Molecular Monitoring of HIV-1 Drug Resistance in Ifakara HIV-1 Cohort, Tanzania

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Pax Jessey Masimba

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PD. Dr. I Felger, Prof. Dr. M. Tanner and Prof. Dr. P. Erb

Basel, den 13 December 2011

Prof. Dr. M. Spiess

Dekan

Dedication

Dedicated to

My wife Phides, my children; Robert, Prosper and Donald, my mother Magdalene

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Acknowledgement

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Summary

HIV-1 resistance is one of the problems affecting success of antiretroviral therapy programmes worldwide. The extent of drug resistance differs in different parts of the globe, countries and regions depending on several factors such as effectiveness of the regimens, ART options available, duration of the ART programmes, degree of drug adherence, infrastructure and logistics support, ART policies and funds to support the programmes.

In Tanzania, ART programmes started officially in the countries in 2004. With more than 1.4 million people affected in the country, the government alone cannot support its ART programmes, as a results it seeks cooperation from other stake holders such as international organizations and agencies and donor countries. With this initiative at least 235 000 (equivalent to 50% of 422, 000) of people in need of ART could get them by 2009. Many studies on efficacy of ART programmes, specifically on drug resistance, have been conducted in developed countries but not in developing countries due to lack of resources. So many tests have been optimized for HIV-1 subtype B which is prevalent in the developed countries but not for non-subtype B which is the main HIV-1 subtype in developing world. Some few studies have been conducted in Tanzania to evaluate the efficacy of the ART regimens and programmes. These studies have evaluated either the virological efficacy and genotypic resistance in patients receiving ART (to evaluate development of acquired resistance) or genotypic resistance in drug naïve patients (to assess primary resistance). These few studies have been conducted in cities or urban areas with referral hospitals and have indeed shown some degree of resistance to ART and have provided some insights into degree of resistance in Tanzania, particularly in the urban areas. Most studies have not reached in the rural areas to investigate the pattern of resistance in these parts of the country.

Summary

This study was conducted to monitor HIV-1 drug resistance in Ifakara HIV/AIDS cohort, a cohort that was established in 2004 as one of the first sites chosen for ART rollout in Tanzania. This cohort is integrated in the Center for Decease and Control of Ifakara Referral Hospital. Ifakara referral hospital serves a population of about 600 000 in Ifakara and Ulanga districts in Morogoro region, South East Tanzania, an area with about 30 000 people estimated to be living with HIV/AIDS. By October, 2011 the cumulative number of patients enrolled in this cohort was 5748 and a total of 3940 had been started on ART. So the first question in this study was to set to answer as to what extent is there a problem of drug resistance in this cohort and if there is a trend over time to this resistance. The other aim was to genotype samples from all patients with suspected clinical resistance. The third component of the study was other was to develop and validate a microarray tool for detection of resistance mutations to reverse transcriptase inhibitors which are the main drugs used as first line drugs in the Ifakara HIV-1 cohort.

To answer the first question, 187 and 200 randomly collected samples in 2005-2007 and 2009 respectively, from drug naïve HIV patients were processed in Ifakara, Tanzania, and CD4 assay was performed. The remaining aliquot were sent to Basel, Switzerland for viral load assay and molecular genotyping to identify resistance mutations known to affect reverse transcriptase inhibitors and protease inhibitors which are the only official class of ART drugs used in Tanzania and Ifakara cohort. The reverse transcriptase and protease genes of the viral DNA were sequenced and the sequences were submitted to Stanford University HIV drug resistance data base to get information on resistance and HIV subtypes.

Major drug resistance mutations were detected in 8.4% and 3.3% of analyzed samples in RT gene in 2005-2007 and 2009 respectively. The observed difference in resistance mutations in 2005-7 and 2009 was not statistically significant. The subtypes identified in 2005-7 were A (28.0

%), C (37.3%), D (24.0%) and CRF01_AE (10.7%). The subtype % frequencies in 2009 were as follows: A (24.2), C (45), D (17.5), CRF01_AE (7.5), B (5) and F (0.8). The pattern of HIV-1 subtypes observed in 2005-7 and 2009 were almost similar with the exception for the occurrence of five isolates of HIV-1 subtype B and one isolate of HIV-1 subtype F in 2009. This pattern was similar to the one observed in other earlier studies in Tanzania which identified HIV-1 subtypes C, A and D as main HIV-1 subtypes in Tanzania. However, as with the other studies, local subtype prevalence variation was also observed.

A follow up sample was also taken from the first samples collected in 2005-7 (patients who had started ART for an average duration of 11 months), so as to assess the development of acquired resistance. A total of 16 samples could be successfully genotyped and major drug resistance mutation to reverse transcriptase inhibitors were detected in two patients (12.5%). In the last analysis from 17 suspected clinical resistance cases 6 patient samples (35.3%) were idenfied to harbour major resistance mutations to reverse transcriptase inhibitors. No major drug resistance mutations to protease inhibitors were detected in all the samples analyzed, but only minor protease resistance mutations were observed. Resistance mutations were present in only one third of patients with suspected resistance, suggesting other factors may have played a role in the observed lower ART response in these patients. In general the ART response was good in the cohort. However, the low level of drug resistance observed likely could affect the ART program in the area.

The microarray was developed and optimized using 4 HIV-1 cloned RT fragments from Swiss HIV cohort patients and 102 samples from Ifakara cohort HIV patients. Overall the microarray had a sensitivity of 92 percent. One main challenge was a high level of failure to produce signals which could be due to either primer mismatch leading to failure in primer extension reaction or

due to failure in the hybridization step. The disadvantages of this microarray are that it is only optimized for Ifakara HIV sequences and it can only test the selected mutations on the array and may not therefore be used as a diagnostic tool. The advantage of this microarray over direct sequencing is that it is relatively cheap and it takes less time to get results. In order to make this microarray suitable for population studies, further optimization is required.

Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
ATV/r	Atazanavir/ritonavir
AZT	Zidovudine
CD4	T-lymphocyte Bearing CD4 Receptor
CDCI	Chronic Disease Centre of Ifakara (Tanzania)
D4T	Stavudine
EFV	Efavirenz
HIV	Human Immunodeficiency Virus
HIV-DR	HIV-Drug Resistance
IHI	Ifakara Health Institute (Tanzania)
3TC	Lamivudine
LPV/r	Lopinavir/ritonavir
MTCT	Mother to Child Transmission (of HIV)
NACP	National AIDS Control Program, Tanzania
NIMR	National Institute for Medical Research (Tanzania)
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NNRTIs	Non-nucloside Reverse Transcripatse Inhibitors
NVP	Nevirapine
PIs	Protease Inhibitors
PLAIDS	People Leaving with HIV/AIDS
PMTCT	Prevention of Mother to Child Transmission (of HIV)
PR	Protease
RT	Reverse Transcriptase
sdNVP	Single-Dose Nevirapine
SNP(s)	Single Nucleotide Polymorphism(s)
TACAIDS	Tanzania Commission for AIDS
TDF	Tenofovir
THIMS	Tanzania HIV and Malaria Indicator Survey
UNAIDS	Joint United Nations Programme on HIV/AIDS

Objectives

WHO	World Health Organization

Chapter 1

Introduction

Introduction

1.1 HIV/AIDS

Acquired Immunodeficiency Syndrome (AIDS) was first reported in 1981. In 1983 its causative agent was discovered and was named Human Immunodeficiency Virus (HIV) [1]. Since then the disease has spread across the globe and has caused tremendous public health problems. The UNAIDS 2010 – Joint United Nations Programme on HIV/AIDS reported in 2010 that an estimated 0.5% of the world's population (about 34 million people) were living with HIV/AIDS (PLAIDS), 68% of whom were living in Sub-Saharan Africa [2].

1.2 HIV-1 structure and its genome organization

1.2.1 The HIV viral envelope

HIV-1 virus is spherical in shape and has a diameter of 1/10,000 of a millimeter. The virus is made up of an outer coat envelope composed of a lipid bilayer membrane that is derived from a host cell when a new virion buds from the cell. The HIV *Env* protein is found throughout the viral envelope and consists of a cap made of glycoprotein 120 (gp120), and transmembrane (TM) gyclycoprotein 41 (gp41) molecules [3].

1.2.2 The viral core

Inside the viral envelope is a capsid core made up of several copies (about 2000) of the viral protein, p24. The capsid surrounds two copies of genomic HIV RNAs. The HIV core contains a nucleocapsid protein (p7), the enzymes reverse transcriptase (RT), protease (PR) and integrase

(IN), and the HIV matrix protein (p17) [3]. The structure of Mature HIV particle is as shown in Figure 1-1.

