06-0680.

[59] Thomson MM, and Najera R: Travel and the introduction of human immunodeficiency virus type 1 non-B subtype genetic forms in to Western Countries. Clin Infec Dis 2001; 32:1732-7.

[60] Bonhoeffer S, Holmes EC, Nowak MA: Causes of HIV diversity. Nature 1995; 376:125.

[61] Nowak R How the parasite disguises itself. Science 1995; 269: 755.

[62] Wolinsky SM, Korber BT, Neumann AU, Daniels M, Kunstman KJ, Whetsell AJ, Furtado MR, Cao Y, Ho DD, Safrit JT: Adaptive evolution of human immunodeficiency virus-type 1 during the natural course of infection. Science 1996; 172:537-42.

[63] Hutchinson JF: The Biology and evolution of HIV. Annu Rev Anthropol 2001; 30:85-108.

[64] Collins KR, Quinones-Mateu ME, Wu M, Luzze H, Johnson JL, Hirsch C, Toossi Z, Arts EJ: Human Immunodeficiency Virus Type 1 (HIV-1) Quasispecies at the sites of *mycobacterium tuberculosis* infection contribute to systemic HIV-1 heterogeneity. J Virol 2002; 76: 1697-1706.

[65] Nowak MA, Anderson RM, McLean AR, Wolfs TF, Goudsmit J, May RM: Antigenic diversity thresholds and the development of AIDS. Science 1991; 15; 254(5034):963-9.

[66] Shankarappa R, Margolick JB, Gange SJ, Rodrigo AG, Upchurch D, Farzadegan H, Gupta P, Rinaldo CR, Learn GH, He X, Haung L, and Mullins JI: Consistent viral evolutionary changes associated with the progression of Human immunodeficiency virus type-1 infection. J Virol 1999; 73:10489-502.

[67] Itescu S, Simonelli PF, Winchester RJ and Ginsberg HS**:** Human immunodeficiency virus type 1 strains in the lungs of infected individuals evolve independently from those in peripheral blood and are highly conserved in the c-terminal region of the envelope V3 loop. Proc Natl Acad Sci USA 1994; 91:11378-11382.

[68] Nakata K, Weiden M, Harkin T Ho D and Rom WN**:** Low copy number and limited variability of proviral DNA in alveolar macrophages from HIV-1-infected patients: evidence for genetic differences in HIV-1 between lung and blood macrophage populations. Mol Med 1995; 1:744-757.

[69] Gupta P, Leroux C, Patterson BK, Kingsley L, Rinaldo C, Ding M, Chen Y, Kulka K, Buchanan W, McKeon B and Montelaro R: Human immunodeficiency virus type 1 shedding pattern in semen correlates with the compartmentalization of viral quasispecies between blood and semen. J Infect Dis 2000; 182:79-87.

[70] Zhu T, Wang N, Carr A, Nam DS, Moor-Jankowski R, Cooper DA and Ho DD: Genetic characterization of human immunodeficiency virus type 1 in blood and genital secretions: Evidence for viral compartmentalization and selection during sexual transmission. J Virol 1996**;** 70:3098-3107.

[71] Wong JK, Ignacio CC, Torriani F, Havlir D, Fitch NJ and Richman DD: In vivo compartmentalization of human immunodeficiency virus: evidence from the examination of *Pol* sequences from autopsy tissues. J Virol 1997; 71:2059-2071.

[72] Korber BT, Kunstman KJ, Patterson BK, Furtado M, McEvilly MM, Levy R and Wolinsky SM: Genetic differences between blood- and brain-derived viral sequences from human immunodeficiency virus type 1-Infected patients: Evidence of conserved elements in the V3 region of the envelope protein of brain-derived sequences. J Virol 1994; 68:7467-7481.

[73] Barrera L: The Basics of Clinical Bacteriology; in Palomino JC, Leão SC, Ritacco V (editors): Tuberculosis 2007 from basic science to patient care. TuberculosisTextbook.com, First Edition, pp 93-112.

[74] Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart Y, Bloom BR: An essential role of Interferon-γ in resistance to *Mycobacterium tuberculosis* infection. J Exp Med 1993; 178:2249-54

[75] Global Tuberculosis Control. A short update to the 2009 report. World Health Organization 2009.

