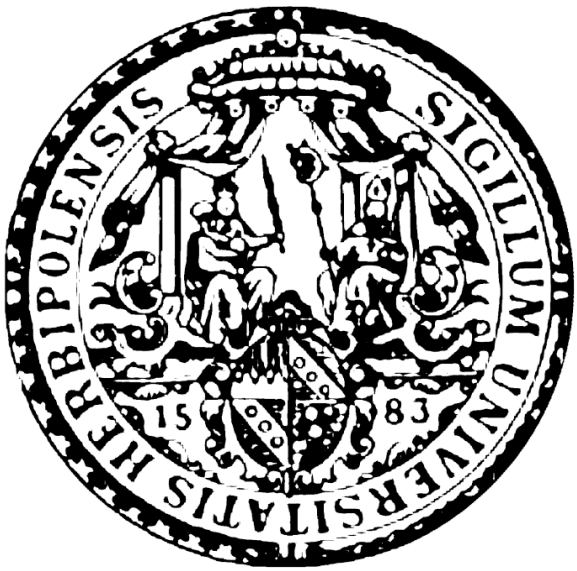
HIV-1 resistance analyses from therapy-naïve patients in South Africa and Tanzania and the characterization of a new HIV-1 subtype C proviral molecular clone



HIV-1 Resistenz-Analysen von nicht-therapierten Patienten aus Südafrika und Tansania und Charakterisierung eines neuen HIV-1 Subtyp C proviralen molekularen Klons

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences, Julius-Maximilians-Universität Würzburg, Section Infection and Immunity.

Submitted by

Graeme Brendon Jacobs

From

Cape Town, South Africa

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Submitted on: _____

Members of the *Promotionskomitee*:

Chairperson: Professor Dr. Thomas Hünig

Primary supervisor: Professor Dr. Axel Rethwilm

Primary supervisor: Dr. Jochen Bodem

Supervisor: Professor Dr. Ulrich Dobrindt

Supervisor: Professor Dr. Wolfgang Preiser

Date of Public Defence: _____

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Würzburg, 2011

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Graeme Brendon Jacobs

Summary

The acquired immunodeficiency syndrome (AIDS) is currently the most infectious disease worldwide. It is caused by the human immunodeficiency virus (HIV). At the moment there are ~33.3 million people infected with HIV. Sub-Saharan Africa, with ~22.5 million people infected accounts for 68% of the global burden. In most African countries antiretroviral therapy (ART) is administered in limited-resource settings with standardised first- and second-line ART regimens. During this study I analysed the therapy-naïve population of Cape Town, South Africa and Mwanza, Tanzania for any resistance associated mutations (RAMs) against protease inhibitors, nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors. My results indicate that HIV-1 subtype C accounts for ~95% of all circulating strains in Cape Town, South Africa. I could show that ~3.6% of the patient derived viruses had RAMs, despite patients being therapy-naïve. In Mwanza, Tanzania the HIV drug resistance (HIVDR) prevalence in the therapy-naïve population was 14.8% and significantly higher in the older population, >25 years. Therefore, the current WHO transmitted HIVDR (tHIVDR) survey that is solely focused on the transmission of HIVDR and that excludes patients over 25 years of age may result in substantial underestimation of the prevalence of HIVDR in the therapy-naïve population. Based on the prevalence rates of tHIVDR in the study populations it is recommended that all HIV-1 positive individuals undergo a genotyping resistance test before starting ART. I also characterized *vif* sequences from HIV-1 infected patients from Cape Town, South Africa as the Vif protein has been shown to counteract the antiretroviral activity of the cellular APOBEC3G/F cytidine deaminases. There is no selective pressure on the HIV-1 Vif protein from current ART regimens and *vif* sequences was used as an evolutionary control. As the majority of phenotypic resistance assays are still based on HIV-1 subtype B, I wanted to design an infectious HIV-1 subtype C proviral molecular clone that can be used for *in vitro* assays based on circulating strains in South Africa. Therefore, I characterized an early primary HIV-1 subtype C isolate from Cape Town, South Africa and created a new infectious subtype C proviral molecular clone (pZAC). The new pZAC virus has a significantly higher transient viral titer after transfection and replication rate than the previously published HIV-1 subtype C virus from Botswana. The optimized proviral molecular clone, pZAC could be used in future cell culture and phenotypic HIV resistance assays regarding HIV-1 subtype C.

Zusammenfassung

Das erworbene Immundefektsyndrom ("acquired immunodeficiency syndrome", AIDS), verursacht durch das Humane Immundefizienzvirus (HIV), ist derzeit die häufigste Infektionskrankheit weltweit. Zirka 33,3 Millionen Menschen sind gegenwärtig mit HIV infiziert, wobei hiervon etwa 22,5 Millionen Infizierte (68%) in den Ländern südlich der Sahara leben. In den meisten dieser Länder ist die antiretrovirale Therapie (ART) in nur zwei standardisierten Medikamentenkombinationen verfügbar. In dieser Arbeit wurden nicht-therapierte Patienten aus Kapstadt (Südafrika) und Mwanza (Tansania) auf resistenzassoziierte Mutationen (RAMs) gegen Protease Inhibitoren, nukleosidische- und nichtnukleosidische Reverse Transkriptase Inhibitoren analysiert. Meine Ergebnisse zeigten, dass in 3,6 % der Patienten RAMs gefunden wurden, obwohl diese nicht vortherapiert waren. In der Patientengruppe aus Tansania wurden sogar in 14,8 % der Patientenviren RAMs gefunden. Dieses Patientenkollektiv war signifikant älter als 25 Jahre und damit außerhalb der von der WHO beobachteten Altersgruppe. Meine Studie legt nahe, dass die WHO-Kriterien zur Überwachung der Übertragung von resistenten HIVs die Weitergabe von resistenten Viren unterschätzt, da Patienten über 25 Jahre ausgeschlossen werden. Weiterhin wurden *vif* Sequenzen von HIV-1 infizierten Patienten aus Kapstadt charakterisiert, da bereits gezeigt wurde, dass das HIV Vif Protein die antiretrovirale Aktivität der Cytidin Deaminase APOBEC3G/F antagonisieren kann. Da jedoch keine Medikamenten induzierte Selektion auf diesen Sequenzen liegt, wurden diese zur Analyse der viralen Evolution verwendet. Phenotypische Resistenzanalysen basieren gegenwärtig meist auf dem HIV Subtyp B, jedoch sind die meisten Infizierten in Südafrika und sogar weltweit mit Subtyp C infiziert. Deshalb war es ein Ziel dieser Arbeit einen proviralen HIV Subtyp C Plasmid zu entwickeln. Dazu wurde das Virus aus einem frühen HIV Subtyp C Isolat kloniert. Das hier neu klonierte Virus (HIV-ZAC) zeigt sowohl einen höheren viralen Titer nach der Transfektion und auch eine höhere Replikationsrate als das zuvor publizierte HIV-1 Suptyp C Virus aus Botswana. Deshalb könnte der von mir optimierte und neu charakterisierte provirale molekulare Klon, pZAC, zukünftig in der Zellkultur und bei phenotypischen HIV Resistenztests als wildtypisches HIV-1 Suptyp C Virus eingesetzt werden.

Statement on individual author contributions and on legal second publication rights.

Publication: **Jacobs, G. B**., Laten, A., van Rensburg, E. J., Bodem, J., Weissbrich, B., Rethwilm, A., Preiser, W. & Engelbrecht S. (2008). Phylogenetic diversity and low level antiretroviral resistance mutations in HIV type 1 treatment-naive patients from Cape Town, South Africa. AIDS Res Hum Retroviruses 24, 1009-1012.					
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Study Design	CS	BW	AR	CK	SK
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Chapter one

1. Introduction and literature review

1.1 Introduction

Today acquired immunodeficiency syndrome (AIDS) is one of the most important infectious disease being the most common cause of death in Africa, above malaria and tuberculosis. AIDS is caused by the retrovirus Human Immunodeficiency Virus (HIV). The UNAIDS estimates that there are currently 33.3 million people infected with HIV/AIDS worldwide. Sub-Saharan Africa remains the heaviest affected region with approximately 22.5 million people infected, which accounts for 68% of the global burden. However, globally since 1999 the number of new infections has fallen by approximately 19%, with antiretroviral therapy (ART) currently being provided to more than 5.0 million people (UNAIDS, 2011).

The genetic subtype distribution of HIV-1 group Major (M), currently responsible for the majority of the AIDS pandemic has become dynamic and unpredictable. Currently HIV-1 group M has been divided into 9 subtypes (A-D, F, G-H, J, K), 49 circulating recombinant forms (CRFs) and numerous unique recombinant forms (URFs). In 2004-2007, subtype C accounted for nearly half (48%) of all global infections, followed by subtypes A (12%), B (11%) and CRF02_AG (8%) (Hemelaar *et al*., 2011).

1.2 History of HIV infection

AIDS was first recognized in 1981 amongst homosexual men in the United States of America (USA) who presented with *Pneumocytis carinii* pneumonia, a rare disease causing lung infections in humans with weakened immune systems (Gottlieb *et al*., 1981a, b). A few of these men developed Kaposi's sarcoma, a previously rarely seen skin cancer caused by Human Herpesvirus 8 (HHV-8) (Friedman-Kien *et al*., 1981; Hymes *et al*., 1981). It was initially thought that the disease was a form of punishment for people participating in high risk behaviour (Shilts, 1987). However, the symptoms and disease was soon recognized in other population groups as well. These included female sexual partners of men (Masur *et al*., 1982), Haitians (Pape *et al*., 1983), infants (Oleske *et al.*, 1983), haemophiliacs (Bloom, 1984) and blood transfusion recipients (Curran *et al.,* 1984). In Africa the first reported outbreak occurred in the heterosexual population of the Democratic Republic of Congo (DRC), previously Zaire (Piot *et al*., 1984). AIDS was initially described as the appearance of

certain rare, dramatic and life-threatening opportunistic infections and associated cancers, which led to a severe depletion of the immune system response (Ammamm *et al*., 1983). The first evidence that AIDS was caused by a retrovirus was discovered in 1983. Barré-Sinoussi and colleagues isolated a retrovirus from a homosexual man who had lymphadenopathy syndrome (LAS), a disease of the lymph nodes. The virus was initially called lymphadenopathy virus (LAV) (Barré-Sinoussi *et al.,* 1983). It was also independently isolated in 1984 by Levy and co-workers who called it AIDS-associated retrovirus (ARV) (Levy *et al.,* 1984). Later on it was confirmed that LAV and ARV were the same virus and responsible for causing AIDS (Ratner *et al*., 1985a, b). To avoid further confusion the International Committee on the Taxonomy of viruses decided to rename the AIDS inducing virus HIV, as it is known today (Coffin *et al*., 1986a, b).

1.3 Origin of HIV

HIV forms part of the *Retroviridae* virus family, genera *Lentivirus* (Sonigo *et al*., 1985). The earliest documented report of a human infection comes from a seropositive patient in Kinshasa, DRC from 1959 (Zhu *et al*., 1998). Molecular clock and phylogenetic analyses estimate that HIV was introduced into the human population during the 1930s with a ± 20 year confidence gap (Hahn *et al*., 2000; Korber *et al*., 2000). HIV is closely related to simian immunodeficiency viruses (SIVs) found in non-human primates and through zoonosis the virus adapted to its human host. SIVs do not usually cause the same dramatic AIDS-defining disease in our non-human primate counterparts (Hahn *et al*., 2000; Silvestri *et al*., 2003). There are more recent reports that indicate that wild chimpanzees can acquire AIDS like diseases from SIVs (Keele *et al*., 2009). Both HIV-1 and HIV-2 are thought to have originated in West-Central Africa (Apetrei *et al*., 2004; Nahmias *et al*., 1986). HIV-1 was transmitted from the common chimpanzee, *Pan troglodytes troglodytes* (Gao *et al*., 1999; Keele *et al*., 2006), while HIV-2 was most likely transmitted from the sooty mangabey, *Cerocebus atys* (Gao *et al*., 1992; Hirsch *et al*., 1989., Keele *et al*., 2006). Transmission events of SIV strains into the human host still frequently occur and it remains unclear why HIV in its current form has become so predominant (Kalish *et al*., 2005, Weiss and Wrangham, 1999).

1.4 HIV diversity

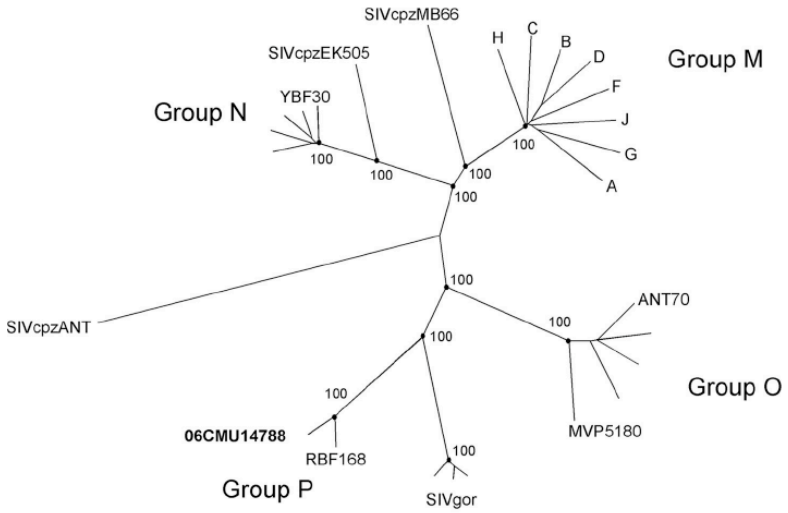


Figure 1.1: HIV-1 Phylogenetic tree derived from nucleotide alignment of genome sequences. The different HIV-1 groups are indicated, rooted with SIVcpzANT. The group M subtypes (A-D, F-H and J) are shown, while reference sequences for groups N, O and P are also marked (Vallari *et al*., 2011).

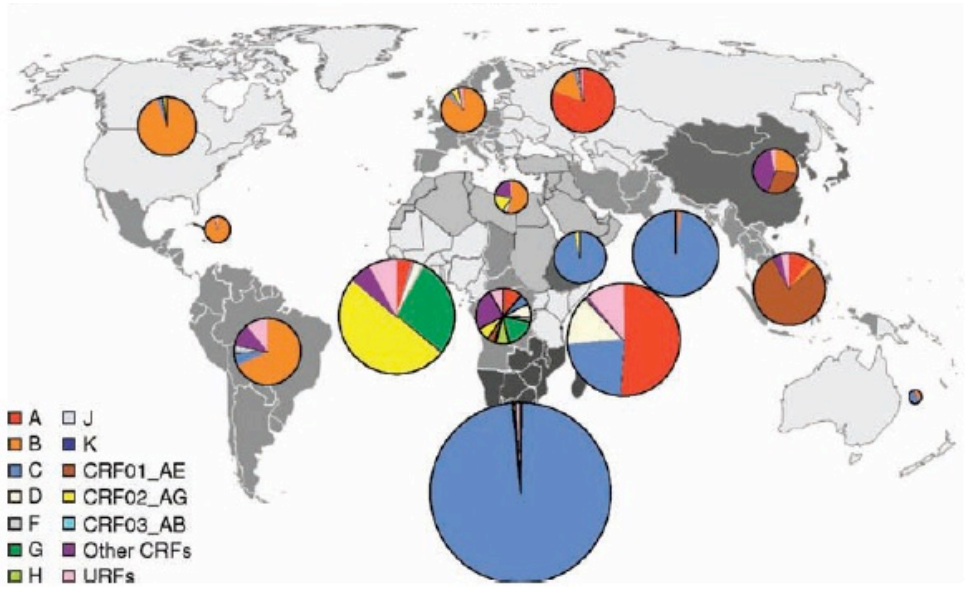


Figure1.2: Current spread of HIV-1 diversity. HIV-1 subtype C accounts for 48% of all currently circulating strains. It is predominantly found in Sub-Saharan Africa and India. HIV-1 subtype B is more predominant in North America and in Europe (Hemelaar *et al*., 2011).

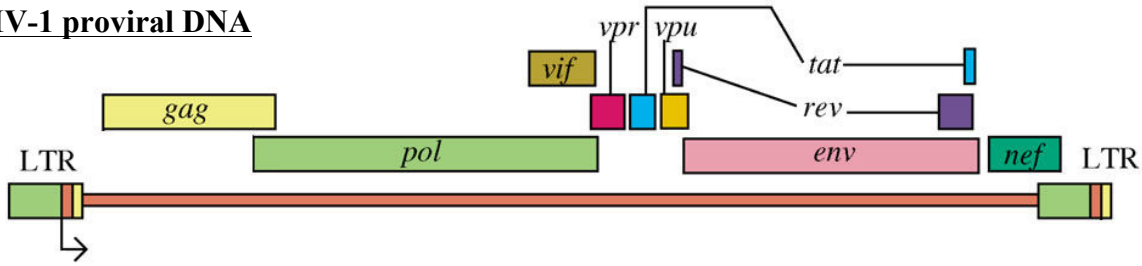
HIV has a high genetic diversity. This is caused by the fast replication cycle of the virus coupled with the high error prone rate of its RT enzyme. RT also further increases HIV diversity by allowing for strains to recombine with each other. There are currently 2 types of HIV that have been identified: HIV-1 and HIV-2. HIV-1 has been divided into four distinct groups M, non-M, non-O (N), outlier (O) and P with group M responsible for the worldwide pandemic we are facing today. HIV-1 groups N, O and P are rare and the degree of their

diversity has not yet been completely differentiated through phylogenetic analysis. However, group N seems to be phylogenetically equidistant from groups M and O (Spira *et al.,* 2003). Group M is currently divided into nine different subtypes (A-D, F-H, J and K) and 49 CRFs. Genetic variation within a subtype is usually 8 to 17%, whereas the variations between different subtypes are 17 to 35% (Korber *et al*., 2001). The highest variation within the genome is seen within the *env* gene, wherease the *pol* gene, encoding for important viral enzymes are the most conserved (Gaschen *et al*., 2002).

HIV-1 group M subtype C (from here on HIV-1 subtype C) is responsible for the majority (currently 48%) of all HIV-1 infections worldwide. HIV-1 subtype B is the most widespread and is especially prevalent in North America, Europe and Australasia. The majority of HIV-1 subtypes can be found in West and Central Africa, where it is believed HIV originated through zoonosis. HIV-2 is less pathogenic than HIV-1 and has mainly been restricted to West Africa. HIV-2 has also been divided into eight subtypes (A-H) based on phylogenetic analysis (Damond *et al.*, 2004).

1.5 The HIV genome, virus structure and viral life cycle

HIV-1 proviral DNA



HIV-1 RNA species

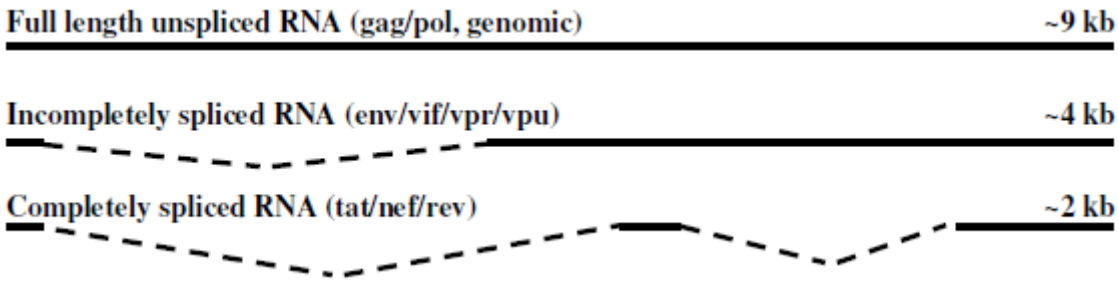


Figure 1.3A: The HIV-1 proviral DNA genome and 1.3B: HIV-1 RNA species. The virus is flanked by the Long terminal repeat (LTR) regions. The *group antigen (gag)* and *envelope (env)* genes are responsible for the virus structure, while *polymerase (pol)* encodes for important viral enzymes . HIV-1 *transcriptional transactivator (tat)* and *regulator of viral expression (rev)* are important for viral transcription and regulation. The accessory genes *virion infectivity factor (vif*), *viral protein R (vpr*), *viral protein U (vpu)* and *negative regulatory factor* (*nef)* play important parts during infectivity and maturation (Nielsen *et al*., 2005).

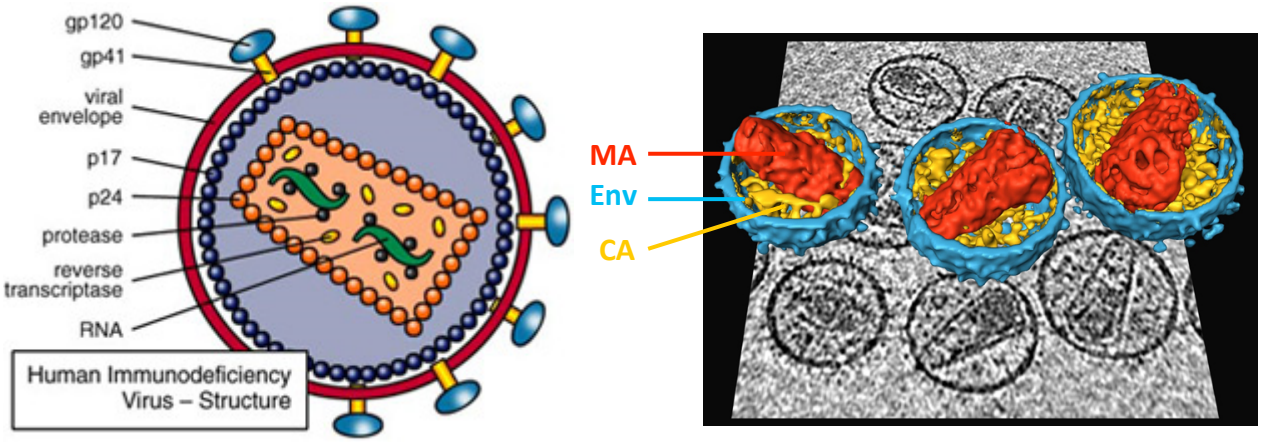


Figure 1.4A: A schematic illustration of the HIV virion and 1.4B: A 3D reconstruction of HIV-1 virions. The viral Envelope (Env) consists of the surface glycoproteins (gp120) anchored with the transmembrane proteins (gp41). The inner Gag Matrix (MA; p17) and Capsid (CA; p24) protein layers are also indicated. The HIV genome consists of two copies of unspliced positive single-stranded molecules indicated in the center. The Pol proteins, Protease (PR) and Reverse Transcriptase (RT) indicated in the diagram are also packaged in the mature virus particle. Source: http://www.asparis.net and http://www.embl.de/research/units/scb/briggs/briggs_1l.jpg.

There are many reviews on the HIV genome, virus structure and viral life cycle. Examples in the literature are: Briggs *et al*., 2003; D'Souza and Summers, 2005; Freed, 2001; Klimas *et al*., 2008; Nisole and Saïb, 2004; Pomerantz and Horn, 2003 and Turner and Summers, 1999. The HIV genome (Figure 1.3), virus structure (Figure 1.4) and life cycle (Figure 1.5) are briefly described here.

1.5.1 HIV structure and genome organization

HIV is an enveloped virus and roughly spherically shaped with a diameter of approximately 120 nm. Its genome consist of two unspliced positive-oriented single-stranded ribonucleic acid (RNA) molecules and encodes for nine genes (*gag*, *pol*, *env*, *vif*, *vpr*, *vpu*, *tat*, *rev* and *nef*) as described below.

1.5.1.1 The HIV LTR

The full-length provirus is approximately 9.2 kb long and is flanked by two LTRs. Although the LTRs do not directly encode for any gene products, except for the partial Nef coding region, they do encode for important structural RNA elements and contain binding sites for important transcription factors. Thus the LTRs are important for the regulation of viral gene expression (Briggs *et al*., 2003). Each LTR consist of a unique 3` region (U3), the terminal

redundancy region (R) and unique 5` region (U5). The U3 promoter enhancer site contains a modulatory negative regulatory element (NRE), an HIV TATA box as well as sequence binding sites for cellular transcription factors such as Nuclear factor (NF)-κβ, Specific Protein 1 (Sp1) and Transcription Factor IID (TFIID) (D'Soza and Summers, 2005; Kashanchi *et al*., 1996). R is the exact region where viral transcription is initiated by the human tRNA and contains the transactivation response (TAR) element sequence. Viral transcript starts at the beginning of R, is capped and proceeds through the viral genome. The R/U5 border in the 3'LTR defines the region where polyadenylation takes place and the polyadenylation signal (AAUAAA) is found within this region. The viral packaging signal, primer binding site (PBS) responsible for RNA initiation and major splice donor (SD) signal involved in the regulation of transcription are also found downstream of U5. Although the LTRs are identical in sequence the 5'LTR acts as the initiation point for transcription and capping of messenger RNA (mRNA) transcripts, whereas sequences in the 3'LTR are responsible for transcription termination and polyadenylation. The LTRs are also responsible for mediating retroviral integration into its host cell genome. The full-length HIV mRNA transcript encodes for nine genes. Their protein products are derived from the primary transcript by means of alternative splicing, ribosomal frameshifting and leaky scanning of initiation codons (Klimas *et al*., 2008; Turner and Summers, 1999).

1.5.1.2 The virus structure and structural genes

HIV's structural genes are encoded by *gag (group antigen)* and *env (envelope)*, as is common in all lentiviral genomes. Recently the secondary structure of HIV-1 RNA genome has also been solved by selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) analysis (Watts *et al*., 2009). The RNA is bound to the nucleocapsid NC (p7) protein and surrounded by enzymes important for viral maturation such as protease (PR), reverse transcriptase (RT) and integrase (IN), encoded by the viral *pol* gene as discussed below. Accessory proteins (Nef, Vif, Vpr) can also be found in the viral ribonucleoprotein core structure. An abundant number of host molecules have also been found in the virus particle, although their importance is still unclear (Maxwell and Frappier, 2007). The RNA molecules are enclosed by a conical shaped capsid layer consisting of roughly 2000 *gag* p24 capsid (CA) molecules. The inner viral surface consists of a p17 matrix (MA) protein shell surrounding the p24 CA and is responsible for the viral integrity. The MA, CA, NC and p6 proteins are cleaved from the precursor Gag (Pr55) protein, encoded by the *gag* gene, as well as the p1 and p2 spacer peptide proteins and cleaved by the viral protease (PR) (Briggs *et al*.,

2003; de Oliveira *et al.*, 2003). The p6 protein binds to HIV-1 Vpr, thereby promoting the incorporation of Vpr proteins into mature virus particles (Paxton *et al*., 1993).

The virus obtains its enveloped phospholipid membrane when budding from the host cell. The lipid bilayer contains several cellular membrane proteins, including major histocompatibility (MHC) antigens derived from the host cell (Arthur *et al*., 1992). The virus Env proteins are embedded within the lipid membrane layer. They consist of the trimeric exposed surface glycoproteins, encoded by *env* gp120. The surface proteins are anchored by the trimeric transmembrane protein, encoded by *env* gp41. These proteins are derived from the *env* gp160 precursor molecule. Env gp120, which binds to CD4+ cells, is further divided into five constant (C1 to C5) and five variable regions (V1 to V5). The variable regions are mostly found within regions encoding disulphide-constrained loops, exposed to the surface and to the host immune system (Leonard *et al*., 1990). The V3 region plays an important role in determining cellular tropism, allowing the virus to either use chemokine receptor type 5 (CCR5) or chemokine receptor type 4 (CXCR4) as its main chemokine co-receptor (Briggs *et al.,* 2003).

HIV *pol* encodes for the viral enzymes protease (PR), reverse transcriptase (RT), RNase H and integrase (IN). The enzymes are formed by cleaving of the precursor Gag-Pol (Pr160) protein by the viral PR (de Oliveira *et al*., 2003). The Pr160 polyprotein is formed by ribosomal frameshifting, the process whereby ribosomes change the open reading frame to allow for alternative translation of mRNA (Hung *et al*., 1998). The HIV-1 PR is part of the aspartyl protease group and responsible for cleavage of the precursor Gag (Pr55) and Gag-Pol (Pr160) molecules and thus viral maturation. Each subunit of the PR homodimer protein contains 99 amino acids with the active site found in the middle of the dimers. The RT enzyme acts as a RNA-DNA-dependant DNA polymerase and is found in all retroviruses. It is responsible for reverse transcribing the viral single stranded RNA genome into a double stranded DNA molecule and helps fold the DNA molecule into its double helix form. The RT protein is a heterodimer and consist of p51 and p66 subunits. (Rodgers *et al*., 1995; Huang *et al*., 1998). The active and DNA-binding site of RT is found within p66, while the p55 subunit merely functions as a support molecule for p66. RNase H helps degrade the HIV RNA once a DNA copy has been transcribed and the RNA is no longer needed. IN (p32) catalyzes the insertion of the newly sythesized HIV DNA molecule into its host genomic DNA. The Pol proein has been an important focus of ART (see below).

1.5.1.3 The accessory genes

Tat is between 86 and 101 amino acids in length, plays a crucial role in both *in vivo* and *in vitro* activation of viral transcription and is absolutely essential for viral replication. Tat promotes HIV-1 elongation via the recognition of the TAR hairpin structure at the 5'-end of the viral transcripts (Zhou *et al*., 1998; Brigati *et al*., 2003). Tat interacts directly with positive transcription elongation factor b (P-TEFb), which is responsible for the regulation of eukaryotic mRNA elongation (Marshall and Price, 1995; Zhou *et al*., 1998). Rev, a 19 kilo-Dalton (kDa) phosphoprotein is responsible for exporting the HIV mRNA products from the nucleus to the cytoplasma, before the mRNA transcripts are spliced by cellular proteins (Strebel, 2003). Rev binds to mRNA transcripts containing a Rev responsive element (RRE). The RRE, encoded as part of the Env gene has a nuclear export signal, allowing for the unspliced export of RNA from the nucleus (Le *et al*., 2002). Without Rev, RNA is spliced into smaller transcripts by the cellular machinery. Rev uses interactions with the host chromosomal region maintenance 1 (Crm1) protein and the small nuclear RNA (snRNA) pathway to export viral transcripts (Strebel, 2003).

The accessory genes include *nef*, *vif*, *vpr* and *vpu*. They are not absolutely essential for viral replication *in vitro*, but play a variety of roles during the life cycle of HIV.

The 192 amino acid (23 kDa) HIV-1 Vif protein helps to counteract antiretroviral activity and is especially active against the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) 3F/G cellular cytidine deaminase family (Arriaga *et al*., 2006; Soros and Greene, 2006). The APOBEC family of proteins act as editing enzymes and causes Cytosine to Uracil editing and leads to the accumulation of Guanine to Adenine mutations in the proviral sense cDNA strand (Malim, 2006). Vif interacts in the producer cell with APOBEC3F/G and recruits it to an ubiquitin ligase complex via a cullin-dependent ubiquitin ligase, Cullin 5 (Cul5). APOBEC3F/G is then ubiquitinated and subsequently degraded by cellular proteasomes. Vif binds to viral genomic RNA and forms part of the nucleoprotein complex (Khan *et al*., 2001) and also forms part of the reverse transcriptase complex which helps control reverse transcription (Carr *et al*., 2008).

The 96 amino (14 kDa) Vpr protein enhances viral expression and interacts with a host of cellular proteins (Bour and Strebel, 2003). Vpr binds to the Gag p6 domain and is directly

incorporated into mature HIV-1 virions (Bachand *et al*., 1999). The protein is responsible for the nuclear transport of the HIV-1 pre-integration complex (PIC) and plays an important role in the extracellular release of virus particles (Romani and Engelbrecht, 2009). Other functions identified include the induction of cellular apoptosis, induction of the G2 cell cycle arrest, modulation of gene expression and the suppression of immune activation (Romani and Engelbrecht, 2009).

Although the Vpu protein (16 kDa) is not found in the mature virus particle, it promotes the extracellular release of virus particles and downregulates CD4 in the Endoplasmic Reticulum (ER) (Schubert *et al*., 1996). These functions are carried out by the two separate domains expressed by the Vpu protein. The N-terminal hydrophobic transmembrane domain functions as a membrane anchor and promotes virus release, while the hydrophilic C-terminal domain contains two amphipathic α-helical domains of opposite polarity and contains sequence motifs critical for CD4 degradation (Schubert *et al*., 1996). Vpu interacts and antagonizes the cellular restriction factor Tetherin [also known as CD317 or bone marrow stromal cell antigen 2 (BST-2)]. The cellular function of Tetherin is still unknown. Viruses lacking Vpu are partially impaired from budding from their host cell and tend to tether at the cellular membrane (Neil *et al*., 2008). Viruses can also however still spread through direct cell-cell interactions (Jolly *et al*., 2010).

The 27 kDA Nef protein is responsible for the establishment of high viral loads during infection, which leads to faster disease progression (Kirchhoff *et al*., 2008). Nef is a multifunctional myristoylated protein which interacts with components of host cell signal transduction pathway as well as the endocytic clathrin-dependent protein pathway. Early in the HIV life cycle Nef is repsonsible for T cell activation and helps establish a persistant viral infection. Nef plays an important role in downregulating CD4, CD28, CXCR4, MHC class I and MHC class II on antigen presenting cells and other target cells, thus enabling the virus to evade the host immune system and help establish latent infection (Bour and Strebel, 2003; Roeth and Collins, 2006). Nef thus helps control responses of HIV-1 infected T cells thereby preventing superinfection, protects against cytotoxic T-lymphocyte (CTL) responses and also facilitates in the release of fully infectious virions (Arhel and Kirchhoff, 2009; Kirchhoff *et al*., 2008; Roeth and Collins, 2006).

1.5.2 The HIV life cycle

HIV infects cells of the immune system such, as CD4+ T-cells, cytotoxic T-lymphocytes (CTLs), CD4+ monocytes and macrophages. The virus can be found in blood plasma, peripheral blood mononuclear cells (PBMCs), lymph nodes, the central nervous system and various other body fluids and cells after infection (Stebbing *et al.*, 2004). The virus enters the host cell via the CD4 receptor molecule and either the CXCR4 or CCR5 chemokine co-receptor; although in certain cases other host cell co-receptors such as CCR3 can also be involved in viral entry (Dash *et al*., 2008; Regoes and Bonhoeffer, 2005; Rucker *et al*., 1997). Binding of Env gp120 to the CD4 molecule leads to a conformational change at the point of attachment, allowing fusion of the membranes and the virological synapses (VS) to form. After successful attachment the viral RNA, along with viral enzymes are released into the host cytoplasm. In the cytoplasm RT reverse transcribes the RNA into the double stranded DNA copy. With the help of IN the newly synthesized viral DNA is imported into the nucleus and incorporated into the host cell genome. Once incorporated the DNA may become dormant, allowing HIV to form latent infection.

Expression of viral proteins is regulated by both viral and cellular proteins and is initiated when Tat binds to the TAR element in the 5'LTR as described above. Both unspliced and spliced mRNA transcripts are exported out of the nucleus with the help of Rev. In the cytoplasm the cellular machinery translates the mRNA transcripts into viral proteins. Viral assembly takes place at the plasma membrane of the host cell. Env gp160 is processed by the ER complex, is transported to the Golgi apparatus and cleaved by cellular furine like proteases into its gp120 and gp41 components. Env gp41 acts as an anchor for gp120 at the plasma membrane. The Pr55 Gag and Pr160 Gag-Pol polyproteins and viral RNA are incorporated into the immature virus particle at the plasma membrane. Gag molecules associated with the membrane attracts two copies of viral RNA and together with cellular and viral proteins trigger budding from the cell surface (Ganser-Pornillos *et al*., 2008). Maturation occurs when the virus buds from the host cell and viral proteins are cleaved by PR into functional proteins and enzymes. The newly formed viruses are free to infect new cells. Infection spreads either through cell-cell interaction via a VS, or through cell-free mediated interactions. The infected host cell is exhausted through continuous immune activation and death of CD4+ T cells paralyses the host immune system (Badley, 2005; Roshal *et al*., 2001).

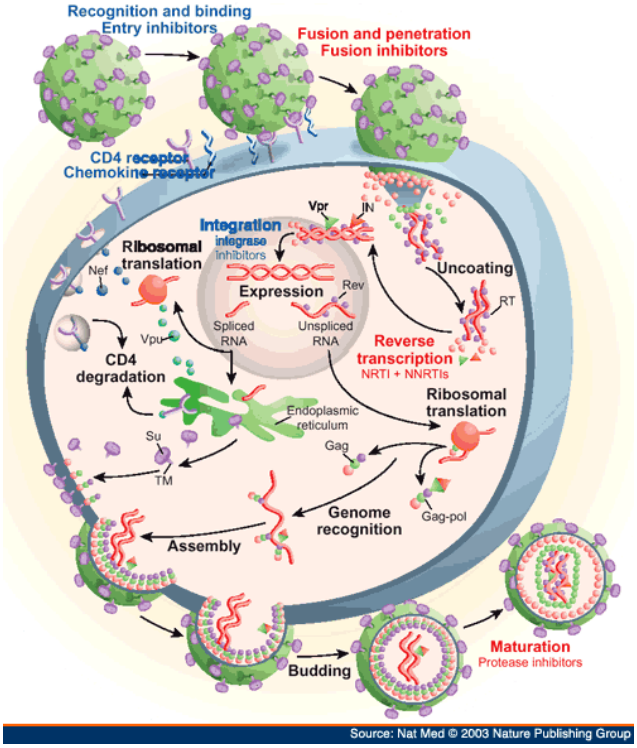


Figure 1.5: The HIV-1 life cycle. HIV replication consists of viral attachment, entry, reverse transcription and integration of the viral DNA into the host DNA / or genome. This is followed by export of the viral proteins as well as viral assembly, budding and maturation of viral particles. The target sites of the three classes of inhibitors, reverse transcription, integration and maturation, are indicated (Pomerantz and Horn, 2003; Turner and Summers, 1999).

1.6 Antiretroviral therapy and resistance

Despite the best efforts, there is still no known cure for HIV/AIDS infection. The HIV-1 life cycle has been a key target in developing efficient antiretroviral drugs against HIV-1 (Figure 1.4) and with the aid of antiretroviral therapy (ART) the life expectancy of infected individuals has dramatically increased.