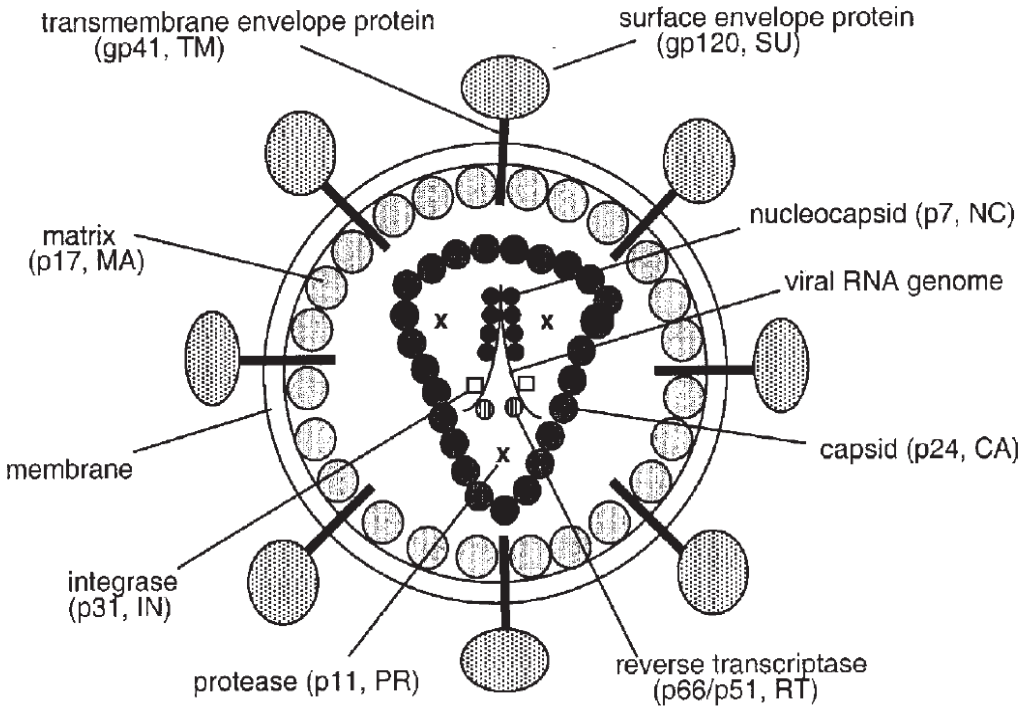

Figure 1-1. A mature HIV-1 particle. The positions of the major viral proteins, the viral envelope, and genomic RNA are shown. Taken from Freed, 2001(Somatic Cell and Molecular Genetics, Vol. 26, Nos. 1/6, November 2001).

1.2.3 HIV-1 genome organization

The HIV genome has 3 major genes that encode structural and non-structural proteins namely *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope glycoprotein). In addition, the HIV has six regulatory genes (tat, rev, nef, vif, vpr, and vpu) that contain information needed to produce proteins that control the ability of HIV to infect a cell, produce new copies of virus, or

responsible for pathogenicity of the virus [3]. The functions of these genes are highlighted below.

The Major genes:

Gag gene-encode Pr55Gag polyprotein. This polyprotein is cleaved by the viral PR enzyme into four mature proteins:

1. Matrix (MA) or p17
2. Capsid (CA) or p24
3. Nucleocapsid (NC) or p7
4. p6

Pol gene-encodes Pr160GagPol polyprotein. This polyprotein is subsequently cleaved by viral protease into PR, RT, and IN enzymes.

Env: The env gylcoproteins are synthesized as a polyprotein precursor and are cleaved by a cellular protease into the surface protein gp120 (interacts with the receptor and co-receptors on the host cell during viral entry process) and a transmembrane (TM) glycoprotein, gp41 (anchors the gp120/gp41 complex in the viral membrane).

The regulatory/accessory genes:

Transcriptional activator (Tat): this is a protein that is involved in regulation of HIV transcription by increasing the efficiency of transcriptional elongation through its interaction with RNA polymerase II and Tat-associated kinase (TAK) complex (cyclin-dependent kinase (cdk9) and cyclin T (cycT)) [4].

Rev: this protein binds to a Rev response element (RRE) present in all unspliced viral mRNAs, and is involved in nuclear export of these mRNAs[4] .

The Viral protein U (*Vpu*): this gene encodes a viral protein that binds CD4 in the endoplasmic reticulum (ER) and targets it for proteolysis by recruitment into the cytosolic ubiquitin-proteasome pathway [5].

Nef: this protein is involved in removal of CD4 on the cell surface by accelerating endocytosis and is also associated with downregulation of expression of major histocompatiblity complex (MHC-I) molecules on the surface of infected host cell [5] [6].

The Viral infectivity factor (*VIF*) promotes infectivity of viral particles by preventing APOBEC3G from being incorporated into HIV-1 particles and thereby preventing premature termination of the viral replication. APOBEC3G is responsible for dC to dU mutations in the viral minus strand DNA during reverse transcription, resulting in G to A substitutions in the viral plus (genomic) strand [7].

Viral protein R (*VPR*), has been proposed to have functions of long terminal repeat transactivation, nuclear import of the preintegration complex (PIC), induction of cell cycle arrest, and apoptosis [8].

The positions of the HIV-1 major genes and genes of regulatory and accessory proteins are as shown in Figure 1-2.

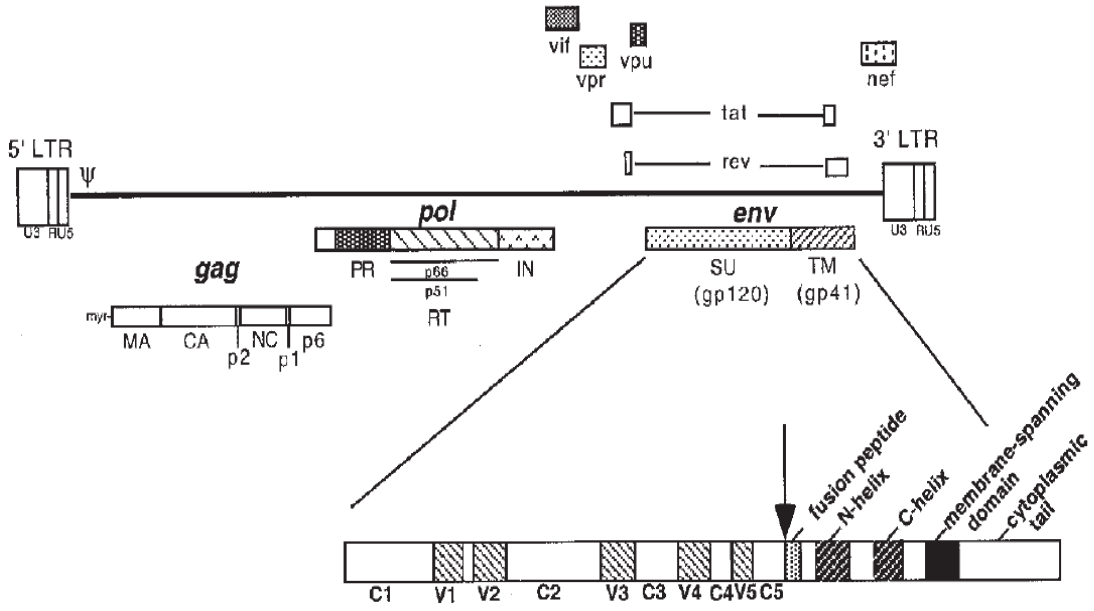

Figure 1-2. Organization of the HIV-1 genome. The positions of the HIV-1 open reading frames gag, pol, env, vif, vpr, vpu, nef, tat, and rev are indicated. The ψ indicates the position of the RNA packaging signal. The Gag spacer peptides (p2 and p1) positions are indicated. The "myr" is a site of Gag N-terminal myristylation. Taken from Freed, 2001 (Somatic Cell and Molecular Genetics, Vol. 26, Nos. 1/6, November 2001).

1.3 HIV-1 replication cycle

HIV replication cycle has several steps involving virus binding to host cell membrane, membrane fusion, uncoating, reverse transcription, nuclear import and integration, transcription of viral genome and synthesis of viral enzymes and structural proteins, assembly, budding and maturation. Only some steps of the replication cycle are of particular relevance for this PhD project and are therefore presented in greater detail below.

1.3.1 Reverse transcription

HIV-1 reverse transcribes each of its two RNA genome into double-stranded DNA just after entry into the host cell. The reverse transcription process involves several steps [15]. These steps have been described by Freed (2001) [3] as shown briefly below:

A primer tRNA binds the primer binding site (pbs). DNA synthesis proceeds to the 5' end of the RNA strand to generate a DNA/RNA hybrid.