[76] Palmero DJ: Tuberculosis and HIV/AIDS; in Palomino JC, Leão SC, Ritacco V (editors): Tuberculosis 2007 from basic science to patient care. TuberculosisTextbook.com, 2007, First Edition, pp 93-112.

[77] Small PM, Shafer RW, Hopewell PC, Singh SP, Murphy MJ, Desmond E, Sierra MF, Schoolnik GK: Exogenous reinfection with multi drug resistant *Mycobacterium tuberculosis* in patients with advanced HIV infection. N Engl J Med 1993; 328: 1137-44.

[78] van Rie A, Warren R, Richardson M, Victor TC, Gie RP, Enarson DA, Beyers N, van Helden PD: Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. N Engl J Med 1999; 341:1174-9.

[79] Villarino ME, Dooley SW, Geiter LJ, Castro KG and Snider Jr DE: Management of persons exposed to multi drug –resistant tuberculosis. Morb Mortal Wkly Rep 1992; 41 (RR-11)**:**61.

[80] Toossi Z: Virological and immunological impact of tuberculosis on human immunodeficiency virus type-1 disease. J Infect Dis 2003; 188:1146-1155.

[81] Vanham G, Edmonds K, Qing L, Hom D, Toossi Z, Jones B, Daley CL, Huebner R, Kestens L, Gigase P, and Ellner JJ: Generalized immune activation in pulmonary tuberculosis: Co-activation with HIV infection. Clin Exp Immunol 1996; 103:30-34.

[82] Goletti D, Weissman D, Jackson RW, Graham NMH, Vlahov D, Klein RS, Munsiff SS, Ortona L, Cauda R and Fauc AS: Effect of *Mycobacterium tuberculosis* on HIV replication. Role of immune activation. J Immunology 1996; 157:1271-1278.

[83] Nakata K, Rom WN, Honda Y, Condos R, Kanegasaki S, Cao Y, Weiden M: *Mycobacterium tuberculosis* enhances HIV-1 replication in the lung. Am J Respir Crit Care Med 1997; 155:996-1003.

[84] Garrait V, Cadranel J, Esvant H, Herry I, Morinet P, Mayaud C, Israël-Biet D: Tuberculosis induces the in-vivo production of cytokines which support replication. J Immunol 1997; 156:1450-1455.

[85] Lawn SD, Shattock RJ, Acheampong JW, Lal RB, Folks TM, Griffin GE, Butera ST: Sustained plasma TNF-alpha and HIV-1 load despite resolution of other parameters of immune activation during treatment of tuberculosis in Africans. AIDS 1999*;* 13:2231-2237.

[86] stART up 2005, Course Documentation. Published & Copyright by HIV Treatment & Research Unit Medical Clinic II / Department for Internal Medicine, Hospital of the Johann Wolfgang Goethe University Frankfurt am Main.

[87] Hall TA : BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999; 41:95-98

[88] HIV Sequence Database. http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html

[89] Korber B: HIV-1 Signature and sequence variation analysis; in Rodrigo AG and Learn GH (editors): Computational analysis of HIV-1 molecular sequences. Dordrecht, Netherlands: Kluwer Academic Publishers, 2000, Chapter 4, pp 55-72.

[90] Efron B and Tibshirani R: Statistical data analysis in the computer age**.** Science 1991, 253:290-395.

[91] Frahm N, Korber BT, Adams CM, Szinger JJ, Draenert R, Addo MM, Feeney ME, Yusim K, Sango K, Brown NV, SenGupta D, Piechocka-Trocha A, Simonis T, Marincola FM, Wurcel AG, Stone DR, Russell CJ, Adolf P, Cohen D, Roach T, StJohn A, Khatri A, Davis K, Mullins J, Goulder PJ, Walker BD, Brander C : Consistent cytotoxic-T-lymphocyte targeting of immunodominant regions in human immunodeficiency virus across multiple ethnicities. J Virol 2004; 78:2187-2000

[92] Gaschen B, Taylor J, Yusim K, Foley B, Gao F, Lang D, Novitsky V, Haynes B, Hahn BH, Bhattacharya T, Korber B : Diversity considerations in HIV-1 vaccine selection. Science 2002; 296:2354-60.