1.6.1 Natural resistance to HIV

Certain rare individuals do not develop AIDS, despite being infected with HIV. They are termed slow or long-term nonprogressors (LTNPs) and they seem to have natural immunity against the virus. They are able to keep the HIV viral load titer to a minimum (O'connell *et al*., 2009). People carrying the CCR5-Δ32 genetic variant sometimes falls within this category. The CCR5-Δ32 allele is found in approximately 10% of the Northern Europe population and has been known to show protection not only against HIV, but also smallpox (Sabeti *et al*., 2005). However the mutation also has a negative effect on T-cell function, while individuals with this mutation also have a higher brisk contracting West Nile virus

(Glass *et al*., 2006). Individuals with certain HLA (Human leukocyte antigen)-type genes, particularly HLA-B*5705 and / or HLA-B*2705 seem also to control HIV infection to a certain extent (Migueles *et al*., 2000). In an Australian cohort individuals were identified carrying Nef-deleted variants, leading to the conclusion that defective HIV can also impair viral replication *in vivo* (Rhodes *et al*., 2000).

Natural host restriction factors such as APOBEC3F/G active against Vif and Tetherin, active against Vpu (discussed above) can also limit HIV-1 viral replication. Other host restriction factor against retrovirus include members of the Tripartite Motif (TRIM) protein family such as TRIM5α and TRIM22. The TRIM family forms part of the host innate immune system. Although TRIM5α is ineffective against HIV-1, it can inhibit murine leukaemia virus (Yap *et al*., 2004). TRIM5α does however successfully block HIV-1 infection in rhesus macaques and Old World monkeys (Stremlau *et al*., 2004). TRIM22 down-regulates HIV-1 transcription from the LTR and prevents viral assembly by blocking HIV-1 Gag export from the nucleus (Barr *et al*., 2008).

1.6.2 Antiretroviral therapy (ART)

ART has dramatically led to the reduction of opportunistic infections, an increased life span and an improved quality of life in many HIV-1 infected individuals. Current therapeutic agents against HIV-1 include viral entry inhibitors, non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), Integrase inhibitors as well as protease inhibitors (PIs) (Johnson *et al.*, 2003). More than 30 drugs have been approved for use in HIV-1 treatment, with many more drugs currently undergoing clinical trials. A list of the Food and Drug Administration (FDA) (USA bureau) approved drugs for use in HIV-1 treatment is available at http://www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm, last accessed 23 August 2011. Most of the current ART drugs attempt to stop viral replication by inhibiting the RT gene, stop virus maturation by inhibiting the PR gene or attempt to stop the virus from entry into the host cell. ART Mutations often lead to the failure of ART in patients infected with HIV-1 (Johnson *et al.*, 2003; Thompson *et al*., 2010). Multi-drug resistant viruses usually arise as a result of selective pressure from these therapeutic agents.

Viral entry inhibitors, also known as fusion inhibitors attempt to stop the virus from entering the host cell. However, Env has evolved to evade the host immune response, and therefore is highly variable, making it very difficult to produce specific entry inhibitors. The currently approved entry inhibitors include Enfuvirtide (Fuzeon), which is active against *env* gp41 and Maraviroc (Celsentri) which acts as a CCR5 antagonist (Pugach *et al*., 2008). Other small molecules and natural ligands targeting the entry receptors CCR5 and CXCR4, such as stromal cell-derived factor-1 (SDF-1) could also be used in future vaccine development strategies (Seibert and Sakmar, 2004). NNRTIs are a set of drugs, which binds and physically interacts with the RT enzyme of HIV-1. Currently available NNRTIs are Delavirdine (DLV), Efavirenz (EFV), Etravirine and Nevirapine (NVP). NVP has been widely used to prevent mother-to-child-transmission (MTCT) during pregnancy and birth (Guay *et al*., 1999; Lallemant *et al*., 2004; Johnson *et al*., 2005). Rilpivirine (Goebel *et al*., 2006) was the latest NNRTI drug approved for HIV-1 therapy by the FDA in May 2011. NRTIs are analogues of the body's own nucleoside or nucleotide molecules and act as alternative substrates for DNA polymerases that bind to the RT. Zidovudine (AZT), an NRTI analogue of thymidine and the first FDA approved drug against HIV/AIDS, was introduced in 1987 (Fischl *et al*., 1987). Didanosine (ddI), an analogue of adenosine was the second approved FDA drug. Other NRTIs include tenofovir (TDF), stavudine (d4T), lamivudine (3TC), abacavir (ABC) and emtricitabine (FTC). FTC and 3TC are structurally similar compounds. TDF is an adenosine analogue, d4T a thymidine analogue, ABC a guanosine analogue while 3TC and FTC are cytidine analogues. Integrase inhibitors actively block retroviral integration into the host genome. Raltegravir (RAL) (Steigbigel *et al*., 2008) is currently the only FDA approved Integrase inhibitor available. Elvitegravir (EVG), a second Integrase inhibitor is still in the Phase III clinical trial phase and has not been approved by the FDA (Shimura *et al*., 2008). Other second generation Integrase inhibitors still being tested in various phases of clinical trials include Dolutegravir (Garrido *et al*., 2011, Hightower *et al*., 2011) and MK-2048 (Bar-Magen *et al*., 2010). The list of currently available PIs are amprenavir (APV), atazanavir (ATV), darunavir (DRV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV) and tipranavir (TPV). LPV is only prescribed in combination with RTV as Kaletra (Walmsley *et al*., 2002). PIs disable the enzymatic function of the protein by binding to the active site and acting as an alternative substrate. The potency of certain PIs allow for their use in monotherapy with certain patients (Cameron *et al*., 2008).

The world health organization (WHO) recommendeds that ART is started when a person has a CD4 cell count below 350 cells per mm^3 or has progressed to the WHO stage II or III disease stage already. People with co-infections are started on therapy immediately. It is recommended that people receiving ART be given two NRTIs (either AZT or TDF) and either a PI or NNRTI at the start of their treatment (WHO, 2010). When the first line therapy fails, second line therapy usually consists of ritonavir-boosted PI and two NRTIs (either AZT or TDF depending on the first line therapy regime). Viruses form patients failing ART are recommended to be analysed genotypic testing before changing the therapy regiment. Guidelines should be adjusted for each individual, although this is only possible in developed countries. To reduce the risk of MTCT pregnant women are given AZT from 28 weeks of pregnancy, a single-dose of NVP during labour and given AZT and 3TC for one week thereafter. The new born baby is also given a single dose of NVP immediately after delivery and AZT for at least a week thereafter. Women who breastfeed should receive a triple ART regiment from 14 weeks of gestation after all exposure to breast milk has ended (Thomas *et al*., 2011). The complete updated treatment guidelines can be found at (http://www.who.int/hiv/pub/arv/adult2010/en/index.html).

1.6.3 Testing for HIV-1 resistance

Current assays that test HIV-1 drug resistance include genotypic and phenotypic assays. With genotypic resistance testing the viral genome is sequenced and scanned for resistance associated mutations. The patient derived viral genome is compared to a database of HIV sequences known to be associated with certain resistance patterns (http://hivdb.stanford.edu). Phenotypic resistance testing, such as the Phenosense GT system (Monogram Biosciences) can be performed by measuring the viral activity in the presence and absence of a drug in question. The assay compares the concentration, usually 50% inhibitory (IC_{50}), of drug needed to inhibit the clinical isolates with that of the wild type reference strain. Although phenotypic tests are more time and labour consuming, the assay directly measures viral enzyme function and more accurately reflects the sensitivity of the virus to antiretroviral compounds. Discordance between genotypic and phenotypic tests have been also been identified (Zolopa, 2006). Previously uncharacterised mutations, especially unknown resistance mutations which form against novel HIV-1 drugs, cannot be predicted by genotypic methods alone.

1.7 Aim of thesis

The primary aim of this thesis was to characterize circulating HIV-1 strains from Cape Town, South Africa. This was done through genotypic methods. Through phylogenetic analyses we analyzed HIV-1 resistance mutations in the treatment naïve patient population. We also characterized HIV-1 Vif sequences from patients derived viruses. Our third aim was to construct an infectious HIV-1 subtype C proviral molecular clone from Cape Town, South Africa having a high replication capacity, which should be used for *in vitro* HIV assays, including phenotypic HIV-1 resistance testing in the future. We also investigated the HIV resistance profile of a treatment naïve cohort in Mwanza, Tanzania.

Chapter two

Chapter Two

2. Materials

2.1 Patient samples

2.1.1 Therapy naïve patients used for HIV-1 genotyping

For HIV-1 genotypic tests Ethylene diamine tetra-acetic acid (EDTA) blood from 140 therapy naïve patients were received from an academic hospital clinic, private clinics, state clinics, the Western Province Blood Transfusion Service (WPBTS) and a sex worker cohort from the Cape Metropole area of South Africa, during the period of 2002 to 2004. These include 81 black females, 34 black males, 5 Caucasian males, 1 Caucasian female as well as 8 males and 11 females of mixed race. These patient samples were also used to characterize HIV-1 *vif* sequences from Cape Town, South Africa. The cohort samples represent different ethnic groups and consist of heterosexuals, homosexuals, bisexuals, and MTCT-infected individuals.

2.1.2 Patient ZAC (R3714)

The retrovirus cohort represents patient samples diagnosed with either HIV or HTLV infection of which samples (plasma and serum) were stored from 1984 to 1995 at the Tygerberg hospital in Cape Town, South Africa. The patient, a South African coloured (mixed race) male born on 22 August 1931 was diagnosed with lymphocyte depleted Hodgkin's lymphoma on 02 March 1989 and diagnosed as HIV-1 positive on 09 March 1989. He travelled frequently to Lusaka, Zambia, where he possibly became infected with the virus. Subsequently, serum and PBMCs were obtained during November 1989 (harvested on 20 and 21 November 1989) and the virus was co-cultured with PBMCs and isolated. High molecular weight DNA was extracted from the HIV-positive cultures through phenol-chloroform extraction and stored. HIV-1 positive cultures were confirmed by RT assay that ranged from 12495 to 35073 counts per minute per millilitre (cpm/ml). The *env* gene was amplified by PCR, sequenced and identified as subtype C (Engelbrecht *et al*., 1995).

2.1.3 Therapy naïve patients from Mwanza, Tanzania

Treatment naïve samples were obtained from the ProCort1 (trial name: ‘‘ProCort1’’; registry: ClinicalTrials.gov; registration number: NCT01299948) clinical trial at the Bugando Medical Centre in Mwanza, Tanzania. The study was approved by the National Institute for Medical Research (Tanzania), Bugando Centre Ethical Board and Ministry of Health (Tanzania). Plasma and PBMCs were collected for each patient at baseline and at 12 later time points over a two year period.

2.2 Equipment, commercial assays, enzymes and chemicals

The list of equipment, chemicals and assays used during this study are listed in this chapter. In Table 2.1 the PCR kits and enzymes used are listed. In Table 2.2 the equipment needed to perform the necessary assays and analysis are presented, while the commercial packages used are given in Table 2.3. Buffers and additional media are listed in Table 2.4. Chemicals needed for buffers were obtained from Merck, Roth and Sigma-Aldrich. Miscellaneous products used are listed in Table 2.5.

Table 2.1: PCR kits and enzymes.

Enzymes	Supplier
Access RT-PCR system	Promega
Antarctic phosphatase	NEB
Expand™ High Fidelity PCR system	Roche Diagnostics
Expand™ Long Template PCR system	Roche Diagnostics
GoTaq™ DNA polymerase	Promega
Herculase® II	Agliotti
Moloney Murine Leukemia Virus (M-MLV) RT	Promega
Phusion® High Fidelity DNA polymerase	NEB
Restriction enzymes	Fermentas, NEB, Promega
Shrimp alkaline phosphatase (SAP)	Fermentas
T4 DNA ligase	Fermentas, NEB

Table 2.2: Equipment used to perform sample assays and analysis.

Equipment	Supplier
ABI prism® 310 genetic analyzer	Applied Biosystems
Biometra® T-personal thermal cycler	Biometra
Gelair flow cabinet BSB4A	Flow Laboratories
Heating block	Peqlab Biotechnologies
Heraeus® CO_2-Auot-Zero incubator	Heraues
Heraeus® Multifuge 1 S-R centrifuge	Heraues
Intas Gel Doc™ system	Bio-Rad
NanoDrop™ system	NanoDrop Technologies
Sartorius PB-11 pH meter	Sartorius
Sonicator-Sonifier® 250	Branson
Sorvall® 90SE ultracentrifuge	Thermo fisher scientific
Sorvall® Evolution RC centrifuge	Thermo fisher scientific
Table top Eppendorf® 5417C centrifuge	Eppendorf
Trans-Blot® SD cell	Bio-Rad
Vortex	A. Hartenstein
Leitz Labovert FS light microscope	Leica
Leitz DM IRE2 fluorescent microscope	Leica

Table 2.3: Commercial kits and assays.

Product	Supplier
BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 1.1	Applied Biosystems
Fugene®6 or HD transfection reagent kit	Roche Diagnostics
Cobas®Amplicor HIV-1 Monitor version 1.5 test kit	Roche Diagnostics
GeneElute™ gel extraction and PCR purification kits	Sigma-Aldrich
GeneRuler™ 1 kb DNA ladder	Fermentas
NucleoBond® PC500 Reagents	Macherey-Nagel
PageRuler™ Prestained Protein Ladder	Fermentas
pcDNA™3.1 Directional TOPO® Expression kit	Invitrogen
Pierce® ECL Western Blot Detection kit	Thermo scientific
PureYield™ Plasmid Midiprep system	Promega
QIAamp® DNA Micro kit	Qiagen
QIAprep® Spin Miniprep kit	Qiagen
RNeasy® Mini extraction kit	Qiagen
TOPO® XL PCR Cloning kit and TA Cloning® kit	Invitrogen
TurboFect™ *in vitro* transfection reagent kit	Fermentas

Table 2.4: Buffers, media and recipes.

Description	Recipe
Alseivers Trypsin Versene (ATV)	8.0g NaCl, 0.27g KCl, 1.15g NaH_2PO_4, 0.2g KH_2PO_4, 0.1g $MgSO_4$ x $7H_2O$, 1.125g Na_2-EDTA, 1.25g Trypsin. Add 1 L distilled H_2O.
Competent cell buffer 1	30 mM Potassium acetate (pH 5.8), 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% Glycerine (v/v).
Competent cell buffer 2	10 mM MOPS [3-(N-morpholino) propanesulfonic acid buffer] (pH 7.0), 10 mM RbCl,75 mM $CaCl_2$, 15 % Glycerine (v/v).
Dulbecos Modified Eagle Medium (DMEM)*	10% Fetal calf serum (FCS) (heat-inactivated), 5% L-Glutamine (500 µg/ml), 0.05% Penicillin (100 µg/ml), 0.05% Streptomycin (100 µg/ml). Add 500 ml H_2O.
DNA loading dye (6 x)	0.125% Bromophenol blue, 40% Sucrose.
Hepes buffered saline (HBS) (2 x)	280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.1.
Luria-Bertani (LB) medium (5 x)	100g broth base, 25g NaCl, 5g α-D-Glucose. Add 1 L H_2O.
LB-Agar	20g LB broth base, 20g Agar, 5g NaCl. Add 1 L H_2O.
MEM (Minimal essential media)*	10% FCS (heat-inactivated), 5% L-Glutamine (50 µg/ml), 0.05% Penicillin (100 µg/ml), 0.05% Streptomycin (100 µg/ml).
Miniprep solution 1, resuspension buffer	50 mM Glucose, 10 mM EDTA (pH 8.0), 25 mM Tris HCl (pH 8.0).
Miniprep solution 2, lysis buffer	0.2 M NaOH, 1% Sodium dodecyl sulfate **(**SDS).
Miniprep solution 3, neutralization buffer	3M Natriumacetate (pH 5.4).
MOPS (10 x)	83.7g MOPS, 13.6g Sodium acetate, 3.7g EDTA.
Phosphate buffer saline (PBS)	137 mM NaCl, 2.07 mM KCl, 4.3 mM Na_2HPO_4 x $2H_2O$, 1.4 mM KH_2PO4, 1.5 mM $CaCl_2$ x $4H_2O$; 1 mM $MgCl_2$ x $6H_2O$.
Radio-Immunoprecipitation Assay (RIPA) – buffer	20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 1% sodium deoxycholate, 1% Triton-X 100, 0.1% SDS.
Roswell Park Memorial Institute (RPMI) – 1640 media*	10% FCS (heat-inactivated), 10 mM HEPES (pH 10.4), 5% GlutaMax (500 µg/ml), 0.05 % Penicillin (100 µg/ml), 0.05 % Streptomycin (100 µg/ml).
SAP reaction buffer	25 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 50% Glycerine.
SDS loading buffer (6 x)	3 ml Glycerine, 1g SDS, 0.375mg bromophenol blue, 3.75 ml ß-Mercaptoethanol. Add 10 ml Tris-HCl with 0.4 % SDS.
SDS running buffer (5 x)	25 mM Tris, 200 mM Glycine, 0.1 % SDS (w/v).
SDS separation gel (4 x)	1.5M Tris HCl (pH 8.8), 0.4% SDS (w/v).
SDS stacking gel (4 x)	0.5M Tris HCl (pH 6.8), 0.4% SDS (w/v).
T4 DNA ligase buffer	50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM Dithiothreitol, pH 7.5.
Tris-Acetate-EDTA (TAE) buffer (50 x)	2 M Tris, 50 mM EDTA (pH 8.0), 5.71% acetic acid (v/v).
Tris-EDTA (TE) buffer	10 mM Tris-HCl (pH 8.0), 1 mM EDTA.
Western Blot buffer	3.04 g Tris, 14.4g Glycine, 100 ml Methanol, Add 1 l ddH_2O.

*DMEM was obtained from Sigma-Aldrich, MEM from Invitrogen and RPMI-1640 from Gibco.
Salts and antibiotics added to the various cell culture mediums are shown in the recipe list.

Table 2.5 Miscellaneous products used.

Material	Supplier
FCS	Gibco
Glassware	Schott
Nitrocellulose membrane	Roth
Laboratory liquids (Acetone, Ethanol, Isopropanol, Methanol, Polyacylamid Terralin)	Roth
Laboratory wear	A. Hartenstein
Neubauer counting chamber	A. Hartenstein
Parafilm	Roth
Pipette tips	A. Hartenstein
Pipettes	Gilson
Plastic material	Costar, Eppendorf, Falcon, Greiner, Nunc, Roth
Sterile filters and filterpaper	Schleicher & Schuell
Fuji medical x-ray film	Fujifilm

2.3 Primers

The primers used for HIV-1 PR and RT genotyping were previously described (Plantier *et al*., 2005). The HIV-1 subtype C full-length sequencing primers were also described before (Rousseau *et al*., 2006). The *vif* genotyping primers are given in Table 2.6. The HIV-1 subtype C primers are given in Table 2.7. The melting temperature (Tm) for each primer is given.

Table 2.6: HIV-1 *vif* genotyping primers.

Primer	Amplification step	Sequence 5'-3')	Tm (^{o}C)
HIV-int1	1st round PCR	WWWYKRGTYWRTWMYRGRRWCAGSAGAG	51.1 – 65.8
HIV-INT6A	1st round PCR	ATNCCTATNCTGCTATGTYGRCAYCCAAT	55.9 – 62.9
INT3S	2nd Round PCR	AGMMAARSYHCTCTGGAACGGTGAAG	56.4 – 64.3
INT5A	2nd Round PCR	CCTATNCTGCTATGTYGACAACCAATKCTGWAAATG	61.0 – 64.4

Table 2.7: HIV-1 subtype C amplification primers.

Primers	Sequence (5'-3')	Tm (^{o}C)
HIV_NgoMIV_F	GAATGCCGGCTGGATGGGCTAGTTTACTCCAAGAGAAGGCAAG	71
CMVstart_NgoMIV	GAATGCCGGCTAGTTATTAATAGTAATCAATTACGGGTC	63
CMF_overlap_F	CAGAGCTGGTTTAGTAACCGGGTCTCTCTAGGTAGACCAGATCTGAGCC CGGGAGCTC	77
CMV_overlap_R	GTGCTCCCGGGCTCAGATCTGGTCTACCTAGAGAGACCCGGTTACTAAA CCAGCTCTG	77
SpeI-R	CTATTTGTTCCTGAAGGGTACTAGTGTTCCTGCTATG	64
SpeI-F	CATAGCAGGAACTACTAGTACCCTTCAGGAACAAATAG	64
PacI-R	CTCTAATTCTTTTAATTAACCAGTCTATTTTTC	54
PacI-F	GAAAAATAGACTGGTTAATTAAAAGAATTAGAG	54
BspEI-R	GTCTTTGTAATACTCCGGATGTAGCTCGCG	63
BspEI-F	CGCGAGCTACATCCGGAGTATTACAAAGAC	63
NotI-R	GAGCGGCCGCACTACCAAAAAGGGTCTGAGGGATCTCTAGTTAC	72

2.4 Plasmids and vectors

The HIV-1 plasmids pNL4-3 (Adachi *et al*., 1986) and pMJ4 (Ndung'u *et al*., 2001) were used during the study. The enhanced Green Fluorescent Protein (eGFP) expression plasmid, pEGFP-C1 was obtained from Clontech. The cloning vectors pUC19, TOPO® XL PCR, pcDNA3.1™ 3.1D/V5-His/lacZ and PCR® II were obtained from Invitrogen.

2.5 Bacterial cells

Bacterial cells were otained from Invitrogen.

***E.coli* Top10 competent cells:** chromosomal genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG.

***E.coli* DH5α competent cells:** chromosomal genotype: F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk -, mk +) phoA supE44 thi-1 gyrA96 relA1 tonA.

2.6 Antibiotics

Ampicillin, Kanamycin, Penicillin, and Streptomycin were obtained from Sigma-Aldrich.

2.7 Culture cell lines

The maintenance of the various cell lines are described in chapter 3.

HEK-293T: Human embryonic kidney (HEK) stem cells expressing a large Simian Vacuolating Virus 40 TAg (SV40 T) antigen on their cell surface (Graham *et al*., 1977; Pear *et al*., 1993).

TZM-bl: A HeLa derived cell line expressing CD4, CCR5 and CXCR4. It contains HIV Tat-inducible Luciferase and ß-galactosidase (X-gal) genes (Derdeyn *et al*., 2000; Wei *et al*., 2002).

MT-4: A Human T-cell Lymphotropic Virus-I (HTLV-I) transformed T-cell line (Harada *et al*., 1985; Larder *et al*., 1989).

PBMCs were isolated from donor blood samples.

2.8 Antibodies

Primary western blot detection antibodies against HIV-1 Gag p24 (Hartl *et al*., 2011), GFP (Sigma-Aldrich) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Sigma-Aldrich) were used. Secondary antibodies were Goat Anti-Rabbit Immunoglobulin G (IgG) and Goat Anti-Mouse IgG and obtained from Jackson Immuno Research.

Chapter three

Chapter three

3. Methods

3.1 Patient sample preparation

The South African samples were collected at Tygerberg Academic hospital, Cape Town, South Africa. Plasma was collected from EDTA blood after centrifugation (Beckman Coulter Allegra™ 6R, Beckman Inc.) at 2000 rpm for 10 minutes at 4°C and subsequently stored at -80°C for future analysis. Viral RNA was extracted using the high throughput M1000 robot extractor (Abbott Diagnostics).

Samples from the Tanzania cohort were collected at the Bugando Medical Centre in Mwanza, Tanzania. Patient PBMCs were isolated from 8 ml of whole blood were collected in cell preparation tubes (Becton Dickenson) with a fixed ficoll gradient. After isolation of PBMCs, the cells were immediately frozen at -80°C in RPMI 1640 medium supplemented with 40% fetal calf serum (FCS) and 10% Dimethyl sulphoxide (DMSO). The EDTA plasma samples were obtained from 6 ml whole blood collected in EDTA Vacutainer (BD) and immediately frozen at -80°C. Plasma was collected as for the South African samples. The PBMC and the plasma samples were shipped frozen at -70°C with temperature log to Würzburg, Germany. DNA was extracted from 1 x 10^6 PBMC using the QIAamp® DNA Micro Extraction Kit (Qiagen). RNA extraction of plasma was performed using the sample preparation kit of the Cobas® Amplicor HIV-1 Monitor version 1.5 test kit (Roche).

3.2 Polymerase chain reaction (PCR)

PCR, developed in 1985 by Kary B. Mullis (Mullis and Faloona, 1987) was used to amplify HIV-1 RNA or DNA from selective patient sample and DNA plasmids. A PCR amplification involves concurrent steps of DNA heat denaturation, primer annealing and DNA extension. These steps are repeated several times during PCR cycling (Saiki *et al*., 1988). The following standard protocol was used to amplify all DNA: One cycle of denaturation at 94°C for 2 minutes, followed by 30 to 35 cycles of denaturing at 94°C for 30 seconds, primer annealing for 30 seconds (according to primer pair Tm) and elongation at 72°C for 30 seconds to 1 minute per kb of target amplified DNA. A final elongation step of 72°C for 3 to 10 minutes was performed, after which the samples were cooled and stored at 4°C or -20°C for longer periods, until used. The reaction consists of 0.5 μM of each specific target primers (listed in

Chapter 2), 200 µM deoxyribonucleoside triphosphates (dNTPs) (Sigma-Aldrich), DNA polymerase with specified reaction buffer, usually obtained from the manufacturer and 10 ng of patient or plasmid DNA in a total reaction mixture of 50 µl in a 0.2 ml thin-wall PCR tube (VWR).

For short fragments, generally less than 1 kb in length, GoTaq™ DNA polymerase (Promega) was used. For larger fragments using a high fidelity enzyme was necessary to limit PCR error mistakes (Hopfner *et al*., 1999). For this the Expand™ High Fidelity PCR system (Roche Diagnostics), Herculase® II PCR system (Agliotti) or the Phusion® High Fidelity DNA polymerase system (NEB) was used. For larger fragments, bigger than 3 kb the Phusion® High Fidelity DNA polymerase system was preferred.

When amplifying RNA, the RNA fragment was first reverse transcribed into complementary DNA (cDNA) using the Moloney Murine Leukemia Virus (M-MLV) RT protocol (Promega). Briefly, 10 ng to 5 µg of total RNA was added with 1 µl of primer (0.5 µg/µl), 2 µl of 10 mM dNTP mix and 1 µl of RevertAID™ Hminus M-MLV RT (200 U/µl) to in a 20 µl reaction volume filled with water in a 0.2 ml thin-wall PCR tube (VWR). The following protocol was used: Incubation at 37°C for 5 minutes, followed by reverse transcription at 42°C for 60 minutes. The reaction was stopped by heating at 70°C for 10 minutes. The Access RT-PCR system (Promega) was also used as a one-step amplification system to amplify small DNA fragments from RNA. The manufacturer's instructions were followed. All PCR steps were performed in a Biometra® T-personal thermal cycler (Biometra).

3.3 Agarose gel electrophoresis

Agarose gel electrophoresis allows for the separation of nucleic acids according their molecular weight size through electrophoresis in an agarose gel with a current of constant strength (Sambrook *et al*., 1989). All PCR products were verified by agarose gel electrophoresis on a 0.8% to 1.5% (w/v) agarose (Roth) gel in 1 x TAE buffer. Samples were mixed with 6 x DNA loading dye (0.125% Bromophenol blue, 40% Sucrose) and approximately 6 µl were loaded per lane on the agarose gel. Electrophoresis reactions were run at 80 V for a 50 ml agarose gel, while 100 V was used for a 150 ml gel. The DNA was stained with ethidium bromide (A. Hartenstein) (0.5 µg/ml) and viewed under a UV light with a wavelength between 280 and 320 nm, with the INTAS Gel Doc™ system (Bio-Rad). Ethidium bromide is a powerful mutagen and should be handled carefully (Ausubel *et al*.,

2003; Sambrook *et al*., 1989; Sharp *et al*., 1973). The GeneRuler™ 1 kb DNA ladder (Fermentas) was used as a molecular weight size marker.

3.4 Purification of nucleic acids

To dispose of excess dNTPs, enzymes and buffers amplified PCR products were purified either by agarose gel extraction using the GeneElute™ gelextraction kit or the GeneElute™ PCR purification kit using the manufacturer's instructions (Sigma-Aldrich). The purification protocols are based on silica membrane spin protocols which allows for binding of nucleic acids to a silica membrane inside a spin column (Vogelstein and Gillespie, 1979; Sambrook *et al*., 1989).

3.5 DNA concentration determination

The NanoDrop™ system (NanoDrop technologies) was used to determine the DNA concentration spectrophotometrically using a 1 µl input sample. DNA measurements are read at a wavelength of 260 nm, while purity is determined by dividing the absorbance at 260 nm with the absorbance at 280 nm (Sambrook *et al*., 1989). Pure DNA has a value between 1.7 and 1.9. Lower values are indicative of protein contamination, while higher values indicate the presence of RNA in the sample.

3.6 Transformation of DNA into bacterial plasmid vectors

Transformation is the process by which bacteria incorporates exogenic material, such as DNA from its surroundings. The standard protocol was followed (Sambrook *et al*., 1989):
Competent cells, previously stored at -80°C were allowed to thaw for 5 to 10 minutes on ice before use. Approximately 0.01 to 0.1 µg of plasmid DNA or 5 to 10 µl of ligated DNA were mixed with 100 µl of competent cells and incubated on ice for 30 minutes. The reaction mixture was heat shocked at 42°C for 90 seconds and incubated on ice for a further 2 minutes. A volume of 900 µl of 1 x LB medium was added to the cells and the reaction was allowed to shake for 1 hour at 37°C. The bacterial cells were pelleted by centrifugation at 3000 rpm for 3 minutes. Approximately 50 to 200 µl of the bacteria were grown on antibiotic specific agar plates overnight at 37°C. For direct large scale preparation of bacterial clones the transformed bacteria were grown in a 250 ml LB-medium containing specific antibiotics, while shaking at 200 rpm overnight.

3.7 Small scale preparation of plasmid DNA (minipreps)

Small scale preparation of plasmid DNA or more commonly referred to as minipreps were isolated from bacterial cultures using the following ethanol precipitation protocol:

Single colonies on agar plates were picked and inoculated in 3 ml of 1 x LB-medium containing antibiotics. Ampicillin was used at a concentration of 100 µg/ml while kanamycin was used at 50 µg/ml. The culture was transferred to a 1.5 ml microcentrifuge tube (Eppendorf) and approximately 1.2 ml of bacteria was pelleted by centrifugation at maximum speed (12000 - 14000) rpm in a table top centrifuge. The supernatant was discarded and the pellet re-suspended by vortexing in 100 µl of miniprep solution 1. Resuspended cells were lysed by adding 200 µl of miniprep solution 2 and incubated at room temperature for 5 minutes, after which 200 µl of miniprep solution 3, neutralization buffer was added. The reaction mixtures were centrifuged at maximum speed for 5 minutes. The supernatant was added to 100% ice cold ethanol (3 x volume) and centrifuged again at maximum speed for 5 minutes. The DNA pellet was washed by adding 500 µl of 70% ethanol to the reaction tube and centrifuging at maximum speed for 2 minutes. After carefully discarding the ethanol supernatant the pellet was air-dried at 55°C for 10 minutes. The DNA was resuspended in 50 µl Tris-EDTA (TE) buffer with Ribonuclease A (RNaseA). RNaseA removes RNA from DNA preparations.

3.8 Large scale preparation of plasmid DNA (Maxipreps)

Maxipreps cultures from transformed DNA or miniprep cultures were grown in 250 ml 1 x LB medium overnight at 37°C and pelleted by centrifugation at 6000 rpm for 10 minutes. The preparations were done with either the PureYield™ Plasmid Midiprep System (Promega) or the NucleoBond® PC500 Reagentkit (Macherey-Nagel) according to the manufacturer's instructions.

3.9 Restriction enzyme digestion

Restriction endonuclease enzymes are enzymes that cut DNA at a specific recognition sequence and are commonly used in laboratories to create recombinant plasmids (Sambrook *et al*., 1989). Restriction enzymes were used to test if recombinant DNA plasmids were correct. The following protocol was followed: Approximately 0.5 µg of DNA was added to specified restriction enzyme buffer with 1 U/µg of enzyme in a 20 µl reaction filled with water. The reaction was incubated for 1 to 2 hours at the specified temperature for each enzyme used. For larger reaction volumes when DNA fragments were needed for ligation

reactions the following protocol was followed: Approximately 5 to 10 µg of DNA were added to specified restriction enzyme buffer with 5 to 10 U/µg of enzyme in a 100 µl reaction filled with water and the reaction was incubated for 2 hours or longer, until the DNA was completely digested. Restriction digestions were analysed by agarose gel electrophoresis as described above.

3.10 Ligation of DNA vectors

In order to ligate DNA cut with restriction enzymes T4 DNA ligase (NEB) was used. T4 DNA ligase catalyzes the formation of phosphodiester bonds between the 5'-phosphate and 3'-hydroxyl termini between double stranded DNA molecules (Sambrook *et al*., 1989). The following protocol was followed: Insert DNA was ligated to vector DNA in a ratio of 1:3, with specified reaction buffer and 2 U of T4 DNA ligase enzyme in a total reaction volume of 20 µl filled with water. The reaction was left for approximately 4 hours at room temperature or incubated at 4°C overnight. The ligated DNA vectors were transformed into *E.coli* TOP10 competent cells as described above.

Before use in ligation reactions vector DNA was sometimes treated with Shrimp Alkaline Phosphatase (SAP, Fermentas) or Antarctic Phosphatase (NEB). Phosphatase is able to catalyze the removal of 5´ phosphate groups from DNA and RNA and is commonly used to stop vectors from self-ligation. This strategy decreases the chance of vector background in cloning strategies. (Sambrook *et al*., 1989; Rina *et al*., 2000). In a 10 µl reaction volume 1 µg of DNA was incubated with 1 U of enzyme and specified reaction buffer at 37°C for 30 minutes. The reaction was heat-inactivated at 65°C for 5 to 10 minutes.

3.11 Preparation of *E.coli* competent cells

For bacterial transformation *E.coli* Top10 and DH5α competent cells (Invitrogen) were used. New competent cells were generated with the following protocol:

Competent cells (100 µl) were inoculated with 5 ml LB medium, without antibiotics and allowed to shake overnight 200 revolutions per minute (rpm) at 37°C. From the overnight culture 1 ml were transferred into a 100 ml LB medium culture, allowed to shake for 2 – 3 hours at 37°C until an Optical Density of 600 (OD_{600}) value of between 0.2 and 0.3 were reached. OD_{600} value calculates the density of the culture and tells you when the logarithmic growth phase is reached. When ready, samples were transferred to sterile 50 ml falcon tubes, incubated on ice for 10 minutes after which they were centrifuged at 6000 rpm for 10 minutes

in a Heraeus® Multifuge 1 S-R centrifuge (Heraeus). The supernatant was removed and pelleted cells were resuspended in 20 ml ice-cold competent cell buffer 1 and left on ice for a further 10 minutes. Resuspended cells were centrifuged for a further 10 minutes at 6000 rpm and pelleted cells were resuspended in 4 ml ice-cold competent cell buffer 2. After 15 minutes competent cells were aliquoted in a volume of 200 µl into 1.5 ml microcentrifuge tubes (Eppendorf), quickly frozen in liquid nitrogen and stored at -80°C until used.

3.12 DNA sequencing

Through DNA sequencing the exact base pair sequence of a DNA fragment can be obtained. The PCR-based sequencing reaction is based on the enzymatic method of Sanger *et al*. (1977). The reaction incorporates both dNTPs as well as dideoxyribo-nucleoside triphosphates (ddNTPs), where the addition of ddNTPs to the DNA strand leads to a chain termination reaction. As the dNTPs are fluorescently labelled with different dyes they can be read on an automated DNA sequencer. In a 5 µl reaction volume 1 µl of BigDye™ version 1.1 terminator enzyme mix (Applied Biosystems), 5 pmol of primer, 500 ng of plasmid DNA and water were added together in a 0.2 ml thin-wall sequencing tube (A. Hartenstein). The following cycle sequencing reaction was performed: Denaturation at 96°C for 10 seconds, primer annealing for 5 seconds at 55°C and an elongation step at 60°C for 4 minutes. Sequences were performed on an automated ABI prism® 310 Genetic Analyzer system (Applied Biosystems).

3.13 Sequence and phylogenetic analyses

In order to study the relationship between various HIV sequences obtained we used phylogenetic analyses. Briefly, molecular phylogenetics is the study of evolutionary relationships among organisms based on their DNA and / or protein sequences. Phylogenetic analyses has become a useful tool to study HIV origin, epidemiology and diversity because of the rapid replication rate and evolution of these viruses (Salemi and Vandamme, 2003). Sequence contigs obtained were edited and assembled using the Lasergene Seqman and MegAlign version 7.0 software packages (DNASTAR Inc.). DNA sequences were aligned using the ClustalW version 2.0 software package (Larkin *et al*., 2007). Phylogenetic trees were generated with the TreeconW for Windows version 1.3b software package (Van de Peer and De Wachter, 1994) or the Mega version 5.0 software package (Tamura *et al*., 2011). The PR and RT derived sequences were screened for mutations associated with drug resistance

with the HIV database Genotypic Resistance Interpretation Algorithm version 4.6.2 on the HIV database maintained by Stanford University, USA (http://hivdb.stanford.edu/index.html). Reference sequence for use in sequence analyses were obtained from the Los Alamos National Laboratory database (http://www.hiv.lanl.gov). Conserved sequences were highlighted with the Bioedit version 7.0.9 (Hall, 1999) and Genedoc version 2.6.002 software packages (Nicholas *et al*., 1997).

3.14 Maintenance of cell lines

HEK-293T and TZM-bl cells were maintained in minimal essential media (MEM) (Invitrogen) at 37°C with a constant (5%) CO_2 level in a Heraeus® CO_2-Auto-Zero incubator (Heraeus). Cells were trypsinised with ATV and diluted (1:10) with fresh media every 2 to 3 days. Cells were maintained in appropiate culture flask (Nunc). Aliquots of cells were frozen away at -80°C with 10% DMSO for future use.

MT-4 cells were maintained in RPMI-1640 media (Gibco) and replaced with fresh media added every 2 to 3 days in a 1:10 dilution. MT-4 cells and maintained in a biosafety level 3 laboratory.