The RNaseH activity of the RT enzyme degrades the RNA portion of the DNA/RNA hybrid to generate a minus-strand strong stop DNA.

The minus-strand strong stop DNA makes the first strand transfer from the 5' to the 3' end of the genome by using short regions of homology called "R" regions.

Minus-strand synthesis occurs by using the 3' end of the minus-strand strong stop DNA as a primer.

Plus-strand synthesis occur using fragments of RNA remaining from minus-strand synthesis as primers. The primary site of priming takes place at a purine-rich sequence, polypurine tract (PPT) and the central PPT.

RNaseH then removes the tRNA from the pbs to allow second-strand transfer to take place.

Plus-strand synthesis proceeds to the end of the minus strand.

The HIV-1 RT enzyme is prone to make errors while copying its genome, due to lacky of proof reading activity, as a result some mutations may develop which may either be harfmful to the virus (replication is hindered) or beneficial (virus becomes more fit, and replicates more efficiently). Some of these mutations may be selected under drug pressure (acquired resistance) and makes antiretroviral drugs less effective [3].

1.4 The HIV-1 molecular epidemiology

1.4.1 HIV-1 subtypes and their distribution

HIV infection is characterised by high levels of virus production and turnover. The reverse transcription of viral RNA into DNA is highly prone to errors introducing on average one mutation for each viral genome transcribed [24] [25]. The high rate of HIV replication, combined with the high mutation rate that occur during the viral replication cycle ensures that patients harbour a complex and diverse mixture of viral genotypes (quasispecies), each differing by one or more mutations. Some of these mutations confer a selective advantage to the virus such as decreased susceptibility to antiretroviral drugs [26]. Globally, the HIV prevalence and

epidemiological pattern is unevenly distributed [27]. The two groups of HIV-2, A and B, are restricted in West Africa [28]. HIV-1 group M viruses are more pathogenic and epidemiologically diverse and account for almost the entire global epidemic. HIV-1 group M is further divided into nine genetically distinct subtypes A, B, C, D, F, G, H, J, and K [15]. In addition, more than 40 circulating recombinant forms (CRFs) have been recognized so far [30]. The most important strains that cause the global epidemic are subtypes A, B, C, D, CRF01_AE and CRF02_AG [29] [27] [31]. The regional and global distribution of HIV-1 subtypes and global prevalence of HIV-1 in 2004-2007 is as shown in Figure 1-3 and Figure 1-4.

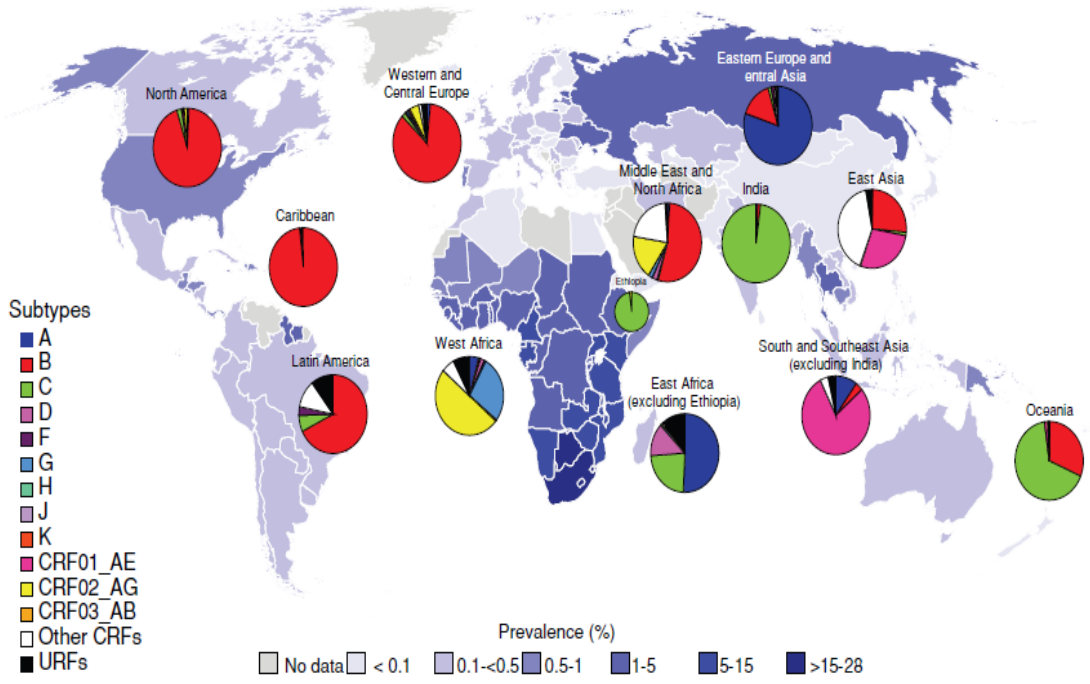

Figure 1-3. The global distribution of HIV-1 subtypes in 2004/7 and HIV-1 prevalence in 2004-2007. Taken from Skar et al, 2011 (Annals of New York Academy of Science. 1230 (2011) 108–118).

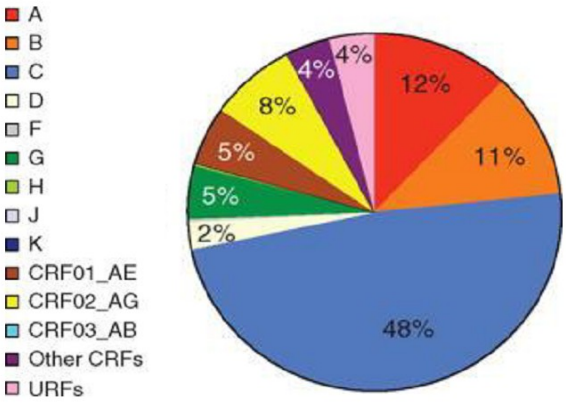

Figure 1-4. Global distribution of HIV-1 subtypes in 2007. Taken from Hemelaar et al., 2011 (AIDS, Volume 25(5), 13 March 2011, p 679–689).

1.4.2 The epidemiological, clinical and diagnostic impact of HIV-1 diversity

The genetic variation of HIV-1 differs between and within subtypes. The within-subtype variation may be as high as 8 to 17%, and the variation between subtypes can be as high as 17 to 35%, depending on the subtypes and genome regions examined [18]. It has been shown that this HIV-1 diversity impacts diagnosis and viral load measurements [33]. Other researchers have observed that the HIV diversity may affect the response to antiretroviral treatment and the emergence of drug resistance [29] [34] [35]. Subtypes may be transmitted at different rates and may exhibit differences in the rate of disease progression [36] [37]. The high diversity of HIV has also been shown to limit the intra- and inter- subtype cross-reactivity of immune responses [38]. Due to a rapid selection of immune escape viral mutants following infection, the viruses cannot be eliminated by the host's immune response [39]. This has hampered efforts towards development of effective vaccines [39].

1.5 HIV-1 antiretroviral therapy

1.5.1 Principles and targets for HIV chemotherapy

HIV drugs interrupt the replication circle of the virus during attachment and fusion to the host cell surface, reverse transcription, integration into host genome and maturation of the virus particles [40]. There are four classes of drugs currently used to treat HIV type 1 (HIV-1) infection. These are: Nucleoside Reverse Transcription Inhibitors (NRTIs), Non-Nucleoside Reverse Transcription Inhibitors (NNRTIs), Protease inhibitors (PIs) and Fusion Inhibitors (FIs). Recently a new drug, Raltegravir, which is an integrase inhibitor, has been approved by Food and Drug Association (FDA) of the United States of America [41]. A summary of important HIV drugs and their targets is shown in Table 1-1. According to current treatment standards, HIV antiretrovirals are combined for triple combination therapy termed highly active antiretroviral therapy (HAART), in order to effectively suppress the viral replication by targeting more than one drug target. Generally drugs are combined as follows:

2 NRTI + 1 NNRTI

2 NRTI + 1 PI

3 NRTI

The World Health Organization (WHO) provides recommendation on first and second line drugs [2]. The WHO recommended that abacavir (ABC) and didanosine (ddI) be considered as backup options whenever there is azidovudine (AZT) or tenofovir (TDF) toxicity or contraindication.