[93] Reza FM: An Introduction to Information Theory. Dover Publications, Inc. NY, 1994.

[94] Yusim K, Kesmir C, Gaschen B, Addo MM, Altfeld M, Brunak S, Chigaev A, Detour V, Korber BT: Clustering patterns of cytotoxic T-lymphocyte epitopes in human immunodeficiency virus type 1 (HIV-1) proteins reveal imprints of immune evasion on HIV-1 global variation. J Virol 2002; 76:8757-68.

[95] Krogh A, Brown M, Mian IS, Sjolander K, and Haussler D: Hidden Markov models in computational biology: Applications to protein modeling. J Mol Biol 1994; 235:1501–1531.

[96] Eddy SR: Profile hidden Markov models. Bioinformatics 1998; 14:755–763.

[97] Durbin R, Eddy SR, Krogh A, and Mitchison GJ: Biological sequence analysis: probabilistic models of proteins and nucleic acids. Cambridge University Press, Cambridge UK; 1998.

[98] Tamura K, Dudley J, Nei M & Kumar S: MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24:1596-1599

[99] Kimura M: A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980; 16:111-120.

[100] Saitou N and Nei M: The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406-425

[101] Tamura K, Nei M and Kumar S: Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A 2004; 101:11030-11035.

[102] Smouse PE, Long JC, and Sokal RR: Multiple regression and correlation extensions of the Mantel test of matrix correspondence. Syst Zool 1986; 35:627-632.

[103] Waddle DM: Matrix correlation tests support a single origin for modern humans. Nature 1994; 368:452-454.

[104] Poss M, Rodrigo AG, Gosink JJ, Learn GH, de Vange Panteleeff D, Martin HL Jr, Bwayo J, Kreiss JK, Overbaugh J : Evolution of envelope sequences from the genital tract and peripheral blood of women infected with clade A human immunodeficiency virus type 1. J Virol 1998; 72:8240-8251.

[105] Eric Bonnet & Yves Van de Peer : ZT: a software tool for simple and partial Mantel tests. J Stat soft 2000; 7:1-12.

[106] Pond SL, Frost SD: Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics 2005; 21:2531-2533.

[107] Kosakovsky Pond SL and Frost SD: Not so different after all: a comparison of methods for detecting amino acid sites under selection. Mol Biol Evol 2005; 22:1208-1222.

[108] Jensen MA, Li FS, van 't Wout AB, Nickle DC, Shriner D, He HX, McLaughlin S, Shankarappa R, Margolick JB, Mullins JI : Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of HIV-1 *env* V3 loop sequences. J Virol 2003; 77:13376-13388.

[109] Sing T, Low AJ, Beerenwinkel N, Sander O, Cheung PK, Domingues F, Büch J, Däumer M, Kaiser R, Lengauer T, Harrigan PR: Predicting HIV co-receptor usage based on genetic and clinical covariates. Antiviral Therapy 2007; 12(7):1097-106.

[110] Sequin-A DNA Sequence Submission and Update Tool. http://www.ncbi.nlm.nih.gov/Sequin/

[111] Myers G, MacInnes K, and Korber B: The emergence of simian/human immunodeficiency viruses. AIDS Res Hum Retroviruses 1992; 8: 373–386.

[112] Fauci AS, Pantaleo G, Stanley S, Weissman D. Immunopathogenic mechanisms of HIV infection. Ann Intern Med 1996; 124:654–63.

[113] Abrahams MR, Anderson JA, Giorgi EE, Seoighe C, Mlisana K, Ping LH, Athreya GS, Treurnicht FK, Keele BF, Wood N, Salazar-Gonzalez JF, Bhattacharya T, Chu H, Hoffman I, Galvin S, Mapanje C, Kazembe P, Thebus R, Fiscus S, Hide W, Cohen MS, Karim SA, Haynes BF, Shaw GM, Hahn BH, Korber BT, Swanstrom R, Williamson C; CAPRISA Acute Infection Study Team; Center for HIV-AIDS Vaccine Immunology Consortium: Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-poisson distribution of transmitted variants. J Virol 2009; 83:3556–3567

[114] Price DA, Goulder PJ, Klenerman P, Sewell AK, Easterbrook PJ, Troop M, Bangham CR, Phillips RE; Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. Proc Natl Acad Sci U S A. 1997; 4; 94:1890-5.