3.15 Isolation and maintenance of PBMCs for cell culture

PBMCs were extracted from donor EDTA blood by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich) to separate them from red blood cells and most granulocytes (Janeway *et al*., 2001). PBMCs were cultivated overnight at 37°C with a constant (5%) CO_2 level in a Heraeus® CO_2-Auot-Zero incubator (Heraeus) with RPMI-1640 media stimulated with phytohemagglutinin-P, (PHA-P) (Sigma-Aldrich) (0.5 mg/ml) and human Interleukin-2 (IL-2) (Sigma-Aldrich) (0.1 mg/ml) before being used for viral kinetics assays. IL-2 is a T-cell growth factor cytokine, while PHA-P is a plant mitogen that upregulate the expression of IL-2 receptors on T-cells (Johnson and Byington, 1990). In a 6-well cell culture plate (Nunc) approximately 5 x 10^5 cells were seeded before using the next day. All cell counts were done with the aid of the Neubauer cell counting chamber (A. Hartenstein).

3.16 Transfection of cells

Transfection is the process by which cells incorporate DNA plasmids through the cell membrane into their cytoplasm. During this study we used the Calcium-Phosphate transfection protocol, Fugene®6 or HD (Roche Diagnostics) or the TurboFect™ *in vitro*

transfection reagent (Fermentas). Calcium phosphate fascilitates the binding of DNA to the cell surface. The DNA subsequently enters the cell via endocytosis (Graham and van der Eb, 1973; Loyter *et al*., 1982). Briefly: In a 6-well cell culture plate (Nunc) 5 x 10^5 cells were seeded in MEM overnight at 37°C with a constant (5%) CO_2 level in a Heraeus® CO_2-Auto-Zero incubator (Heraeus). The next day fresh media was added. For Calcium-Phosphate transfection 2.5mM of $CacCl_2$ was added to 2 x HBS solution. Approximately 4 µg of DNA was transfected and the culture incubated for 2 to 3 days to allow for the expression of proteins. For TurboFect™ 6 µl transfection reagent was added to 4 µg of DNA with Dulbecos Modified Eagle Medium (DMEM) (Sigma-Aldrich). With Fugene®6 or HD 3 µl transfection reagent was added to 1 µg of DNA with DMEM (Sigma-Aldrich).

3.17 Western Blot analyses

To detect the expression of proteins specific antibodies were used in western blot detection assays as follows: From cell cultures supernatant was removed as described above. Cells were lysed with RIPA buffer and sonification (Sonicator-Sonifier® 250, Branson). Sonification breaks the cell membranes and releases the proteins into the supernatant (Sambrook *et al*., 1989). SDS loading buffer (6 x) was added to each sample. Samples were heated at 95°C for 5 minutes, centrifuged in a table top centrifuge at 8000 rpm for 5 minutes and loaded onto a polyacrylamide gel for separation. Polyacrylamide gels separates proteins according to seize. The polyacrylamide gel consists of a separation gel, layered on a stacking gel for loading of samples. The composition of the gel is given in Table 3.1. The Rotiphorese® acrylamid / bis-acrylamid Gel 40 (29:1) solution as the polyacrylamid source was obtained from Roth. Ammonium persulfate (APS) was obtained from Merck and Tetramethylethylenediamine (TEMED) from Sigma-Aldrich.

Table 3.1: Composition of SDS gel (8.0 cm by 10 cm)

Reagents	Separation gel (12.5%)	Stacking gel
Rotiphorese® Gel 40 (29:1)	4.68 ml	0.65 ml
4 x SDS buffer	3.75 ml	1.25 ml
dd H_20	6.57 ml	3.0 ml
10% APS	150 µl	100 µl
TEMED	25 µl	10 µl

The proteins are separated according to size by running the gel at a constant voltage (100 V for a small scale gel) in SDS-running buffer. The PageRuler™ Prestained Protein Ladder (Fermentas) was used as a size marker control. From the polyacrylamide gel proteins were transferred to a 0.2 µM Roti® nitrocellulose membrane (Roth) with the Trans-Blot® SD cell system (Bio-Rad). To block non-specific proteins from binding to the nitrocellulose membrane, the membrane was washed with 5% milk-PBS solution [5g milk powder (Roth) in 100 ml PBS]. The nitrocellulose membrane was incubated overnight at 4°C in 5% milk-PBS solution, while constantly shaking. The following concentrations of antibodies were used. GFP (1:6000), anti-p24 (1:4000) and GAPDH (1:4000). The following day the nitrocellulose membrane was washed three times with PBS and secondary antibody added (1:10000 in PBS). After an hour incubation at room temperature while shaking, a final wash was performed (3 x with PBS). Proteins blots were developed with the Pierce® ECL Western Blot Detection Kit (Thermo scientific) in a dark room environment on Fuji medical x-ray film (Fujifilm).

3.18 Determination of viral infectivity

In order to determine the viral titre after transfection supernatant from transfected cells were titred onto TZM-bl cells. TZM-bl cells (1 x 10^4) were seeded the day before use in a 96-well cell culture plate (Nunc) and allowed to grow overnight at 37°C with a constant (5%) CO_2 level in a Heraeus® CO_2-Auto-Zero incubator (Heraeus). The next day 1 ml of supernatant from the transfected HEK 293T cells was centrifuged at 1500 rpm for 5 minutes at room temperature in a centrifuge. From the supernatant 100 µl was titrated per well on the TZM-bl plate and diluted down (1:10; 1:100 and 1:1000). The rest of the supernatant was stored at -80°C for future use. Two days later cells were fixed with methanol and acetone (1:1) for 5 minutes, washed 3 x with PBS and stained with X-gal staining buffer:

X-gal staining buffer

2.5% X-gal (Sigma-Aldrich) in Dimethylformamide (DMF) (Roth)
2 mM $MgCl_2$ (Sigma-Aldrich)
4 mM potassium ferricyanide ($K3[Fe(CN)_6]$) (Sigma-Aldrich)
4 mM potassium ferrocyanide ($K4[Fe(CN)_6]$) (Sigma-Aldrich)
Fill to desired volume with PBS.

Infectious HIV-1 cells turn blue because of the LacZ promoter is induced by HIV-1 tat. As a control GFP containing cells were stained with 4% paraformaldehyde and quantified under the fluorescent microscope. The Multiplicity of Infectivity (MOI) was determined for each set of transfection reactions to use in growth infectivity assays. To determine virus growth kinetics MT-4 cells or PBMCs were infected with an MOI of 0.05 and cells were cultured for up to 8 days. Fresh media was added every 4 days.

Chapter four

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Phylogenetic Diversity and Low Level Antiretroviral Resistance Mutations in HIV Type 1 Treatment-Naive Patients from Cape Town, South Africa

Graeme B. Jacobs,[1,2] Annette Laten,[1] Estrelita Janse van Rensburg,[3] Jochen Bodem,[2] Benedikt Weissbrich,[2] Axel Rethwilm,[2] Wolfgang Preiser,[1] and Susan Engelbrecht[1]

Abstract

We analyzed the HIV-1 *pol* gene from patients in Cape Town to determine the genetic diversity of HIV-1 in the region and to assess the baseline HIV-1 resistance level of treatment-naive patients. Plasma was collected prior to the national antiretroviral therapy (ART) program. RNA was extracted, followed by RT-PCR and automated DNA sequencing of the viral protease (PR) and reverse transcriptase (RT) coding region. Genotyping was done through phylogenetic analysis. The sequences were inspected for resistance-associated mutations against PR and RT inhibitors. A total of 140 *pol* sequences were analyzed, of which 133 (95%) belong to HIV-1 subtype C, five (3.6%) were subtype B, and one each was subtype G and CRF02_AG. Five sequences (3.6%) had resistance-associated mutations. These include three (2.1%) NNRTI mutations. With the progression of the national ART program, it is important to monitor the resistance profile of naive and treatment-experienced patients.

By the end of 2007 approximately 33.2 million people were living with HIV and an estimated 2.5 million new infections were acquired in the year. An estimated 2.1 million lives were lost due to HIV/AIDS. Sub-Saharan Africa remains the worst affected geographic area. There are currently approximately 22.5 million individuals in this region infected with HIV-1.[1] In South Africa, where about 6.3 million people are infected with HIV, HIV/AIDS has become the leading cause of death.[2] Current therapeutic treatment regimes against HIV-1 include protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), as well as viral entry inhibitors.[3] As a result of selective pressure from these antiretroviral drugs HIV-1 develops mutations that cause the failure of antiretroviral therapy (ART) regimens. In the South African public sector first line ART consists of two NRTIs, stavudine (d4T) and lamivudine (3TC), and either efavirenz (EFV) or nevirapine (NVP) as an NNRTI. Second line therapy consists of two NRTIs, didanosine (ddI) and zidovudine (AZT), and either lopinavir or ritonavir as PIs.[4] In the districts of Cape Town the ART rollout program was implemented as early as 2001, especially in resource-limited settings.[5] This was followed by the national rollout program in 2004. There are approximately 213,000 people benefiting from the national program.[6] Therapy is usually initiated in individuals who have a CD4 cell count of 200 cells/mm^3 or WHO stage IV AIDS-defining illness. Previous studies have shown that as many as 10% of the untreated HIV-1-infected population may have strains resistant to current available therapeutic agents. With indications that HIV-1-resistant strains might preexist before the start of ART,[7,8] it will become crucial to monitor HIV-1 diversity and the emergence of new resistant strains in the population. During this study we analyzed the *pol* protease (PR) and reverse transcriptase (RT) coding region of HIV-1 for genetic diversity and also for any possible baseline-resistant associated mutations against PIs, NRTIs, and NNRTIs.

EDTA blood samples from 140 patients were received from 14 different clinics from the Cape Metropole area of South Africa during the period of 2002 to 2004. Our cohort was obtained from an academic hospital clinic, private clinics, state clinics, the Western Province Blood Transfusion Service (WPBTS), and a sex worker cohort in Cape Town. Two of the clinics were near long distance truck routes. The cohort samples represent different ethnic groups and consist of heterosexuals, homosexuals, bisexuals, and mother-to-

[1]Division of Medical Virology, National Health Laboratory Service (NHLS) and University of Stellenbosch, Tygerberg 7505, Cape Town, South Africa.
[2]Institute for Virology and Immunobiology, University of Würzburg, 97078 Würzburg, Germany.
[3]Department of Oral Pathology, School of Dentistry, Faculty of Health Sciences, University of Pretoria, Pretoria 0002, South Africa.

child transmission (MTCT)-infected individuals. These include 81 African females, 34 African males, 5 white males, 1 white female, as well as 8 males and 11 females of mixed race. All individuals stated that they were drug naive.

Plasma was collected after centrifugation and subsequently stored at −80°C for analysis. RNA was extracted using the high-throughput M1000 robot extractor (Abbott Diagnostics). Reverse transcriptase polymerase chain reaction (RT-PCR) amplification was performed on the *pol* PR and RT coding region of HIV-1 using primers previously described.[9] The Access RT-PCR system (Promega, Madison, WI) was used for cDNA synthesis and first round PCR amplification, followed by a nested PCR using the Expand High Fidelity PCR system (Roche Diagnostic, Mannheim, Germany). Positive amplification products were sequenced with an automated ABI prism 3100 Genetic Analyzer using the BigDye Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, Foster City, CA). Sequence contigs were edited, assembled, and aligned using the Lasergene Seqman and MegAlign software packages (DNASTAR Inc., Madison, WI). The TreeconW software package was used to generate neighbour-joining phylogenetic trees using the Kimura two-parameter, followed by bootstrap analysis of 1000 replicates.[10] Reference sequences were obtained from the Los Alamos National Laboratory database (http://www.hiv.lanl.gov). Genotyping was performed using the REGA HIV-1 subtyping tool[11] as well as the HIV-1 BLAST analysis tool (http://www.hiv.lanl.gov).

The PR and RT-derived sequences were screened for mutations associated with drug resistance with the HIVdb Drug Resistance Interpretation program version 4.3.2 on the HIV database maintained by Stanford University (http://hivdb.stanford.edu/index.html). We also used the Calibrated Population Resistance (CPR) tool Version 3.0 beta for both alignment sets to confirm the prevalence of drug resistance.

A total of 140 partial *pol* sequences were analyzed during the study. These include 81 samples for which both the PR and RT coding region sequences were available. The 920 basepair region covers the HIV-1 genome between 2246 and 3165 basepairs, relative to the HXB2 reference strain. Besides the 81 complete *pol* PR and RT sequences, an additional 49 PR (region 2247–2639) and 10 RT (region 2544–3165) sequences were analyzed. Subtyping was confirmed through BLAST analyses, the Rega HIV-1 subtyping tool, and phylogenetic analysis. All the sequences are presented on the neighbor-joining phylogenetic trees in Fig. 1a (PR tree) and Fig. 1b (RT tree). The trees represent HIV-1 group M reference sequences and bootstrap values greater than 75% are indicated. The CRF02_AG reference sequences were included in Fig. 1a to analyze possible subtype G and AG recombinant strains. There are 127 sequences that cluster with subtype C reference sequences, four with subtype B, one with CRF02_AG, and one with subtype G in the figure. With the BLAST analysis and Rega HIV-1 subtyping tool TV1679 was confirmed to belong to subtype G while TV1612 was also confirmed to belong to CRF02_AG. In Fig. 1b there are 87 RT sequences that cluster with the subtype C reference sequences and four that cluster with the subtype B reference sequences. Of the 140 samples analyzed, a total of 133 sequences (95.0%) belong to HIV-1 subtype C, five (3.6%) sequences that belong to subtype B, and one (0.7%) each of subtype G and CRF02_AG.

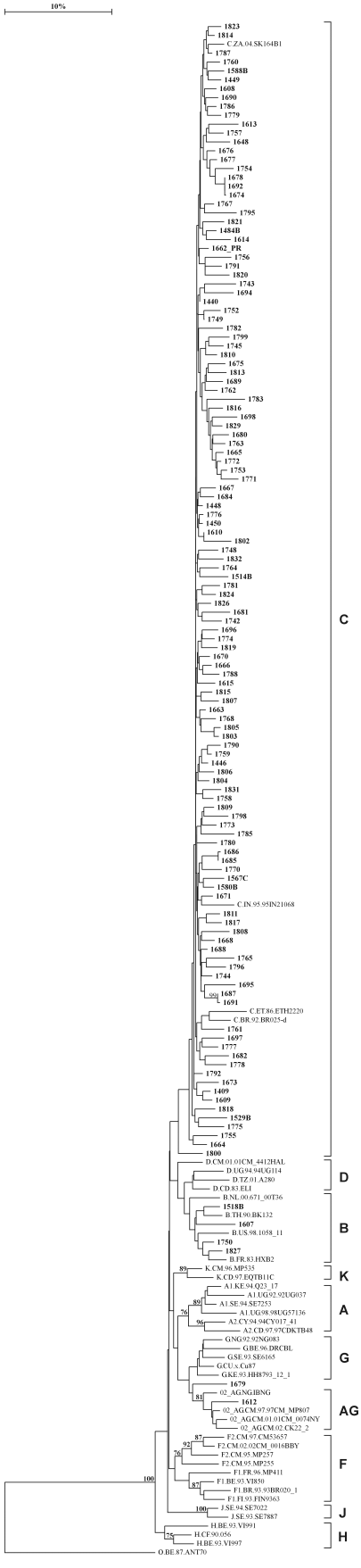
The *pol* PR and RT sequences were analyzed for drug-resistant-associated mutations on the HIV drug resistance database maintained by Stanford University (http://hivdb.stanford.edu), last accessed 10 January 2008. Five (3.6%) of the cohort samples had drug-associated mutations. Three samples had NNRTI mutations. The NNRTI mutations can cause possible resistance to nevirapine (NVP), EFV, and delavirdine (DLV). These include the major mutations Y181C and Y188C as well as the minor F227L mutation. Y181C can lead to an increased susceptibility of AZT and tenofovir (TDF). It occurs in more than 5% of patients with virologic failure and also reduces EFV susceptibility. The Y188H/C mutation reduces NVP susceptibility and is less common than the Y188L mutation. Y188L is found in 5% of patients receiving NVP and failing therapy. The F227L mutation usually occurs with V106A and in combination reduces NVP susceptibility. Other minor mutations identified include the V118I NRTI mutation as well as the L33I PI mutation. V118I is found in more or less 2% of untreated persons. It is associated with low-level resistance to 3TC when it appears with the E44A/D mutation and multiple thymidine analogue mutations (TAMs). The L33I polymorphism increases in frequency in persons receiving PIs. Typically an L33F mutation causes resistance to ritonavir (RTV), amprenavir (APV), lopinavir (LPV), tipranavir (TPV), darunavir (DRV), and atazanavir (ATV). A wide variety of polymorphisms not currently associated with any form of resistance were also observed. These include the PI polymorphisms K20M/R (n = 34, 27.4%), M36I (n = 105, 84.7%), L63P/V (and numerous others) (n = 60, 42.9%), V77I (n = 14, 11.3%), and I93L (n = 122, 98.4%).

HIV-1 group M subtype C is currently the most prevalent subtype in the Cape Metropole of South Africa. This was confirmed by the analysis of 140 HIV-1 sequences obtained from the region. Five percent of the sequences identified comprise other HIV-1 subtypes. HIV-1 diversity may have future implications for viral pathogenesis, viral diagnostic assays, as well as antiretroviral resistance testing as previously described.[12]

Very little is currently known about the impact of large scale ART on the resistance profile of non-B subtypes. Only a few studies have investigated the impact of HIV-1 diversity on drug resistance in South Africa.[8,13,14] In general, primary resistance mutations among HIV-1 subtype C treat-

FIG. 1. A neighbor-joining HIV-1 *pol* PR (**a**) and RT (**b**) phylogenetic tree. Reference sequences, spanning the *pol* PR and RT coding region of HIV-1, were obtained from the HIV-1 database (http://www.hiv.lanl.gov/). The sequences represent HIV-1 group M subtypes A1, A2, B, C, D, F1, F2, G, H, J, and K as well as CRF02_AG. The trees were drawn with the TreeconW software package. A distance scale is included and the significance of the tree was tested by performing bootstrap analysis with 1000 replicates. Values greater than 75% are indicated. The majority (n = 133, 95%) of sequences from Cape Town, South Africa cluster with the HIV-1 subtype C reference sequences. Five subtype B (3.6%) as well as one (0.7%) G and one (0.7%) CRF02_AG sequences are also presented on the phylogenetic trees.

a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D.TZ.01.A280
D.CD.83.ELI
B.NL.00.671_00T36
B.TH.90.BK132
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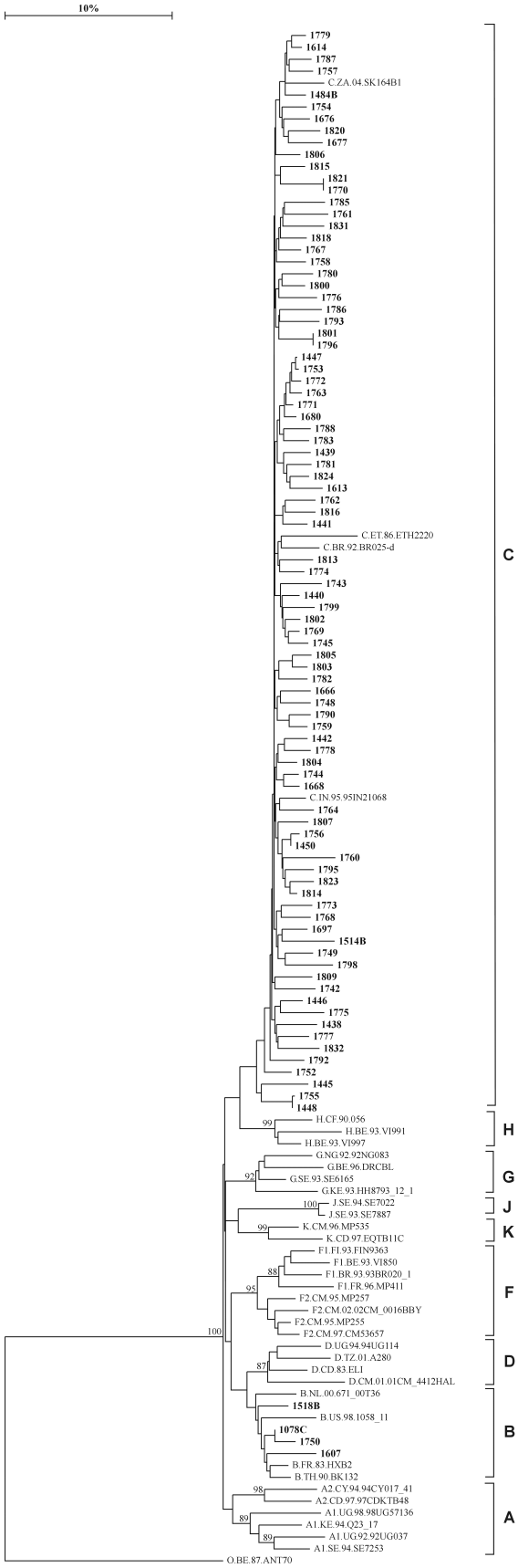
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ment-naive patients seem to be rare in Sub-Saharan Africa. These studies concluded that large-scale resistance testing prior to the onset of ART might not be necessary. However, the impact of various newly identified polymorphisms will have on large-scale treatment is still unknown.

HIV-1 strains associated with drug resistance can either be acquired intrapersonally through selective pressure or transmitted from person to person.[15] During this study HIV-1 sequences from treatment-naive patients were analyzed. Mutations identified include possible low-level resistance to 3TC as well as resistance to NVP, EFV, and DLV. In South Africa HIV-1-infected pregnant women are often treated with a single dose NVP or a combination of 3TC and AZT to prevent or reduce the occurrence of MTCT.[16] The majority of mutations identified occurred in African females of child-bearing age and the possibility exists that these patients received NVP during pregnancy. More concerning, however, is the possibility that the resistant virus was transmitted and is spreading in the population. This is especially important in resource-poor settings where the availability of drug regimens is limited.[16] Persons who begin ART with a preexisting resistant strain transmitted to them have a higher potential of early virologic failure and a higher risk of failing ART.[15]

The HIV-1 genetic diversity in Cape Town, South Africa is not homogeneous. Non-C HIV-1 subtypes are frequently being observed. A small percentage of the HIV-1-infected population we studied also harbors strains that are known to be resistant to some of the current therapeutic reagents. As the South African national ART program progresses, these resistant strains may become more prevalent in the future. It is thus important to continuously monitor for genetic diversity and drug resistance mutations in the HIV-1-infected population in South Africa.

Sequence Data

The sequences reported here were submitted to GenBank and are available under the following accession numbers: EF602162 to EF602301.

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References

1. UNAIDS/WHO working group on global HIV/AIDS and STD surveillance: AIDS epidemic update. Geneva, Switzerland, December 2007 (www.unaids.org).
2. Hosegood V, Vanneste AM, and Timaeus IM: Levels and causes of adult mortality in rural South Africa: The impact of AIDS. AIDS 2004;18:663–671.
3. Johnson VA, Brun-Vezinet F, Clotet B, Conway B, D'Aquila RT, Demeter LM, Kuritzkes DR, Pillay D, Schapiro JM, Telenti A, Richman DD, and the International AIDS Society–USA Drug Resistance Mutations Group: Drug resistance mutations in HIV-1. Top HIV Med 2003;11:215–221.
4. South African Department of Health/Directorate Health Systems Research: National Antiretroviral Treatment Guidelines. Directorate Health Systems Research, Department of Health, Pretoria, South Africa, 2004 (http://www.doh.gov.za/).
5. WHO: Antiretroviral therapy in primary health care: Experience of the Khayelitsha programme in South Africa, case study. World Health Organization, Geneva, Switzerland, 2004 (www.who.int/hiv/amds/case8.pdf).
6. South African Department of Health/Directorate Health Systems Research: National HIV and Syphilus Antenatal sero-prevalence survey in South Africa, 2005. Directorate Health Systems Research, Department of Health, Pretoria, South Africa, 2006 (http://www.doh.gov.za/).
7. Arasteh K, Gölz J, Marcus U, RockStroh J, Salzberger B, and Vielhaber B: Deutsch-Österreichische Leitlinien zur antiretroviralen Therapie der HIV-infektion. Teilaktualisierung, Stand Juni, 2005 (http://www.rki.de/).
8. Bessong PO, Mphahlele J, Choge IA, Obi LC, Morris L, Hammarskjold ML, and Rekosh DM: Resistance mutational analysis of HIV type 1 subtype C among rural South African drug-naive patients prior to large-scale availability of antiretrovirals. AIDS Res Hum Retroviruses 2006;22:306–312.
9. Plantier JC, Dachraoui R, Lemee V, Gueudin M, Borsa-Lebas F, Caron F, and Simon F: HIV-1 resistance genotyping on dried serum spots. AIDS 2005;19(4):391–397.
10. Van de Peer Y and De Wachter R: TREECON for Windows: A software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Applic Biosci 2005;10:569–570.
11. de Oliveira T, Deforche K, Cassol S, Salminen M, Paraskevis D, Seebregts C, Snoeck J, van Rensburg EJ, Wensing AM, van de Vijver DA, Boucher CA, Camacho R, and Vandamme AM: An automated genotyping system for analysis of HIV-1 and other microbial sequences. Bioinformatics 2005;21:3797–3800.
12. Kantor R: Impact of HIV-1 pol diversity on drug resistance and its clinical implications. Curr Opin Infect Dis 2006;19(6):594–606.
13. Gordon M, De Oliveira T, Bishop K, Coovadia HM, Madurai L, Engelbrecht S, Janse van Rensburg E, Mosam A, Smith A, and Cassol S: Molecular characteristics of human immunodeficiency virus type 1 subtype C viruses from KwaZulu-Natal, South Africa: Implications for vaccine and antiretroviral control strategies. J Virol 2003;77(4):2587–2599.
14. Pillay C, Bredell H, McIntyre J, Gray G, and Morris L: HIV-1 subtype C reverse transcriptase sequences from drug-naive pregnant women in South Africa. AIDS Res Hum Retroviruses 2002;18(8):605–610.
15. Shafer RW, Rhee SY, Pillay D, Miller V, Sandstrom P, Schapiro JM, Kuritzkes DR, and Bennett D: HIV-1 protease and reverse transcriptase mutations for drug resistance surveillance. AIDS 2007;21:215–223.
16. Moodley D, Moodley J, Coovadia H, Gray G, McIntyre J, Hofmyer J, Nikodem C, Hall D, Gigliotti M, Robinson P, Boshoff L, Sullivan JL; South African Intrapartum Nevirapine Trial (SAINT) Investigators: A multicenter randomized controlled trial of nevirapine versus a combination of zidovudine and lamivudine to reduce intrapartum and early postpartum mother-to-child transmission of human immunodeficiency virus type 1. J Infect Dis 2003;187:725–735.

Address reprint requests to:
Susan Engelbrecht
Department of Pathology
Division of Medical Virology
P. O. Box 19063
Tygerberg 7505, South Africa

E-mail: susanen@sun.ac.za

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Sequence Note

Molecular Analysis of HIV Type 1 *vif* Sequences from Cape Town, South Africa

Graeme B. Jacobs,[1,2] Markus Nistal,[2] Annette Laten,[1] Estrelita Janse van Rensburg,[3] Axel Rethwilm,[2] Wolfgang Preiser,[1] Jochen Bodem,[2] and Susan Engelbrecht[1]

Abstract

South Africa has the highest number of HIV-1-infected individuals in the world, with HIV-1 subtype C prevailing. However, HIV-1 subtype C accessory genes are rarely characterized in the country. These genes are important for establishing viral pathogenesis. The Vif protein has been shown to counteract the antiretroviral activity of APOBEC3G/F cytidine deaminases. In this study an additional 50 HIV-1 *vif* sequences are characterized. These include 48 HIV-1 subtype C and 2 HIV-1 subtype B sequences. Highly conserved HIV-1 subtype C motifs are outlined. The previously identified RLRR (90–93) motif does not seem to be conserved among our newly analyzed sequences. Conserved motifs can be useful for developing new vaccine strategies or antiretroviral drugs.

South Africa currently has the highest number of people living with HIV/AIDS.[1] The majority of new infections in the country are acquired via heterosexual transmission and HIV-1 subtype C prevails. This subtype is responsible for more than half of all infections worldwide.[2] Therefore recent vaccine development has focused on using HIV-1 subtype C gene domains, such as *gag*, *pol*, *tat*, *env*, and *nef* polypeptides, as part of various vaccine constructs.[3–5] However, very few studies have focused on characterizing the accessory genes of *vif*, *vpr*, and *vpu* of HIV-1 strains in South Africa,[6,7] although these proteins play an important role in viral pathogenesis. The HIV-1 Vif accessory protein counteracts innate antiretroviral activity, especially against the APOBEC3F/G cellular cytidine deaminase family.[8,9] In the absence of Vif, APOBEC3 is incorporated into the virion by direct interaction with the nucleocapsid. During the reverse transcriptase reaction APOBEC3F/G causes cytidine (C)-to-uridine (U) deamination of the first strand, leading to an accumulation of G to A mutations in the proviral sense cDNA strand.[10] Vif interacts in the producer cell with APOBEC3F/G and recruits it to a ubiquitin ligase complex via a cullin-dependent ubiquitin ligase, Cullin 5 (Cul5). APOBEC3F/G is then ubiquitinilated and subsequently degraded by cellular proteasomes. Vif has also been shown to be part of the HIV-1 reverse transcription complex, thus helping control reverse transcription.[11] In this study we describe the molecular analysis of 50 HIV-1 *vif* sequences from HIV-1 patients in Cape Town, South Africa.

The study cohort consists of 28 African females, 14 African males, two white males, as well as three males and three females of mixed race. To amplify the *vif* gene RNA was extracted using the high-throughput M1000 robot extractor (Abbott Diagnostics). Polymerase chain reaction (PCR) amplification was performed using degenerative primers meant to amplify all HIV-1 group M circulating strains in the cohort. Primers HIV-int1 (5′- WWWYKRGTYWRTWMYRGRRWCAGSAGAG-3′) and HIV-INT6A (5′-ATNCCTATNCTGCTATGTYGRCAYCCAAT-3′) were used for cDNA synthesis and initial amplification combined with the Access RT-PCR system (Promega, Madison, WI), using 5 μl as the template input. The following PCR cycle conditions were used: a hot start at 65°C for 30 s followed by reverse transcription at 48°C for 50 min, continued by one cycle of denaturation at 94°C for 2 min and 40 cycles of heat denatura-

[1]Division of Medical Virology, National Health Laboratory Service (NHLS) and University of Stellenbosch, Tygerberg 7505, Cape Town, South Africa.
[2]Institute for Virology and Immunobiology, University of Würzburg, 97078 Würzburg, Germany.
[3]Department of Oral Pathology, School of Dentistry, Faculty of Health Sciences, University of Pretoria, Pretoria 0002, South Africa.

tion at 94°C for 20 s, primer annealing at 50°C for 30 s, and elongation at 68°C for 1 min. The final elongation was done at 68°C for 5 min. A nested amplification using 2–5 μl input was performed with Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany). Primers INT3S (5′-AGMMARSYHCTCTGGAACGGTGAAG-3′) and INT5A (5′-CCTATNCTGCTATGTYGACAACCAATKCTGWAAATG-3′) were used in the nested round of amplification. Cycle conditions were as follows: one cycle of denaturation at 94°C for 2 min followed by 40 cycles of denaturing at 94°C for 30 s, primer annealing at 50°C for 30 s, and elongation at 68°C for 1 min, with a final elongation step at 68°C for 5 min.

The PCR products were purified using the enzymes exonuclease 1 (*Exo*1) and shrimp alkaline phosphatase (SAP) (Amersham Pharmacia Biotech). The BigDye Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, Foster City, CA) was used to directly sequence the purified PCR products with an automated ABI prism 3100 Genetic Analyzer. The Lasergene Seqman and MegAlign software packages (DNASTAR Inc., Madison, WI) were used to edit, assemble, and align the acquired nucleotide and amino acid sequences. A neighbor-joining phylogenetic tree was constructed with the TreeconW for Windows, version 1.3b using the Kimura two-parameter.[12] Conserved regions were highlighted with the Bioedit version 7.0.9.0 and Genedoc version 2.6.002 software packages.

The amplified *vif* genomic region lies between nucleotides 5041 and 5619, relative to the HIV-1 HXB2 reference strain. Phylogenetic analysis of the *vif* gene (576 bp) is shown in Fig. 1. Two sequences (TV1607 and TV1750) clustered with subtype B, whereas the other 48 sequences clustered with subtype C. Subtype C was mainly spread via heterosexual transmission in the cohort ($n = 46$, 95.8%), with two (4.2%) homosexual transmissions recorded. The two subtype B sequences originated from a homosexual white male and a heterosexual male of mixed race.

An alignment with the consensus subtype C sequence compared with the 48 new subtype C sequences and the subtype B Vif amino acid sequences is represented in Fig. 2. Important conserved regions are highlighted. This analysis revealed that the Vif-APOBEC3F/G amino acid interaction domain (40–71) and especially the essential HH motif (42/43) are conserved.[13] Just one sequence (TV1607) contained an amino acid insertion at position 63 (aspartic acid), similar to those found in the subtype F2 reference sequences of unknown function. All known important sites for the Vif-APOBEC3 interaction were found to be conserved.[13] Furthermore, we could show that the region from 100 to 142, including the zinc-binding HCCH motif, which was shown to mediate interaction with Cul5, was conserved in all samples as well.[14]

The S95 and T96 phosphorylation sites, the SLQYLA viral BC box binding motif (144–149), and the PPLP (161–164) Vif dimerization sites are also highly conserved among the *vif* sequences analyzed. Interestingly, the subtype C consensus motif RLRR (90–93) does not seem to be conserved in our sequences, as previously described.[7] The motif is thought to code for a nuclear localization inhibitory signal, allowing the Vif protein to remain in the cellular cytoplasm to effectively perform its functions. HIV-1 mutants with Vif maintained in the nuclease have been shown to have a reduced viral replication capacity and a reduction in the levels of infectivity.[7]

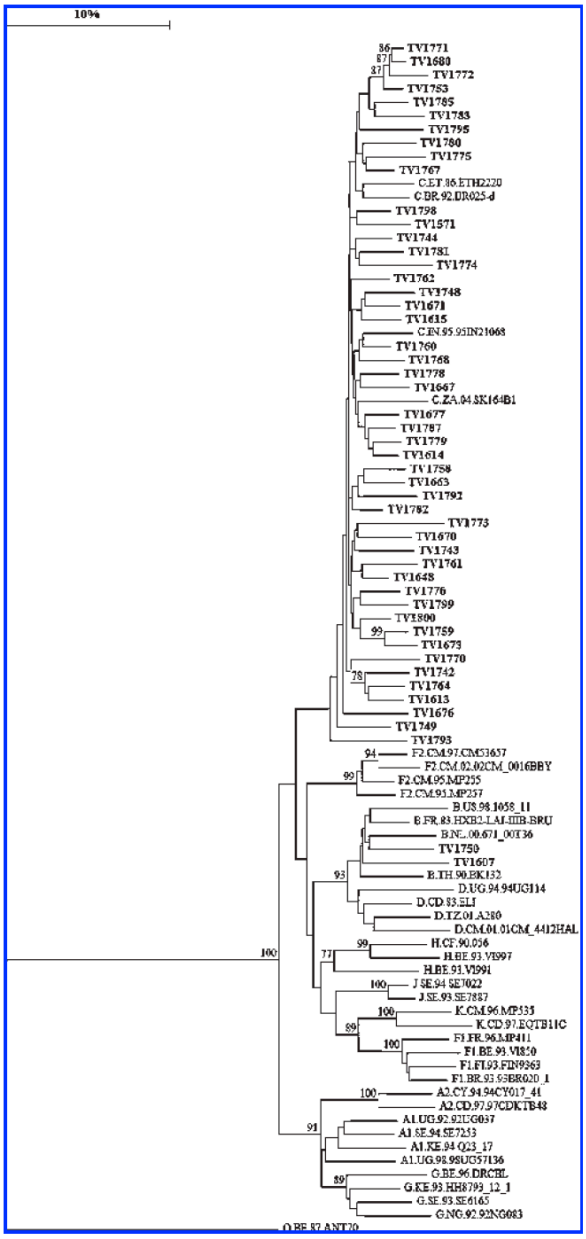

FIG. 1. A neighbor-joining HIV-1 *vif* phylogenetic tree. Reference sequences of HIV-1 group M subtypes A1, A2, B, C, D, F1, F2, G, H, J, and K were obtained from the HIV-1 database (http://www.hiv.lanl.gov/). The tree includes all 50 vif sequences, 48 of which belong to HIV-1 subtype C and two to HIV-1 subtype B. The tree is based on the 576 vif nucleotide sequence, which lies between the genomic region of 5041 and 5619, relative to HXB2. The alignment was created with the MegAlign software packages (DNASTAR Inc., Madison, WI), while the tree was drawn with the TreeconW software package. A distance scale is included with bootstrap values greater than 70% also shown.