Table 1-1. Important HIV-1 Drugs and their Targets

Drug	Group	Target
*Zidovudine	NRTIs	RT enzyme
*Didanosine		
Zalcitabine		
*Stavudine		
*Lamivudine		
*Abacavir		
Emtricitabine		
Tenofovir disoproxil		
Saquinavir	PIs	PR Enzyme
*Ritonavir		
Indinavir		
Amprenavir		
Nelfinavir		
*Lopinavir		
Atazanavir		
*Nevirapine	NNRTIs	RT Enzyme
Delavirdine		
*Efavirenz		
Raltegravir	IN inhibitors	IN enzyme
Enfuvirtide	Fusion Inhibitor	Viral gp41
Maraviroc	Chemokine Antagonists	Chemokine Receptor 5 (CCR5)

* Drugs that are used in Tanzania and in Ifakara Cohort

1.5.2 Nucleoside Reverse Transcription Inhibitors

The NRTIs blocks the action of HIV RT enzyme [42]. Chemically modified nucleosides and nucleotides (analogs) are incorporated in place of natural nucleotide into the viral DNA during reverse transcription of viral RNA into cDNA. These modified nucleotides lack an OH group at

the 3` position of the ribose. Their incorporation terminates the replication of HIV DNA, thus inhibiting the multiplication of the virus [26] [43].

1.5.3 Non-Nucleoside Reverse Transcription Inhibitors

NNRTIs target RT, but in a different way than NRTIs do. The NNRTIs help stop HIV replication by attaching to the hydrophobic pocket located close to the catalytic site of the RT, preventing it from transcribing viral RNA into DNA [44] [45].

1.5.4 Protease inhibitors

PIs target the HIV PR enzyme which cleaves the gag-pol polyprotein of the virus into essential functional proteins during the maturation process of the virion. If the polyprotein is not cleaved, the virus fails to mature and is incapable of infecting a new cell. PIs bind to the active site in the protease enzyme where protein cleaving occurs, and so prevent the release of individual mature particles [46].

1.5.5 Fusion Inhibitors

Viral entry is a complex process that involves binding of viral envelope protein, gp120 to CD4 receptors on the target cell followed by binding to a co-receptor, CCR5 or CXCR4. These interactions induce a conformational change on gp 120 which exposes the transmembrane protein gp41. Gp41 has an N- terminal domain, two heptad repeats, HR1 and HR2, and a C-terminal domain. After exposure of gp41, the HR2 domain zips around HRI and together they form a six-helix bundle. This conformation change in gp41 brings the viral and target cell membranes together and allows fusion to proceed. Inhibitors such as Enfuvirtide or T-1249 are peptides that mimic HR2 and bind to HR1. This inhibits the HR2 zipping process and prevents fusion [47] [48] [49].

1.5.6 Integrase Inhibitors

Integration of HIV-1 proviral DNA into the host cell genome occurs in three steps that involve incision of proviral and host DNA and ligation by the integrase enzyme [50]. The first step is the processing of the 3′ ends of proviral DNA in which the enzyme removes two nucleotides from each end leaving the dinucleotide ``CA`` at the 3′ ends. Moreover, integrase cleaves the human DNA at the site of integration 5 bases apart. In a second step, termed strand transfer, the integrase enzyme joins the previously processed 3′ ends to the 5′ ends of target DNA at the site of integration. The third step is an enzymatic removal of two unpaired nucleotides at the 5`-ends of the viral DNA by cellular enzymes [41]. Integrase inhibitors bind to the strand transfer complex of integrase blocking the insertion of the processed HIV-1 DNA strand into the host DNA [51] [52].

1.6 The Reverse Transcriptase Enzyme

HIV-1 RT is a heterodimer of a 66-kDa molecular mass subunit (p66) and a 51-kDa molecular mass subunit (p51). DNA polymerase and RNase H catalytic activities are both conferred by the larger p66 subunit [53]. Figure 1-5 depicts the interaction of HIV-1 RT with dNTPs, NNRTI. This image also shows the primer- template RNA interaction discussed above in section 1.3.1

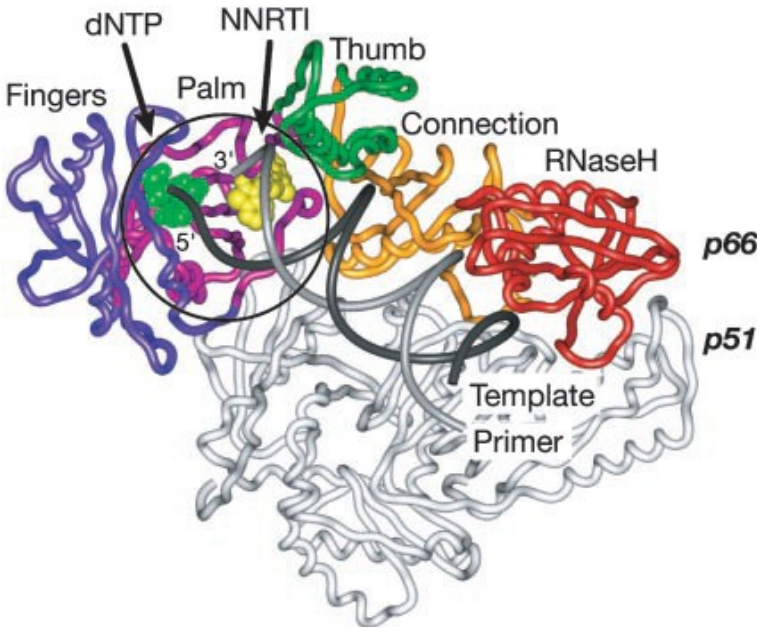

Figure 1-5. HIV-1 RT enzyme with substrates. dNTP (green), NNRTI (yellow), the DNA primer (light gray), template (dark gray), fingers (blue), palm (purple), thumb (green), connection (yellow), and RNaseH (red) the p66 subunit, and p51 subunit (white). The region circled is the polymerase active site and NNRTI-binding pocket. Taken from Pata et al., 2004 (PNAS, July 20, 2004, Vol. 101, No. 29, p 10548–10553)

1.7 The Protease Enzyme

The HIV PR is a C2-symmetric homodimeric enzyme consisting of two monomers of 99 amino acid each [54]. The HIV PR has an extended beta-sheet known as the flap, which constitutes in part the substrate binding site. Each flexible flap contains side chains that extend outward (Met46, Phe53), hydrophobic chains extending inward (Ile47, Ile54), and a glycine rich region (Gly48, 49, 51, 52). The dimeric active site triad `AspThrGly' is in amino acid positions 25 to 27 and 25´ to 27´, respectively. Each monomer contains an aspartic acid residue (Asp-25 and Asp-25´) that is essential for catalysis. The two residues of Asp25 and Asp25´ do interact directly with the substrate or the inhibitor.

1.8 The HIV-1 Intergrase

The HIV-1 integrase enzyme is comprised of three domains; catalytic core domain, N-terminal domain and C-terminal domain. The catalytic core domain contains a D,D-35-E motif, comprising residues Asp64, Asp116, and Glu152. It has been proposed that coordination of divalent metal ion to these residues plays a key role in catalysis [41] [56]. It is also thought that the C-terminal domain binds DNA nonspecifically [58] or interacts with a subterminal region just inside the very ends of the viral DNA end [59].

1.9 HIV-1 drug resistance

1.9.1 Primary and acquired drug resistance

HIV resistance to antiretroviral therapy can be divided into two categories, namely primary and secondary resistance. Primary resistance reflects acquisition of a drug-resistant strain of HIV by a newly infected person, while secondary or acquired resistance develops after a period of HIV treatment [60].

1.9.2 Mechanism of resistance to NRTIs

There are two mechanisms that have been observed to be responsible for development of HIV-1 drug resistance. For nucleoside and nucleotide analogues resistance is brought about by either impairment of the incorporation of these molecules into the synthesised DNA strand (Figure 1-1) or their removal from the terminated DNA strand (Figure 1-7) [42] [61] [62]. The impairment of drug incorporation is due to mutations in the catalytic site of the RT enzyme which compromise attachment of the base analogues at the active site of the enzyme [40]. They include M184V mutation (main mutation that confers resistance to lamivudine), Q151M complex of mutations

(confer resistance to most NRTIs such as stravudine and didanosine but not lamivudine and tenofovir), and the K65R mutation (confers resistance to most NRTIs such as tenofovir and abacavir but not zidovudine). The mutations at codons 41, 67, 70, 210, 215, and 219 of reverse transcriptase enzyme are collectively referred to as thymidine analog mutations (TAMs), or nucleotide excision mutations. The TAMs are removed from the terminated DNA strand by pyrophosphorolysis. The removal of the analogue from the terminated DNA chain involves pyrophosphate or adenosine 5′-triphosphate acting as an acceptor molecule for the removal of the drug [62] [63]. In addition, a six base pair insertion at position 69 (T69S) of the reverse transcriptase enzyme contributes to class-wide resistance [64].