[115] Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, Nelson JA, Gairin JE, Hahn BH, Oldstone MB, Shaw GM: Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med 1997; 3:205-11.

[116] Huber M, Trkola A: Humoral immunity to HIV-1: neutralization and beyond. J Intern Med 2007;262:5-25.

[117] Brumme ZL, Walker BD: Tracking the culprit: HIV-1 evolution and immune selection revealed by single-genome amplification. J Exp Med 2009; 206:1215-8.

[118] Carding SR, Allan W, McMickle A, Doherty PC: Activation of cytokine genes in T cells during primary and secondary murine influenza pneumonia. J Exp Med 1993; 177:475-82.

[119] Grossman Z, Paul WE: Adaptive cellular interactions in the immune system: the tunable activation threshold and the significance of subthreshold responses. Proc Natl Acad Sci U S A 1992; 89:10365-9.

[120] Unutmaz D, Pileri P, Abrignani S: Antigen-independent activation of naive and memory resting T cells by a cytokine combination. J Exp Med 1994; 180:1159-64.

[121] Ostrowski MA, Krakauer DC, Li Y, Justement SJ, Learn G, Ehler LA, Stanley SK, Nowak M, Fauci AS: Effect of immune activation on dynamics of human immunodeficiency virus replication and on the distribution of viral quasispecies. J Virol 1998; 72:7772-7784.

[122] Collins KR, Mayanja-Kizza H, Sullivan BA, Quinones-Mateu ME, Toossi Z, and Arts EJ : Greater diversity of HIV-1 quasispecies in HIV-infected individuals with active tuberculosis. J Acquir Immune Defic Syndr 2000; 24:408-417.

[123] Kizza HM, Rodriguez B, Quinones-Mateu M, Mirza M, Aung H, Yen-Lieberman B, Starkey C, Horter L, Peters P, Baseke J, Johnson JL, Toossi Z: Persistent replication of human immunodeficiency virus type 1 despite treatment of pulmonary tuberculosis in dually infected subjects. Clin Diagn Lab Immunol 2005; 12: 1298-1304

[124] Kalou M, Sassan-Morokro M, Abouya L, Bile C, Maurice C, Maran M, Tossou O, Roels T, Greenberg AE, Wiktor SZ, and Nkengasong : Changes in HIV RNA viral load, CD4+ T-cell counts and levels of immune activation markers associated with anti-tuberculosis therapy and cotrimoxazole prophylaxis among HIV-infected tuberculosis patients in Abidjan, Cote d Ivoire. J Med Virol 2005; 75:202-208

[125] Morris L, Martin DJ, Bredell H, Nyoka SN, Sacks L, Pendle S, Page-Shipp L, Karp CL, Sterlings TR, Quinn TC, Chaisson RE: Human Human

immunodeficiency virus-1 RNA levels and CD4 lymphocyte counts, during treatment for active tuberculosis, in South African patients. J Infect Dis 2003; 187:1967-71.

[126] Sulkowski MS, Chaisson RE, Karp CL, Moore RD, Margolick JB, and Quinn TC : The effect of acute infectious illnesses on plasma human immunodeficiency virus (HIV) type 1 load and the expression of serologic markers of immune activation among HIV-infected adults. J Infect Dis 1998; 178:1642-1648.

[127] Bush CE, Donovan RM, Markowitz NP, Kvale P and Saravolatz LD: A study of HIV RNA viral load in AIDS patients with bacterial pneumonia. J Acquir Immune Defici Syndr Hum Retrovirol 1996; 13:23-26

[128] Domingo E, Holland JJ: RNA virus mutations and fitness for survival. Annu Rev Microbiol 1997; 51:151-78.

[129] Feeney ME, Tang Y, Roosevelt KA, Leslie AJ, McIntosh K, Karthas N, Walker BD, Goulder PJ: Immune escape precedes breakthrough human immunodeficiency virus type 1 viremia and broadening of the cytotoxic T-lymphocyte response in an HLA-B27-positive long-term-nonprogressing child. J Virol 2004; 78:8927-30.