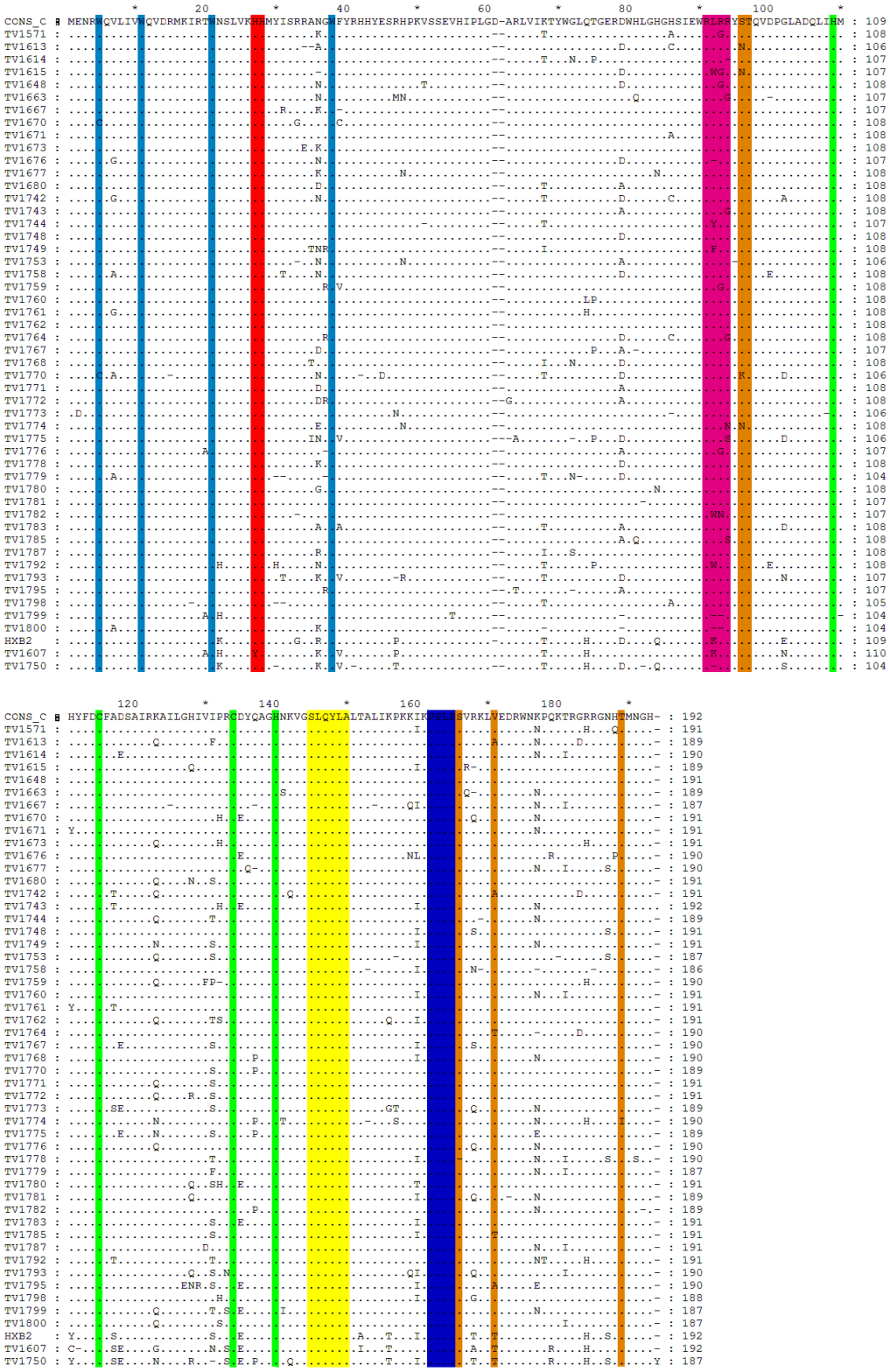

FIG. 2. Alignment of the deduced Vif amino acids from Cape Town, South Africa. The sequences are compared to a consensus subtype C reference sequence and the subtype B HXB2 reference strain is included as well. Dots represent identical residues while dashes represent insertions and deletions. Protein domains and conserved regions are shaded as follows: light blue (1–35), tryptophan-rich stretch, important for APOBEC3G recognition; red (42, 43), conserved Vif APOBEC3F/G interaction domain; pink (90–93), RLRR nuclear localization inhibitory signal; orange, S95, T96, S165, T170V, and T188 phosphorylation sites; green, HCCH (H108, C114, C133, and H139) zinc-binding motif; yellow (144–149), SLQYLA motif involved in APOBEC3F/G antiviral activity interactions; dark blue (161–164), PPLP vif dimerization domain.

All the sequences analyzed contain the S144, S165, and T188 phosphorylation sites, while only two subtype C samples contained the T170 phosphorylation site. Both the subtype B sequences contained the T170 phosphorlation sites, as does the HXB2 reference strain. The Vif protein is usually phosphorylated on these sites by cellular kinases, such as mitogen-activated protein kinase (MAPK). The maintenance of these phosphorylation sites is important for viral replication and helps promote HIV infectivity.[15] One sample (TV1768) has a two amino acid deletion at position 19 (arginine) and 20 (threonine) of its Vif protein of so far unknown function. As these mutations are not in the functional or structural domain motifs, the affect they will have on the Vif protein functionality is uncertain.

The Vif protein of HIV-1 protects the virus against antiviral activity and plays an important role in reverse transcription. The Vif protein and APOBEC3F/G cellular host interactions have been studied extensively.[8,9] Previously analyzed HIV-1 *vif* sequences from South Africa indicate that highly conserved motifs within the Vif protein of HIV-1 subtype C are present.[6,7] However, the previous studies describe only a limited number of new HIV-1 subtype C *vif* sequences, 16 sequences from Scriba *et al*.[6] and 20 sequences from Bell *et al*.[7] The analysis of 50 additional *vif* sequences in this study confirms the presence of these conserved functional motifs. The previously identified RLRR (90–93) motif, however, does not seem to be conserved among the newly analyzed sequences, nor is it conserved among all HIV-1 group M isolates. Therefore more functional studies need to be done to completely understand the role of the Vif protein in viral pathogenesis. Conserved motifs that have been identified can possibly be used for future vaccine development or can be possible targets for new antiviral drugs. It is important to fully characterize all circulating HIV-1 strains in South Africa.

Sequence Data

The sequences reported here were submitted to GenBank and are available under the following accession numbers: EU269071 to EU269459.

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References

1. UNAIDS/WHO working group on global HIV/AIDS and STD surveillance: AIDS epidemic update, December 2007 (www.unaids.org).
2. McCutchan FE: Global epidemiology of HIV. J Med Virol 2006;78:S7–S12.
3. Burgers WA, van Harmelen JH, Shephard E, Adams C, Mgwebi T, Bourn W, Hanke T, Williamson AL, and Williamson C: Design and preclinical evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. J Gen Virol 2006;87:399–410.
4. Harari A, Bart PA, Stöhr W, Tapia G, Garcia M, Medjitna-Rais E, Burnet S, Cellerai C, Erlwein O, Barber T, Moog C, Liljestrom P, Wagner R, Wolf H, Kraehenbuhl JP, Esteban M, Heeney J, Frachette MJ, Tartaglia J, McCormack S, Babiker A, Weber J, and Pantaleo G: An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. J Exp Med 2008;205(1):63–67.
5. Gómez CE, Nájera JL, Jiménez V, Bieler K, Wild J, Kostic L, Heidari S, Chen M, Frachette MJ, Pantaleo G, Wolf H, Liljeström P, Wagner R, and Esteban M: Generation and immunogenicity of novel HIV/AIDS vaccine candidates targeting HIV-1 Env/Gag-Pol-Nef antigens of clade C. Vaccine 2007;12;25(15):2863–2885.
6. Scriba TJ, Treurnicht FK, Zeier M, Engelbrecht S, and van Rensburg EJ: Characterization and phylogenetic analysis of South African HIV-1 subtype C accessory genes. AIDS Res Hum Retroviruses 2001;17(8):775–781.
7. Bell CM, Connell BJ, Capovilla A, Venter WD, Stevens WS, and Papathanasopoulos MA: Molecular characterization of the HIV type 1 subtype C accessory genes vif, vpr, and vpu. AIDS Res Hum Retroviruses 2007;23(2):322–330.
8. Arriaga ME, Carr J, Li P, Wang B, and Saksena NK: Interaction between HIV-1 and APOBEC3 sub-family of proteins. Curr HIV Res 2006;4(4):401–409.
9. Soros VB and Greene WC: APOBEC3G and HIV-1: Strike and counterstrike. Curr Infect Dis Rep 2006;8(4):317–323.
10. Malim MH: Natural resistance to HIV infection: The Vif-APOBEC interaction. C R Biol 2006;329(11):871–875.
11. Carr JM, Coolen C, Davis AJ, Burrell CJ, and Li P: Human immunodeficiency virus 1 (HIV-1) virion infectivity factor (Vif) is part of reverse transcription complexes and acts as an accessory factor for reverse transcription. Virology 2008;1;372(1):147–156.
12. Van de Peer Y and De Wachter R: TREECON for Windows: A software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Appl Biosci 1994;10:569–570.
13. Mehle A, Wilson H, Zhang C, Brazier AJ, McPike M, Pery E, and Gabuzda D: Identification of an APOBEC3G binding site in human immunodeficiency virus type 1 Vif and inhibitors of Vif-APOBEC3G binding. J Virol 2007;81(23):13235–13241.
14. Mehle A, Thomas ER, Rajendran KS, and Gabuzda D: A zinc-binding region in Vif binds Cul5 and determines cullin selection. J Biol Chem 2006;23;281(25):17259–17265.
15. Yang X and Gabuzda D: Mitogen-activated protein kinase phosphorylates and regulates the HIV-1 Vif protein. J Biol Chem 1998;273(45):29879–29887.

Address reprint requests to:
Susan Engelbrecht
Department of Pathology
Division of Medical Virology
P.O. Box 19063
Tygerberg 7505, South Africa

E-mail: susanen@sun.ac.za

Construction of a high titer Infectious and novel HIV-1 subtype C proviral clone from South Africa, pZAC.

Graeme B. Jacobs[1], Anita Schuch[1], Tanja Schied[1], Wolfgang Preiser[2], Axel Rethwilm[1], Eduan Wilkinson[2], Susan Engelbrecht[2] and Jochen Bodem[1*]

[1]Institute for Virology and Immunbiology, University of Würzburg, Versbacher Strasse 7, 97078 Würzburg, Germany

[2]Division of Medical Virology, Stellenbosch University and NHLS, Tygerberg, 7505, Cape Town, South Africa

Summary

Human Immunodeficiency Virus type 1 (HIV-1) subtype C, spread mainly via heterosexual transmission is currently the most predominant HIV-1 subtype worldwide. Cell culture studies of Sub-Saharan African subtype C proviral clones (pMJ4 and pHIV1084i) are hampered by their low replication capacity. We describe here the modification of pMJ4, leading to a proviral clone with a higher replication rate in cell culture. Furthermore, an early primary HIV-1 subtype C isolate from Cape Town, South Africa is characterized and a new infectious subtype C proviral clone (pZAC) is created. The new pZAC clone has a higher viral titer than the original pMJ4 clone. Characteristics of pZAC Env gp120, such as a shortened compact V1 loop and an elongated V4 sequence, favors an enhanced viral replication rate *in vitro*. The newly characterized infectious HIV-1 subtype C clone, pZAC can be useful for future *in vitro* studies.

The UNAIDS estimates that there are currently 33.3 million people infected with HIV/AIDS worldwide. Since 1999 the number of new infections has fallen by approximately 19%, with more than 5.0 million people now receiving antiretroviral therapy. Sub-Saharan Africa remains the most severely affected region with approximately 22.5 million people infected, which accounts for 68% of the global burden. Although the rate of new infections in Sub-Saharan Africa has steadily decreased, 1.8 million in 2009 compared to 2.2 million in 2001, the total number of people with new diagnosed infections in this region continues to rise (UNAIDS, 2011).

The genetic subtype distribution of HIV-1 group M, currently responsible for the majority of the AIDS pandemic, has become dynamic. Currently HIV-1 group M has been divided into 9 subtypes (A-D, F, G-H, J, K), 49 circulating recombinant forms (CRFs) and numerous unique recombinant forms (URFs). In 2004-2007, subtype C accounted for nearly half (48%) of all global infections, followed by subtypes A (12%), B (11%) and CRF02_AG (8%) (Hemelaar *et al*., 2011).

HIV-1 subtype C is predominant in Eastern and Southern Africa as well as in India. It accounts for approximately 95% of all HIV-1 infections in Southern Africa (Jacobs *et al*., 2009). It is also increasing in frequency in countries such as China and Brazil (Hemelaar *et al*., 2011). HIV-1 subtype C has unique characteristics, which distinguishes it from other subtypes. These include the presence of an extra NF-κß enhancer binding site in the long terminal repeat (LTR), a prematurely truncated Rev protein and a 5 amino acid insertion of the 5'end of the Vpu open reading frame (McCormick-Davis *et al*., 2000; Rodenburg *et al*., 2001). Curiously, these differences on a molecular level result in a lower replication-fitness in primary CD4+ T cells and peripheral blood mononuclear cells (PBMCs), making it more difficult to study *in vitro* (Ariën *et al*., 2005). Recently Iordanskiy *et al*., (2010) suggested that characteristics of the reverse transcriptase (RT) polymerase domain of HIV-1 subtype C strongly affect the replication capacity of these viruses in cell culture, compared to that of HIV-1 subtype B. However, these genetic differences do not seem to influence the transmission efficiency of subtype C viruses *in vivo*. In addition, HIV-1 subtype C has a relatively high transmission fitness in dendritic cells (Ball *et al*., 2003).

Almost all *in vitro* HIV-1 cell culture assays are based on HIV-1 subtype B strains. The infectious proviral subtype B strains (for example pNL4-3) have been used in HIV-1 studies

for the last 25 years of HIV research (Adachi *et al*., 1986). Only recently has the focus shifted in developing HIV-1 antiretroviral reagents targeting multiple strains of HIV-1 (Hemelaar *et al*., 2011).

There are currently four HIV-1 subtype C proviral infectious clones described, two from Sub-Saharan Africa (Grisson *et al*., 2004; Ndung'u *et al*., 2001) and two from India (Dash *et al*., 2008; Mochizuki *et al*., 1999). During the course of this study we cultured a HIV-1 subtype C isolate from Cape Town, South Africa using peripheral blood mononuclear cells (PBMCs). We characterized the full-length sequence and used the strain to improve the replication capacity of a previously characterized infectious HIV-1 subtype C proviral molecular clone pMJ4, originating from Botswana (Ndung'u *et al*., 2001).

The pMJ4 proviral clone has a low replication rate and grows slowly on PBMCs, although it replicates better in CCR5 cell lines (Ndung'u *et al*., 2001). After transfection of Human Embryonic Kidney (HEK) 293 T-cells, with further titration onto the TZM-Bl indicator cell line, which is dependent on Tat-activation of the HIV-1 LTR (Wei *et al*., 2002), pMJ4 has significant lower viral titers compared to pNL4-3 (Fig. 1c). To improve the initial gene expression the 5'-U3 was replaced with the CMV-IE promoter by PCR, as previously described for NL4-3 (Bohne & Kräusslich, 2004), using restriction sites *NgoM*IV and *Spe*I. The resulting plasmid was abbreviated as pcMJ4. The CMV-IE-promoter has been shown to enhance lentiviral expression and is frequently used as a promoter in plasmid expression vectors (Bohne & Kräusslich, 2004). Transfection of HEK 293T cells, with further titration on TZM-Bl cells showed that pcMJ4 produced a 4-fold higher viral titer than the parental MJ4 plasmid (Fig. 1c). In order to compare Gag amounts of pcMJ4 with pMJ4 a p24 Western Blot analysis was performed (Hartl *et al*., 2011) and pcMJ4 clearly showed an increase in Gag expression levels (Fig. 1d). Hence, the pcMJ4 clone was used further as a HIV-1 subtype C expression control plasmid.

To further establish a fast replicating HIV-1 subtype C proviral clone, the HIV-1 positive sample from patient ZAC (previously named R3714, supplementary data; Engelbrecht *et al*., 1995) was obtained from one of the earliest documented cases of heterosexual transmission of HIV-1 subtype C in South Africa, during 1989. The primary isolate was cultured in PBMCs and HIV-1 positive high molecular weight (HMW) DNA stored for further analysis (Engelbrecht *et al*., 1995). We first replaced the *env* of MJ4 with that of our primary isolate,

ZAC using standard cloning techniques and a proofreading Herculase II polymerase (Stratagene) (Fig. 1a). The 3.2 kb PCR product was amplified from the HMW DNA of ZAC with primers containing the restriction enzyme recognition sites for *Pac*I and *BspE*I. This corresponds to position 6198 and 9393 relative to the reference HXB2 genome. Clones were screened by restriction enzyme digestion and sequenced to confirm the presence of the correct insert. Forty-eight hybrid clones were transfected and titrated on TZM-Bl cells as described above. However, only 5 (10.4 %) of proviral clones produced significant viral titers. The 5 clones are identical in sequence and showed approximately a 10 fold increase in viral titer after titration on TZM-Bl, compared to that of pcMJ4 (Fig. 1c), confirmed by Gag p24 Western Blot analysis (Fig. 1d). The clone was designated pcMJ4/ZACenv. We decided to use pcMJ4/ZACenv to create the new infectious subtype C pZAC proviral clone.

The 5' fragment of ZAC was amplified in two further parts encompassing the *gagpol* and LTR-*gag* region (Fig. 1a). The *gagpol* region was replaced using restriction sites *Spe*I (corresponding to position 1507 of HXB2) and *Pac*I (corresponding to position 6198 of HXB2), while the CMV-IE LTR-*gag* sequence from ZAC was added as for pcMJ4. The new proviral clone was designated pcZAC. The 3'-U5 was replaced using *BspE*I and the vector located *Not*I restriction site and the 5'-U3 CMV-IE was replaced with the ZAC derived 5'-U3 sequence. The final clone (without the CMV-IE promoter) was named pZAC. The full-length pcZAC and pZAC clones have slightly lower viral titers on TZM-Bl cells compared to the pcMJ4-ZACenv proviral clone (Fig. 1b). The sequence from pZAC has typical African HIV-1 subtype C characteristics. These include 3 Nf-κB sites in the LTR, a premature Rev stop codon and a 5 amino acid insertion in the Vpu transmembrane domain. The Indian subtype C strains lack this 5 amino acid insertion.

The full-length ZAC sequence on nucleotide level was found to be 91.9% similar to HIV1084i and 91.4% similar to MJ4; 91.6% compared to Indie_C1 and 89.3% compared to D24. To analyse the phylogenetic relationships of the full-length infectious HIV-1 subtype C clones a neighbour-joining phylogenetic tree was constructed with Mega version 5.0 using the Maximum Composite Likelihood method (Fig. 1b) (Tamura *et al*., 2011). The Indian and Africa HIV-1 subtype C strains exhibited two unique phylogenetic clusters (Fig. 1b). The new ZAC sequence had a close phylogenetic relationship with the HIV-1 subtype C sequences from Sub-Saharan Africa.

An alignment of the Env gp120 is given in Fig. 2. The ZAC Env gp120 sequence were 80.8% similar to MJ4, 79.2% to HIV1084i, 78.2% to Indie_C1 and 76.2% to D24. The sequences had a two amino acid insert on position 24 and 25 in the hydrophobic core of the signal peptide sequence, not seen in the MJ4 and HIV1084i African strains, although the Indian infectious sequences have a similar insert. Furthermore, ZAC had a shortened, very compact V1 loop similar to HIV1084i and a slightly larger V4 loop compared to MJ4 (3 amino acids) and HIV1084i (7 amino acids). Furthermore, ZAC also had more potential N-linked glycosylation sites (26), compared to that of MJ4 (23) while NL4-3 had 24 and Indie-C1 had 27 and D24 had 31.

We cultivated our infectious viruses on PBMCs for the indicated time points, initial multiplicity of infection (MOI) of 0.05, for up to 8 days (Fig. 3). pMJ4/ZACenv and pZAC grew significantly better in PBMCs than the original MJ4 clone. For virus titer we infected TZM-Bl cells as described above. pZAC peaks at days 4-6, similarly as described for the Indian HIV-1 subtype C clones (Dash *et al*., 2008; Mochizuki *et al*., 1999). However, MJ4 (as well as HIV1084i, Grisson *et al*., 2004) only peaks in PBMC cell culture at days 8-12. With the ZAC *env* sequence, the pMJ4 strain also peaks at days 4-6, although the viral titer is not as high as NL4-3 (Fig. 3).

HIV-1 subtype C remains the predominate subtype worldwide and is especially prevalent in Sub-Saharan Africa, where the HIV/AIDS epidemic is at the highest. However, *in vitro* studies with infectious proviral HIV strains have shown that HIV-1 subtype C generally has a lower replication rate compared to that of the infectious HIV-1 subtype B (pNL4-3) strain (Grisson *et al*., 2004; Ndung'u *et al*., 2001). This is also true for other non-subtype B strains and it has been difficult to obtain infectious proviral molecular clones from primary HIV-1 isolates for non-subtype B strains. It has also been reported that HIV-1 subtype C may have lower levels of pathogenic fitness when compared to other HIV-1 group M strains (Abraha *et al*., 2009).

The nucleotide sequence diversity between *env* genes in the same subtype can range from a few percent to as high as 15% (Gaschen *et al.*, 2002). HIV-1 subtype C Env gp120 has unique characteristics distinguishing it from other HIV-1 strains. It has the most compact V1-V2 described sequences of all the HIV-1 strains. It also has a relatively conserved Env gp120 V3 loop containing the well-preserved GPGQ motif on the tip of the V3 crown, with the

corresponding virus showing preference to using CCR5 as its major co-receptor irrespective of the stage of disease progression (Ariën *et al.*, 2005).

With the exchange of pZAC *env* sequence in pMJ4 we could improve the infectivity and replication rate of the original subtype C proviral clone. The pZAC *env* gp120 sequence has a shorter V1 sequence than that of the described infectious HIV-1 subtype C clones, subsequently making the V1 loop more compact. Walter *et al*., (2009) previously described that the shortening of the V1 loop in HIV-1 strains increases viral interactions with CD4, leading to more stable binding of the virus with CD4, which enhances viral entry and subsequently improves viral infectivity. Glycosylation patterns of the V2 loop facilitate *env* interactions with CD4 and CCR5 and have been shown to affect viral replication kinetics. Thus, the higher number of glycosylation sites in the newly described *env* gp120 sequence, compared to that of MJ4, may also play a role in improving viral infectivity as seen in the *in vitro* cell culture assays (Ly & Stamatatos, 2000).

The ZAC sequence also has a larger V4 region, compared to the other infectious African subtype C strains. In this respect it is more similar to the Indian C-type strains. Larger V4 sequences may play an important role in the ability of infectious viruses to bind more efficiently to co-receptor molecules and it has been shown that they probably enhance the repertoire of co-receptor usage of HIV-1 subtype C (Walter *et al*., 2009). The replication kinetics of the new infectious pZAC clone is much higher for a Subtype C strain of African origin than previously described.

The gold standard to study and to develop antiviral drugs against HIV-1 diversity remains *in vitro* cell culture assays. In Europe and America this has ultimately been based on the HIV-1 subtype B infectious strains. As HIV-1 subtype C predominate in Sub-Saharan Africa we aimed to construct an infectious subtype C molecular clone representative of strains in this region and which can be used in *in vitro* cell culture assays comparable to that of HIV-1 subtype B. The newly characterized infectious HIV-1 subtype C strain, pZAC should be useful in those studies.

Sequence data

The ZAC sequence reported here was submitted to GenBank and is available under the following accession number: JN188292.

Acknowledgements

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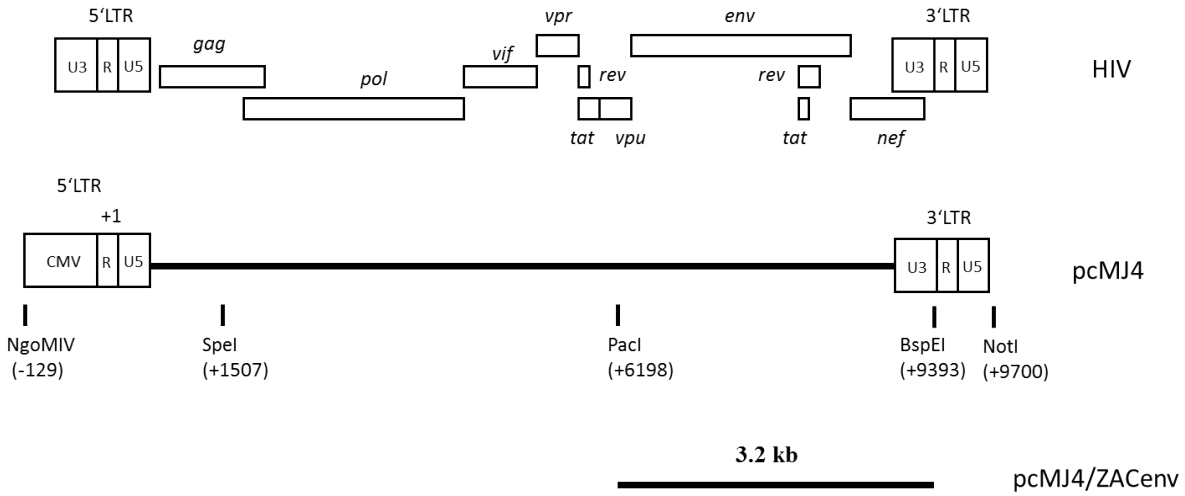


Figure 1A: Modification of pMJ4 with the use of marked restriction enzyme sites.

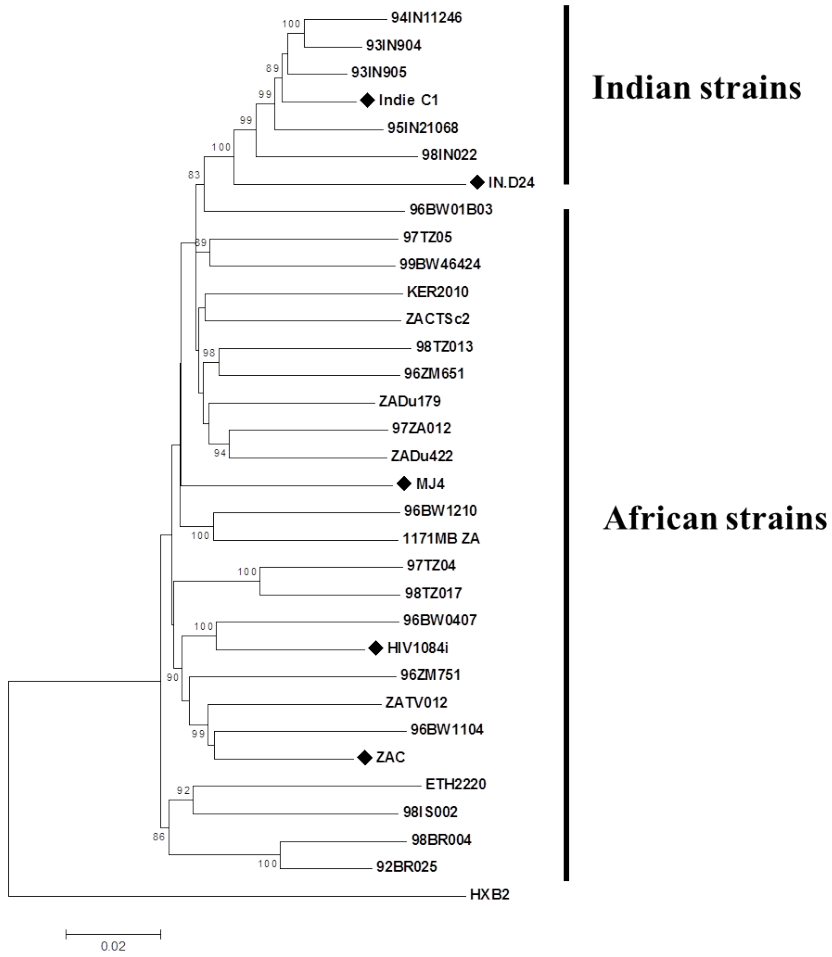


Figure 1B: A neigbour-joining HIV-1 subtype C phylogenetic tree. Infectious clones are indicated with a ♦.

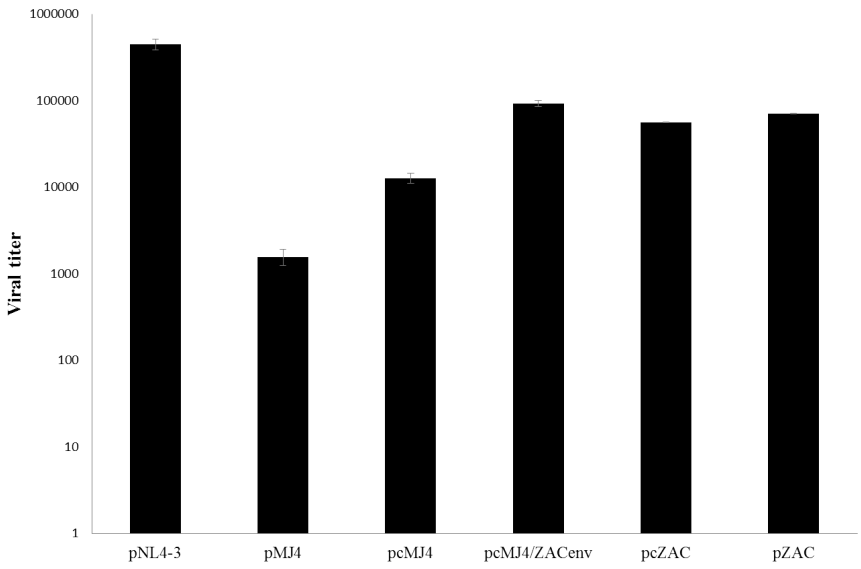


Figure 1C: Transient viral titer on TZM-Bl

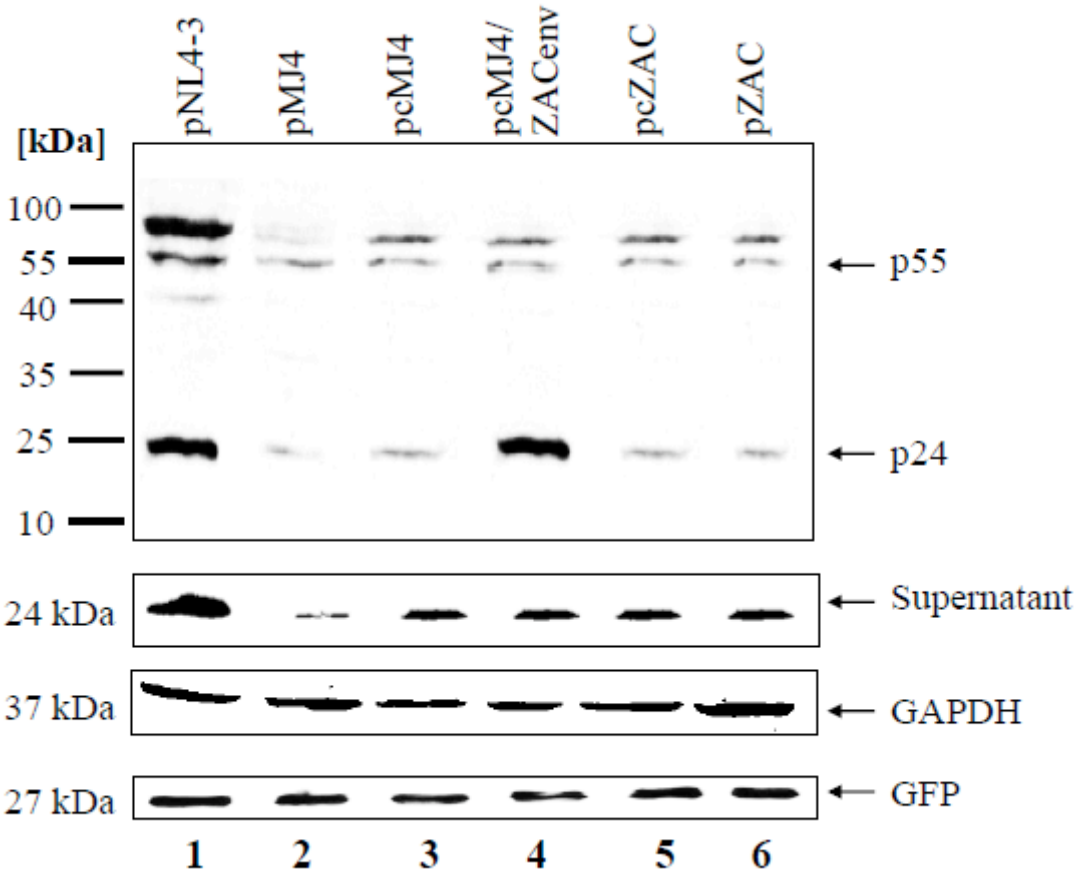
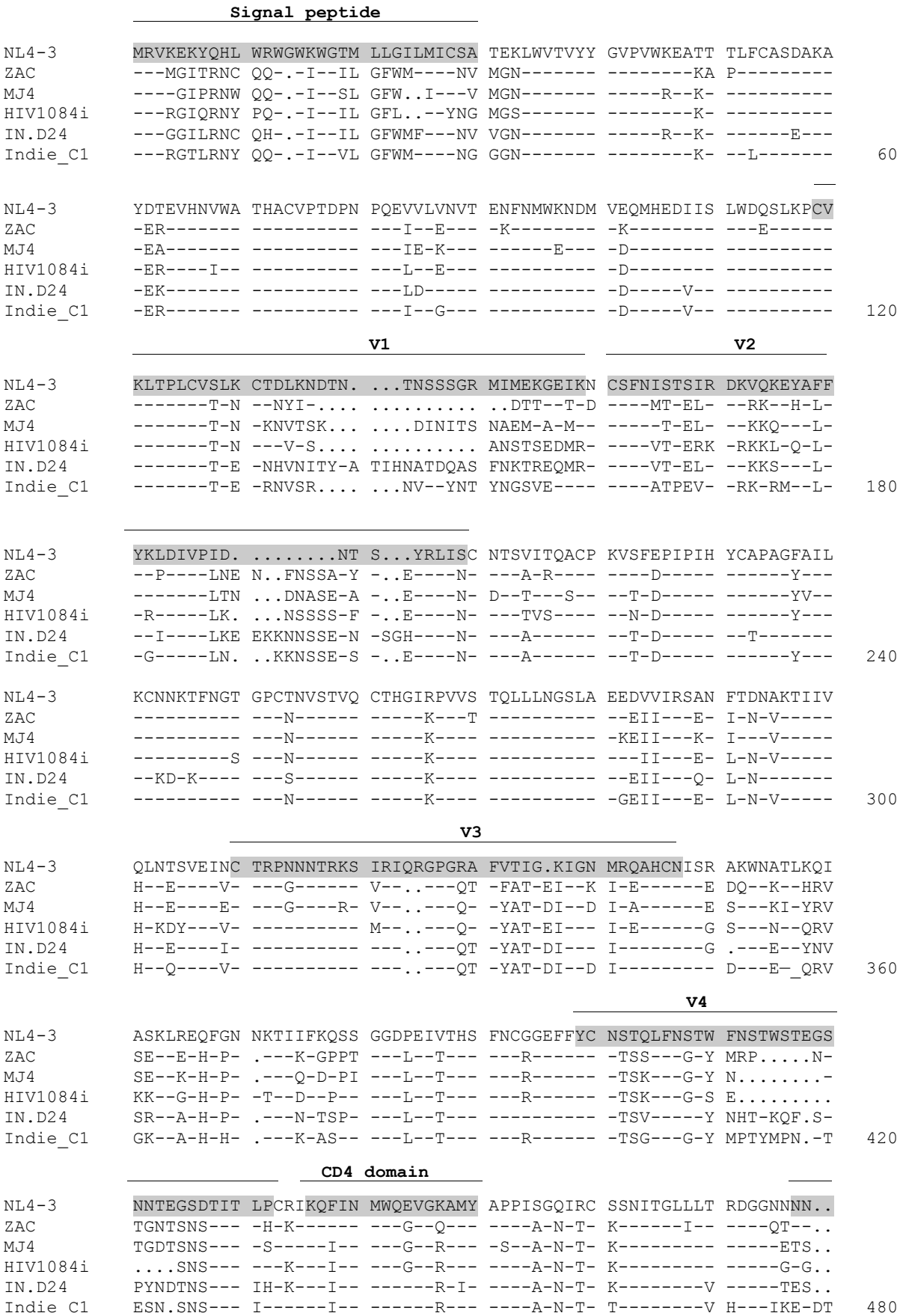


Figure 1D: Transient protein expression of HIV clones.

Fig. 1. Molecular characterization of pZAC, an infectious proviral clone from Cape Town, South Africa. (a) Cloning strategy used during the study. The U3 promoter of pMJ4 was replaced with a CMV-IE promoter, as indicated. The new proviral clone, pcMJ4, was used as a backbone for further characterization of pZAC. The enzyme restriction sites used for cloning are indicated. **(b) Phylogenetic analysis of HIV-1 subtype C infectious clones.** A Neighbour-Joining tree was drawn from the infectious HIV-1 subtype C clones, compared to a HIV-1 subtype C reference set (dataset obtained from BioAfrica.net). Evolutionary distances were calculated using the Maximum Composite Likelihood method, with a bootstrap test of 10000 replicates. The branch scale, indicating the evolutionary distance, is indicated. The Indian and African strains form two unique phylogenetic clusters, with the newly described ZAC sequence showing similarity to the Botswana HIV-1 subtype C sequences. **(c)** and **(d) Transient virus titer on TZM-Bl and Western Blot analyses of infectious proviral clones**. After transfection of HEK 293T cells, cultured supernatant were titrated on HeLa TZM-Bl indicator cells to determine the transient viral titer of HIV proviral clones. HIV-1 Gag was detected with an anti-p24 specific anti-serum. GAPDH was used as loading control while GFP served as indicator of transfection efficiency. The pZAC-derived clones have a higher viral titer on TZM-Bl cells, as confirmed by Western Blot analysis, compared to that of both pMJ4 and pcMJ4. Titers and standard deviation were derived from three independent experiments.