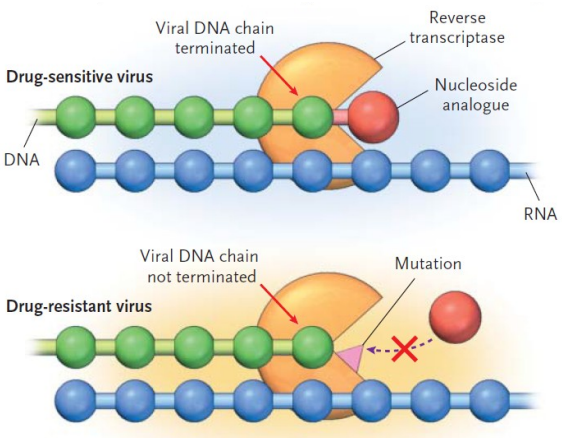

Figure 1-6. Resistance to NRTIs by interference with the incorporation of a nucleoside analogue. Taken from Clavel and Hance, 2004 (New England Journal of Medicine, 2004; 350:1023-1035).

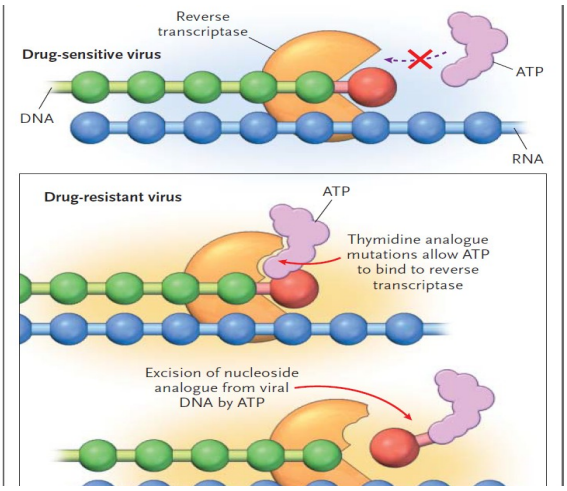

Figure 1-7. Resistance to NRTIs by ATP-mediated excision of the nucleoside analogue. Taken from Clavel and Hance, 2004 (New England Journal of Medicine, 2004; 350:1023-1035).

1.9.3 Mechanism of resistance to NNRTIs

Development of resistance to Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) occurs as a result of mutations in a hydrophobic pocket located adjacent to the catalytic site of the reverse transcriptase enzyme which affect the binding of these agents to the enzyme [65]. The binding of the inhibitor to the hydrophobic pocket of reverse transcriptase interferes with its ability to synthesise DNA [66]. Resistance to nevirapine, for example, is mainly due to Y181C mutation, but other mutations, such as Y188C, K103N, G190A, and V106A, do also take part. Efavirenz resistance is mainly due the K103N mutation, and to a small extent Y188L mutation [67] [68] [69].

1.9.4 Mechanism of resistance to PIs

Resistance to PIs is due to amino acid substitutions inside or adjacent to the substrate-binding domain of the protease enzyme which reduces the affinity of the inhibitors to the enzyme [70].

These amino acid changes modify the number and the nature of the points of contact between the inhibitors and the protease, resulting into overall reduction in their affinity for the enzyme. The V82A mutation, for example, reduces the size of an amino acid residue in the protease that is more important for binding most inhibitors than for binding the natural viral substrate. Protease inhibitors have been designed to bind the protease with maximal affinity and tend to occupy more space inside the active site cavity than do natural substrates. Unlike the inhibitors, the natural substrates of the protease have a less tight interaction with the catalytic site. Resistance mutations in the protease result in an overall enlargement of the catalytic site of the enzyme affecting the binding of inhibitors than the natural templates [71].

1.9.5 Mechanism of resistance to INIs

Resistance to raltegravir is caused by mutations involving amino acid residues 148 (Q148H/K/R), 155 (N155H) and to a lesser extent 143 (Y143C/H/R) of integrase [72] [73]. Q148 is located on the active site flexible loop that consist of residues 140 to 148, involved in catalytic core domain–DNA contacts [74] [75]. The N155H and Y143R/C mutations have been shown to reduce the replication capacity of the virus by impairing strand transfer activity and to some extent 3′ processing activity [76].

1.9.6 Resistance to Fusion Inhibitors

Resistance to fusion inhibitors occurs as a result of mutations at positions 36-45 in the HR1 domain, particularly in the 3 conserved amino acid positions 36, 37 and 38 of gp41 [79]. As a result, fusion inhibitors are unable to bind to gp 41 due to conformational changes in gp41 [80].

1.10 Monitoring of HIV-1 drug resistance

1.10.1 Reasons for HIV-1 Drug resistance monitoring

In resource-limited settings where antiretroviral treatment (ART) is being scaled-up, the World Health Organization (WHO) recommends the surveillance of transmitted HIV drug resistance [81]. The purpose of monitoring HIV Drug Resistance (HIVDR) emerging in populations is to assess the effectiveness of ART programmes and minimising the spread of HIVDR following the use of antiretroviral treatment. Where drug resistance emerges, the data collected will inform the guidelines on appropriate population-based first- and second-line regimens, indications for time-of-regimen switch on a population basis, and specific actions to improve outcomes of ART programmes. Phenotypic and genotypic assay are two types of assays available to measure ARV drug resistance. A third, assay is a virtual Phenotype, in which genotyping data from the patient's virus are used to interrogate a large database of previously determined and paired HIV genotypes and phenotypes.

1.10.2 Phenotypic drug resistance testing

The phenotypic assays are based on generation of a recombinant HIV-1. To generate a recombinant HIV-1 for assaying PRIs and RTIs resistance, the PR and RT genes of HIV-1 are amplified from a patient's sample and the amplified products are inserted into a plasmid containing a HIV backbone where the PR and RT genes had been deleted [82]. The fold change in drug concentration required to inhibit the recombinant HIV-1 is compared to that required for a wild type reference strain. The result is then expressed as IC50, which is defined as the concentration of the drug required to inhibit the virus replication by 50% [83].

1.10.3 Genotypic Drug Resistance Testing

The HIV-1 genotypic tests assess known mutations associated with drug resistance in the HIV-1 drug targets such as PR and RT [84]. The RNA extracted from replicating HIV-1, is amplified by PCR and the PCR products are then analyed after performing a sequencing reaction or hybridisation-based method. The advantage of sequencing is that it determines the entire set of mutations when present in the gene which is analyzed while hybridization examines only the specific mutations of interest in that gene [84].

1.10.4 Dideoxynucleotide sequencing

This is the standard methodology utilized for testing ARV drug resistance worldwide. The deoxynucleotide triphosphates (dNTPs) and dideoxynucleotide triphosphates (ddNTPs) are added per each sequencing reaction in the presence of specific primers [85]. The incorporation of ddNTPs during DNA synthesis leads to chain termination, finally generating numerous single stranded DNA of varying lengths, differing from each other by one nucleotide length. The DNA strands containing labelled primers or ddNTPs are separated by capillary electrophoresis and detected by fluorometric methods in an automated sequencer. This direct sequencing of the PCR amplified RT gene is known as viral population-based sequencing. The sequences generated are edited with sequencing analysis software and submitted online to the HIV-1 drug resistance data bases such as Stanford University HIV drug resistance database for prediction of drug resistance [85].

1.10.5 Hybridization based methods

The specific drug-resistance mutations from amplified RNA can be analyzed in a microarray. The process involves several steps: amplification of target viral RNA by RT-PCR, PCR, in vitro transcription using labelled ddNTPs, hybridization of the labelled amplicons with DNA probes on the microarray and the scanning of the probe arrays with a microarray scanner. The data from the scanned array are then analysed by using a special microarray software [86].

1.10.6 SNPs Genotyping

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variations which involve a single base pair mutation in a DNA, [87]. There are number of methods that can be used for SNP genotyping and each of these technologies has its advantages and disadvantages when weighed in terms of robustness, cost, high-throughput, simplicity, specificity and sensitivity [88].

1.11 DNA microarray technology

1.11.1 Basic concept of DNA microarray

A DNA microarray consists of a solid support, usually glass, with unique nucleic acid sequences (probes) bound at discrete positions on the solid support. Microarray technology uses the principles of DNA hybridization. Sequences from a labelled target are recognised by the specific and complementary oligonucleotide probes on the microarray [89]. The probes are DNA sequence bound to the solid-surface support in the microarray, while the target is the unknown sequence of interest to be analyzed. In general terms, probes are synthesized and immobilized as discrete features, or spots. Each feature contains millions of copies of identical probes. The target

is fluorescently labeled and then hybridized to the probe microarray. A successful hybridization reaction between the labeled target and the immobilized probe will result in an increase of fluorescence intensity over a background level, which can be measured using a fluorescent scanner [90]. Microarrays fall into five categories; printed microarrays, in-situ synthesized oligonucleotide microarrays, high-density bead arrays, electronic microarrays and suspension bead arrays [90].