[130] Geels MJ, Jansen CA, Baan E, De Cuyper IM, van Schijndel GJ, Schuitemaker H, Goudsmit J, Pollakis G, Miedema F, Paxton WA, van Baarle D : CTL escape and increased viremia irrespective of HIV-specific CD4+ T-helper responses in two HIV-infected individuals. Virology 2006; 345:209-19.

[131] Nowak MA, May RM, Phillips RE, Rowland-Jones S, Lalloo DG, McAdam S, Klenerman, P, Koppe B, Sigmund K, Bangham CR, and McMichael AJ: Antigenic oscillations and shifting immunodominance in HIV-1 infections. Nature 1995; 375:606–611.

[132] Flynn JL: Immunology of tuberculosis and implications in vaccine development. Tuberculosis (Edinb) 2004; 84:93-101.

[133] Wallis RS, Vjecha M, Amir-Tahhmasseb M, Okwara A, Byekwaso F, Nyole S, Kabengera S, Mugerwa RD, and Ellner JJ: Influence of tuberculosis on human immunodeficiency virus (HIV-1): enhanced cytokine expression and elevated β2-microglobulin in HIV-1-associated tuberculosis. J Infect Dis 1993; 167:43-48.

[134] Hoshino Y, Tse DB, Rochford G, Prabhakar S, Hoshino S, Chitkara N, Kuwabara K, Ching E, Raju B, Gold JA, Borkowsky W, Rom WN, Pine R, Weiden M: Mycobacterium tuberculosis-induced CXCR4 and chemokine expression leads to preferential X4 HIV-1 replication in human macrophages. J Immunol 2004; 172:6251-6258.

[135] Wolday D, Tegbru B, Kassu A, Messele T, Coutinho R, Van-Barle D, Miedema F: Expression of chemokine receptors CCR5 and CXCR4 on CD4+ T-cells and plasma chemokine levels during treatment of active tuberculosis in HIV-1 co-infected patients. J Acquir Immune Defic Syndr 2005; 39(3):265-71.

[136] Li WH, Tanimura M, Sharp PM: Rates and dates of divergence between AIDS virus nucleotide sequences. Mol Biol Evol 1988; 5:313-330.

[137] Korber B, Theiler J, Wolinsky S: Limitations of a molecular clock applied to considerations of the origin of HIV-1. Science 1998; 280:1868-1871.

[138] Leitner T, Albert J: The molecular clock of HIV-1 unveiled through analysis of a known transmission history. Proc Natl Acad Sci 1999; 96:10752-10757.

[139] Salemi M, Strimmer K, Hall WW, Duffy M, Delaporte E, Mboup S, Peeters M, Vandamme AM: Dating the common ancestor of SIVcpz and HIV-1 group M and the origin of HIV-1 subtypes using a new method to uncover clock-like molecular evolution. FASEB J 2001; 15:276-278.

[140] Yusim K, Peeters M, Pybus OG, Bhattacharya T, Delaporte E, Mulanga C, Muldoon M, Theiler J, Korber B: Using human immunodeficiency virus type 1 sequences to infer historical features of the acquired immune deficiency syndrome epidemic and human immunodeficiency virus evolution. Philos Trans R Soc Lond B Biol Sci 2001; 356:855-866.

[141] Moriuchi M, Moriuchi H, Turner W, Fauci AS: Exposure to bacterial products renders macrophages highly susceptible to T-Tropic HIV-1. J Clin Invest 1998; 102:1540–1550.

[142] Orenstein JM, Fox C, Wahl SM: Macrophages as a source of HIV during opportunistic infections. Science 276:1857–1861.

[143] Kinsey NE, Anderson MG, Unangst TJ, Joag SV, Narayan O, Zink MC, Clements JE: Antigenic variation of SIV: mutations in V4 alter the neutralization profile. Virology 1996; 22:114-21.

[144] Teeraputon S, Louisirirojchanakul S, Auewarakul P: N-linked glycosylation in C2 region of HIV-1 envelope reduces sensitivity to neutralizing antibodies. Viral Immunol 2005; 18:343-353.

[145] Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM: Antibody neutralization and escape by HIV-1. Nature 2003; 422:307-312.