Signal peptide
V1
V2
V3
V4
CD4 domain
NL4-3 MRVKEKYQHL WRWGWKWGTM LLGILMICSA TEKLWVTVYY GVPVWKEATT TLFCASDAKA
ZAC ---MGITRNC QQ-.-I--IL GFWM----NV MGN------- --------KA P---------
MJ4 ----GIPRNW QQ-.-I--SL GFW..I---V MGN------- -----R--K- ----------
HIV1084i ---RGIQRNY PQ-.-I--IL GFL..--YNG MGS------- --------K- ----------
IN.D24 ---GGILRNC QH-.-I--IL GFWMF---NV VGN------- -----R--K- ------E---
Indie_C1 ---RGTLRNY QQ-.-I--VL GFWM----NG GGN------- --------K- --L------- 60
NL4-3 YDTEVHNVWA THACVPTDPN PQEVVLVNVT ENFNMWKNDM VEQMHEDIIS LWDQSLKPCV
ZAC -ER------- ---------- ---I--E--- -K-------- -K-------- ---E------
MJ4 -EA------- ---------- ---IE-K--- ------E--- -D-------- ----------
HIV1084i -ER----I-- ---------- ---L--E--- ---------- -D-------- ----------
IN.D24 -EK------- ---------- ---LD----- ---------- -D-----V-- ----------
Indie_C1 -ER------- ---------- ---I--G--- ---------- -D-----V-- ---------- 120
NL4-3 KLTPLCVSLK CTDLKNDTN. ...TNSSSGR MIMEKGEIKN CSFNISTSIR DKVQKEYAFF
ZAC -------T-N --NYI-.... ....DTT--T-D ----MT-EL- --RK--H-L-
MJ4 -------T-N -KNVTSK... ....DINITS NAEM-A-M-- -----T-EL- --KKQ---L-
HIV1084i -------T-N ---V-S.... .... ANSTSEDMR- ----VT-ERK -RKKL-Q-L-
IN.D24 -------T-E -NHVNITY-A TIHNATDQAS FNKTREQMR- ----VT-EL- --KKS---L-
Indie_C1 -------T-E -RNVSR.... ...NV--YNT YNGSVE---- ----ATPEV- -RK-RM--L- 180
NL4-3 YKLDIVPID. ....NT S...YRLISC NTSVITQACP KVSFEPIPIH YCAPAGFAIL
ZAC --P----LNE N..FNSSA-Y -..E----N- ---A-R---- ----D----- ------Y---
MJ4 -------LTN ...DNASE-A -..E----N- D--T---S-- --T-D----- ------YV--
HIV1084i -R-----LK. ...NSSSS-F -..E----N- ---TVS---- --N-D----- ------Y---
IN.D24 --I----LKE EKKNNSSE-N -SGH----N- ---A------ --T-D----- --T-------
Indie_C1 -G-----LN. ..KKNSSE-S -..E----N- ---A------ --T-D----- ------Y--- 240
NL4-3 KCNNKTFNGT GPCTNVSTVQ CTHGIRPVVS TQLLLNGSLA EEDVVIRSAN FTDNAKTIIV
ZAC ---------- ---N------ -----K---T ---------- --EII---E- I-N-V-----
MJ4 ---------- ---N------ -----K---- ---------- -KEII---K- I---V-----
HIV1084i ---------S ---N------ -----K---- ---------- ---II---E- L-N-V-----
IN.D24 --KD-K---- ---S------ -----K---- ---------- --EII---Q- L-N-------
Indie_C1 ---------- ---N------ -----K---- ---------- -GEII---E- L-N-V----- 300
NL4-3 QLNTSVEINC TRPNNNTRKS IRIQRGPGRA FVTIG.KIGN MRQAHCNISR AKWNATLKQI
ZAC H--E----V- ---G------ V--..---QT -FAT-EI--K I-E------E DQ--K--HRV
MJ4 H--E----E- ---G----R- V--..---Q- -YAT-DI--D I-A------E S---KI-YRV
HIV1084i H-KDY---V- ---------- M--..---Q- -YAT-EI--- I-E------G S---N--QRV
IN.D24 H--E----I- ---------- ---..---QT -YAT-DI--- I--------G .---E--YNV
Indie_C1 H--Q----V- ---------- ---..---QT -YAT-DI--D I--------- D---E-_QRV 360
NL4-3 ASKLREQFGN NKTIIFKQSS GGDPEIVTHS FNCGGEFFYC NSTQLFNSTW FNSTWSTEGS
ZAC SE--E-H-P- .---K-GPPT ---L--T--- ---R------ -TSS---G-Y MRP.....N-
MJ4 SE--K-H-P- .---Q-D-PI ---L--T--- ---R------ -TSK---G-Y N.........
HIV1084i KK--G-H-P- -T--D--P-- ---L--T--- ---R------ -TSK---G-S E.........
IN.D24 SR--A-H-P- .---N-TSP- ---L--T--- ---------- -TSV-----Y NHT-KQF.S-
Indie_C1 GK--A-H-H- .---K-AS-- ---L--T--- ---R------ -TSG---G-Y MPTYMPN.-T 420
NL4-3 NNTEGSDTIT LPCRIKQFIN MWQEVGKAMY APPISGQIRC SSNITGLLLT RDGGNNNN..
ZAC TGNTSNS--- -H-K------ ---G--Q--- ----A-N-T- K------I-- ----QT--..
MJ4 TGDTSNS--- -S-----I-- ---G--R--- -S--A-N-T- K--------- -----ETS..
HIV1084i ....SNS--- ---K---I-- ---G--R--- ----A-N-T- K--------- -----G-G..
IN.D24 PYNDTNS--- IH-K---I-- ------R-I- ----A-N-T- K--------V -----TES..
Indie_C1 ESN.SNS--- I------I-- ------R--- ----A-N-T- T--------V H---IKE-DT 480

```
                V5
NL4-3       ..GSEIFRPG GGDMRDNWRS ELYKYKVVKI EPLGVAPTKA KRRVVQREKR
ZAC         --TN-T---A ---------- --------EV K---L---T- -----E----
MJ4         ---I-----A ---------- --------E- K---L----S -----E----
HIV1084i    --.T------ ---------- ---------- ----I----- -----E-G--
IN.D24      -NNT------ ---------- --------EV K---I---A- -----E----
Indie_C1    ENKT------ ---------- --------E- K-------A- -----E----  530
```

Fig. 2. Amino Acid alignment of Env gp120 of the HIV-1 subtype C infectious clones. The variable regions (V1-V5) are marked as well as the CD4 binding domain. pZAC has a shortened V1 loop and a slightly enlarged V4 loop, compared to that of pMJ4 and pHIV1084i.

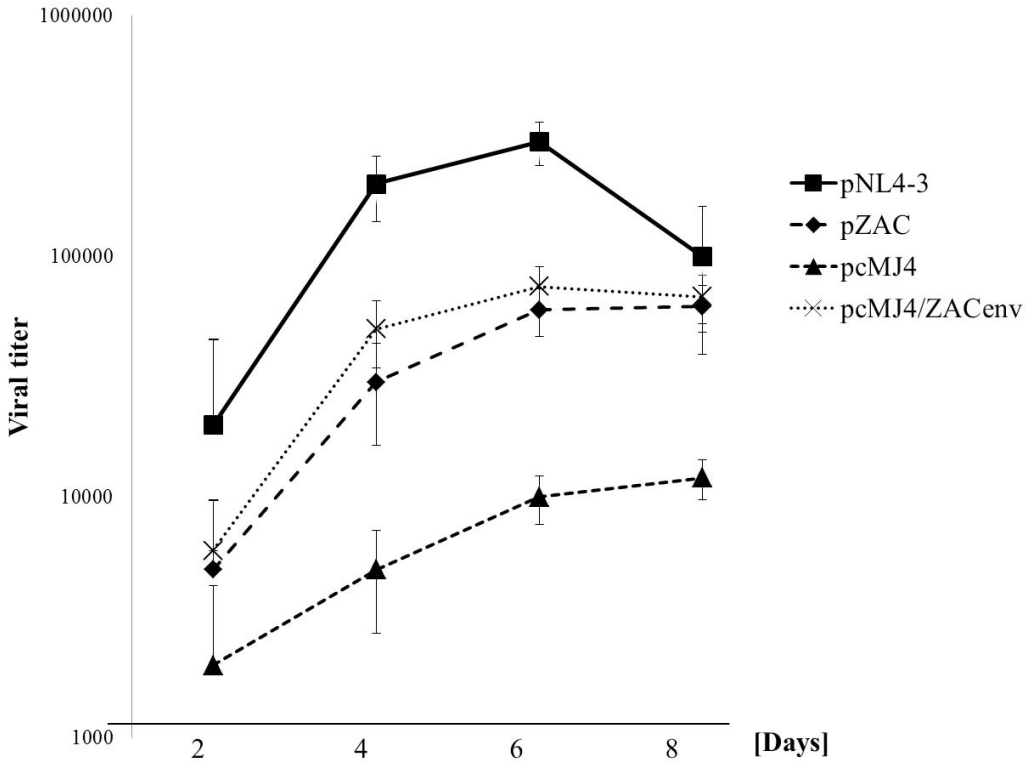


Fig. 3

Fig. 3. Growth kinetics on PBMCs. Infectious viruses (MOI: 0.05) were cultured for 8 days and supernatant were titrated on TZM-Bl HeLa cells to determine the viral titer as indicated. Experiments were done in triplicate. NL4-3 had the highest replication capacity, whereas MJ4/ZACenv and ZAC replicated better.

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PLoS one

HIV Drug Resistance (HIVDR) in Antiretroviral Therapy-Naïve Patients in Tanzania Not Eligible for WHO Threshold HIVDR Survey Is Dramatically High

Christa Kasang[1], Samuel Kalluvya[2,3], Charles Majinge[2], August Stich[4], Jochen Bodem[1], Gilbert Kongola[3], Graeme B. Jacobs[1], Mathias Mlewa[2], Miriam Mildner[1], Irina Hensel[1], Anne Horn[1], Wolfgang Preiser[5], Gert van Zyl[5], Hartwig Klinker[6], Eleni Koutsilieri[1], Axel Rethwilm[1], Carsten Scheller[1]*, Benedikt Weissbrich[1]

1 Institute of Virology and Immunobiology, University of Würzburg, Würzburg, Germany, **2** Bugando Medical Centre, Mwanza, Tanzania, **3** BUCHS, Mwanza, Tanzania, **4** Medical Mission Institute, Würzburg, Germany, **5** Division of Medical Virology, Department of Pathology, National Health Laboratory Service, University of Stellenbosch, Cape Town, South Africa, **6** Division of Infectious Diseases, Department of Internal Medicine, University of Würzburg, Würzburg, Germany

Abstract

Background: The World Health Organization (WHO) has recommended guidelines for a HIV drug resistance (HIVDR) survey for resource-limited countries. Eligibility criteria for patients include age below 25 years in order to focus on the prevalence of transmitted HIVDR (tHIVDR) in newly-infected individuals. Most of the participating sites across Africa have so far reported tHIVDR prevalences of below 5%. In this study we investigated whether the rate of HIVDR in patients <25 years is representative for HIVDR in the rest of the therapy-naïve population.

Methods and Findings: HIVDR was determined in 88 sequentially enrolled ART-naïve patients from Mwanza, Tanzania (mean age 35.4 years). Twenty patients were aged <25 years and 68 patients were aged 25–63 years. The frequency of HIVDR in the study population was 14.8% (95%; CI 0.072–0.223) and independent of NVP-resistance induced by prevention of mother-to-child transmission programs. Patients >25 years had a significantly higher HIVDR frequency than younger patients (19.1%; 95% CI 0.095–0.28) versus 0%, P = 0.0344). In 2 out of the 16 patients with HIVDR we found traces of antiretrovirals (ARVs) in plasma.

Conclusions: ART-naïve patients aged over 25 years exhibited significantly higher HIVDR than younger patients. Detection of traces of ARVs in individuals with HIVDR suggests that besides transmission, undisclosed misuse of ARVs may constitute a significant factor in the generation of the observed high HIVDR rate. The current WHO tHIVDR survey that is solely focused on the transmission of HIVDR and that excludes patients over 25 years of age may therefore result in substantial underestimation of the prevalence of HIVDR in the therapy-naïve population. Similar studies should be performed also in other areas to test whether the so far reported optimistic picture of low HIVDR prevalence in young individuals is really representative for the rest of the ART-naïve HIV-infected population.

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Competing Interests: The authors have declared that no competing interests exist.

* E-mail: scheller@vim.uni-wuerzburg.de

Introduction

The World Health Organization (WHO), together with the Joint United Nation Programme on AIDS (UNAIDS) and other partners pursue the goal of providing worldwide access to ART [1]. In most African countries the ART roll-out [2] takes place in a context of a medical infrastructure with limited resources and a low number of available antiretroviral drugs and HIV infection is treated with standardised first- and second-line ART regimens.

Since 2004, a nation-wide care and treatment program has been implemented for HIV-infected individuals in Tanzania. Patients receive their antiretroviral medication free of charge in more than 200 nationwide Care and Treatment Centers (CTCs) and by the end of 2007 more than 165.000 eligible patients have been started on ART. Patients eligible for ART belong to one of the following three groups: a) patients with WHO clinical stage irrespective of their CD4 counts, b) patients with WHO clinical stage 3 and CD4 counts below 350/ml and c) all patients with CD4 counts <200/ml, regardless of their clinical symptoms [3]. The first-line regimens consist of a combination of zidovudine/lamivudine (AZT/3TC) or stavudine/lamivudine (d4T/3TC) together with nevirapine (NVP) or efavirenz (EFV). After therapy

failure, a second-line regimen that includes abacavir/didanosine (ABC/ddI) in combination with liponavir or saquinavi boosted with ritonavir (LPV/r or SQV/r) is used [3]. Neither viral load monitoring nor resistance testing are routinely done. HIV-positive pregnant women have been offered participation in a prevention of mother-to-child transmission (PMTCT) programme since 2004 consisting of one single dose of NVP for the mother at onset of labour, followed by a single dose of NVP for the newborn within 72 hours after delivery. Since 2009, a combination therapy of AZT, NVP and 3TC is recommended for PMTCT at sites that have the possibility to offer and monitor this ARV regimen [3].

Although ARV therapy is now available free of charge for eligible patients, ARVs are also sold on the black market in Tanzania. We do not know to which extent this phenomenon contributes to self-medication, especially because these drugs are quite expensive when not being prescribed. However, the fear of stigmatization when receiving ARV regimens at HIV CTCs as well as a general lack of knowledge of HIV treatment guidelines could be factors promoting unadvised medication.

The large-scale availability of first- and second-line ART regimens in a resource-limited area bears the risk of an unnoticed evolution of drug-resistant quasispecies in treated patients and their subsequent transmission into the ART-naïve population [4]. The WHO therefore recommends the monitoring of HIVDR among newly infected individuals in order to estimate the extent to which transmission of drug resistant HIV occurs, classifying HIVDR prevalence into three groups (low: <5%, moderate 5–15%, and high >15%) [5]. Mandatory criteria for inclusion into the WHO-initiated transmitted HIVDR (tHIVDR) surveillance are a) age below 25 years, b) no previous pregnancies, and c) no previous ART use [6]. Their rationale is to exclude people with long-standing infections, infected before ART was widely available in the country, or patients previously exposed to antiretrovirals (ARVs), e.g. through participation in PMTCT programs [5].

So far, the reported prevalence of tHIVDR in eligible patient populations is below 5% across Africa [7,8,9,10,11,12] and only for some areas moderate HIVDR rates have been reported in some studies [11]. These rather optimistic findings are in sharp contrast to observations from sub-Saharan Africa, that a significant proportion of HIV patients who start first-line ART encounter early virological failure within the first 12 months of therapy [13,14,15]. This may suggest that the true prevalence of HIVDR in patients presenting for ART initiation might be significantly higher than estimated from tHIVDR data.

Therefore, we hypothesized that HIVDR prevalence may differ between people eligible for WHO tHIVDR surveillance and the rest of the HIV-infected population. A systematic underestimation of the prevalence of HIVDR would misguide health care decision makers in resource-limited countries and result in fatal consequences for ART efficacy. In this study we determined the frequency of HIVDR in ART-naïve patients in Tanzania aged below and above the WHO-initiated tHIVDR survey limit of 25 years of age.

Methods

Ethics statement

The study was approved by the National Institute for Medical Research (Tanzania), Bugando Centre Ethical Board, and Ministry of Health (Tanzania). All patients gave written informed consent. All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki.

Patients

In this study we investigated samples from HIV patients enrolled in the clinical trial ProCort1 (trial name: "ProCort1"; registry: ClinicalTrials.gov; registration number: NCT01299948). ProCort1 is a randomized, double-blinded placebo-controlled trial testing low-dose (5 mg/day) prednisolone on HIV disease progression in ART-naive patients who are not yet eligible for ART. According to ProCort1 inclusion criteria, patients were HIV positive but ART-naive with CD4+ cell counts >300 cells/µl and no other clinical conditions that require ART. For the ProCort1 trial, a total number of 416 consecutively enrolled HIV patients were initially screened. 90 patients presented as screening failures, mostly (about 80%) because of CD4 counts below 300 cells/µl. The other screening failures were due to TB-coninfections, abnormal blood parameters (HB, ALT, AST, creatinin) or diabetes. Plasma and peripheral blood mononuclear cells (PBMC) were collected from every patient at baseline and at 12 later time points covering a time span of two years. The study was performed at the Bugando Medical Centre in Mwanza (Tanzania).

Samples and bulk sequencing reaction

75 samples of DNA isolated from frozen PBMC and 55 samples of RNA isolated from frozen plasma collected from 120 randomly-selected patients (from 10 patients both DNA and RNA were isolated) enrolled in the ProCort study were amplified as described [16] by HIV-Pol-specifc PCR or RT-PCR, respectively. A positive PCR reaction was yielded from 46 plasma samples and 48 PBMC samples, corresponding to a recovery rate of 84% (46/55) from plasma and 64% (48/75) from PBMC samples. PBMCs used for these experiments had a low viability, probably a result of suboptimal freezing conditions during transport or storage, which may explain the low PCR recovery rate from these samples. Bulk-sequencing of the PCR products was performed as described [16]. Sequences were submitted to GenBank, accession numbers HM572334–HM572422.

HIV drug resistance and subtype analysis

For analysis of resistance-associated mutations (RAM), the FASTA files were submitted to Stanfords HIV drug resistance database [17]. The 2009 HIVDR surveillance database [18] was used to identify mutations eligible for the WHO HIVDR surveillance analysis. HIV subtype analysis was performed by phylogenetic analysis of the FASTA files as described [16].

Therapeutic Drug Monitoring (TDM)

Plasma samples collected at baseline from patients with RAM were subjected to TDM for the detection of Efavirenz, Nevirapine, Nelfinavir, Saquinavir, Atazanavir, and Lopinavir as described [19,20]. TDM is a well-established investigation in HIV-patients during antiretroviral treatment and part of international guidelines. The methods to determine plasma concentrations of HIV-protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) are highly specific and sensitive. In the HPLC/GC methods used in the study, the limit of detection (LOD) of nevirapine was determined at 2 ng/ml, the lower limit of quantification (LLQ) of nevirapine was reached at a concentration of 10 ng/ml. For efavirenz the LOD was 3 ng/ml, and the LLQ was 25 ng/ml.

Statistical Analysis

Patients in different age groups were sorted according to the absence or presence of HIVDR in a 2×2 contingency table. Frequency of HIVDR in different age groups was compared using

Fisher's exact probability test. $P<0.05$ was considered as statistically different. 95% confidence intervals (CI) were calculated using GraphPad Prism Software. 95% CIs were truncated at 0.000 if lower CI was negative.

Results

Patient characteristics

The sequences analyzed in this study derive from 88 ART-naïve HIV-infected patients in Mwanza, Tanzania, enrolled in the clinical trial ProCort1 (mean age 35.4 years, 78% female) (Table 1). The mean duration since HIV diagnosis was 1.3 years. Twenty patients were younger than 25 years and thus fulfilled the age criteria of the WHO tHIVDR surveillance program, while 68 patients (77%) were aged above 25. This corresponds to the typical age distribution of ART-naïve patients in Mwanza.

High frequency of drug-resistant HIV

With bulk sequencing reactions from PBMC-derived DNA (46 cases) and plasma-derived RNA (42 cases) we detected HIV-pol mutations associated with drug resistance in 16 of 88 patients (18.2%; 95% CI 0.100–0.264) (Table 2). Only four of the 16 RAM were initially detected from plasma and 12 from PBMC. In order to investigate if there is any difference in RAM frequency in the two compartments, we sequenced plasma specimens from five patients who were initially tested positive for RAM with PBMC-derived DNA and found the same RAM also in plasma. In five additional cases we retested RAM-negative patients who were initially tested in plasma and found no sequence differences in PBMC (data not shown). Three of the 16 samples had RAM associated with polymorphic positions (T98G; T69N; one patient with both E44D and V118I) that are excluded from WHO tHIVDR surveillance [18]. We hence use the term "WHO-defined HIVDR" to refer to mutations listed by WHO for tHIVDR surveillance [18] and the term "HIVDR" on its own to refer to all RAM according to the Stanford Database, irrespective of their association with polymorphic sites [17]. The frequency of "WHO-defined HIVDR" was 14.8% (13/88, 95% CI 0.072–0.223) in the entire study population (Fig. 1A, white bar).

Age-dependent frequency of drug-resistant HIV

The occurrence of RAM was highly correlated with the age (Fig. 1). WHO-defined HIVDR frequency was 0% (0/20) in patients aged under 25 but 19.1% (13/68; 95% CI 0.095–0.28) in patients older than 25 years (Fig. 1A), which is statistically significantly different ($p = 0.0344$, Fishers exact test).

As depicted in figure 1B, frequency of HIVDR seems to peak in middle-aged patients, although the number of patients in each age-group is too small in order to reach statistical significance. Some of the patients presented with two or three different RAM (Fig. 1C). This "RAM burden", i.e. the number of different RAM contributing to resistance against ARVs per individual, correlates with impairment of a greater spectrum of classes of ARVs (Fig. 1D).

Clinical impact of the RAM

The resistance pattern detected could severely limit the options for ART once the patients become eligible for antiviral therapy. About every fifth patient (21.6%; 8/37) in the group 25–39 years has a predicted reduced susceptibility towards the local first-line ART regimens according to Stanford Database (Fig. 2A). Without prior resistance testing, prescribing this regimen would result in insufficient suppression of viral replication, likely leading to a rapid development of further mutations conferring resistance to other components of the regimen and – finally – to complete virological failure. Even more concerning are the findings that the efficacy of the local second-line regimens is also affected by the detected mutation profile: a) 24% (9/37) of the patients aged between 25–39 have RAM that correspond to low- and high-level resistance towards ARVs included in the local second-line regimens according to Stanford Database (Fig. 2B) and b) 8 out of 9 patients (89%) in this age group with predicted reduction of first-line efficacy also have a predicted reduction of second-line efficacy (Fig. 2C). Related to the whole study population, these numbers

Table 1. Demographic patient characteristics of the study population.

	PCR-negative	PCR-positive	PCR-positive	PCR-positive
		all	Age<25 years	Age≥25 years
total n = 120	**n = 32**	**n = 88**	**n = 20**	**n = 68**
Age (years)	35.0±12.2	35.2±11.3	22.6±1.5	38.9±10.1
	(21.1–59.0)	(18.6–63.7)	(18.7–24.9)	(25.2–63.7)
Gender	77% female	78% female	95% female	75% female
CD4+ T-Cell count (cells/μl)	664±299	490±222	534±215	478±223
	(224–1435)	(99–1056)	(140–1037)	(99–1056)
time since first diagnosis (years)	1.30±2.75	1.30±1.75	0.77±0.83	1.45±1.92
	(0.05–15.09)	(0.04–9.97)	(0.05–2.99)	(0.04–9.97)
Sequencing from PBMC (%) compared to plasma	--	52%	50%	53%
Clinical stage (CDC) A1	56%	33%	45%	29%
Clinical stage (CDC) A2	38%	51%	45%	53%
Clinical stage (CDC) B1	0%	9%	10%	9%
Clinical stage (CDC) B2	6%	7%	0%	9%

The study population consisted of 120 ART-naive HIV-1 infected adults. PBMC- or plasma samples from 88 patients yielded amplicons for the bulk sequencing reaction. Data are expressed as means ± S.D. and range in parentheses. Patients with CD4 counts <200/ml at sample date initiated ART if CD4 counts remained below 350/ml four weeks later.

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Table 2. HIVDR in the Mwanza cohort.

Age (years)	Sample number	NRT RAM	NNRTI RAM	PI RAM	Affected drug (low-level resistance)	Affected drug (high-level resistance)	TDM	time since HIV test (years)
20.5	TZ.09.032647		A98G[1]		NVP		neg.	0.31
25.4	TZ.08.029746			**D30N**	ATV	NFV	neg.	0.58
26.4	TZ.08.017184	**M41L**			D4T, AZT, ddI, ABC		neg.	1.05
28.0	TZ.08.014495	**M184I**			ABC	3TC, FTC	**EFV**	0.33
30.2	TZ.08.022112	**M184I**	**G190E**		ABC	3TC, FTC, NVP, EFV	neg.	8.3
32.2	TZ.09.001336			**G73S**	SQV, NFV, ATV		neg.	0.28
32.4	TZ.09.001332	**M184I**	**K103N**		ABC	3TC, NVP, EFV, DLV	neg.	0.18
33.2	TZ.08.017176	**M41L**			D4T, AZT, ddI, ABC		**NVP**	2.50
33.7	TZ.08.023280	**V75A**			D4T, ddI		neg.	1.63
36.7	TZ.08.017197	**T215I**	**K103N**	**V82T**	D4T, AZT, ddI, ABC, SQV/r, LPV/r, ATV, NFV	NVP, EFV, DLV	n.d.	2.81
38.7	TZ.09.001323		**G190E**	**G73S**	SQV/r, NFV,ATV, DLV	NVP, EFV	neg.	1.06
42.6	TZ.09.006017			**V82A**	LPV/r, SQV/r, ATV, NFV		neg.	4.60
44.9	TZ.10.002035	T69N[2]			ddI		negative	1.14
46.7	TZ.08.017195		**K103N**			NVP, EFV, DLV	n.d.	0.38
46.8	TZ.10.003309	E44D[3] V118I[3]			3TC		neg.	0.15
51.4	TZ.08.017193		**Y188H**		NVP, EFV, DLV		neg.	0.04

RAM according to the Stanford HIV Drug Resistance Database [17] in 16/88 baseline samples. Mutations associated with a score of 60 were attributed as high-level resistance-associated mutations (RAM) and mutations with a score of 10–35 were attributed as low-level RAM. Mutations listed for WHO HIVDR surveillance [18] are indicated in bold. Superscripted numbers identify the reasons for excluding the respected mutations from the HIVDR list: 1) nonpolymorphic, but at highly polymorphic position; 2) polymorphic position in subtypes B, F, CRF01_AE; 3) polymorphic in multiple subtypes. ABC: Abacavir; ATV: Atazanavir; AZT: Zidovudine; ddI: Didanosine; DLV: Delaviridine; D4T: Stavudine; EFV: Efavirenz; FTC: Emtricitabine; LPV: Lopinavir; NFV: Nelfinavir; NVP: Nevirapine; SQV: Saquinavir; 3TC:Lamivudine [29]. Underlined drugs are part of the local first line regimens, double line underlined drugs are part of local second line regimens. Data derived from 88 sequenced samples. Therapeutic drug monitoring (TDM) was performed from plasma samples collected at baseline for NNRTIs (Efavirenz and Nevirapine) and PIs (Nelfinavir, Saquinavir, Atazanavir and Lopinavir).

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could translate into complete ART failure (first- and second-line) in about 9% (8/88) of all patients.

Long-term persistence of RAM

In order to determine the long-term persistence of RAM in the 16 patients with baseline RAM, plasma specimens collected up to 21 month later were analyzed. Three different scenarios were observed: In five patients the initially-detected RAM persisted, in six patients RAM detected at baseline became undetectable later, and in five patients RAM that were undetected at baseline were found in follow-up samples (Table 3).

Therapeutic drug monitoring

Baseline plasma samples of 14 patients with RAM were screened for the presence of antiretroviral substances. We detected traces of efavirenz (160 ng/ml, sample number TZ.08.014495) and nevirapine (202 ng/ml, sample number TZ.08.017176) in two samples (Table 2). These drugs are, however, unrelated to the RAM detected in these patients (M184I and M41L). For one patient (TZ.08.017195) in which we detected K103 that confers resistance to EFV or NVP and was stable for at least 18 months, we screened additional plasma samples donated at 3, 6, 12 and 18 months after initial sampling and found no traces of either drug.

HIV-1 subtype distribution

Subtype analysis identified seven different clusters that can be divided into different HIV subtype groups, including A1 (34% of the samples), B (1%), C (26%), D (28%), CRF10_CD (4%) and recombinants A1D (7%) (Fig. 3). These findings are in line with previous reports from the area [10,21].

Discussion

The frequency of RAM in our ART-naïve study group is much higher than expected from hitherto reported prevalence rates of below 5% from WHO tHIVDR threshold surveys across Africa [22]. This this difference is probably mainly attributed to the different criteria for choosing patients for HIVDR surveillance: The WHO surveillance criteria define eligible patients as presumably recently infected patients below 25 years of age with CD4+ T-cell counts above 500 cells/µl, and – if female - restricted to women without prior history of pregnancies to avoid PMTCT-induced resistance. Our study population, in contrast, consists of HIV patients with a probably slightly longer history of infection (1.30±1.75 years since diagnosis) with a mean age of 35.4 years, a mean baseline CD4+ T-cell count of 483 cells/µl and amongst females predominantly multigravidae (Table 1).

By applying the age criteria of WHO HIVDR surveillance to our analysis, we would have concluded that the prevalence of drug resistant HIV in the Mwanza region is below 5% similar to what was reported for the Dar es Salaam region in Tanzania in 2008 [23] and from most other HIVDR surveillance sites across Africa so far [7,8,9,10,11,12]. However, the true frequency of HIVDR in our study population is much higher: 14.7%, if mutations at polymorphic sites are excluded and even 18.2% if they are included.

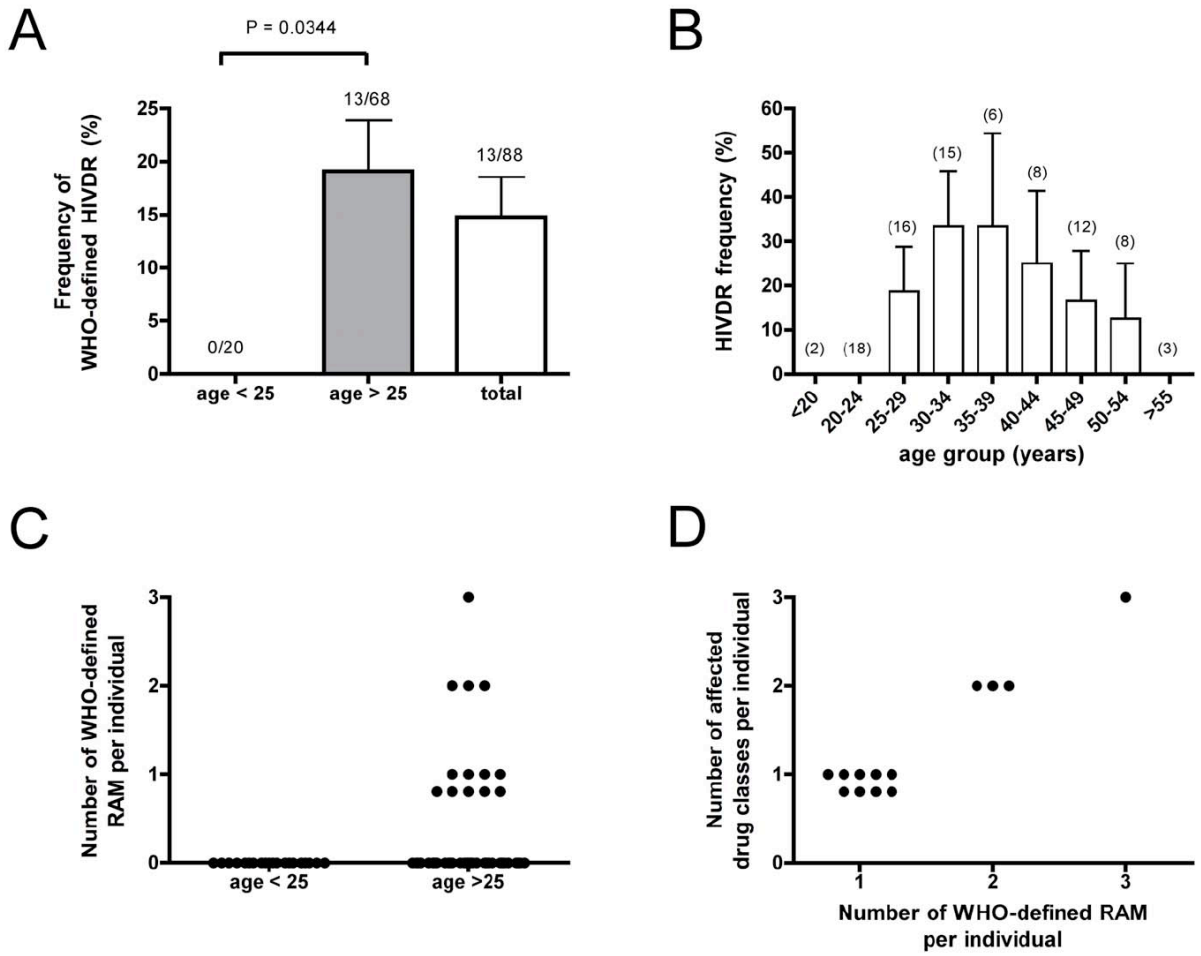


Figure 1. The frequency of HIVDR is age-dependent. HIVDR was determined by bulk sequencing from ART-naïve patients. A.: Frequency of WHO-defined HIVDR in patients aged under 25 years (n = 20, left bar), patients aged over 25 years (n = 68, middle bar), and in the total study population (n = 88, right white bar). B: Frequency of WHO-defined HIVDR peaks in different age groups. Numbers in brackets indicate the number of individuals tested in each age group. Data as means ± S.E.M.. For statistical analysis, Fisher's exact test was performed. Differences with a $P<0.05$ were regarded as statistically significant. C: Number of WHO-defined RAM per individual ("RAM burden"). D: Number of affected antiretroviral drug classes (NRTI, NNRTI, PI) per individual in relation to the number of WHO-defined RAM per individual.
doi:10.1371/journal.pone.0023091.g001

This alarmingly high frequency of HIVDR could have been generated by two different ways: a) by direct transmission of drug resistant viruses from sexual partners carrying the resistant virus or b) by selection from the natural pool of quasispecies in each individual patient following undisclosed antiretroviral drug experience. It is reasonable to suggest that both factors may have contributed to the observed high frequency of RAM in our Tanzanian study group.

Intra-patient generated drug resistance in ART-naive patients can either result from episodes of NVP monotherapy of pregnant women during PMTCT or from undisclosed (self-) medication with ARVs. To exclude PMTCT-generated NVP resistances from tHIVDR surveillance, the WHO criteria exclude multigravidae, which results in excluding many, if not the majority of women.

In our study group we identified seven patients with NVP resistance (Table 4). We can exclude PMTCT as a possible cause for the occurrence of the NVP resistance mutation in five of them (71%), because the patients are either male (two cases) or the pregnancies date before HIV infection was diagnosed (three cases). In one additional case we were unable to ascertain the patient's PMTCT status and in only one case we positively identified a patient who actually received PMTCT. Therefore, the vast majority of NVP resistance in our study group is obviously unrelated to PMTCT. The two NVP resistance mutations that can be potentially attributed to PMTCT have, however, no influence on the overall HIVDR frequency in our study group, as the respective patients (TZ.09.001332, TZ.09.001323) present with multiple RAM against other ARVs that are completely unrelated to the Tanzanian PMTCT program. Since these other mutations (M184I and G73S, respectively) must have been acquired independently of PMTCT, there is no reason to exclude these two patients from the analysis.

As for undisclosed self-medication, all patients who presented with RAM repeatedly reported that they were treatment-naïve (except for those who participated in PMTCT programs) and also their medical records – if available - were negative in this respect,

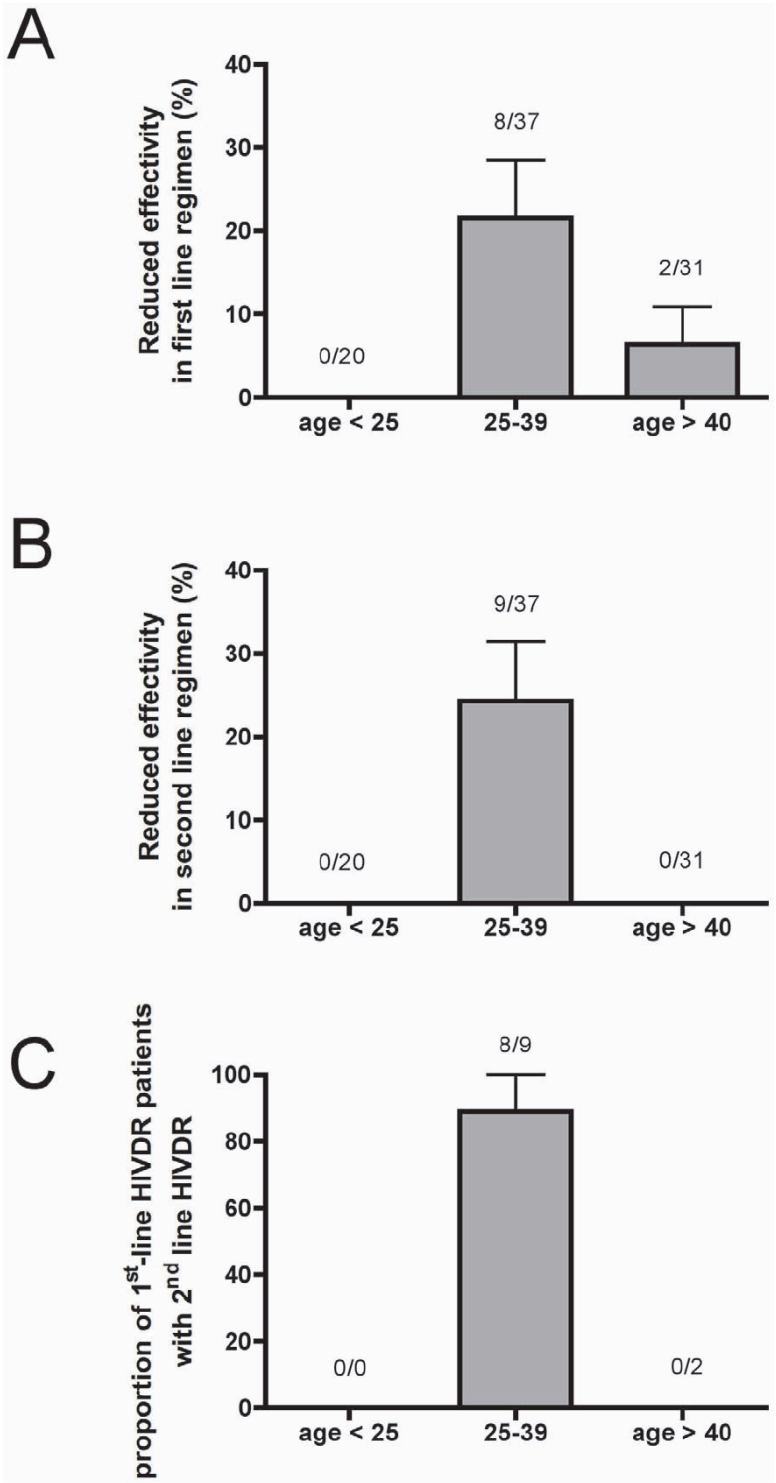


Figure 2. HIVDR affects efficacy of local first- and second-line ART regimens. Effects of HIVDR (high- and low-level resistances) on drugs included in the local antiretroviral regimens. A: Effects on local first-line ART regimen. HIVDR that affects at least one of the drugs included in first-line therapy (AZT/D4T plus 3TC plus NVP/EFV) scored positive. B: Effects on local second-line ART regimen. HIVDR that affects at least one of the drugs included in second-line therapy (ddI/ABC plus LPV/SQV plus RTV) scored positive. C: Proportion of patients with HIVDR that affects first-line ART regimen who also carry HIVDR that affects second-line ART regimen.
doi:10.1371/journal.pone.0023091.g002

Table 3. Long-term persistence of RAM.