1.11.2 Application of microarrays in research and diagnostics

Microarrays are potential molecular tools for research, drug discovery and diagnostics [95]. Microarray technology has been used to investigate the differential gene expression of pathogens, to the detection and identification of various pathogens, pathogen discovery, antimicrobial resistance monitoring, and strain typing [90].

1.12 The Global statistics of HIV and AIDS

The UNAIDS 2010 – Joint United Nations Programme on HIV/AIDS reported in 2010 that about 34 million people were living with HIV/AIDS globally, and Sub-Saharan Africa alone accounted for 68% of this global burden. In this report, Tanzania was among the top five countries with the highest number of PLAIDS that accounted 1.4 million people. The disease is estimated to cause 1.8 million deaths per year globally and a global GDP loss of USD 52.3 billion [1]. The HIV/AIDS statistics in 2009 is summarized in and Figure 1-8.

Table 1-2. Regional HIV/AIDS statistics in 2009. Source: Global report, WHO/UNAIDS (2010).

Region	Adult and children living with HIV	Adult % prevalence (15-49)	Adult and child deaths due to AIDS
Sub-Saharan Africa	22.5 million	5.0	1.3 million
Middle East, North Africa	460 000	0.2	24 000
South and South-East Asia	4.1 million	0.3	260 000
East Asia	770 000	0.1	36 000
Central and South America	1.4 million	0.5	58 000
Caribbean	240 000	1.0	12 000
Eastern Europe, Central Asia	1.4 million	0.8	76 000
Western and Central Europe	820 000	0.2	8500
North America	1.5 million	0.5	26 000
Oceania	57 000	0.3	1400
Total	**33.3 million**	**0.8**	**1.8 million**

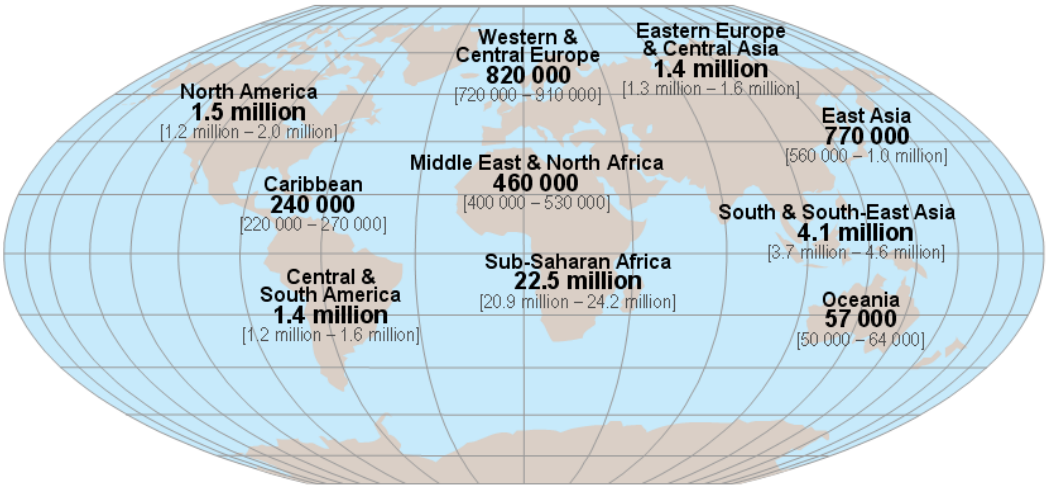

Figure 1-8. Adults and children estimated to be living with HIV/AIDS in 2009. Source: Global Report, WHO/UNAIDS (2010).

1.13 Statistics of HIV/AIDS in Africa

The North Africa region is least affected with HIV/AIDS area in Africa, and there are few statistics for prevalence of HIV/AIDS in this region. However, it was estimated in 2009 that the number of people living with HIV/AIDS in North Africa and Middle East was 400 000 - 530 000. The prevalence of HIV/AIDS in West and Central Africa in adults in 2009 was estimated at around 2% in Benin, Burkina Faso, Democratic Republic of the Congo, Gambia, Ghana, Guinea, Liberia, Mali, Mauritania, Niger, Senegal, and Sierra Leone, while in Cameroon Gabon, Central African Republic, Nigeria and Côte d'Ivoire was 5.3%, 5.2%, 4.7%, 3.6% and 3.4%, respectively. East and Southern Africa region is the most heavily affected area by the HIV epidemic accounting for almost 34% of people living with HIV/AIDS globally in 2009 [2].

1.14 HIV/AIDS in Tanzania

The first HIV case in Tanzania was reported in Kagera region in 1983 [92]. The disease has spread in all regions of Tanzania and by 2008 already 1.4 million people were infected by this virus. HIV/AIDS in Tanzania is caused by HIV-1 and the main subtypes involved are C, A, D and CRFs [92]. The prevalence of disease in Tanzania was estimated at 5.8% in 2008 but the prevalence is not uniform across the regions and age groups, being higher in the mainland regions than in the coastal areas and Zanzibar [93]. The most affected age group is between 15-49 years of age (35-39 years), and women are more affected than men [92]. The regional distribution of HIV-1 in Tanzania in 2003-4 and 2007 is as shown in Figure 1-9.

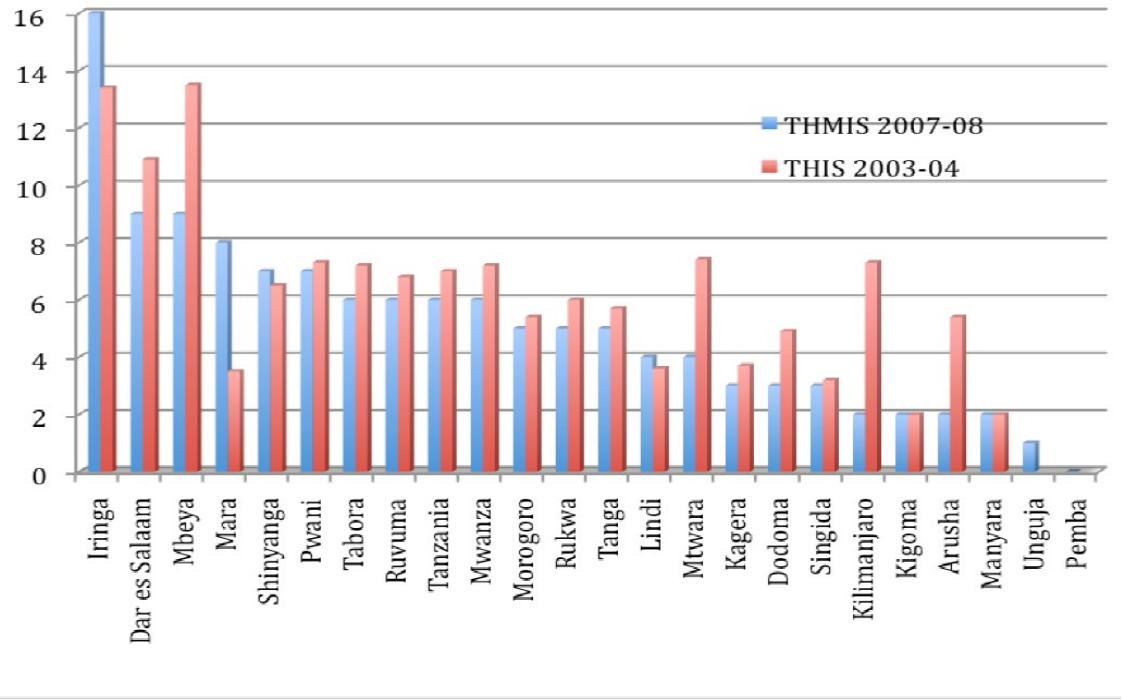

Figure 1-9. Trends by Region in HIV Prevalence in Tanzania between 2003-4 and 2007.

Source: Tanzania HIV/AIDS and Malaria Indicator Survey (THIMS) 2007-08 (http://www.tacaids.go.tz/dmdocuments/THMIS%202007-08.pdf).

1.14.1 HIV-1 Transmission in Tanzania

In Tanzania, as in many other countries of Sub-Saharan Africa, the main routes of HIV-1 transmission are:

sexual intercourse with infected partner

exposure to infected blood and blood products

mother to child transmission during pregnancy, delivery or breast feeding

to a lesser extent, transmission from body fluids other than blood such as genital secretions, cerebral-spinal fluid (CSF), pleural fluid and amniotic fluid [92].