[146] Moore PL, Gray ES, Choge IA, Ranchobe N, Mlisana K, Abdool Karim SS, Williamson C, Morris L; CAPRISA 002 Study Team: The C3-V4 region is a major target of autologous neutralizing antibodies in human immunodeficiency virus type 1 subtype C infection. J Virol 2008; 82:1860-9.

[147] Pollakis G, Kang S, Kliphuis A, Chalaby MI, Goudsmit J, Paxton WA: N-linked glycosylation of the HIV type-1 gp120 envelope glycoprotein as a major determinant of CCR5 and CXCR4 coreceptor utilization. J Biol Chem 2001; 276(16):13433-41.

[148] Polzer S, Dittmar MT, Schmitz H, Meyer B, Müller H, Kräusslich HG, Schreiber M: Loss of N-linked glycans in the V3-loop region of gp120 is correlated to an enhanced infectivity of HIV-1. Glycobiology 2001; 11: 11–19.

[149] Polzer S, Dittmar MT, Schmitz H, Schreiber M: The N-linked glycan g15 within the V3 loop of the HIV-1 external glycoprotein gp120 affects coreceptor usage, cellular tropism, and neutralization. Virology 2002; 304; 70–80.

[150] Lucas S and Nelson AM: Pathogenesis of tuberculosis in human immunodeficiency virus-infected people. In: Tuberculosis: pathogenesis, protection, and control. Bloom BR (editor). ASM Press: Washington, DC, 1994. pp. 503-513.

[151] Badri M, Ehrlich R, Wood R, Pulerwitz T and Maartens G: Association between tuberculosis and HIV-1 disease progression in a high tuberculosis prevalence area. Int J Tuberc Lung Dis 2001; 5:225-232.

[152] Salazar-Gonzalez JF, Bailes E, Pham KT, Salazar MG, Guffey MB, Keele BF, Derdeyn CA, Farmer P, Hunter E, Allen S, Manigart O, Mulenga J, Anderson JA, Swanstrom R, Haynes BF, Athreya GS, Korber BT, Sharp PM, Shaw GM, Hahn BH : Deciphering human immunodeficiency virus type 1 transmission and early envelop diversification by single-genome amplification and sequencing. J Virol 2008; 82:3952-3970.

Appendix

Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral therapy
ARVs	AIDS-associated retroviruses
bp	base pair
BLAST	Basic local alignment searching tool
Cat No	Catalogue number
CCR5	CC Chemokine Receptor 5
CDC	Center for disease control
cDNA	complementary DNA
CRF	Circulating Recombinant Form
CXCR4	CXC chemokine Receptor 4
dn	the relative proportion of non-synonymous substitution
DNA	Deoxyribonucleic acid
DDBJ	DNA Data Bank of Japan
ds	the relative proportion of synonymous substitution
Env	envelop
EPTB	Extra pulmonary tuberculosis
E.coli	*Escherichia Coli*
EMBL	European Molecular Biology Laboratory
EU	European Union
FEL	fixed effects likelihood
Gag	Group specific antigen
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HBCs	high-burden countries
HIV	Human Immunodeficiency virus
HIVCENTER	HIV Treatment and Research Unit

HMMs	Hidden Markov models
HXB2	Name of HIV-1 M subtype B reference strain
HTLV-III	human T-cell leukemia virus III
ICTV	International Committee on Taxonomy of Viruses
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
LAV	Lymphadenopathy associated virus
LB	Luria-Bertani
MIP-1	Macrophage Inflammatory Proteins
MEGA	Molecular evolutionary genetics analysis
MTB	*Mycobacterium tuberculosis*
Nef	Negative factor
NFκB	Nuclear factor Kappa B
NSI	Non-syncytium Inducing
NJ	Neighbour joining
NCBI	National Center for Biotechnology Information
NTB	HIV-1/non TB patient groups
PBMC	Peripheral Blood Mononuclear cells
PCR	Polymerase chain reaction
Pol	polymerase
POP	performance optimized polymer
PND	principal neutralizing domains
PNGs	Potential N-linked glycosylation sites
PTB	Pulmonary tuberculosis
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
REL	random effects likelihood
Ref. no.	Reference number

Rev	Regulator of virion protein
R/H	Rapid/High
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
SI	Syncytium Inducing
S/L	Slow/low
SIV	Simian Immunodeficiency Virus
SLAC	Single Likelihood Ancestor Counting
SDF-α	stromal-derived factor-&alpha
Tat	transactivator of transcription
TB	Tuberculosis
TB -	HIV-1/TB (for this particular study)
TP	Time point (for this particular study)
TNFα	Tumor necrosis factor α
URFs	unique recombinant forms
Vif	Virion infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

Acknowledgement

First of all, I would like to thank the Almighty God, for His endless blesses and for making me motivated and courageous to finish the job.