	Sample number	RAM at baseline	Time to Follow-up 1 (months)	RAM at Follow-up 1	Time to Follow-up 2 (months)	RAM at Follow-up 2
	TZ.09.032647	**A98G**	**5**	**A98G**	n.a.	n.d.
	TZ.08.017184	**M41L**	**11**	**M41L**	**18**	**M41L**
persistent RAM	TZ.08.017176	**M41L**	**12**	**M41L**	**21**	**M41L**
	TZ.08.017195	**K103N**	**13**	**K103NS**, Y188HY	**18**	**K103N**
	TZ.10.003309	**E44D, V118I**	**21**	**E44D, V118I**	n.a.	n.d.
	TZ.08.029746	**D30N**	12	none	n.a.	n.d.
	TZ.09.006017	**V82A**	18	none	n.a.	n.d.
RAM disappeared	TZ.08.022112	**M184I, G190E**	15	Q151QR	18	none
	TZ.09.001336	**G73S**	18	V179T	n.a.	n.d.
	TZ.08.023280	**V75A**	12	T69ST	14	T69ST
	TZ.08.017193	**Y188H**	6	L10V	n.a.	n.d.
	TZ.08.022112	M184I, G190E	**15**	**Q151QR**	18	none
	TZ.09.001336	G73S	**18**	**V179T**	n.a.	n.d.
new RAM	TZ.08.023280	V75A	**12**	**T69ST**	**14**	**T69ST**
	TZ.08.017193	Y188H	**6**	**L10V**	n.a.	n.d.
	TZ.08.017195	K103N	**13**	K103NS, **Y188HY**	18	K103N
	TZ.08.014495	M184I	6	no PCR product	n.a.	n.d.
	TZ.09.001323	G190E, G73S	18	no PCR product	n.a.	n.d.
unclear	TZ.09.001332	M184I, K103N	n.a.	n.d.	n.a.	n.d.
	TZ.08.017197	T215I, K103N, V82T	n.a.	n.d.	n.a.	n.d.
	TZ.10.002035	T69N	n.a.	n.d.	n.a.	n.d.

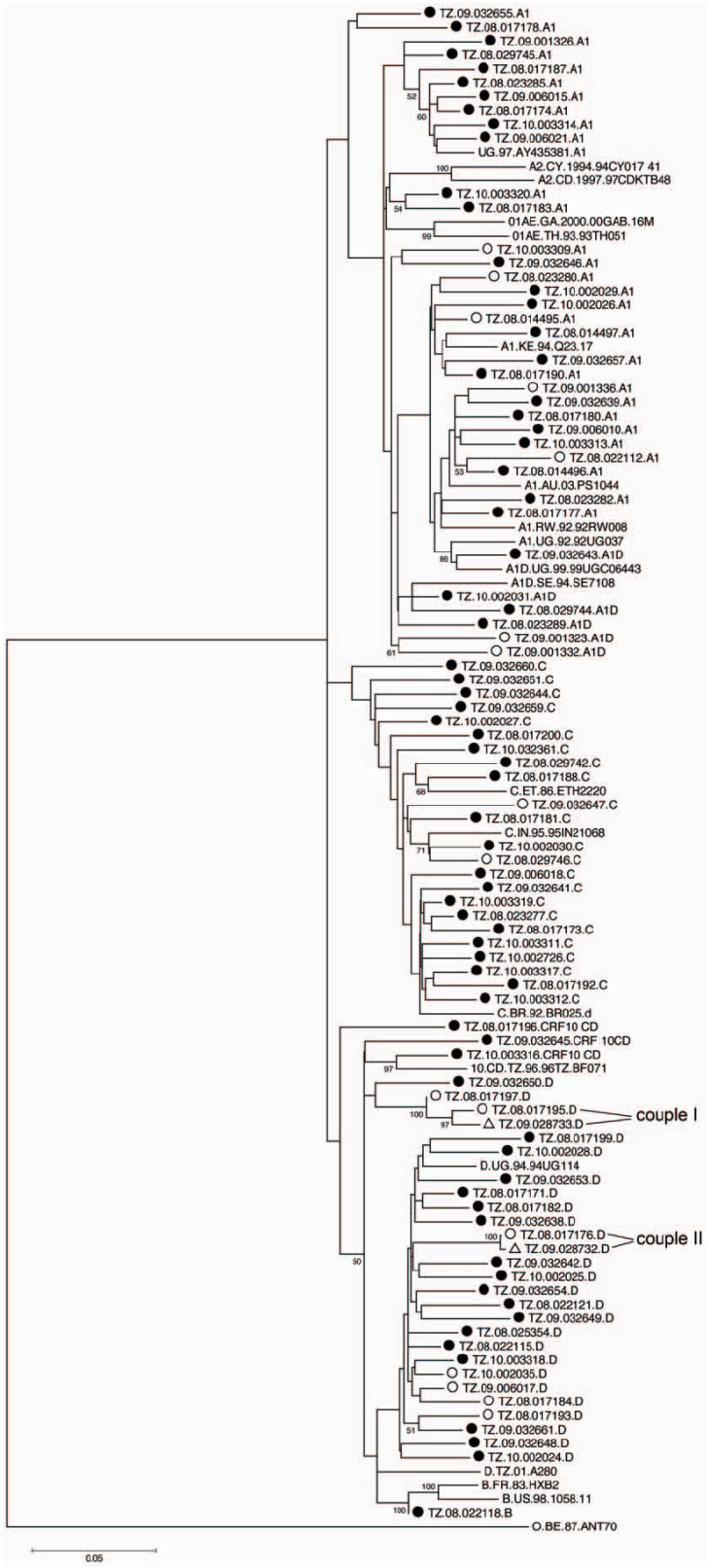
Of the 16 patients who presented with RAM at baseline, we analyzed plasma specimens collected at later time points ("follow-up 1", "follow-up 2") for the presence of RAM. We detected three different scenarios, including persistent RAM, disappearing RAM, and newly emerging RAM (some samples appear in more than one scenario). Time to follow-up sample is indicated in months. In some cases, no PCR product from plasma samples could be generated. The investigation for long-term stability of RAM is insofar incomplete as plasma specimens collected at later time points were not available ("n.a.") for all patients, referred to as "n.d." (not determined).
doi:10.1371/journal.pone.0023091.t003

suggesting that not only the majority of NVP mutations but also a great proportion of the other detected mutations were acquired by transmission.

In an attempt to challenge the given disclosure about the patients' drug status, we used TDM as a forensic method in order to determine whether the patient has taken NNRTIs or PIs within the past 1–2 weeks, the time window in which we can detect these drugs in plasma samples [24,25]. Of the 16 patients who presented with RAM, traces of ARVs were detected in two (Table 2), indicating that at least some of the patients gave a false statement regarding their drug status. We can almost exclude that the patients studied here received ARVs by CTCs, as their health status was still good enough to be eligible for ART and any clinically-advised antiretroviral medication would have been documented in their medical records – which were negative in this regard. To our knowledge, this is the first reported evidence for undisclosed self-medication with ARVs in Africa, a phenomenon that has unfortunately not yet been addressed by systematic analyses. The detected ARVs were, however, not related to the RAM found in these patients, so that the undisclosed medication with ARVs might just have been an epiphenomenon and the detected RAM may nonetheless have been acquired by transmission.

According to WHO criteria, these two patients are not eligible for tHIVDR survey, because they were (as found by experimental evidence) not ARV naïve. Even if we excluded these patients from our analysis, HIVDR frequency would still be 12.5% (11/88) and therefore much higher than anticipated from previous reports from Tanzania [23]. However, we did not exclude the patients from our analysis for the following reasons: a) First and foremost, the aim of our study is not to fulfil current WHO criteria as good as possible and to see how close we can get to previous-published results, but rather to challenge these very criteria by looking into a therapy-naïve population as it is, i.e. not being filtered by additional criteria concerning age or drug naivety (not to be mistaken with therapy naivety!). In our case, the frequency of HIVDR in patients who have never before received medically-advised antiretroviral therapy is in the range of 15% and this constitutes a severe medical problem for future antiretroviral therapy with predefined regimens - no matter how many patients we exclude from our analysis for not fulfilling WHO criteria for whatever reasons. b) The availability of ARVs on the black market is a known phenomenon in many sub-Saharan countries and there is no reason to believe that undisclosed self-medication is a phenomenon restricted to our study population. It is rather plausible to assume that self-medication with ARVs (be it from black-market supplies or by drug-sharing within a family) is inevitably connected to ART roll-out programs and surveys to monitor the prevalence of HIVDR in therapy-naïve patients should not exclude this source of drug resistance. c) WHO criteria do not include forensic methods to control whether the studied populations are really drug-naïve, and to our knowledge, drug monitoring has not been done in any of the tHIVDR surveys published so far.

In five out of the 16 RAM carriers we observed the emergence of new RAM at later time points (Table 3). It seems very unlikely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O.BE.87.ANT70
0.05

Figure 3. Phylogenetic analysis of RT and PR sequences from the Tanzanian cohort. A neighbour-joining phylogenetic tree [30] was constructed from the 88 patient derived HIV-1 sequences from the Tanzania cohort and the two partner-derived sequences. Reference sequences were obtained from the Los Alamos HIV sequence database. The analyzed 1302 bp region includes the complete Protease and Reverse Transcriptase coding region. The tree was constructed using Mega software version 4, and the evolutionary distances were calculated using the Kimura 2-parameter method. The bootstrap consensus tree was inferred from 50000 replicates and values greater than 70% are indicated on the branch lengths. The scale at the bottom left indicates the calculated genetic distances between the branches of the phylogenetic tree. Circles represent the 88 samples from our cohort. Black-dotted circles are without RAM, open-circled sequences are with RAM, open triangles are sequences with RAM from HIV-infected partners of two study subjects, which were not included in the determination of HIVDR as these patients received ART. Sequences without symbols are subtype reference sequences derived from Los Alamos database. The subtype is indicated at the end of each sequence name. Relative subtype frequency: A1: 34%, A1D: 7%, C: 26%, CRF10_CD: 4%, D: 28%, B: 1%. Sequences isolated from two couples (couple I, couple II) with NVP resistances.
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that these newly-detected RAM originate from superinfections with drug-resistant viruses but rather that the emergence of these RAM was either caused by recent misuse of ARVs or - probably the more plausible interpretation – simply reflects a false-negative result of the bulk sequencing method at baseline determination (for discussion of bulk sequencing sensitivity see also below). In three cases the RAM detected at baseline remained stable for more than 18 months. Two of these patients carried the mutation M41L and one carried the mutation K103N. For both mutations a long-term stability has been reported even in the absence of antiviral medication [26].

In four of the 16 patients presenting with RAM, plasma samples could be obtained from their HIV-infected partners. Two of these could be sequenced and had the same RAM as their partners (K103N detected in TZ08.017195 and the partner's sequence TZ09.028733, and M41L detected in TZ08.017176 and the partner's sequence TZ09.028732). In a phylogenetic analysis these sequences clustered in direct neighbourhood to the partner's sequences (marked with "couple I" and "couple II" in Fig. 3). As it is rather unlikely that a potential misuse of an ARV generates exactly the same RAM profile in both partners (many different RAMs can become selected by a given ARV), it is reasonable to assume that the respective RAM was received by transmission, although we cannot say in which direction the transmission occurred.

Irrespective of whether the observed high frequency of RAM is predominantly caused by transmission or by selection with undisclosed medication with ARVs, such a high frequency of RAM in patients who are likely to start ART in the near future translates into an expected reduction of first- and second-line ART regimen efficacy. The full extent of the problem may be even greater, as we generated our data from bulk sequencing reactions, which are known to underestimate the number of RAM, because they fail to reliably detect mutations if the respective quasispecies contribute less than 20–30% to the total viral population [27]. More sensitive techniques, such as real time PCR-based resistance testing, show that bulk sequencing considerably underestimates the true frequency of HIVDR [28].

If the observed level of HIVDR in our study group is representative of other regions of Africa, this would affect the settings in which ART roll-out programmes are being implemented. The current WHO surveillance criteria bear the danger of answering a merely academic question (i.e. the transmission rate of resistant viruses in people aged under 25), whereas the clinical reality has to deal with pre-existing HIVDR in all patients eligible for ART, irrespective of their origin (transmitted or intrapatient-selected) and irrespective of the patient's age. Beyond that, it is questionable whether the under-25 year olds are a representative "sentinel group" for HIVDR transmission in the rest of the population, because important social parameters that presumably affect the rate of resistance transmission, such as promiscuity or the ART status of the sexual partners are likely to significantly differ between different age groups, which may compromise the current resistance surveys. Moreover, the (so far excluded) patients over 25 years of age represent a significant proportion in the group of patients eligible for ART. Over the years at Bugando Medical Centre, the proportion of those older than 25 years amongst patients eligible for ART has been between 70 to 80 percent. This is similar to the age distribution in our study group, which in this respect quite accurately pictures the characteristics of patients seeking for ART at Bugando Medical Center. Our study population was, however, not intentionally designed to represent the whole HIV-infected population in Mwanza, so that an extrapolation of our data to the whole population in Mwanza must be taken with caution. Moreover, the patients investigated in this study have been reenrolled for an interventional clinical trial

Table 4. NVP resistance and PMTCT.

Sample number	Gender	PMTCT with NVP	Birth dates of children	First HIV diagnosis	NVP resistance caused by PMTCT
TZ.09.032647	female	none	2007	24.08.08	**no**
TZ.08.022112	male	n.a.	n.a.	24.09.04	**no**
TZ.09.001332	female	unknown	unknown	23.08.07	**unclear**
TZ.08.017197	female	none	1989, 1993, 1995	16.04.07	**no**
TZ.09.001323	female	yes	1990, 1992, 1994, 1996, 2004, 2006	26.01.06	**possible**
TZ.08.017195	female	none	1992, 1993, 1995, 1997, 1999	22.05.07	**no**
TZ.08.017193	male	n.a.	n.a.	15.09.05	**no**

Patients with NVP resistance mutations were analyzed regarding PMTCT (with NVP monotherapy) as a possible cause for the emergence of the mutation. PMTCT as a cause for the mutation's appearance is discussed as "possible" if the mother received PMTCT; it is discussed as "unclear" if the PMTCT status and the dates of birth of the children are unknown; for plausibility reasons PMTCT was excluded ("no") as a trigger of the NVP mutation if the patient is either male or if a female patient presented with unknown PMTCT history combined with the HIV infection being diagnosed only since the date of birth of the youngest child.
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("ProCort1"), which may have caused a selection bias (like, for instance, patients who agree to join an interventional trial may or may not be more prone for self-medication with ARVs in the past or to keep information about ARV misuse undisclosed). We therefore refer to the term "frequency" rather than "prevalence" when describing the rate of RAM in our patient population.

WHO recommends the analysis of at least 34–47 specimens (34 if no mutations were found, ≥47 if one or more mutations are detected within the first 34 sequences) of consecutively enrolled patients for the threshold survey method in order to categorize HIVDR as <5%, 5–15% or >15% [18]. Here we analyzed 88 samples and the determined frequency of HIVDR is therefore quite robust. Although we did find a frequency of HIVDR in the range of 5–15% in our study group – a finding that in the context of an expected low HIVDR rate is remarkable in itself – the most important aspect of our study is that this relatively high frequency of HIVDR would have remained completely unnoticed, if we strictly applied the current age criteria (<25) of the WHO threshold survey method to our analysis. The difference in HIVDR between the two age groups was so pronounced that it reached statistical significance despite the relatively low number of only 20 studied patients aged below 25 years. On the other hand, the estimation of a 0% HIVDR frequency in patients <25 years remains relatively uncertain, as the number of analyzed sequences in this subgroup is smaller than recommended.

The most substantial argument in favor of an age restriction of HIVDR surveys to patients younger than 25 years is probably the intention to exclude as much as possible older infections that reflect HIVDR transmission rates of the past rather than the current situation. In our study, the mean time since HIV diagnosis of the patients under 25 years old was 0.77 (±0.19 SD) years with a range of 0.05–2.99 years, compared to 1.45 (±0.23 SD) years for patients aged above 25 years ($p = 0.1285$, non-significant) (Table 1). Moreover, 10 of 13 (77%) patients with HIVDR had a known infection time within the range of the young age group, and 6 of 13 (46%) had infection times even below the average of the young group (Table 2). This is in fact not very astonishing, as young people in Tanzania are considered to be at substantial risk of HIV infection by sexual intercourse already at the age of 15 [3] which is still ten years away from the 25 years limit. Combined with an asymptomatic phase of infection that can last on the one hand for several years but on the other hand turns into a symptomatic disease within less than a decade, it is not very surprising that the asymptomatic (hence therapy-naïve) individuals under and over 25 years of age do not significantly differ in time of infection. In the light of this argument it appears that the age-restriction to under-25 years old is not very effective in terms of selecting recent infections. (The same argument would also apply for the intention to minimize chances of being exposed to ARVs.) Moreover, given that the over-25 years old – at least in our study - significantly differ in HIVDR frequency compared to young patients, this age-restriction may turn out to be even counterproductive in an attempt to determine representative HIVDR prevalence rates.

Considering the high frequency of HIVDR in our study population, we recommend the implementation of ART baseline resistance testing in the Mwanza area, as HIVDR prevalence in this area may have reached a critical level. In this regard, we encourage the development of cheap, robust and easy-to-use methods for detection of the commonest resistance-associated mutations as well as the regular monitoring of viral load during ART also in resource-limited settings. This would allow the selection of suitable ARVs from the available repertoire and prevent early therapy failure.

In conclusion, the results of this study demonstrate that the frequency of HIVDR in our study population is much higher than anticipated from the so-far published data of low tHIVDR levels in Tanzania and sub-Saharan Africa in general, and – even more important – that a restriction to patients <25 years of age as implemented in the current criteria of the WHO-initiated HIVDR threshold survey would have dramatically failed to detect this. The reason for this discrepancy is that the frequency of HIVDR correlated with age, for it was totally absent in young individuals, but surprisingly high in individuals being older than 25 years. Sporadic and undisclosed misuse of ARVs may have contributed to the observed high resistance rate. This indicates that the exclusive focus on transmission of HIVDR in under 25-years old as recommended by the current WHO HIVDR threshold survey, instead of a broader approach that is open to HIVDR accumulation by different mechanisms and across all age groups, may fail to adequately monitor the effects of ART role out programs on the spread of HIV drug resistance into the therapy-naive population. We therefore recommend to perform similar studies in other resource-limited areas to test whether the so far reported optimistic picture of low HIVDR prevalence in young individuals is really representative for the rest of the ART-naïve HIV-infected population.

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Author Contributions

Conceived and designed the experiments: CS BW JB WP GvZ HK SK CM AS AR EK CK. Performed the experiments: CK GBJ MMildner IH AH. Analyzed the data: CS BW CK HK. Contributed reagents/materials/analysis tools: EK BW SK CM GK HK AR CS. Wrote the paper: CS EK AR WP GvZ BW SK JB GBJ. Study conduct in the field: SK CM AS GK MMlewa. Clinical data management: CK.

References

1. Gilks CF, Crowley S, Ekpini R, Gove S, Perriens J, et al. (2006) The WHO public-health approach to antiretroviral treatment against HIV in resource-limited settings. Lancet 368: 505–510.
2. WHO progress report (2010) http://www.who.int/hiv/pub/2010progressreport/report/en/index.html. Accessed 2011 May 15th.
3. National Guidelines for the management of HIV and AIDS in Tanzania.
4. Deeks SG (2008) Transmitted minority drug-resistant HIV variants: a new epidemic? PLoS Med 5: e164.
5. Bennett DE, Bertagnolio S, Sutherland D, Gilks CF (2008) The World Health Organization's global strategy for prevention and assessment of HIV drug resistance. Antivir Ther 13 Suppl 2: 1–13.
6. Bennett DE, Myatt M, Bertagnolio S, Sutherland D, Gilks CF (2008) Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment. Antivir Ther 13 Suppl 2: 25–36.
7. Kamoto K, Aberle-Grasse J (2008) Surveillance of transmitted HIV drug resistance with the World Health Organization threshold survey method in Lilongwe, Malawi. Antivir Ther 13 Suppl 2: 83–87.
8. Maphalala G, Okello V, Mndzebele S, Gwebu P, Mulima N, et al. (2008) Surveillance of transmitted HIV drug resistance in the Manzini-Mbabane corridor, Swaziland, in 2006. Antivir Ther 13 Suppl 2: 95–100.
9. Ayouba A, Lien TT, Nouhin J, Vergne L, Aghokeng AF, et al. (2009) Low prevalence of HIV type 1 drug resistance mutations in untreated, recently

infected patients from Burkina Faso, Cote d'Ivoire, Senegal, Thailand, and Vietnam: the ANRS 12134 study. AIDS Res Hum Retroviruses 25: 1193–1196.
10. Ndembi N, Lyagoba F, Nanteza B, Kushemererwa G, Serwanga J, et al. (2008) Transmitted antiretroviral drug resistance surveillance among newly HIV type 1-diagnosed women attending an antenatal clinic in Entebbe, Uganda. AIDS Res Hum Retroviruses 24: 889–895.
11. Aghokeng AF, Vergne L, Mpoudi-Ngole E, Mbangue M, Deoudje N, et al. (2009) Evaluation of transmitted HIV drug resistance among recently-infected antenatal clinic attendees in four Central African countries. Antivir Ther 14: 401–411.
12. Nyombi BM, Holm-Hansen C, Kristiansen KI, Bjune G, Muller F (2008) Prevalence of reverse transcriptase and protease mutations associated with antiretroviral drug resistance among drug-naive HIV-1 infected pregnant women in Kagera and Kilimanjaro regions, Tanzania. AIDS Res Ther 5: 13.
13. Harries AD, Zachariah R, van Oosterhout JJ, Reid SD, Hosseinipour MC, et al. (2010) Diagnosis and management of antiretroviral-therapy failure in resource-limited settings in sub-Saharan Africa: challenges and perspectives. Lancet Infect Dis 10: 60–65.
14. Barth RE, van der Loeff MF, Schuurman R, Hoepelman AI, Wensing AM (2010) Virological follow-up of adult patients in antiretroviral treatment programmes in sub-Saharan Africa: a systematic review. Lancet Infect Dis 10: 155–166.
15. Barth RE, Wensing AM, Tempelman HA, Moraba R, Schuurman R, et al. (2008) Rapid accumulation of nonnucleoside reverse transcriptase inhibitor-associated resistance: evidence of transmitted resistance in rural South Africa. AIDS 22: 2210–2212.
16. Jacobs GB, Laten A, van Rensburg EJ, Bodem J, Weissbrich B, et al. (2008) Phylogenetic diversity and low level antiretroviral resistance mutations in HIV type 1 treatment-naive patients from Cape Town, South Africa. AIDS Res Hum Retroviruses 24: 1009–1012.
17. Stanford University Drug Resistance Database. http://hivdb.stanford.edu/. Accessed 2011 May 15.
18. Bennett DE, Camacho RJ, Otelea D, Kuritzkes DR, Fleury H, et al. (2009) Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. PLoS ONE 4: e4724.
19. Langmann P, Heinz W, Klinker H, Schirmer D, Guhl C, et al. (2008) High performance liquid chromatographic method for the determination of HIV-1 protease inhibitor tipranavir in plasma of patients during highly active antiretroviral therapy. Eur J Med Res 13: 52–58.
20. Langmann P, Weissbrich B, Desch S, Vath T, Schirmer D, et al. (2002) Efavirenz plasma levels for the prediction of treatment failure in heavily pretreated HIV-1 infected patients. Eur J Med Res 7: 309–314.
21. Herbinger KH, Gerhardt M, Piyasirisilp S, Mloka D, Arroyo MA, et al. (2006) Frequency of HIV type 1 dual infection and HIV diversity: analysis of low- and high-risk populations in Mbeya Region, Tanzania. AIDS Res Hum Retroviruses 22: 599–606.
22. World Health Organization (WHO) HIVDR status report. http://www.who.int/hiv/topics/drugresistance/status/en/index.html. Accessed 2011 May 15.
23. Somi GR, Kibuka T, Diallo K, Tuhuma T, Bennett DE, et al. (2008) Surveillance of transmitted HIV drug resistance among women attending antenatal clinics in Dar es Salaam, Tanzania. Antivir Ther 13 Suppl 2: 77–82.
24. Haas DW, Gebretsadik T, Mayo G, Menon UN, Acosta EP, et al. (2009) Associations between CYP2B6 polymorphisms and pharmacokinetics after a single dose of nevirapine or efavirenz in African americans. J Infect Dis 199: 872–880.
25. Smith PF, DiCenzo R, Morse GD (2001) Clinical pharmacokinetics of non-nucleoside reverse transcriptase inhibitors. Clin Pharmacokinet 40: 893–905.
26. Pao D, Andrady U, Clarke J, Dean G, Drake S, et al. (2004) Long-term persistence of primary genotypic resistance after HIV-1 seroconversion. J Acquir Immune Defic Syndr 37: 1570–1573.
27. Gunthard HF, Wong JK, Ignacio CC, Havlir DV, Richman DD (1998) Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples. AIDS Res Hum Retroviruses 14: 869–876.
28. Johnson JA, Li JF, Wei X, Lipscomb J, Irlbeck D, et al. (2008) Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naive populations and associate with reduced treatment efficacy. PLoS Med 5: e158.
29. Joint United Nations Programme on HIV/AIDS () Financial resources required to achieve universal access to HIV prevention, treatment, care and support. September 2007. (Accessed 3 April 2008.) Available from http://data.unaids.org/pub/ Report/2007/20070925_advocacy_grne2_en.pdf.
30. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425.

Chapter five

Chapter five

5. Discussion

5.1 HIV in South Africa

There are currently 5.7 million people infected with HIV in South Africa, making it the country with the highest number of infections in the world. This accounts for almost 12% of the total population (UNAIDS, 2011). The annual antenatal survey estimates that approximately 29.3% of women between 15 and 49 are infected. High variations are also seen between the provinces of South Africa, with the Western Cape having the lowest prevalence (16.1%) and Kwazulu-Natal the highest (38.7%) (South Africam Department of Health, 2010). The early South African HIV-1 epidemic was dominated by HIV-1 subtypes B and D, associated with the homosexual population (Engelbrecht *et al.*, 1995). This has been replaced by the fast spreading subtype C epidemic which is more commonly found in the heterosexual population (Jacobs *et al*., 2009; Van Harmelen *et al.*, 2003). Recently more and more non-B, non-C HIV and other recombinant strains have also been identified in the South African population (Wilkinson and Engelbrecht, 2009).

By May 2011 there were an estimated 1.4 million people receiving ART in South Africa, with the number still increasing. Current therapy guidelines state that therapy be initiated at a CD4+ cell count of 350 cells per mm^3 or below. Treatment should be given to all infants under the age of one, regardless of their CD4 count and all infected pregnant women should participate in a PMTCT prevention program. In the Western world it is recommended that a resistance profile be done on all HIV-1 infected individuals before the start of ART, as approximately 10% of patients have primary drug resistant strains (Arasteh *et al*., 2005). However, because of the high number of people infected in South Africa and the financial burden of treating everyone, such genetic test for everyone is currently impossible. During this study we investigated the genetic diversity of HIV-1 in Cape Town, South Africa for the period 2002 to 2004. We found that 95% of circulating strains belong to HIV-1 subtype C, 3.6% belong to subtype B with a subtype G and CRF02_AG strain also being identified. RAMs were also identified in five sequences (3.6%). These include three (2.1%) NNRTI mutations, one NRTI (0.7%) mutation and one PI (0.7%) leading to resistance against to 3TC, NVP, EFV, and DLV. Bessong *et al*., (2006) also identified resistance to NVP (5.7%) and 3TC (8.5%) in rural settings in South Africa. People starting therapy with pre-existing HIV resistant strains often have a higher chance for failing ART (Shafer *et al*., 2007). With the

progression of the national ART program, it is important to monitor the resistance profile of naïve and treatment-experienced patients.

5.2 HIV-1 subtype C

HIV-1 subtype C is currently the most prevalent HIV-1 subtype worldwide and therefore there has been much focus on the development of a subtype C candidate vaccine (Van Harmelen *et al.*, 2003). HIV-1 subtype C was first discovered in North East Africa, particularly Malawi and Ethiopia in the early 1980s (McCormack *et al*., 2002; Salminen *et al*., 1996). The oldest documented case, confirmed by DNA sequencing comes from a Malawian patient infected in 1983 (McCormack *et al*., 2002). The most common ancestor of HIV-1 subtype C dates back to the 1960's, which is consistent with data that HIV-1 group M originated in the 1930's (Travers *et al*., 2004). It has since spread throughout the world and has become the most dominant subtype in Sub-Saharan Africa (Gordon *et al.*, 2003) as well as East and Central Africa (Vidal *et al.*, 2000). There have been reports of subtype C in numerous countries, such as Brazil, China, India and Russia (Osmanov *et al.*, 2002). In many of these countries subtype C variants with intersubtype recombination have also been characterized (Pollakis *et al.*, 2003).

HIV-1 subtype C has very unique genetic characteristics which distinguishes it from other HIV-1 subtypes. These include the presence of extra NF-κβ enhancer copies in the LTR, Tat and Rev prematurely truncated proteins and a 15 bp insertion at the 5` end of the *vpu* reading frame. Subtype C also has a relatively conserved *env* gp120 V3 loop, with the virus showing preference to using CCR5 as its major co-receptor irrespective of the stage of disease progression (Ariën *et al.*, 2005), compared to subtype B which switches to CXCR4 and syncytia inducing during late stage of infection. It has been hypothesised that differences seen in the LTR promoter may be responsible for this rapid expansion of subtype C. The efficiency by which subtype C is transmitted from one person to another has been suggested as a contributing factor to subtype C predominance. However, subtype C does not have a higher fitness level compared to the other HIV-1 subtypes (Ariën *et al.*, 2005) and it is still exactly unclear why HIV-1 subtype C has become so predominant. Improved tourism and migration to and from countries with a high HIV-1 subtype C prevalence rate are probably also contributing to the spread of subtype C variants worldwide (de Oliveira *et al*., 2010).

5.3 HIV-1 in Tanzania

The HIV prevalence in adults (15 – 49) in Tanzania is currently 5.7% (UNAIDS, 2011). By the end of 2010 approximately 200 000 patients in the country were receiving ART through CTCs. HIV-1 subtypes A, C and D, and recombinants thereof are frequently being detected (Herbinger *et al*., 2006; Ndembi *et al*., 2008), as with our own observations. During this study we analysed viral strains from treatment naïve patients from Mwanza, Tanzania. HIVDR was determined in 88 sequentially enrolled ART-naïve patients (mean age 35.4 years). The frequency of HIVDR in the study population was 14.8% (95%; CI 0.072–0.223) and independent of NVP-resistance induced by PMTCT programs. Patients > 25 years had a significantly higher HIVDR frequency than younger patients (19.1%; 95% CI 0.095–0.28) versus 0%, P = 0.0344). This alarmingly high frequency of HIVDR could have been generated either by direct transmission of drug resistant viruses from sexual partners or through the natural pool of quasispecies in each individual patient following undisclosed ART. Both factors probably contributed to the observed high frequency of RAM in our Tanzanian study group.

5.4 HIV-1 diversity, ART and HIV-1 resistance

An ideal HIV vaccine should be active against all currently circulating strains. However, this is highly unlikely as HIV has an extremely high genetic diversity and easily mutates to escape the immune system (Nickle *et al*., 2007). ART has achieved success by keeping the viral titer under control. The life expectancy of individuals on ART has dramatically increased over the last few years, with therapy regimes continually improving. However, as a result of selective pressure from therapeutic drugs, HIV develops mutations which causes resistance to ART drugs (Johnson *et al*., 2003; Thompson *et al*., 2010). Most of what we know about HIV-1 resistance is based on observations with HIV-1 subtype B, as this is the most common subtype found in Europe and North America (Hemelaar *et al*., 2011). Only recently has Nauwelaers *et al*., (2011) developed a synthetic HIV-1 subtype C phenotypic assay, comparable to that of subtype B. It has been shown that genotypic resistance profiles may differ between HIV-1 subtype B and non-B subtypes. An example is the K65R RT protein change, which accumulates more easily in treatment failure patients of HIV-1 subtype C. HIV-1 subtype C isolates have a higher IC_{50} baseline value for the PI ATV, compared to the NL4-3 HIV-1 subtype B laboratory strain. Other RAMs not typical of subtype B include the RT change V106M and the PI changes I93L and M89I/V (Martinez-Cajas *et al*., 2008). It has also been found that some HIV-1 strains have lower pathogenic fitness levels than other

strains (Abraha *et al*., 2009). Therefore, it is still unclear to what extent HIV-1 genetic diversity will play a role in the ultimate successful treatment of HIV/AIDS patients. We should keep monitoring the HIV-1 genetic strains worldwide, as well as be aware of the RAMs which HIV strains may develop while patients are on treatment. During this study the HIV prevalence in the treatment naïve populations from Cape Town, South Africa and Mwanza, Tanzania were carefully investigated.

5.5 Vif function and diversity

Little is known about the influence of Vif diversity in HIV-1 pathogenesis. Vif is an accesory HIV-1 protein that blocks the antiretroviral activty of the APOBEC3F/G protein family (Conticello *et al*., 2003). Thus by helping degrade APOBEC3F/G, HIV infectivty in the producer cell is significantly enhanced. Vif also prohibits APOBEC3F/G being packaged into viral particles, although the exact mechanism is still unknown (Argyris and Pomerantz, 2004). Viruses lacking Vif are susceptible to being hypermutated, leading to non-infectious virions being produced (Simon *et al*., 2005). Some reports also suggest that long-term nonprogressors have a higher number of mutated or defective Vif proteins (Hassaïne *et al*., 2000; Yamada and Iwamoto, 2000). Therefore, during this study we also investigated HIV-1 *vif* sequence diversity in Cape Town, South Africa. As Vif-host interactions might be considered for future vaccine strategies, it is important to investigate the diversity of these genes (Miller *et al*., 2007).

5.6 Development of infectious HIV proviral molecular clones

Much of what we have learned about HIV biology has been with the use of studying infectious HIV proviral molecular clones in *in vitro* assays. Initially infectious clones were generated through lambda phage cloning, however this was a laborious and time consuming process (Adachi *et al*., 1986; Gao *et al*., 1998). New infectious molecular clones are known being created through long range Polymerase Chain Reaction (PCR) methods, as the fidelity of polymerase enzymes has been improved dramatically over the last few years (Cheng *et al*., 1995; Hogrefe and Borns, 2011; Michael and Kim, 1999). With PCR cloning techniques however, HIV genome errors often have to be fixed first before the proviral molecular clone is infectious, as a large number of primary HIV isolates circulating are often non-infectious (Ariën *et al*., 2005). Chimeric hybrid clones or simian human immunodeficiency viruses (SHIVs) have also been widely used to study HIV and SIV, especially in animal studies (Song *et al*., 2006; Smith *et al*., 2010).

During the course of this study we used a cultured South African HIV-1 subtype C strain to improve the replication capacity of a previously described proviral molecular clone MJ4 (Ndung'u *et al*., 2001). We added a CMV-driven promoter to pMJ4 to improve expression levels of virion proteins, which led to an approximate fourfold transient increase / production of infectious virus. The newly developed pcMJ4 clone was subsequently used to characterise the patient derived sequence of ZAC and to develop the more pathogenic pZAC infectious molecular clone from Cape Town, South Africa. The new infectious clone should be used in *in vitro* assays concerning HIV-1 subtype C.

5.7 Future perspectives

The high level diversity of HIV-1 has made it very difficult to obtain a vaccine against an epidemic that has plagued us for the last twenty years, although antiretroviral therapy continues to improve. The gold standard to study HIV-1diversity remains *in vitro* cell culture assays. In Europe and America this has ultimately been based on the HIV-1 subtype B infectious strains. As HIV-1 subtype C predominates in Sub-Saharan Africa we aimed to construct an infectious subtype C proviral molecular clone representative of strains in this region and which can be used in *in vitro* cell culture assays comparable to that of HIV-1 subtype B. The newly characterized infectious HIV-1 subtype C strain, pZAC can be used in pathogenesis studies and help to characterise currently circulating as well as drug resistance mutations of HIV-1 subtype C.

Although the HIVDR in the treatment naïve population in Cape Town, South Africa, at the time of this study was below 5%, in rural South Africa the levels were reported as high as 8.5%. In Tanzania we found that the HIVDR in the total population was 14.8%, much higher than has been previously reported. Therefore we recommend that all HIV-1 patients should undergo a HIV-1 genotyping test before the start of ART, although this is not always possible in resource-limited settings.

Chapter six

6. References

Abraha, A., Nankya, I. L., Gibson, R., Demers, K., Tebit, D. M., Johnston, E., Katzenstein, D., Siddiqui, A., Herrera, C. & other authors (2009). CCR5- and CXCR4-tropic subtype C human immunodeficiency virus type 1 isolates have a lower level of pathogenic fitness than other dominant group M subtypes: implications for the epidemic. *J Virol* **83,** 5592-5605.

Adachi, A., Gendelman, H. E., Koenig, E., Folks, T., Willey, R., Rabson, A. & Martin, M. A. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol* **59,** 284-291.

Aghokeng, A. F., Vergne, L., Mpoudi-Ngole, E., Mbangue, M., Deoudje, N., Mokondji, E., Nambei, W. S., Peyou-Ndi, M. M., Moka, J. J., Delaporte, E. & Peeters, M. (2009). Evaluation of transmitted HIV drug resistance among recently-infected antenatal clinic attendees in four Central African countries. *Antivir Ther* **14,** 401-411.