1.14.2 Risk factors for HIV-1 transmission in Tanzania

According to the UNGASS 2010 report [93], the main risk factors driving HIV-1 transmission in Tanzania are:

Age group: 35-39 years adults more affected than other age groups

Risky sexual acts: unprotected sex, commercial sex

Poverty/wealth: prevalence higher among richest and lower in poorest individuals

Socio-cultural norms and practices: gender inequality, early marriages, female circumcision, sharing injection needles among infected drugs users and other infected individuals

Promiscuity: prevalence higher in individuals with multiple sexual partners

sex: females more affected than males

marital status: prevalence higher in widowed women, divorced and separated women and lower in those who have never married

Location: individuals in urban areas more affected than those in rural areas, prevalence also higher in areas of high mobility like roadsides, prevalence higher in mainland Tanzania than in Zanzibar.

1.14.3 HIV-1 Treatment in Tanzania

Tanzania has a national guideline for management of HIV/AIDS through NACP. The NACP treatment guidelines are adopted from WHO guidelines. The care and treatment programme was initiated country-wide in 2004 and so far about 600 000 individuals require ART and have been

enrolled in care and treatment clinic (CTC) centres. Only 235 092 (55.6%) out of 422 632 eligible ART patients were on ART in 2009 at 563 CTCs throughout the country [92]. The NACP-recommended ART regimens for 2009 are summarized in Table 1-3.

Table 1-3. Recommended first and second line ART regimens in adults and children in Tanzania.
Source: NACP, 2009 (National Guidelines for the management of HIV and AIDS, 2009).

Group	First line regimen	Second line regimen
Adults	AZT + 3TC + NVP / EFV d4T + 3TC + NVP / EFV TDF + FTC + EFV / NVP TDF + 3TC + EFV / NVP	ABC + ddI+ LPV/r or ATV/r TDF + 3TC / FTC + LPV/r or ATV/r
Children	AZT + 3TC + NVP / EFV d4T + 3TC + NVP / EFV ABC + 3TC + EFV / NVP	ABC + ddI + LPV/r

1.14.4 HIV-1 Drug Resistance in Tanzania

Only few studies have been conducted in some regions of Tanzania to establish prevalence of resistance mutation either in drug naïve or in individuals on ART. These studies have been based either in the urban areas and referral hospitals like Dar es Salaam, Kilimanjaro and Kagera [94] [95] [96].

The results from these studies have indicated slight differences in the prevalence of drug resistance mutations in drug naïve (primary or transmitted mutations) patients. In Dar es Salaam for example, Mosha and coworkers (2011) found a prevalence of 7% resistance to NRTIs and

9% resistance to NNRTIs in a study involving 13-25 year old youths attending the Infectious Disease Centre's voluntary counselling clinic in Dar es Salaam from July 2004 to June 2005 [94]. Nyombi and coworkers (2008) found a prevalence of 3% mutations for NRTIs and 4% to NNRTIs in infected pregnant women from Kagera and Kilimanjaro in 2005 [95]. In each of these two studies NNRTI resistance seemed to be slightly higher than that of NRTI resistance. In the same study, a prevalence of 1.6 % for NRTI and 11.5% for NNRTI resistance was observed in women who had received single dose NVP (sdNVP). In another study conducted at Haydom Lutheran hospital in North East Tanzania between November 2007 and June 2008, resistance mutations were detected at a prevalence of 3.9% in patients who had received ART for one year and 8.4% in patients that were on ART for two years [96]. These data suggest that the rate of resistance development is not uniform in different parts of Tanzania.

1.14.5 HIV-1 subtypes in Tanzania

Previous studies in Tanzania on HIV-1 sub-types are also limited and have been restricted to cities and towns with referral hospitals. In these studies the dominant subtypes observed were C, A, D and CRFs [97] [98]. Table 1-4 summarizes the previous data on HIV-1 sub-types in Tanzania. The data shows that the distribution of subtype varies within Tanzania. For example, in Mbeya sub-type C is dominant followed by recombinant forms of sub-types C and A, subtype A prevails in Dar es Salaam, Kilimanjaro and Kagera. Subtype C is found at high prevalence in all the studied regions.

Table 1-4. Reported HIV-1 Subtype distribution in Tanzania

	Dar es Salaam	Kagera	Kilimanjaro	Mbeya

Year		July 2004 to January 2005	September to December 2005	September to December 2005	September 2002 to April 2003
Sub-type percent Prevalence	A	36	31.8	36.0	18
	C	36	27.3	25.0	43
	D	14	19.7	20.0	3
	CRFs	14	21.2	19.0	36
Gene analysed		Pol	Pol	Pol	Pol/Env
Sample size		44	66	100	487
Reference cited		[94]	[98]	[98]	[99]

1.15 STUDY RATIONALE

Although it is universally recognised that combined ART has dramatically reduced HIV-related mortality [100], one major concern is the emergence and transmission of HIV drug resistant strains at the population level as a consequence of uncontrolled ART usage and genetic mutations in the drug targeted genes [101] [102]. This could lead to the failure of basic ART programmes as well as strategies to prevent HIV morbidity and mortality [103]. Monitoring of HIV-1 drug resistance is recommended in patients initiating drugs or failing their first regimen in many countries in the developed world [87]. In developing countries like Tanzania, lack of resources limit the use of drug resistance assays in HIV-1 patients, as a result there is a gap in data about the drug resistance situation in these countries. This thesis work was intended to evaluate drug resistance mutations in both drug naïve patients and in patients on ART so as to generate resistance data that will reveal the resistance situation in a HIV-1 cohort in a rural setting of Tanzania.

Although there are several commercially available methods for assaying HIV-1 drug resistance, antiretroviral drug resistance assays have largely been optimized based on HIV-1 subtype B which is most prevalent sub-type in Western Europe, North America and Australia. In Tanzania, as in many other Sub-Saharan countries, the HIV-1 subtypes present are non-B sub-types. The standard genotyping method is sequencing which genotypes a single sample and requires 5-7 sequencing reactions per sample. This makes it expensive to use in poor countries like Tanzania. This work intended to adapt and validate a microarray as an alternative technique for genotyping local subtypes present in Tanzania. Microarray technology allows simultaneous analysis of multiple SNPs through differential hybridization of target DNA to a library of oligonucleotide probes arrayed on a small solid surface such as glass [105]. A microarray platform was previously developed at Swiss Tropical and Public Health Institute, Basel (Swiss TPH), for detection of SNPs in drug resistance marker genes of malaria parasites [106]. The mini-sequencing principle provides highly specific base calling, while the hybridization between panels of tags with reverse complementary anti-tags on the chip facilitate parallel genotyping of many SNPs at a time. The aim was to adapt this technique to genotyping SNPs in HIV-1. Such a tool would make it possible to genotype patient samples at relatively lower cost. Due to relative low cost of microarray genotyping, it is logical to make efforts to use such a technology in resource poor countries like Tanzania. To minimize costs of microarray-based typing only those drug resistance mutations in the RT gene were targeted which are of relevance for antiretrovirals currently in Tanzania. The rather restricted selection of HIV drugs available in Tanzania limits the numbers of SNPs to be detected. Development of such a genotyping tool could potentially provide a device for monitoring HIV-1 drug resistance in resource poor settings. Its use in molecular epidemiological studies and for surveillance will generate valuable data for decision

making on optimal and locally adapted treatment guidelines as well as for monitoring the spread of resistance.

1.16 THE STUDY SITE

This study was conducted in a HIV-1 cohort in Kilombero and Ulanga districts of Morogoro region in Tanzania. This cohort was named KIULARCO. The cohort study was approved by ethics review bodies of Tanzania, Ifakara Health Institute (IHI) institutional review board, National Institute for Medical Research, NIMR, and the Ethics Committee of the University and State of Basel, EKBB. Patients were enrolled at the Chronic Disease Clinic of Ifakara, (CDCI), which serves as a Care and Treatment Centre for HIV/AIDS patients and is affiliated with the Ifakara Health Institute (IHI) and St Francis Referral Hospital (SFRH). The SFRH is the major health facility in Kilombero and Ulanga districts and serves a population of about 600 000 individuals. The first HIV-1 cases were reported in 1988 (M. Stoeckle, personal communication), five years later after the first HIV cases were found in Tanzania [92]. An estimated number of 30 000 individuals have been infected with HIV-1 virus in these 2 districts [107]. As of October 2011, the cumulative number of patients enrolled in CDCI was 5748, of which 3664 (63.7%) were females and 2084 (36.3%) were males and the number of people under ART was 3940 (1395 males and 2545 females).

1.17 AIMS AND OBJECTIVES

1.17.1 AIMS

This thesis work aimed at establishing the prevalence of HIV-1 drug resistance in drug naïve patients and patients under ART in a rural setting of Tanzania (KIULARCO cohort) as well as

adapting and validating a low cost genotyping tool (microarray) for monitoring HIV-1 drug resistance in Tanzania. All genotyping procedures targeted specifically HIV subtypes present in Tanzania. The focus was on SNPs associated with RT resistance against first and second line drugs used in Tanzania.