Next my heartfelt thanks go to the following people:

1) Prof. Dr. Hans Wilhelm Doerr for allowing me to work in his institute under his supervision; his encouragement, advice and help in all the way through and his support to solve any problem which I was confronted.

2) Prof. Dr. Schlomo Staszewski, my former boss and supervisor, although he retired prematurely because of health problem, I am always thankful to him for giving me this chance to register as his PhD student, and to work in close collaboration with HIVCENTER. For enabling me make possible contacts with Institute of Medical Virology and his institution, HIVCENTER.

3) Dr. Zehava Grossman (National HIV Reference Laboratory, Central Virology, Sheba Medical Center, Tel Hashomer, Israel) for helping me to establish contact with Prof. Dr. Schlomo Staszewski.

4) Prof. Dr. Lutz G. Gürtler for all his expert advice, very thorough discussions on my thesis and for his day to day help for my intellectual growth; for sponsoring me to present my work in an international presentation; for his encouragement, support and kindness.

5) Prof. Dr. Jindrich Cinatl, for his advices, reading the pre manuscript, and giving me helpful comments; and for allowing me to work in the experimental virology laboratory.

6) Prof. Dr. Peter Kraiczy for allowing me to work in his gene technic laboratory; for his nice behaviour and welcoming atmosphere and for all the technicians who are working in his laboratory.

7) Dr. Tessa Lennemann, without her support, this work or thesis won’t be possible; besides, the work for being a very nice friend.

8) PD Dr. Martin Stürmer, for all his help from the start, for his advice and for facilitating to work in his HIV laboratory and also to his technician Ms. Margarethe Podporski for her excellent technical help.

9) To all HIVCENTER medical doctors and staffs esp. to Dr. Christoph Stephan Dr. Markus Bickel, Ms. Claudia Wengenroth, Mr. Guido Heidenreich and all the rest.

10) To Dr. Jonas Schmidt-Chanasi for helping me to establish the cloning procedure and for his nice behaviour.

11) To all friends and colleagues who are/were working under the institute of medical virology, only because of space limitation otherwise I would have listed their names.

12) To my friends, esp. Nazreth Tegafaw and her families and friends for being a great friend just like a sister; Kinfe Gebreyes for his encouragement since the beginning; Beide-Mariam Ejigu, for reading the pre-manuscript and for all his valuable comments.

13) To Ethiopian Orthodox Medihane-Alem Frankfurt church choir, for being very pleasant friends just like families.

14) To all my families in Addis, esp. for my caring parents, my sweet young brother, Surafel Biru, and to my elder brother Endalikachew Biru.

15) Last but not least, to the beautiful and peaceful country, Germany, and her nation, for being a nice host and home for me for the past five years.

Lebenslauf

Persönliche Daten:

Name	Tsigereda Biru Leulebirhan
Adresse	Rheinland str. 14, 60529 Frankfurt
Telefon	+4915208790127 (Handy)
Email	tsigereda76@yahoo.com
Nationalität	Äthiopisch
Geburtsdatum	23-07-1976

Ausbildung:

Dezember, 2006- Promotionsstudium Anfertigung einer Dissertationshrift zur„ characterization of The Longitudinal HIV-1 Quasispecies Evolution in HIV-1 Infected Individuals Co-infected with *Mycobacterium tuberculosis"* mit dem Ziel der Erlangung des akademischen Grades "Dr.rer.med"