Ammamm, A. J., Abrams, D. I., Conant, M., Chudwin, D., Cowan, M., Volberding, P., Lewis, B. & Casavant, C. (1983). Acquired immune dysfunction in homosexual men: Immunologic profiles. *Clin Immunol Immunopathol* **27,** 315-320.

Apetrei, C., Robertson, D. L. & Marx, P. A. (2004). The history of SIVS and AIDS: epidemiology, phylogeny and biology of isolates from naturally SIV infected non-human primates (NHP) in Africa. *Front Biosci* **9,** 225-254.

Arasteh, K., Gölz, J., Marcus, U., RockStroh, J., Salzberger, B., & Vielhaber, B. (2005). Deutsch-Österreichische Leitlinien zur antiretroviralen Therapie der HIV-infektion. *Teilaktualisierung, Stand Juni* (http://www.rki.de/).

Argyris, E. G. & Pomerantz, R. J. (2004). HIV-1 Vif versus APOBEC3G: newly appreciated warriors in the ancient battle between virus and host. *Trends Microbiol* **12,** 145-148.

Arhel, N. J. & Kirchhoff, F. (2009). Implications of Nef: host cell interactions in viral persistence and progression to AIDS. *Curr Top Microbiol Immunol* **339,** 147-75.

Ariën, K. K., Abraha, A., Quiñones-Mateu, M. E., Kestens, L., Vanham, G. & Arts, E. J. (2005). The replicative fitness of primary human immunodeficiency virus type 1 (HIV-1) group M, HIV-1 group O, and HIV-2 isolates. *J Virol* **79,** 8979-8990.

Arriaga, M. E., Carr, J., Li, P., Wang, B., & Saksena, N. K. (2006). Interaction between HIV-1 and APOBEC3 sub-family of proteins. *Curr HIV Res* **4,** 401-409.

Arthur, L. O., Bess, J. W. Jr, Sowder, R. C. 2nd, Benveniste, R. E., Mann, D. L., Chermann, J. C. & Henderson, L. E. (1992). Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. *Science* **18,** 1935-1938.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (2003). Current protocols in Molecular Biology. *John Wiley and Sons* New York, USA.

Ayouba, A., Lien, T. T., Nouhin, J., Vergne, L., Aghokeng, A. F., Ngo-Giang-Huong, N., Diop, H., Kane, C. T., Valéa, D. & other authors (2009). Low prevalence of HIV type 1 drug resistance mutations in untreated, recently infected patients from Burkina Faso, Cote d'Ivoire, Senegal, Thailand, and Vietnam: the ANRS 12134 study. *AIDS Res Hum Retroviruses* **25,** 1193-1196.

Bachand, F., Yao, X. J., Hrimech, M., Rougeau, N. & Cohen, E. (1999). Incorporation of Vpr into human immunodeficiency virus type 1 requires a direct interaction with the p6 domain of the p55 Gag precursor. *J Biol Chem* **274,** 9083-9091.

Badley, A. D. (2005). In vitro and in vivo effects of HIV protease inhibitors on apoptosis. *Cell Death Differ* **12,** 924-931.

Ball, S. C., Abraha, A., Collins, K. R., Marozsan, A. J., Baird, H., Quiñones-Mateu, M. E., Penn-Nicholson, A., Murray, M., Richard, N. & other authors (2003). Comparing the ex vivo fitness of CCR5-tropic human immunodeficiency virus type 1 isolates of subtypes B and C. *J Virol* **77,** 1021-1038.

Bar-Magen, T., Sloan, R. D., Donahue, D. A., Kuhl, B. D., Zabeida, A., Xu, H., Oliveira, M., Hazuda, D. J. & Wainberg, M. A. (2010). Identification of novel mutations responsible for resistance to MK-2048, a second-generation HIV-1 integrase inhibitor. *J Virol* 2010 **84,** 9210-9216.

Barr, S. D., Smiley, J. R. & Bushman, F. D. (2008). The interferon response inhibits HIV particle production by induction of TRIM22. *PLoS Pathog* 2008 **4,** e1000007.

Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyve, M. T., Chamaret, S., Gruist, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F. & other authors (1983). Isolation of a T- lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220,** 868-871.

Barth, R. E., van der Loeff, M. F., Schuurman, R., Hoepelman, A. I. & Wensing, A. M. (2010). Virological follow-up of adult patients in antiretroviral treatment programmes in sub-Saharan Africa: a systematic review. *Lancet Infect Dis* **10,** 155-166.

Barth, R. E., Wensing, A. M., Tempelman, H. A., Moraba, R., Schuurman, R. & Hoepelman, A. I. (2008). Rapid accumulation of nonnucleoside reverse transcriptase inhibitor-associated resistance: evidence of transmitted resistance in rural South Africa. *AIDS* **22,** 2210-2212.

Bell, C. M., Connell, B. J., Capovilla, A., Venter, W. D., Stevens, W. S. & Papathanasopoulos, M. A. (2007). Molecular characterization of the HIV type 1 subtype C accessory genes vif, vpr, and vpu. *AIDS Res Hum Retroviruses* **23,** 322-330.

Bennett, D. E., Bertagnolio, S., Sutherland, D. & Gilks, C. F. (2008). The World Health Organization's global strategy for prevention and assessment of HIV drug resistance. *Antivir Ther* **13 Suppl 2**, 1-13.

Bennett, D. E., Camacho, R. J., Otelea, D., Kuritzkes, D. R., Fleury, H., Kiuchi, M., Heneine, W., Kantor, R., Jordan, M. R. & other authors (2009). Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. *PLoS ONE* **4,** e4724.

Bennett, D. E., Myatt, M., Bertagnolio, S., Sutherland, D. & Gilks, C. F. (2008). Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment. *Antivir Ther* **13 Suppl 2,** 25-36.

Bessong, P. O., Mphahlele, J., Choge, I. A., Obi, L. C., Morris, L., Hammarskjold, M. L., & Rekosh, D. M. (2006). Resistance mutational analysis of HIV type 1 subtype C among rural South African drug-naive patients prior to large-scale availability of antiretrovirals. *AIDS Res Hum Retroviruses* **22,** 306-312.

Bloom, A. L. (1984). Acquired immunodeficiency syndrome and other possible immunological disorders in European haemophiliacs. *Lancet* **1,** 1452-1455.

Bohne, J. & Kräusslich, H. G. (2004). Mutation of the major 5' splice site renders a CMV-driven HIV-1 proviral clone Tat-dependent: connections between transcription and splicing. *FEBS Lett* **563,** 113-118.

Bour, S. & Strebel, K. (2003). The HIV-1 Vpu protein: a multifunctional enhancer of viral particle release. *Microbes Infect* **5,** 1029-1039.

Brigati, C., Giacca, M., Noonan, D. M., & Albini, A. (2003). HIV Tat, its TARgets and thecontrol of viral gene expression. *FEMS Microbiol Lett* **220,** 57-65.

Briggs, J. A. G., Wilk, T., Welker, R., Kräusslich, H. G., Stephen, D. & Fuller, S. D. (2003). Structural organization of authentic, mature HIV-1 virions and cores. *EMBO J* **22,** 1707-1715.

Burgers, W. A., van Harmelen, J. H., Shephard, E., Adams, C., Mgwebi, T., Bourn, W., Hanke, T., Williamson, A. L. & Williamson, C. (2006). Design and preclinical evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. *J Gen Virol* **87,** 399-410.

Cameron, D. W., da Silva, B. A., Arribas, J. R., Myers, R. A., Bellos, N. C., Gilmore, N., King, M. S., Bernstein, B. M., Brun, S. C. & Hanna, G. J. (2008). A 96-week comparison of lopinavir-ritonavir combination therapy followed by lopinavir-ritonavir monotherapy versus efavirenz combination therapy. *J Infect Dis* **198,** 234-240.

Carr, J. M., Coolen, C., Davis, A. J., Burrell, C. J. & Li, P. (2008). Human immunodeficiency virus 1 (HIV-1) virion infectivity factor(Vif) is part of reverse transcription complexes and acts as an accessory factor for reverse transcription. *Virology* **1,** 147-156.

Cheng, S., Chen, Y., Monforte, J. A., Higuchi, R. & Van Houten B. (1995). Template integrity is essential for PCR amplification of 20- to 30-kb sequences from genomic DNA. *PCR Methods Appl* **4,** 294-298.

Coffin, J. M., Haase, A. & Levy, J. A. (1986a). What to call the AIDS virus? *Nature* **321,** 10.

Coffin, J. M., Haase, A., Levy, J. A., Montagnier, L., Oroszlan S, Teich, N., Temin, H., Toyoshima, K., Varmus, H., Vogt, P. & Weiss, R. (1986b). Human immunodeficiency viruses. *Science* **232,** 697.

Conticello, S. G., Harris, R. S. & Neuberger, M. S. (2003). The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. *Curr Biol* **13,** 2009-2013.

Curran, J. W., Lawrence, D. N., Jaffe, H., Kaplan, J.E., Zyla, L. D., Chamberland, M., Weinstein, R., Lui, K. J., Schonberger, L. B. & Spira, T. J. (1984). Acquired immunodeficiency syndrome (AIDS) associated with transfusions. *N Eng J Med* **310,** 69-74.

Damond, F., Worobey, M., Campa, P., Farfara, I., Colin, G., Matheron, S., Brun-Vezinet, F., Robertson, D. L. & Simon, F. (2004). Identification of a highly divergent HIV type 2 and proposal for a change in HIV type 2 classification. *AIDS Res Hum Retroviruses* **20,** 666-672.

Dash, P. K., Siddappa, N. B., Mangaiarkarasi, A., Mahendarkar, A. V., Roshan, P., Anand, K. K., Mahadevan, A., Satishchandra, P., Shankar, S. K. & other authors (2008). Exceptional molecular and coreceptor-requirement properties of molecular clones isolated from an Human Immunodeficiency Virus Type-1 subtype C infection. *Retrovirology* **5,** 25.

Deeks, S. G. (2008). Transmitted minority drug-resistant HIV variants: a new epidemic? *PLoS Med* **5,** e164.

de Oliveira, T., Deforche, K., Cassol, S., Salminen, M., Paraskevis, D., Seebregts, C., Snoeck, J., van Rensburg, E. J., Wensing, A. M. & other authors (2005). An automated genotyping system for analysis of HIV-1 and other microbial sequences. *Bioinformatics* **21,** 3797-3800.

de Oliveira, T., Engelbrecht, S., Janse van Rensburg, E., Gordon, M., Bishop, K., zur Megede, J., Barnett, S. W. & Cassol, S. (2003). Variability at human immunodeficiency virus type 1 subtype C protease cleavage sites: an indication of viral fitness? *J Virol 2003* **77,** 9422-9430.

de Oliveira, T., Pillay, D. & Gifford, R. J., for the UK Collaborative Group on HIV Drug Resistance (2010). The HIV-1 Subtype C Epidemic in South America Is Linked to the United Kingdom. *PLoS ONE* **5,** e9311.

Derdeyn, C. A., Decker, J. M., Sfakianos, J. N., Wu, X., O'Brien, W.A., Ratner, L., Kappes, J.C., Shaw, G. M. & Hunter, E. (2000). Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. *J Virol* **74,** 8358-8367.

D'Souza, V. & Summers, M. F. (2005). How Retroviruses select their genomes. *Nature Reviews Microbiology* **3,** 643-655.

Engelbrecht, S., Laten, J. D., Smith, T-L., & J van Rensburg, E. (1995). Identification of *env* subtypes in fourteen HIV type 1 isolates from South Africa. *AIDS Res Hum Retroviruses* **11,** 1269-1271.

Fischl, M. A., Richman, D. D., Grieco, M. H., Gottlieb, M. S., Volberding, P. A., Laskin, O. L., Leedom, J. M., Groopman, J. E., Mildvan, D. & Schooley, R. T. (1987). The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. *N Engl J Med* **317,** 185-191.

Freed, E. O. (2001). HIV-1 replication. *Somat Cell Mol Genet* **26,** 13-33.

Friedman-Kien, A. E., Laubenstein, L., Marmor, M., Hymes, K., Green, J., Ragaz, A., Gottleib, J., Muggia, F., Demopoulos, R., & other authors. (1981). Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men - New York City and California. *MMWR* **30,** 305-308.

Gao, F., Bailes, E., Robertson, D. L., Chen, Y., Rodenburg, C. M., Michael, S. F., Cummins, L. B., Arthur, L. O., Peeters, M. & other authors (1999). Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397,** 436-441.

Gao, F., Robertson, D. L., Carruthers, C. D., Morrison, S. G., Jian, B., Chen, Y., Barré-Sinoussi, F., Girard, M., Srinivasan, A. & other authors (1998). A comprehensive panel of near-full-length clones and reference sequences for non-subtype B isolates of human immunodeficiency virus type 1. *J Virol* **72,** 5680-5698.

Gao, F., Yue, L. & White, A. T. (1992). Human infection by genetically diverse SIVSM-related HIV-2 in west Africa. *Nature* **358,** 495-499.

Garrido, C., Soriano, V., Geretti, A. M., Zahonero, N., Garcia, S., Booth, C., Gutierrez, F., Viciana, I. & de Mendoza, C. (2011). Resistance associated mutations to dolutegravir (S/GSK1349572) in HIV-infected patients - impact of HIV subtypes and prior raltegravir experience. *Antiviral Res* **90,** 164-167.

Gaschen, B., Taylor, J., Yusim, K., Foley, B., Gao, F., Lang, D., Novitsky, V., Haynes, B., Hahn, B. H., Bhattacharya, T. & Korber, B. (2002). Diversity considerations in HIV-1 vaccine selection. *Science* **296,** 2354-2360.

Gilks, C. F., Crowley, S., Ekpini, R., Gove, S., Perriens, J., Souteyrand, Y., Sutherland, D., Vitoria, M., Guerma, T. & De Cock, K. (2006). The WHO public health approach to antiretroviral treatment against HIV in resource- limited settings. *Lancet* **368,** 505-510.

Glass, W. G., McDermott, D. H., Lim, J. K., Lekhong, S., Yu, S. F., Frank, W. A., Pape, J., Cheshier, R. C. & Murphy, P. M. (2006). CCR5 deficiency increases risk of symptomatic West Nile virus infection. *J Exp Med* **203,** 35-40.

Goebel, F., Yakovlev, A., Pozniak, A. L., Vinogradova, E., Boogaerts, G., Hoetelmans, R., de Béthune, M. P., Peeters, M. & Woodfall, B. (2006). Short-term antiviral activity of TMC278-a novel NNRTI in treatment-naive HIV-1-infected subjects. *AIDS* **20,** 1721-1726.

Gómez, C. E., Nájera, J. L., Jiménez, V., Bieler, K., Wild, J., Kostic, L., Heidari, S., Chen, M., Frachette, M. J. & other authors (2007). Generation and immunogenicityof novel HIV/AIDS vaccine candidates targeting HIV-1 Env/Gag-Pol-Nef antigens of clade C. *Vaccine* **12,** 2863-2885.

Gordon, M., de Oliveira, T., Bishop, K., Coovadia, H. M., Madurai, L., Engelbrecht, S., Janse van Rensburg, E., Mosam, A., Smith, A. & Cassol, S. (2003). Molecular characteristics of human immunodeficiency virus type 1 subtype C viruses from KwaZulu-Natal, South Africa: Implications for vaccine and antiretroviral control strategies. *J Virol* **77,** 2587-2599.

Gottlieb, M. S., Schanker, H. M., Fan, P. T., Saxon, A. & Weisman, J. D. (1981a). Pneumocystis Pneumonia - Los Angeles. *MMWR* **30,** 250-252.

Gottlieb, M. S., Schroff, R., Schanker, H. M., Weisman, J. D., Fan, P. T., Wolf, R. A. & Saxon, A. (1981b). Pneumocytis Carinii pneumonia and mucosal candidiasis in previously healthy homosexual men. *N Eng J Med* **305,** 1425-1431.

Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36,** 59-74.

Grisson, R. D., Chenine, A. L., Yeh, L. Y., He, J., Wood, C., Bhat, G. J., Xu, W., Kankasa, C. & Ruprecht, R. M. (2004). Infectious molecular clone of a recently transmitted pediatric human immunodeficiency virus clade C isolate from Africa: evidence of intraclade recombination. *J Virol* **78,** 14066-14069.

Guay, L. A., Musoke, P., Fleming, T., Bagenda, D., Allen, M., Nakabiito, C., Sherman, J., Bakaki, P., Ducar, C. & other authors (1999). Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. *Lancet* **354,** 795-802.

Gunthard, H. F., Wong, J. K., Ignacio, C. C., Havlir, D. V. & Richman, D. D. (1998). Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples. *AIDS Res Hum Retroviruses* **14,** 869-876.

Hahn, B. H., Shaw, G. M., De Cock, K. M. & Sharp, P. M. (2000). AIDS as a Zoonosis: Scientific and public health implications. *Science* **287,** 607-614.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* **41,** 95-98.

Harada, S., Koyanagi, Y. &Yamamoto, N. (1985). Infection of human T-lymphotropic virus type-I (HTLV-I)-bearing MT-4 cells with HTLV- III (AIDS virus): chronological studies of early events. *Virology* **146,** 272-281.

Harari, A., Bart, P. A., Stöhr, W., Tapia, G., Garcia, M., Medjitna-Rais, E., Burnet, S., Cellerai, C., Erlwein, O. & other authors (2008). An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. *J Exp Med* **205,** 63-67.

Harries, A. D., Zachariah, R., van Oosterhout, J. J., Reid, S. D., Hosseinipour, M. C., Arendt, V., Chirwa, Z., Jahn, A., Schouten, E. J. & Kamoto, K. (2010). Diagnosis and management of antiretroviral-therapy failure in resource- limited settings in sub-Saharan Africa: challenges and perspectives. *Lancet Infect Dis* **10,** 60-65.

Hartl, M. J., Bodem, J., Jochheim, F., Rethwilm, A., Rösch, P. & Wöhrl, B. M. (2011). Regulation of foamy virus protease activity by viral RNA: a novel and unique mechanism among retroviruses. *J Virol* **85,** 4462-4469.

Hassaïne, G., Agostini, I., Candotti, D., Bessou, G., Caballero, M., Agut, H., Autran, B., Barthalay, Y. & Vigne R. (2000). Characterization of human immunodeficiency virus type 1 vif gene in long-term asymptomatic individuals. *Virology* **276,** 169-180.

Hemelaar, J., Gouws, E., Ghys, P. D. & Osmanov, S. WHO-UNAIDS Network for HIV Isolation and Characterisation. (2011). Global trends in molecular epidemiology of HIV-1 during 2000-2007. *AIDS* **25,** 679-689.

Herbinger, K. H., Gerhardt, M., Piyasirisilp, S., Mloka, D., Arroyo, M. A., Hoffmann, O., Maboko, L., Birx, D. L., Mmbando, D., McCutchan, F. E. & Hoelscher M. (2006). Frequency of HIV type 1 dual infection and HIV diversity: analysis of low- and high-risk populations in Mbeya Region, Tanzania. *AIDS Res Hum Retroviruses* **22,** 599-606.

Hightower, K. E-, Wang. R-, Deanda. F., Johns, B. A., Weaver, K., Shen, Y., Tomberlin, G. H., Carter, H. L. 3rd., Broderick, T. & other authors (2011). Dolutegravir (S/GSK1349572) Exhibits Significantly Slower Dissociation than Raltegravir and Elvitegravir from Wild Type and Integrase Inhibitor-Resistant HIV-1 Integrase-DNA Complexes. *Antimicrob Agents Chemother* Aug 2011.

Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H. & Johnson, P. R. (1989). An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339,** 389-392.

Hogrefe, H. H. & Borns, M. C. (2011). Long-range PCR with a DNA polymerase fusion. *Methods Mol Biol*. **687,** 17-23.

Hopfner, K. P., Eichinger, A., Engh, R. A., Laue, F., Ankenbauer, W., Huber, R. & Angerer, B. (1999). Crystal structure of a thermostable type B DNA polymerase from Thermococcus gorgonarius. *Proc Natl Acad Sci USA* **96,** 3600-3605.

Hosegood, V., Vanneste, A. M., & Timaeus, I. M. (2004). Levels and causes of adult mortality in rural South Africa: The impact of AIDS. *AIDS* **18,** 663-671.

Huang, H., Chopra, R., Verdine, G. L. & Harrison, S. C. (1998). Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* **282,** 1669-1675.

Hymes, K. B., Cheung, T., Greene, J. B., Prose, N. S., Marcus, A., Ballard, H., William, D. C. & Laubenstein, L. J. (1981). Kaposi's sarcoma in homosexual men - a report of eight cases. *Lancet* **2,** 598-600.

Iordanskiy, S., Waltke, M., Feng, Y. & Wood, C. (2010). Subtype-associated differences in HIV-1 reverse transcription affect the viral replication. *Retrovirology* **7,** 85.

Jacobs, G. B., Loxton, A. G., Laten, A., Robson B., van Rensburg, E. J. & Engelbrecht, S. (2009). Emergence and diversity of different HIV-1 subtypes in South Africa, 2000-2001. *J Med Virol* **81,** 1852-1859.

Janeway, C. A., Travers, P., Walport, M. & Shlomchik, M. (2001). Immunobiology: The immune system in health and disease. *Fith Edition Garland Publishing* New York, USA.

Johnson, V. A., Brun-Vezinet, F., Clotet, B., Conway, B., D'Aquila, R. T., Demeter, L. M., Kuritzkes, D. R., Pillay, D., Schapiro, J. M. & other authors (2003). Drug resistance mutations in HIV-1. *Top HIV Med* **11,** 215-221.

Johnson, V.A., & Byington, R.E. (1990). Infectivity assay (virus yield assay). In: Techniques in HIV Research (Aldovani, A., and Walker, B.D., eds.). *Stockton Press*, New York, N.Y., 71-76.

Johnson, J. A., Li, J. F., Morris, L., Martinson, N., Gray, G., McIntyre, J. & Heneine, W. (2005). Emergence of drug-resistant HIV-1 after intrapartum administration of single-dose nevirapine is substantially underestimated. *J Infect Dis* **192,** 16-23.

Johnson, J. A., Li, J. F., Wei, X., Lipscomb, J., Irlbeck, D., Craig, C., Smith, A., Bennett, D. E. & Monsour, M., (2008). Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naive populations and associate with reduced treatment efficacy. *PLoS Med* **5,** e158.

Joint United Nations Programme on HIV/AIDS. Financial resources required to achieve universal access to HIV prevention, treatment, care and support. September 2007. (Accessed 3 April 2008.) Available from http://data.unaids. org/pub/ Report/2007/20070925_advocacy_grne2_en.pdf.

Jolly, C., Booth, N. J. & Neil, S. J. (2010). Cell-cell spread of human immunodeficiency virus type 1 overcomes tetherin/BST-2-mediated restriction in T cells. *J Virol* **84,** 12185-12199.

Kalish, M. L., Wolfe, N. D., Ndongmo, C. B., McNicholl, J., Robbins, K. E., Aidoo, M., Fonjungo, P. N., Alemnji, G., Zeh, C., Djoko, C. F. & other authors (2005). Central African hunters exposed to simian immunodeficiency virus. *Emerg Infect Dis* **11,** 1928-1930.

Kamoto, K. & Aberle-Grasse, J. (2008). Surveillance of transmitted HIV drug resistance with the World Health Organization threshold survey method in Lilongwe, Malawi. *Antivir Ther* **13 Suppl 2,** 83-87.

Kantor, R. (2006). Impact of HIV-1 pol diversity on drug resistance and its clinical implications. *Curr Opin Infect Dis* **19,** 594-606.

Kashanchi, F., Khleif, S. N., Duvall, J. F., Sadaie, M. R., Radonovich, M. F, Cho, M., Martin, M. A., Chen, S. Y., Weinmann, R. & Brady, J. N. (1996). Interaction of human immunodeficiency virus type 1 Tat with a unique site of TFIID inhibits negative cofactor Dr1 and stabilizes the TFIID-TFIIA complex. *J Virol* **70,** 5503-5510.

Keele, B. F., Jones, J. H., Terio, K. A, Estes, J. D., Rudicell, R. S., Wilson, M. L., Li, Y., Learn, G. H., Beasley, T.M. & other authors (2009). Increased mortality and AIDS-like immunopathology in wild chimpanzees infected with SIVcpz. *Nature* **460,** 515-519.

Keele, B. F., Van Heuverswyn, F., Li, Y., Bailes, E., Takehisa, J., Santiago, M. L., Bibollet-Ruche, F., Chen, Y., Wain, L. V. & other authors (2006). Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. *Science* **313,** 523-526.

Khan, M. A., Aberham, C., Kao, S., Akari, H., Gorelick, R., Bour, S. & Strebel, K. (2001). Human immunodeficiency virus type 1 Vif protein is packaged into the nucleoprotein complex through an interaction with viral genomic RNA. *J Virol* **75,** 7252-7265.

Kirchhoff, F., Schindler, M., Specht, A., Arhel, N. & Münch, J. (2008). Role of Nef in primate lentiviral immunopathogenesis. *Cell Mol Life Sci* **65,** 2621-2636.

Klimas, N., Koneru, A. O. & Fletcher, M. A. (2008). Overview of HIV. *Psychosom Med* **70,** 523-530.

Korber, B., Gaschen, B., Yusim, K., Thakallapally, R., Kesmir, C. & Detours, V. (2001). Evolutionary and immunological implications of contemporary HIV-1 variation. *Br Med Bull* **58,** 19-42.

Korber, B., Muldoon, M., Theiler, J., Gao, F., Gupta, R., Lapedes, A., Hahn, B. H., Wolinsky, S. & Bhuttacharga, T. (2000). Timing the ancestor of the HIV-1 pandemic strains. *Science* **288,** 1789-1795.

Lallemant, M., Jourdain, G., Le Coeur, S., Mary, J. Y., Ngo-Giang-Huong, N., Koetsawang, S., Kanshana, S., McIntosh, K. & Thaineua, V; Perinatal HIV Prevention Trial (Thailand) Investigators. (2004). Single-dose perinatal nevirapine plus standard zidovudine to prevent mother-to-child transmission of HIV-1 in Thailand. *N Engl J Med* **15,** 217-228.

Langmann, P., Heinz, W., Klinker, H., Schirmer, D., Guhl, C., Leyh, M. & Winzer, R. (2008). High performance liquid chromatographic method for the determination of HIV-1 protease inhibitor tipranavir in plasma of patients during highly active antiretroviral therapy. *Eur J Med Res* **13,** 52-58.

Langmann, P., Weissbrich, B., Desch, S., Väth, T., Schirmer, D., Zilly, M. & Klinker H. (2002). Efavirenz plasma levels for the prediction of treatment failure in heavily pretreated HIV-1 infected patients. *Eur J Med Res* **7,** 309-314.

Larder, B. A., Kemp, S. D., Purifoy, D. J. (1989). Infectious potential of human immunodeficiency virus type 1 reverse transcriptase mutants with altered inhibitor sensitivity. *Proc Natl Acad Sci USA* **86,** 4803-4807.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23,** 2947-2948.

Le, S. Y., Zhang, K. & Maizel, J. V. Jr. (2002). RNA molecules with structure dependent functions are uniquely folded. *Nucleic Acids Res* **30,** 3574-3582.

Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N. & Gregory, T. J. (1990). Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J Biol Chem* **265,** 10373-10382.

Levy, J. A., Hoffman, A. D., Kramer, S. M., Landis, J. A., Shimabukuro, J. M. & Oshiro, L. S. (1984). Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* **225,** 840-842.

Loyter, A., Scangos, G. A. & Ruddle, F. H. (1982). Mechanisms of DNA uptake by mammalian cells: fate of exogenously added DNA monitored by the use of fluorescent dyes. *Proc Natl Acad Sci USA* **79,** 422-426.

Ly, A. & Stamatatos, L. (2000). V2 Loop Glycosylation of the Human Immunodeficiency Virus Type 1 SF162 Envelope Facilitates Interaction of This Protein with CD4 and CCR5 Receptors and Protects the Virus from Neutralization by Anti-V3 Loop and Anti-CD4 Binding Site Antibodies. *J Virol* **74,** 6769-6776.

Malim, M. H. (2006). Natural resistance to HIV infection: The Vif-APOBEC interaction. *C R Biol* **329,** 871-875.

Maphalala, G., Okello, V., Mndzebele, S., Gwebu, P., Mulima, N., Dlamini, S., Nhlabatsi, B., Ginindza, T., Ghebrenegus, Y. & other authors (2008). Surveillance of transmitted HIV drug resistance in the Manzini-Mbabane corridor, Swaziland, in 2006. *Antivir Ther* **13 Suppl 2,** 95-100.

Masur, H., Michelis, M. A., Wormser, G. P., Lewin, S., Gold, J., Tapper, M. L., Giron, J., Lerner, C. W., Armstrong, D. & other authors (1982). Oppurtunistic infection in previously healthy women. *Ann Intern Med* **97,** 533-539.

Martinez-Cajas, J. L., Pai, N. P., Klein, M. B. & Wainberg, M. A. (2009). Differences in resistance mutations among HIV-1 non-subtype B infections: a systematic review of evidence (1996-2008). *J Int AIDS Soc* **12,** 11.

Maxwell, K. L. & Frappier, L. (2007). Viral proteomics. *Microbiol Mol Biol Rev* **71,** 398-411.

McCormack, G. P., Glynn, J. R., Crampin, A. C., Sibande, F., Mulawa, D., Bliss, L., Broadbent, P., Abarca, K., Pönnighaus, J. M., Fine, P. E. & Clewley, J. P. (2002). Early evolution of the human immunodeficiency virus type 1 subtype C epidemic in rural Malawi. *J Virol* **76,** 12890-12899.

McCormick-Davis, C., Dalton, S. B., Singh, D. K. & Stephens, E. B. (2000). Comparison of Vpu sequences from diverse geographical isolates of HIV type 1 identifies the presence of highly variable domains, additional invariant amino acids, and a signature sequence motif common to subtype C isolates. *AIDS Res Hum Retroviruses* **16,** 1089-1095.

McCutchan, F. E. (2006). Global epidemiology of HIV. *J Med Virol* **78,** S7-S12.

Mehle, A., Thomas, E. R., Rajendran, K. S. & Gabuzda, D. (2006). A zincbinding region in Vif binds Cul5 and determines cullin selection. *J Biol Chem* **23,** 17259-17265.

Mehle, A., Wilson, H., Zhang, C., Brazier, A. J., McPike, M., Pery, E. & Gabuzda D. (2007). Identification of an APOBEC3G binding site in human immunodeficiency virus type 1 Vif and inhibitors of Vif-APOBEC3G binding. *J Virol* **81,** 13235-13241.

Migueles, S. A., Sabbaghian, M. S., Shupert, W. L., Bettinotti, M. P., Marincola, F. M., Martino, L., Hallahan, C. W., Selig, S. M., Schwartz, D., Sullivan, J. & Connors, M. (2000). HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci USA* **97**, 2709-2714.

Miller, J. H., Presnyak, V. & Smith, H. C. (2007). The dimerization domain of HIV-1 viral infectivity factor Vif is required to block virion incorporation of APOBEC3G. *Retrovirology* **4,** 81.

Mochizuki, N., Otsuka, N., Matsuo, K., Shiino, T., Kojima, A., Kurata, T., Sakai, K., Yamamoto, N., Isomua, S. & other authors (1999). An infectious DNA clone of HIV type 1 subtype C. *AIDS Res Hum Retroviruses* **15,** 1321-1324.

Moodley, D., Moodley, J., Coovadia, H., Gray, G., McIntyre, J., Hofmyer, J., Nikodem, C., Hall, D., Gigliotti, M. & other authors (2003). A multicenter randomized controlled trial of nevirapine versus a combination of zidovudine and lamivudine to reduce intrapartum and early postpartum mother-to-child transmission of human immunodeficiency virus type 1. *J Infect Dis* **187,** 725-735.

Mullis, K. B. & Faloona, F. A. (1987). Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. *Methods Enzymol* **155,** 335-350.

Nahmias, A. J., Weiss, J., Yao, X., Lee, F., Kodsi, R., Schanfield, M., Matthews, T., Bolognesi, D., Durack, D. & other authors (1986). Evidence for human infection with an HTLV III/ LAV like virus in central Africa, 1959. *Lancet* **1,** 1279-1280.

National Guidelines for the management of HIV and AIDS in Tanzania.

Nauwelaers, D., Van Houtte,. M., Winters, B., Steegen K., Van Baelen, K., Chi, E., Zhou, M., Steiner, D., Bonesteel, R., Aston, C. & Stuyver, L. J. (2011). A synthetic HIV-1 subtype C backbone generates comparable PR and RT resistance profiles to a subtype B backbone in a recombinant virus assay. **PloS ONE** *6,* e19643.

Ndembi, N., Lyagoba, F., Nanteza, B., Kushemererwa, G., Serwanga, J., Katongole-Mbidde, E., Grosskurth, H. & Kaleebu P.; Uganda HIV Drug Resistance Working Group. (2008). Transmitted antiretroviral drug resistance surveillance among newly HIV type 1-diagnosed women attending an antenatal clinic in Entebbe, Uganda. *AIDS Res Hum Retroviruses* **24,** 889-895.

Ndung'u, T., Renjifo, B. & Essex, M. (2001). Construction and analysis of an infectious human Immunodeficiency virus type 1 subtype C molecular clone. *J Virol* **75,** 4964-4972.

Neil, S. J., Zang, T. & Bieniasz, P. D. (2008). Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* **451,** 425-430.

Nicholas, K. B., Nicholas, H. B. Jr. & Deerfield, D. W. II. (1997). GeneDoc: Analysis and Visualization of Genetic Variation, *EMBNEW.NEWS* **4,** 14.

Nickle, D, C., Rolland, M., Jensen, M. A., Pond, S. L., Deng, W., Seligman, M., Heckerman, D., Mullins, J. I. & Jojic, N. (2007). Coping with viral diversity in HIV vaccine design. *PLoS Comput Biol* **3,** e75.

Nisole, S. & Saïb, A. (2004). Early steps of retrovirus replicative cycle. *Retrovirology* **1,** 1-20.

Nyombi, B. M., Holm-Hansen, C., Kristiansen, K. I., Bjune, G. & Muller, F. (2008). Prevalence of reverse transcriptase and protease mutations associated with antiretroviral drug resistance among drug-naive HIV-1 infected pregnant women in Kagera and Kilimanjaro regions, Tanzania. *AIDS Res Ther* **5,** 13.

O'Connell, K. A., Bailey, J. R. & Blankson, J. N. (2009). Elucidating the elite: mechanisms of control in HIV-1 infection. *Trends Pharmacol Sci* **30,** 631-637.

Oleske, J., Muimefor, A., Cooper, R. Jr., Thomas, K., dela Cruz, A., Ahdieh, H., Guerrero, I., Joshi, V. V. & Desposito, F. (1983). Immune deficiency syndrome in children. *JAMA*. **249,** 2345-2351.

Osmanov, S., Pattou, C., Walker, N., Schwarlander, B., Esparza, J. & the WHO-UNAIDS Network for HIV Isolation and Characterisation. (2002). Estimated global distribution and regional spread of HIV-1 genetic subtypes in the year 2002. *J Acquir Immune Defic Syndr* **29,** 184-190.

Pao, D., Andrady, U., Clarke, J., Dean, G., Drake, S., Fisher, M., Green, T., Kumar, S., Murphy, M. & other authors (2004). Long-term persistence of primary genotypic resistance after HIV-1 seroconversion. *J Acquir Immune Defic Syndr* **37,** 1570-1573.

Pape, J. W., Liautaud, B., Thomas, F., Mathurin, J. R., St Amand, M. M., Boncy, M., Pean, V., Pamphile, M., Laroche, A. C. & Johnson, W. D. Jr. (1983). Characteristics of the acquired immunodeficiency syndrome (AIDS) in Haiti. *N Engl J Med* **309,** 945-950.

Paxton, W., Connor, R. I. & Landau, N. R. (1993). Incorporation of Vpr into human immunodeficiency virus type 1 virions: Requirement for the p6 region of gag and mutational analysis. *J Virol* **67,** 7229-7237.

Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* **90,** 8392-8396.

Pillay, C., Bredell, H., McIntyre, J., Gray, G. & Morris, L. (2002). HIV-1 subtype C reverse transcriptase sequences from drugnaivepregnant women in South Africa. *AIDS Res Hum Retroviruses* **18,** 605-610.

Piot, P., Ouinn, T. C., Taelman, H., Feinsod, F. M., Minlangu, K. B., Wobin, O., Mbendi, N., Mazebo, P., Ndangi, K. & Stevens, W. (1984). Acquired immunodeficiency syndrome in a heterosexual population in Zaire. *Lancet* **2,** 65-69.

Plantier, J. C., Dachraoui, R., Lemee, V., Gueudin, M., Borsa-Lebas, F., Caron, F. & Simon, F. (2005). HIV-1 resistance genotyping on dried serum spots. *AIDS* **19,** 391-397.

Pollakis, G., Abebe, A., Kliphuis, A., De Wit, T. F., Fisseha, B., Tegbaru, B., Tesfaye, G., Negassa, H., Mengistu, Y. & other authors (2003). Recombination of HIV type 1C (C'/C") in Ethiopia: possible link of EthHIV-1C' to subtype C sequences from the high-prevalence epidemics in India and Southern Africa. *AIDS Res Hum Retroviruses* **19,** 999-1008.

Pomerantz, R. J. & Horn, D. L., (2003). Twenty years of therapy for the HIV-1 infection. *Nature Medicine* **9,** 867-873.

Pugach, P., Ketas, T. J., Michael, E. & Moore, J. P. (2008). Neutralizing antibody and anti-retroviral drug sensitivities of HIV-1 isolates resistant to small molecule CCR5 inhibitors. *Virology* **377,** 401-407.

Ratner, L., Gallo, R. C. & Wong-Staal, F. (1985a). HTLV-III, LAV, ARV are variants of same AIDS virus. *Nature* **313,** 636-637.

Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, A., Whitehorn, E. A. & other authors (1985b). Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* **313,** 277-284.

Regoes, R. R. & Bonhoeffer, S. (2005). The HIV coreceptor switch: a population dynamical perspective. *Trends Microbiol* **13,** 269-277.