April 2005 - Juli 2006: Master (M.Sc)- äquivalenter Postgraduierten-Kurs: Cologne University Bioinformatics Center (CUBIC)Thesis (Abschlußarbeit): Automated prediction of Potential drug targets and their implications on Metabolic network of *P.falciparum*" (Automatische Vorhersage von potentiellen Medikamenten-Zielen und deren Implementierung in metabolischen Netzwerken von *P. falciparum*)"

Betreuer: Prof. Dr. D.Schomburg; Dr. Syed Asad Rahman

Feb 2003 – März 2005: Master(M.Sc) in Addis Ababa Universität, Wissenschaftliche Fakultät, Biologie Departement angewandter Microbiologie Thesis: (Abschlußarbeit): "Virological and Immunological Features of Human Immunodeficiency Virus Type-1 (Hiv-1) Pre and Post Treatment of Tuberculosis in Co-infected Patients" (Virologische und immunologische Funktionen des menschlichen Immundefizienz-Virus Typ-1 Vor- und Nachbehandlung von Tuberkulose in co-infizierten Patienten) **Betreuer:** Prof. Dr. Beyene Petros

Sep 1994 – Juli 1998: Bacchelor(B.Sc) Addis Ababa Universität, Wissenschaftliche Fakultät, in Biologie Thesis (Abschlußarbeit): "AIDS awareness among Junior High school students in Addis Ababa" (AIDS Bewußtsein unter Mittelstufe-Schülern in Addis Ababa) **Betreuer:** Prof. Dr.

Sep 1982 - Mai 1994: vorschulische und schulische Ausbildung Addis Ababa, Ãthiopien.

ARBEITS-ERFAHRUNGEN

Jan 2003 - März 2005 Mitarbeiter im Forschungsbereich der Infektionskrankheiten-Abteilung des äthiopischen Forschungsinstitut für Gesundheit und Ernährung ("Ethiopian Health and Nutrition Research Institute, EHNRI")

Jan 2000 – Jan 2003 "Junior" Forscher im Forschungsbereich der Infektionskrankheiten- Abteilung des äthiopischen Forschungsinstitut für Gesundheit und Ernährung("Ethiopian Health and Nutrition Research Institute, EHNRI")

Juli 1998 – Feb 1999 Freiwilliger Mitarbeiter im äthiopisch-niederländischen AIDS Forschungsprojekt (Ethio-Netherlands AIDS Research Project, ENARP) unter dem äthiopischen Forschungsinstitut für Gesundheit und Ernährung ("Ethiopian Health and Nutrition Research Institute, EHNRI").

VERÖFFENTLICHUNGEN

- Aklilu,M. ,Messele,T. ,Tsegaye,A.,**Biru,T**.,Mariam,D.H.,Benthem,B.V.,Coutino,R., Rinke de Wit,T., Fontanet, A.(2000) “Factors Associated with HIV-1 Infection among Sex workers of Addis Ababa,Ethiopia. *AIDS*15:87-96”
- Messele,T., Rinke de Wit,T., Brouwer,M., Aklilu,M., **Birru,T.,** Fontanet,A., Maker,H.S., Hamann,D.(2001) “No Difference in Vitro Susceptibility to HIV Type-1 Between High Risk HIV Negative Ethiopian Commercial Sex Workers and Low Risk Control Subjects. *AIDS Research and Human Retroviruses* Vol.17,No.5 pp.433-441”
- Tilahun Woldemichael, Asfaw Debella, Dawit Abebe, Geremew Tassew, **Tsigereda Biru,** Yared Makonnen, Frehiwot Teka, Daniel Melaku. (2006). Screening of Some Medicinal Plants of Ethiopia for Their Molluscicidal Activities and Phytochemical Constituents. *Pharmacologyonlin*e 3: 245-258.

Frankfurt a.M., den 01 Juli 2010

Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

"Characterization of The Longitudinal HIV-1 Quasispecies Evolution in HIV-1 Infected Individuals Co-infected with *Mycobacterium tuberculosis*"

in dem Fachbereich Medizin, Der Johann Wolfgang Goethe-Universität, Zentrum der Hygiene Institut für Medizinische Virologie Frankfurt am Main

unter Betreuung und Anleitung von Prof. Dr.med.H.W.Doerr mit Unterstützung durch PD Dr. Martin Stürmer, ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Frankfurt a.M., den 01 Juli 2010