Rhodes, D. I., Ashton, L., Solomon, A., Carr, A., Cooper, D., Kaldor, J. & Deacon, N. (2000). Characterization of three nef-defective human immunodeficiency virus type 1 strains associated with long-term nonprogression. Australian Long-Term Nonprogressor Study Group. *J Virol* **74,** 10581-10588.

Rina, M., Pozidis, C., Mavromatis, K., Tzanodaskalaki, M., Kokkinidis, M. & Bouriotis, V. (2000). Alkaline phosphatase from the Antarctic strain TAB5. Properties and psychrophilic adaptations. *Eur J Biochem* **267,** 1230-1238.

Rodenburg, C. M., Li, Y., Trask, S. A., Chen, Y., Decker, J., Robertson, D. L., Kalish, M. L., Shaw, G. M., Allen, S. & other authors (2001). Near-full length clones and reference sequences for subtype C sequences of HIV type 1 from three different continents. *AIDS Res Hum Retroviruses* **17,** 161-168.

Rodgers, D. W., Gamblin, S. J., Harris, B. A., Ray, S., Culp, J. S., Hellmig, B., Woolf, D. J., Debouck, C. & Harrison, S. C. (1995). The structure of unliganded reverse transcriptase from the human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* **92,** 1222-1226.

Roeth, J. F. & Collins, K. L. (2006). Human immunodeficiency virus type 1 Nef: adapting to intracellular trafficking pathways. *Microbiol Mol Biol Rev* **70,** 548-563.

Romani, B. & Engelbrecht, S. (2009). Human immunodeficiency virus type 1 Vpr: functions and molecular interactions. *J Gen Virol* **90,** 1795-1805.

Roshal, M., Zhu, Y. & Planelles, V. (2001). Apoptosis in AIDS. *Apoptosis* **6,** 103-116.

Rousseau, C. M., Birditt, B.A., McKay, A. R., Stoddard, J. N., Lee, T. C., McLaughlin, S., Moore, S. W., Shindo, N., Learn, G. H. & other authors (2006). Large-scale amplification, cloning and sequencing of near full-length HIV-1 subtype C genomes. *J Virol Methods* **136,** 118-125.

Rucker, J., Edinger, A. L., Sharron, M., Samson, M., Lee, B., Berson, J. F., Yi, Y., Margulies, B., Collman, R. G. & other authors (1997). Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. *J Virol* **71,** 8999-9007.

Sabeti, P. C., Walsh, E., Schaffner, S. F., Varilly, P., Fry, B., Hutcheson, H. B., Cullen, M., Mikkelsen, T. S., Roy, J. & other authors (2005). The case for selection at CCR5-Delta32. PLoS *Biol* **3,** e378.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239,** 487-491.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4,** 406–425.

Salemi, M. & Vandamme, A. (2003). The Phylogenetic Handbook: A practical approach to DNA and protein phylogeny. *Cambridge University Press* Cambridge, United Kingdom.

Salminen, M. O., Johansson, B., Sonnerborg, A., Ayehunie, S., Gotte, D., Leinikki, P., Burke, D. S. & McCutchan, F. E. (1996). Full-length sequence of an Ethiopian human immunodeficiency virus type 1 (HIV-1) isolate of genetic subtype C. *AIDS Res Hum Retroviruses* **12,** 1329-1339.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular cloning: A laboratory Manual second edition. *Cold Spring Harbor Laboratory Press* New York, USA.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74,** 5463-5467.

Schubert, U., Bour, S., Ferrer-Montiel, A. V., Montal, M., Maldarell, F. & Strebel, K. (1996). The two biological activities of human immunodeficiency virus type 1 Vpu protein involve two separable structural domains. *J Virol* **70,** 809-819.

Scriba, T. J., Treurnicht, F. K., Zeier, M., Engelbrecht, S. & van Rensburg, E. J. (2001). Characterization and phylogenetic analysis of South African HIV-1 subtype C accessory genes. *AIDS Res Hum Retroviruses* **17,** 775-781.

Shafer, R. W., Rhee, S. Y., Pillay, D., Miller, V., Sandstrom, P., Schapiro, J. M., Kuritzkes, D. R. & Bennett, D. (2007). HIV-1 proteaseand reverse transcriptase mutations for drug resistance surveillance. *AIDS* **21,** 215-223.

Sharp, P. A., Sugden, B. & Sambrook, J. (1973). Detection of two restriction endonuclease activities in Haemophilus parainfluenzae using analytical agarose--ethidium bromide electrophoresis. *Biochemistry* **12,** 3055-3063.

Shilts, R. M. (1987). And the band played on: Politics, people and the AIDS epidemic. *St. Martin's Press* New York, USA.

Shimura, K., Kodama, E., Sakagami, Y., Matsuzaki, Y., Watanabe, W., Yamataka, K., Watanabe, Y., Ohata, Y., Doi, S. & other authors (2008). Broad antiretroviral activity and resistance profile of the novel human immunodeficiency virus integrase inhibitor elvitegravir (JTK-303/GS-9137). *J Virol* **82,** 764-774.

Seibert, C. & Sakmar, T. P. (2004). Small-molecule antagonists of CCR5 and CXCR4: a promising new class of anti-HIV-1 drugs. *Curr Pharm* **10,** 2041-2062.

Silvestri, G., Sodora, D. L., Koup, R. A., Paiardini, M., O'Neil, S. P., McClure, H. M., Staprans, S. I. & Feinberg, M. B. (2003). Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. *Immunity* **18,** 441-452.

Simon, V., Zennou, V., Murray, D., Huang, Y., Ho, D. D. & Bieniasz, P. D. (2005). Natural variation in Vif: differential impact on APOBEC3G/3F and a potential role in HIV-1 diversification. *PLoS Pathog* **1,** e6.

Smith, J. M., Dauner, A., Li, B., Srinivasan, P., Mitchell, J., Hendry, M., Ellenberger, D., Butera, S. & Otten, R. A. (2010). Generation of a dual RT Env SHIV that is infectious in rhesus macaques. *J Med Primatol* **39,** 213-223.

Smith, P. F., DiCenzo, R. & Morse, G. D. (2001). Clinical pharmacokinetics of non-nucleoside reverse transcriptase inhibitors. *Clin Pharmacokinet* **40,** 893-905.

Somi, G. R., Kibuka, T., Diallo, K., Tuhuma, T., Bennett, D. E., Yang, C., Kagoma, C., Lyamuya, E. F., Swai, R. O. & Kassim, S. (2008). Surveillance of transmitted HIV drug resistance among women attending antenatal clinics in Dar es Salaam, Tanzania. *Antivir Ther* **13 Suppl 2,** 77-82.

Song, R. J., Chenine, A. L., Rasmussen, R. A., Ruprecht, C. R., Mirshahidi, S., Grisson, R. D., Xu, W., Whitney, J. B., Goins, L. M. & other authors (2006). Molecularly cloned SHIV-1157ipd3N4: a highly replication-competent, mucosally transmissible R5 simian-human immunodeficiency virus encoding HIV clade C Env. *J Virol* **80,** 8729-8738.

Sonigo, P., Alizon, M., Staskus, K., Klatzmann, D., Cole, S., Danos, O., Retzel, E., Tiollais, P., Haase, A. & Wain-Hobson, S. (1985). Nucleotide sequence of the Visna Lentivirus: relationship to the AIDS virus. *Cell* **42,** 369-382.

Soros, V. B. & Greene, W. C. (2006). APOBEC3G and HIV-1: Strike and counterstrike. *Curr Infect Dis Rep* **8,** 317-323.

South African Department of Health/Directorate Health Systems Research. (2004). National Antiretroviral Treatment Guidelines. Directorate Health Systems Research, Department of Health, Pretoria, South Africa (http://www.doh.gov.za/).

South African Department of Health/Directorate Health Systems Research. (2006). National HIV and Syphilus Antenatal sero-prevalence survey in South Africa, 2005. Directorate Health Systems Research, Department of Health, Pretoria, South Africa (http://www.doh.gov.za/).

South African Department of Health/Directorate Health Systems Research. (2010). National HIV and Syphilus Antenatal sero-prevalence survey in South Africa, 2009. Directorate Health Systems Research, Department of Health, Pretoria, South Africa (http://www.doh.gov.za/).

Spira, S., Wainberg, M. A., Loemba, H., Turner, D. & Brenner, B. G. (2003). Impact of clade diversity on HIV-1 virulence, antiretroviral drug sensitivity and drug resistance. *J Antimicrobial Chemotherapy* **51,** 229-240.

Stanford University Drug Resistance Database. http://hivdb.stanford.edu/. Accessed 2011 May 15th.

Stebbing, J., Gazzard, B. & Douek, D. C. (2004). Where does HIV live? *N Engl J Med* **350,** 1872-1880.

Steigbigel, R. T., Cooper, D. A., Kumar, P. N., Eron, J. E., Schechter, M., Markowitz, M., Loutfy, M. R., Lennox, J. L., Gatell, J. M. & other authors (2008). Raltegravir with optimized background therapy for resistant HIV-1 infection. *N Engl J Med* **359,** 339-354.

Strebel, K. (2003). Virus-host interactions: role of HIV proteins Vif, Tat, and Rev. *AIDS* **17,** S25-S34.

Stremlau, M., Owens, C. M., Perron, M. J., Kiessling, M., Autissier, P. & Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* **427,** 848-853.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol Evol* in press.

Thompson, M. A, Aberg, J. A, Cahn, P., Montaner, J. S., Rizzardini, G., Telenti, A., Gatell, J. M., Günthard, H. F., Hammer, S. M. & other authors (2010). Antiretroviral treatment of adult HIV infection: 2010 recommendations of the International AIDS Society-USA panel. *JAMA* **304,** 321-333.

Travers, S. A., Clewley, J. P., Glynn, J. R., Fine, P. E., Crampin, A. C., Sibande, F., Mulawa, D., McInerney, J. O. & McCormack, G. P. (2004). Timing and reconstruction of the most recent common ancestor of the subtype C clade of human immunodeficiency virus type 1. *J Virol* **78,** 10501-10506.

Turner, B. G. & Summers, M. F. (1999). Structural biology of HIV. *J Mol Biol* **285,** 1-32.

UNAIDS/WHO working group on global HIV/AIDS and STD surveillance. (2007). AIDS epidemic update. Geneva, Switzerland, December 2007 (www.unaids.org).

UNAIDS/WHO working group on global HIV/AIDS & STD surveillance. (2011). AIDS epidemic update. Geneva, Switzerland, December 2010 (www.unaids.org).

Vallari, A., Holzmayer, V., Harris, B., Yamaguchi, J., Ngansop, C., Makamche, F., Mbanya, D., Kaptué, L., Ndembi, N. & other authors (2011). Confirmation of putative HIV-1 group P in Cameroon. *J Virol* 2011 **85,** 1403-1407.

Van de Peer, Y. & De Wachter, R. (1994). TREECON for Windows: A software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**, 569-570.

van Harmelen, J. H., Shephard, E., Thomas, R., Hanke, T., Williamson, A. L. & Williamson, C. (2003). Construction and characterisation of a candidate HIV-1 subtype C DNA vaccine for South Africa. *Vaccine* **21,** 4380-4389.

Vidal, N., Peeters, M., Mulanga-Kabeya, C., Nzilambi, N., Robertson, D., Ilunga, W., Sema, H., Tshimanga, K., Bongo, B. & Delaporte, E. (2000). Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. *J Virol* **74,** 10498-10507.

Vogelstein, B. & Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. *Proc Natl Acad Sci USA* **76,** 615-619.

Walmsley, S., Bernstein, B., King, M., Arribas, J., Beall, G., Ruane, P., Johnson, M., Johnson, D., Lalonde, R. & other authors (2002). Lopinavir-ritonavir versus nelfinavir for the initial treatment of HIV infection. *N Engl J Med* **346,** 2039-2046.

Walter, B. L., Armitage, A. E., Graham, S. C., de Oliveira, T., Skinhøj, P., Jones, E. Y., Stuart, D. I., McMichael, A. J., Chesebro, B. & Iversen, A. K. (2009). Functional characteristics of HIV-1 subtype C compatible with increased heterosexual transmissibility. *AIDS* **23,** 1047-1057.

Watts, J. M., Dang, K. K., Gorelick, R. J., Leonard, C. W., Bess, J. W. Jr, Swanstrom, R., Burch, C. L. & Weeks, K. M. (2009). Architecture and secondary structure of an entire HIV-1 RNA genome. *Nature* **460,** 711-716.

Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M., Saag, M. S., Wu, X., Shaw, G. M. & Kappes, J. C. (2002). Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob Agents Chemother* **46,** 1896-1905.

Weiss, R. A. & Wrangham, R. W. (1999). From pandemic to pandemic. *Nature* **397,** 385-386.

WHO. (2004). Antiretroviral therapy in primary health care: Experienceof the Khayelitsha programme in South Africa, casestudy. World Health Organization, Geneva, Switzerland (www.who.int/hiv/amds/case8.pdf).

WHO. (2010). Antiretroviral therapy for HIV infection in adults and adolescents. Recommendations for a public health approach 2010 revision (http://www.who.int/hiv/pub/arv/adult2010/en/index.html).

WHO progress report. (2010). http://www.who.int/hiv/pub/2010progressreport/report/en/index.html. Accessed 2011 May 15th.

WHO HIVDR status report. http://www.who. int/hiv/topics/drugresistance/status/en/index.html. Accessed 2011 May 15th.

Wilkinson, E. & Engelbrecht, S. (2009). Molecular characterization of non-subtype C and recombinant HIV-1 viruses from Cape Town, South Africa. *Infect Genet Evol* **9,** 840-846.

Yamada, T. & Iwamoto, A. (2000). Comparison of proviral accessory genes between long-term nonprogressors and progressors of human immunodeficiency virus type 1 infection. *Arch Virol* **145,** 1021-1027.

Yang, X. & Gabuzda, D. (1998). Mitogen-activated protein kinase phosphorylates and regulates the HIV-1 Vif protein. *J Biol Chem* **273,** 29879-29887.

Yap, M. W., Nisole, S., Lynch, C. & Stoye, J. P. (2004). Trim5alpha protein restricts both HIV-1 and murine leukemia virus. *Proc Natl Acad Sci USA* **101,** 10786-10791.

Zhou, Q., Chen, D., Pierstorff, E. & Luo, K. (1998). Transcription elongation factor P-TEFb mediates Tat activation of HIV-1 transcription at multiple stages. *EMBO* **17,** 3681-3691.

Zhu, T., Korber, B. T., Nahmias, A. J., Hooper, E., Sharp, P. M. & Ho, D. D. (1998). An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. *Nature* **391,** 594-597.

Zolopa, A. R. (2006). Incorporating drug-resistance measurements into the clinical management of HIV-1 infection. *J Infect Dis* **194,** S59-S64.

List of publications

Jacobs, G. B., Laten, A., van Rensburg, E. J., Bodem, J., Weissbrich, B., Rethwilm, A., Preiser, W. & Engelbrecht S. (2008). Phylogenetic diversity and low level antiretroviral resistance mutations in HIV type 1 treatment-naive patients from Cape Town, South Africa. *AIDS Res Hum Retroviruses* **24,** 1009-1012.

Jacobs, G. B., Nistal, M., Laten, A., van Rensburg, E. J., Rethwilm, A., Preiser, W., Bodem, J. & Engelbrecht S. (2008). Molecular analysis of HIV type 1 vif sequences from Cape Town, South Africa. *AIDS Res Hum Retroviruses* **24,** 991-994.

Jacobs, G. B., Schuch, A., Schied, T., Preiser, W., Rethwilm, A., Wilkinson, E., Engelbrecht, S. & Bodem, J. (2011). Construction of a high titer Infectious and novel HIV-1 subtype C proviral clone from South Africa, pZAC. Submitted to *J Gen Virol* 29 June 2011.

Kasang, C., Kalluvya, S., Majinge, C., Stich, A., Bodem, J., Kongola, G., Jacobs, G. B., Mllewa, M., Mildner, M., Hensel, I., Horn, A., Preiser, W., van Zyl, G., Klinker, H., Koutsillieri, E., Rethwilm, A., Scheller, C. & Weissbrich, B. (2011). HIV drug resistance (HIVDR) in antiretroviral therapy-naïve patients in Tanzania not eligible for WHO threshold HIVDR survey is dramatically high. *PLoS ONE* **6,** e23091.

Kozisek, M. Jacobs, G. B., Saskova, K. G., Henke, S., Schuch, A., Bucholz, B., Kräusslich, H., Rezacova, P., Konvalinka, J. & Bodem, J. Mutations in HIV-1 gag and pol compensate for the loss of viral fitness of HIV-1 from highly mutated Protease. In preparation.

List of abbreviations

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
APS	Ammonium persulfate
APV	Amprenavir
ART	Antiretroviral therapy
ARV	AIDS-associated retrovirus
ARV drugs	Antiretroviral drugs
ATV	Atazanavir
ATV media	Alseivers Trypsin Versene media
AZT	Zidovudine
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BST-2	Bone marrow stromal cell antigen 2
C1 to C5	Constant regions 1 to 5
CA	Capsid protein
CD	Cluster of differentiation (CD4, CD8, CD317)
CCR5	Chemokine receptor type 5
cDNA	Complementary Deoxyribonucleic acid
CI	Confidence intervals
CMV	Cytomegalovirus

cpm/ml	counts per minute per milliliter
CPR	Calibrated population resistance
CRF	Circulating recombinant form
CRS	Cis-acting repressive sequences
CTCs	Care and Treatment Centers
CTL	Cytotoxic T lymphocytes
CXCR4	Chemokine receptor type 4
d4T	Stavudine
Da	Dalton
ddI	Didanosine
ddNTPs	Dideoxyribo-nucleoside triphosphates
DLV	Delavirdine
DMEM	Dulbecos Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
dNTPs A, G, C, T	Adenine, Guanine, Cytosine, Thymidine
DRC	Democratic Republic of Congo
DRV	Darunavir
EDTA	Ethylene diamine tetra-acetic acid
EFV	Efavirenz
env	Envelope gene
Env	Envelope protein

ER	Endoplasmic Reticulum
FCS	Fetal calf serum
FDA	Food and Drug Administration
FTC	Emtricitabine
gag	Group antigen gene
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green flourescent protein
gp	Glycoprotein
HBS	Hepes buffered saline
HEK	Human embryonic kidney
HHV8	Human herpesvirus 8
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HIVDR	HIV drug resistance
HLA	Human leukocyte antigen
HMW	High molecular weight
HTLV	Human T-lymphotropic virus
IDV	Indinavir
IE	Internal early
IN	Integrase protein
INS	Inhibitory / Instability Ribonucleic acid sequences
kb	Kilo – base pairs
LANL	Los Alamos National Library

LAS	Lymphadenopathy syndrome
LAV	Lymphadenopathy virus
LB	Luria-Bertani
LLQ	Lower limit of quantification
LOD	Limit of detection
LPV	Lopinavir
LTNPs	Long-term nonprogressors
LTR	Long terminal region
M-MLV	Moloney murine leukemia virus
MA	Matrix protein
MAPK	Mitogen-activated protein kinase
MEM	Minimal essential media
MHC	Major histocompatibility
MOI	Multiplicity of infectivity
mRNA	Messenger Ribonucleic acid
MTCT	Mother-to-child transmission
NC	Nucleocapsid protein
nef	Negative factor gene
NF-κβ	Nuclear factor κβ
NVP	Nevirapine
NFV	Nelfinavir
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRE	Negative regulatory element
NRTIs	Nucleoside / nucleotide reverse transcriptase inhibitors

OD	Optical density
PBMCs	Peripheral blood mononuclear cells
PBS	Primer binding site
PBS buffer	Phosphate buffer saline buffer
PCR	Polyemerase chain reaction
PI	Protease inhibitor
PIC	Pre-integration complex
PMTCT	Prevention of mother-to-child transmission
pol	Polymerase gene
PR	Protease enzyme
RAM	Resistance associated mutation
rev	Regulator of viral expression gene
RIPA	Radio-Immunoprecipitation assay
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RRE	Rev responsive element
RT	Reverse Transcriptase enzyme
RT-PCR	Reverse transcription polymerase chain reaction
RTV	Ritonavir
SAP	Shrimp alkaline phosphatase
SD	Splice donor
SDS	Sodium dodecyl sulfate
SIV	Simian immunodeficiency virus

SHAPE	Selective 2'-hydroxyl acylation analyzed by primer extension
snRNA	Small nuclear Ribonucleic acid
SQV	Saquinavir
SU	Surface glycoproteins
TAE	Tris-Acetate-EDTA
Taq	*Thermus aquaticus*
TAMs	Thymidine analogue mutations
TAR	Transactivation response
tat	Transcriptional transactivator gene
TB	Tuberculosis
TDF	Tenofovir
TDM	Therapeutic drug monitoring
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
tHIVDR	Transmitted HIV drug resistance
TM	Transmembrane protein
TPV	Tipranavir
TRIM	Tripartite motif
U3	Unique 3` region
U5	Unique 5` region
UNAIDS	United Nations AIDS association
URF	Unique recombinant forms
USA	United States of America
V1 to V5	Variable regions 1 to 5

V3	Third variable
vif	Virion infectivity factor gene
vpr	Viral protein R gene
vpu	Viral protein U gene
VS	Virological synapses
WHO	World Health Organisation
WPBTS	Western Province Blood Transfusion Service
X-gal	ß-galactosidase (5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid)

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Curriculum Vitae – Graeme Brendon Jacobs

Current address	:	Scwhabenstrasse 1A Apartment 64 Versbach 97078 Würzburg Germany
Address in South Africa	:	250 Ninth Avenue Eikendal Kraaifontein 7570 Cape Town
Telephone number (Germany)	:	
Telephone number (South Africa)	:	
Email	:	
Internet	:	http://www.gk-1522.uni-wuerzburg.de/

Personal Details

Surname	:	Jacobs
First names	:	Graeme Brendon
Identity number	:	
Passport number	:	
Date of Birth	:	14 April 1982
Marital Status	:	Not married
Language	:	Afrikaans & English (Speak, read and write) German (Basic skills course)
Drivers License (South Africa)	:	Yes, Code 08 (South Africa)

Education

1999
Senior Certificate with exemption at D.F.Malan High School, Bellville, Cape Town
Subjects: Afrikaans First Language HG. English Second Language HG, Mathematics HG, Biology HG, History HG, Physical Science SG

2000 – 2002
B.Sc (Molecular and Cellular Biology), University of Stellenbosch, South Africa
Subjects: Microbiology, Genetics, Biochemistry and Computer Literacy

2003
B.Sc (Hons) in Medical Sciences, Medical Virology, University of Stellenbosch, South Africa

2004 – 2005
M.Sc in Medical Virology, University of Stellenbosch, South Africa

2006 – 2007
Employed as a scientific research assistant at the Institute of Medical Virology, University of Stellenbosch, South Africa

October 2007 – March 2008
Employed as a scientific research assistant at the Institute of Virology and Immunobiology, University of Würzburg, Germany

2008 – Current
Ph.D student at the Institute of Virology and Immunobiology, University of Würzburg, Germany.

Project title: Design of an infectious HIV-1 subtype C molecular clone for phenotypic resistance testing

Publications

1. **Jacobs GB**, de Beer C, Fincham JE, Adams V, Dhansay MA, van Rensburg EJ and Engelbrecht S. Serotyping and genotyping of HIV-1 infection in residents of Khayelitsha, Cape Town, South Africa. J Med Virol. 2006 Dec; 78(12): 1529-36.

2. **Jacobs GB**, Loxton AG, Laten A and Engelbrecht S. Complete genome sequencing of a non-syncytium-inducing HIV type 1 subtype D strain from Cape Town, South Africa. AIDS Res Hum Retroviruses. 2007 Dec; 23(12): 1575-8.

3. **Jacobs GB**, Laten A, van Rensburg EJ, Bodem J, Weissbrich B, Rethwilm A, Preiser W, Engelbrecht S. Phylogenetic diversity and low level antiretroviral resistance mutations in HIV type 1 treatment-naive patients from Cape Town, South Africa. AIDS Res Hum Retroviruses. 2008 Jul; 24(7): 1009-12.

4. **Jacobs GB**, Nistal M, Laten A, van Rensburg EJ, Rethwilm A, Preiser W, Bodem J, Engelbrecht S. Molecular analysis of HIV type 1 vif sequences from Cape Town, South Africa. AIDS Res Hum Retroviruses. 2008 Jul; 24(7): 991-4.

5. Engelbrecht S, **Jacobs GB**, Loxton AG, Laten A and Robson BA. AIDS Vaccine 2008. Cape Town, South Africa. October 13-15, 2008: Abstracts from Conference Proceedings: AIDS Research and Human Retroviruses. Volume 24, Supplement 1.

6. **Jacobs GB**, Loxton AG, Laten A, Robson B, van Rensburg EJ and Engelbrecht S. Emergence and diversity of different HIV-1 subtypes in South Africa, 2000-2001. J Med Virol. 2009 Nov; 81(11): 1852-9.

7. Kasang C, Kalluvya S, Majinge C, Stich A, Bodem J, Kongola G, **Jacobs GB**, Mllewa M, Mildner M, Hensel I, Horn A, Preiser W, van Zyl G, Klinker H, Koutsillieri E, Rethwilm A, Scheller C & Weissbrich B. (2011). HIV drug resistance (HIVDR) in antiretroviral therapy-naïve patients in Tanzania not eligible for WHO threshold HIVDR survey is dramatically high. 2011 *PLoS ONE* **6,** e23091.

Recent courses attended
(University of Würzburg, Germany)

Winter semester 2007/2008: German language course (Grundstuffe 1)
5 July 2008: Hottest life Science Symposium
16 – 17 September 2008: Writing for publication
16 – 17 October 2008: DAAD orientation-seminar
24 – 26 July 2009: IRTG Summer School: From Bioinformatics to rational drug design
8 – 10 October 2009: Dynamic microscopy workshop
03 – 04 November 2009: Research project management
18 February 2010 – Information Technology in Life Sciences

Recent conferences attended

International Research Training Group (IRTG 1522) Symposium on HIV/AIDS and associated infectious diseases. 27 – 28 February 2009. Cape Town, South Africa. Oral presentation on HIV-1 resistance testing in South Africa.

19th Annual meeting of the society of Virology. 18 – 21 March 2009. Leipzig, Germany. Poster presentation: Development of HIV-1 phenotypic resistance assay for a South African environment

4th International student symposium: Revolution Research. 26 – 27 March 2009: Würzburg, Germany. Poster presentation: Development of HIV-1 phenotypic resistance assay for a South African environment

SFB479 International Symposium: Living with pathogens. 16 – 18 July 2009. Würzburg, Germany. Just attendance.

2nd International Symposium of the IRTG 1522. 14th – 15th May 2010. Kloster Banz, Bad Staffelstein, Germany. Oral presentation. Generation of a HIV-1 subtype C proviral clone.

5th International student symposium: Chiasma. 13 – 14 October 2010: Würzburg, Germany. Poster presentation: Development of HIV-1 phenotypic resistance assay for a South African environment.

EMBO HIV/AIDS Global Exchange Lecture Course. 30 January – 05 February 2011. Stellenbosch University, Cape Town, South Africa. Oral and poster presentation. Generation of a HIV-1 subtype C proviral clone.

References

Professor Dr. Wolfgang Preiser
Division of Virology, Faculty of Health Sciences
Stellenbosch University
Cape Town
South Africa

Tel: 021 938 9353 / 4
E-mail: preiser@sun.ac.za

Professor Dr. Axel Rethwilm
Institute for Virologie und Immunbiologie
University of Würzburg
Würzburg
Germany

Tel: (+49) 093120149554 / 8
E-mail: axel.rethwilm@mail.uni-wuerzburg.de

Graeme Brendon Jacobs

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Appendix

Appendix A: Gemeinsam gegen HIV by Robert Emmerich. Published online on the University of Würzburg news page (www.idw-online.de/pages/de/news267754).

Gemeinsam gegen HIV

Robert Emmerich Stabsstelle Öffentlichkeitsarbeit
Julius-Maximilians-Universität Würzburg

27.06.2008 09:16

In keinem anderen Land der Welt sind so viele Menschen mit HIV infiziert wie in Südafrika. Rund 15 Prozent der 48 Millionen Einwohner tragen das Virus in sich. Um die Erforschung des Erregers weiter voranzubringen, haben sich Wissenschaftler der Universität Würzburg mit Kollegen in Südafrika zusammengetan: In einem neu eingerichteten Internationalen Graduiertenkolleg befassen sie sich mit HIV/Aids und assoziierten Infektionskrankheiten. Die Deutsche Forschungsgemeinschaft (DFG) fördert die Arbeiten in den kommenden viereinhalb Jahren mit rund 2,2 Millionen Euro.

Der Südafrikaner Graeme Jacobs (rechts) macht derzeit seine Doktorarbeit am Würzburger Lehrstuhl für Virologie. Dessen Inhaber Professor Axel Rethwilm (Mitte) pflegt schon seit Längerem Kontakte nach Südafrika. Aus diesen wissenschaftlichen Kooperationen ist das erste deutsch-afrikanische Graduiertenkolleg entstanden, das im Oktober an den Start geht. Links Jochen Bodem, der den südafrikanischen Doktoranden betreut.
Foto: Robert Emmerich

In Graduiertenkollegs arbeiten Doktoranden in einem koordinierten Forschungs- und Studienprogramm gemeinsam an einem Thema. Dabei werden sie von mehreren Hochschullehrern betreut. Das neue Kolleg, das die DFG der Universität Würzburg im Mai bewilligt hat, ist das erste deutsch-afrikanische Graduiertenkolleg überhaupt. Sein Sprecher in Würzburg ist der Virologe Axel Rethwilm, in Südafrika der Virologe Wolfgang Preiser von der Universität Stellenbosch.

An der Uni Würzburg stehen unter dem Dach des neuen Graduiertenkollegs ab Oktober Stipendien für elf naturwissenschaftliche und zwei medizinische Doktoranden zur Verfügung. Hinzu kommen in Südafrika zwölf Stipendien für Naturwissenschaftler, die dort ab Januar voraussichtlich von der National Research Foundation finanziert werden.

Mehrere Würzburger Arbeitsgruppen wirken an dem Projekt mit: Die Infektionsimmunologen Heidrun Moll und Joachim Morschhäuser bringen ihre Expertise über den Parasiten Leishmania und den krank machenden Pilz Candida ein. Beide Erreger haben besonders leichtes Spiel bei HIV-Infizierten, weil deren Immunabwehr geschwächt ist. Mit im Kolleg ist auch die Malaria-Expertin Gabriele Pradel. Wenn sich HIV-Patienten zusätzlich mit Malaria infizieren, wirken beide Erreger synergistisch; sie setzen den Betroffenen im Verbund also deutlich stärker zu als jeweils im Alleingang. Auch Infektionen mit Würmern und Staphylokokken sind im Zusammenhang mit HIV problematisch. Mit diesen Themen befassen sich Klaus Brehm, Matthias Frosch und Bhanu Sinha.

Manfred Lutz und Thomas Hünig, der schon seit Längerem mit Wissenschaftlern in Südafrika kooperiert, bringen zwei Projekte aus dem Bereich der Immunologie ein. Die Virologin Eleni Koutsilieri erforscht die Auswirkungen des HI-Virus auf das Gehirn - denn der Erreger bewirkt nicht nur eine Immunschwäche, sondern auch direkt eine schwere neurologische Erkrankung, bei der die Nervenzellen im Gehirn zunehmend dahinschwinden. Die Virologin Sibylle Schneider-Schaulies ergründet generell die Mechanismen, mit denen Viren das Immunsystem unterdrücken. Schließlich betreuen die Virologen Jochen Bodem und Axel Rethwilm zwei Projekte, die sich mit der Variabilität von HIV und der Entwicklung medikamentenresistenter Viren beschäftigen.

Appendix A continue:

Neben diesen grundlagenorientierten Vorhaben gehört auch ein klinisches Projekt ins Programm. Verantwortlich dafür ist Hartwig Klinker, dessen Team auf dem Gebiet des therapeutischen Drug-Monitoring bei HIV-Patienten in Deutschland führend ist: Die Würzburger haben Methoden entwickelt, um die Konzentration der Anti-HIV-Medikamente im Blut zu bestimmen. Dadurch ließen sich Medikamentenkombination und Dosis den jeweiligen Erfordernissen so gut anpassen, dass die Effizienz der Therapie deutlich verbessert wurde. Das soll künftig auch den Patienten in Südafrika zu Gute kommen.

"Jede unserer Arbeitsgruppen kooperiert mit einem Forschungsteam in Südafrika", sagt Axel Rethwilm. Die Kontakte sollen, außer über Internet und E-Mail, auch persönlich stattfinden: "In unserem Budget ist viel Geld für Reisen vorgesehen, so dass häufig Besuche bei den Partnern vor Ort möglich sind."

Das Graduiertenkolleg geht auf das Jahr 2005 zurück. Damals vereinbarte eine bayerische Delegation, zu der auch Rethwilm und Klinker gehörten, in Südafrika ein gemeinsames Aktionsprogramm zur Aids-Bekämpfung mit der Region Westkap. Bayern und Westkap sind seit 1995 Partnerregionen; Schwerpunkte der Zusammenarbeit liegen in den Bereichen Soziales, Bildung sowie wirtschaftliche und wissenschaftliche Zusammenarbeit. "Entsprechend hat der Freistaat am Entstehen des Graduiertenkollegs auch finanziell mitgewirkt", sagt Rethwilm.

Ein Ausblick des Würzburger Virologen: "Mit rein medizinischer Forschung wird man das HIV-Problem in Südafrika nicht in den Griff bekommen." Denn die starke Verbreitung des Virus habe vor allem auch soziale Gründe: "Viele Südafrikaner in den Townships wissen nicht, ob sie in den nächsten Tagen genug Nahrung zum Überleben haben werden. Wenn es um HIV geht, denken diese Menschen nicht in einer Perspektive von mehreren Jahren voraus."

Weitere Informationen: Prof. Dr. Axel Rethwilm, T (0931) 201-49554, rethwilm@vim.uni-wuerzburg.de

URL dieser Pressemitteilung: http://idw-online.de/pages/de/news267754

Appendix B: Optimismus auch in schwierigen Situationen.

Studierende aus Südafrika in Deutschland by Sabine Hellman, Lemmens Medien GmbH, Bonn (http://laenderprofile.gate-germany.de/de/).

Optimismus auch in schwierigen Situationen

Studierende aus Südafrika in Deutschland

Laut Statistischem Bundesamt waren an deutschen Hochschulen im Wintersemester 2008/2009 156 Studierende aus Südafrika eingeschrieben (Bildungsausländer). Das sind nur 0,7 Prozent aller afrikanischen Studierenden, die sich in diesem Zeitraum in Deutschland aufgehalten haben. Die am häufigsten gewählten Fachbereiche waren die Rechts-, Wirtschafts- und Sozialwissenschaften (47 Studierende), die Sprach- und Kulturwissenschaften (31 Studierende) sowie die Ingenieurwissenschaften (27 Studierende). 47,4 Prozent der Studierenden waren Frauen (vgl. S. 11).

„It doesn't really matter where you are: students are students!" Graeme Jacobs (27) sieht über die Unterschiede deutscher und südafrikanischer Studentenkulturen gelassen hinweg. Als PhD-Stipendiat des DAAD forscht er seit Oktober 2008 im internationalen Graduiertenkolleg *HIV/Aids and associated infectious diseases in Southern Africa*, einem Kooperationsprojekt der Universitäten Würzburg, Stellenbosch und Kapstadt (vgl. S. 19). Aufgefallen ist ihm allerdings, dass Forschung in Deutschland straffer und zielorientierter organisiert sei. Alles würde etwas strikter gehandhabt, was er in einer wissenschaftlichen Institution auch für sinnvoll hält. Was wird er mitnehmen aus Deutschland, wenn er 2011 die Heimreise antritt? Vor allem erhofft er sich durch die hier geknüpften Kontakte einen Mehrwert für spätere Studierende in Südafrika. Er strebt eine akademische Laufbahn an und möchte gerne dazu beitragen, dass der wissenschaftliche Austausch weiter ausgebaut wird. Im Alltag stellt die uneingeschränkte Mobilität für Jacobs die größte Veränderung dar. Was aus europäischer Sicht selbstverständlich ist, gestaltet sich in Afrika deutlich schwieriger: Reisen zwischen den Staaten werden stärker reglementiert und sind nicht frei von Gefahren. Auch die Bewegungsfreiheit in den Ländern selbst ist wesentlich eingeschränkter als in Deutschland.

Um seinem Bachelor of Commerce Honours, den er an der südafrikanischen Rhodes University gemacht hat, einen Master of Management Sciences hinzuzufügen, hat sich Trevor Surridge (27) an der privaten European Business School in Östrich-Winkel eingeschrieben. Hier kann er das Studium auch auf Englisch absolvieren. Ohne die finanzielle Unterstützung durch insgesamt drei Stipendiengeber wäre das für ihn allerdings nicht bezahlbar gewesen. Die Alternative, ein fachlich entsprechend ausgerichteter Masterkurs an einer öffentlichen Hochschule, wurde nur in deutscher Sprache angeboten und setzte entsprechend hohe Sprachkenntnisse voraus.

Optimismus, der auch in schwierigen Situationen nicht verloren geht – das ist für Gerhard Werle, Professor für deutsches und internationales Strafrecht, Strafprozessrecht und juristische Zeitgeschichte an der Humboldt-Universität zu Berlin, die Haupteigenschaft südafrikanischer Studierender, und zwar über alle ethnischen Gruppen hinweg. Im Rahmen einer Hochschulpartnerschaft mit der University of the Western Cape fliegt Werle regelmäßig nach Kapstadt, um dort als Gastdozent am Südafrikanisch-Deutschen Fachzentrum für Entwicklungsforschung und Strafjustiz zu unterrichten. Aus seiner Perspektive besteht das momentan wichtigste Ziel darin, die Bildungsressourcen in Südafrika selbst zu stärken. ◀

Sabine Hellmann, Lemmens Medien GmbH, Bonn

Arbeitsgruppe Virologie am Institut für Virologie und Immunbiologie an der Universität Würzburg; Graeme Jacobs steht in der Mitte.

Südafrikanische Studierende im Ausland

Gastland (Top 5 in 2007)	Anzahl der Studierenden
USA	1.702
Großbritannien	1.699
Australien	707
Deutschland	280
Kuba	243
Studierende im Ausland insgesamt	5.746

Quelle: UNESCO Global Education Digest 2009