1.17.2 OBJECTIVES

To establish prevalence of drug resistance mutations in HIV-1 patients in KIULARCO cohort.

To optimize and validate an existing prototype genotyping chip for detection of SNPs in the RT gene.

1.17.2.1 SPECIFIC OBJECTIVES

To sequence the RT and Protease genes of baseline samples of KIULARCO patients in two time points; in 2005-2007 and 2009, so as to identify sequence diversity and estimate prevalence of mutations relevant for HIV-1 drug resistance.

To asses development of drug resistance mutations after commencing ART in KIULARCO in 2004. A follow up sample from the above patients were used.

To investigate DR-mutations in all patients presenting with clinical, or immunological treatment failure in the period 2005-2009.

To improve a prototype microarray for detection of DR-SNPs in the HIV-1 RT gene by optimizing all experimental steps.

To validate the HIV-DR-chip comparing microarray based typing with sequencing results from >100 KIULARCO patients.

Chapter 2

Prevalence of Drug-Resistance Mutations and HIV-1 Subtypes in a HIV-1 COHORT in rural Tanzania

Prevalence of Drug-Resistance Mutations and HIV-1 Subtypes in a HIV-1 COHORT in Rural Tanzania

Pax Masimba[1,2], Elimsaada Kituma[1,2],Thomas Klimkait[3], Edit Horvath[1,5], Marcel Stoeckle[2], Christoph Hatz[1,2], Erick Mossdorf[1], Emmanuel Mwaigomole[4,5], Salim Khamis[4,5], Boniphace Jullu[5], Salim Abdulla[5], Marcel Tanner[1,2], Ingrid Felger[1,2 *]

1 Swiss Tropical and Public Health Institute, Basel, Switzerland

2 University of Basel, CH-4003 Basel, Switzerland

3 Institute for Medical Microbiology, University of Basel, Switzerland

4 St. Francis Designated District Hospital, Ifakara, United Republic of Tanzania

5 Ifakara Health Institute, Ifakara, United Republic of Tanzania

* Corresponding author

Ingrid Felger

Swiss Tropical and Public Health Institute,

Department of Medical Parasitology and Infection Biology,

Socinstrasse 57,

4051, Basel, Switzerland

E-mail: Ingrid.Felger@unibas.ch

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1.18 ABSTRACT

1.18.1 Background

The development of resistance mutations in drug-targeted genes compromises success of antiretroviral therapy (ART) programmes. Genotyping of these mutations enables adjusted therapeutic decisions both at individual and population level. We investigated over time prevalence of HIV-1 primary drug resistance mutations in treatment naive patients and described the HIV-1 subtype distribution in a cohort in rural Tanzania at the beginning of the ART roll out in 2005 and later in 2009.

1.18.2 Methods

Viral RNA was reverse transcribed from 387 baseline plasma samples from treatment naïve patients over a period of five years. The reverse transcriptase (RT) and protease genes were PCR amplified and directly sequenced to identify the HIV-1 subtypes and single nucleotide polymorphisms associated with drug resistance (DR-SNPs).

1.18.3 Results

The major DR-SNPs in 2005 in the RT gene were K103N (5.0%), Y181C (2.5%), M184V (2.5%), G190A (1.7%) and M41L, K65KR, K70KR and L74LV (0.8%). In 2009 only K103N (3.3%), M184V and T215FY (0.8%) were detected. Initial frequencies of subtypes C, A, D and CRF01_AE were 37%, 28%, 24% and 11%, respectively. Later similar frequencies were found except for the rare observation of two additional subtypes, B and F, highlighting subtype diversity and stable subtype frequency in the area.

1.18.4 Conclusion

DR-SNPs were found at initiation of the cohort despite very low previous ART use in the area. Statistically frequencies of major mutations did not change significantly over the five year interval we studied. These mutations could reflect primary resistance and indicate a possible risk for treatment failure.

1.19 INTRODUCTION

Although it is universally recognised that combined antiretroviral therapy (ART) has dramatically reduced HIV-related mortality [1] [2], there are still some controversies by HIV-1 experts over the benefits of wide scale use of these drugs in resource poor settings in terms of controlling morbidity and mortality on one hand and minimizing emergence and transmission of resistance strains on the other hand. One major concern raised by one group is that the rapid and not appropriately controlled scaling up of ART, may accelerate the selection of drug resistance mutations and transmission of HIV drug resistant strains in the population. This could impair basic ART programmes as well as strategies to reduce HIV morbidity and mortality [3]. In view of the above, we aimed at investigating key resistance mutations in the reverse transcriptase (RT) and protease genes following ART rollout in a rural setting in Ifakara Tanzania.

In Tanzania, HIV prevalence in 2009 in adults 15-49 years of age was estimated to be 5.7%, corresponding to 1.5 million people, of whom 6.6% were women and 4.6% were men. The National ART programme started in 2004 [4]. The default first line drugs in Tanzania are Zidovudine/Stavudine, Lamivudine and Nevirapine/Efavirenz and the second line drugs are Abacavir, Didanosine and Lopinavir/Ritonavir or Indinavir/Ritonavir. In 2009 more than 454,000 Tanzanian HIV-1 patients were in need of ART (according to the 2002 WHO criteria of

initiation of ART) and about 235,000 received ART [5]. However, according to more recent, 2010 WHO criteria for initiation of ART [6], only one third of eligible patients in Tanzania are actually on ART. With many stakeholders supporting ART in the country, the Ministry of Health and Social Welfare (MoHSW) of Tanzania plans to further expand the ART coverage through its National Aids Control Programme (NACP).

In late 2004 a HIV cohort was established in Morogoro, rural Tanzania. The aim of this Kilombero-Ulanga- Antiretro-viral-Cohort (KIULARCO) was to implement care and treatment of HIV/AIDS patients according to the Tanzania National AIDS Control Care Programme (NACP) guidelines, to strengthen infrastructure, provide education of staff, to conduct research on optimal strategies for delivering treatment, and to conduct follow-up care in a resource limited and rural setting in Tanzania [4] [7].

To date, a total of 5748 HIV infected individuals have been enrolled at the CDCI. After a patient is enrolled, biomedical data are collected longitudinally. Blood samples are routinely collected at enrolment, before initiation of ART, and at different time points during visits to the clinic. In patients under ART clinical or immunological parameters are assessed at some point during follow up visits. Details on population and structure of the KIULARCO cohort have been described previously [7] [8].

The term “primary HIV-1 drug resistance” defines a new infection with a resistant virus strain. The presence of single nucleotide polymorphism associated with drug resistance (DR-SNP) plays an important role in treatment outcome. To investigate primary drug resistance, HIV-1 isolates from drug naive persons are examined in a cross sectional survey conducted at the beginning of the ART roll out in the cohort. A comparative analysis with samples collected in

subsequent years generally makes it possible to examine whether the level of primary resistance is increasing over time as a consequence of increased drug pressure in the study area. An increased level of primary resistance has been reported from other African countries, e.g. Nigeria [9]. Molecular monitoring of the prevalence of DR-mutations allows surveillance of transmitted resistance and may provide a valuable public health tool required in making decisions regarding HIV-1 treatment.

Studies on the transmission of primary HIV-1 drug resistance in Tanzania are few and limited to urban areas with large HIV-1 sentinel centres, e.g. Mbeya, Kagera, Kilimanjaro and Dar es Salaam [10] [11]. Additional data from the situation in rural areas are needed, as 80% of Tanzanians live outside urban centres. Thus completed surveillance data will add to a more precise and reliable picture of the HIV drug resistance situation in Tanzania.

The genetic diversity among HIV-1 subtypes is extensive. The median percentage of amino acid differences within a subtype from the Los Alamos database was found to be 17% in Env and 8% in Gag, whereas the inter-subtype differences were 25% and 17%, respectively [13]. In contrast to the highly variable Env, the pol sequence is more conserved [12] [13]. Different HIV-1 subtypes exhibit differences in the chance and routes of virus transmission, pathogenesis of the disease as well as in the kinetics and mechanisms of drug resistance development, thus potentially affecting HIV-1 disease management [3] [14] [15].

This study aimed to investigate the prevalence of HIV-1 drug resistance mutations in treatment naive patients and to establish HIV-1 subtypes in the KIULARCO Cohort in Ifakara Tanzania between 2005-7 and 2009.

1.20 Materials and Methods

1.20.1 Study Site and Subjects

The KIULARCO study was approved by ethics review bodies of Tanzania, Ifakara Health Institute (IHI) institutional review board, National Institute for Medical Research, NIMR, and the Ethics Committee of the University and State of Basel, EKBB. Patients were enrolled at the Chronic Disease Clinic of Ifakara, (CDCI) which serves as a Care and Treatment Centre for HIV/AIDS patients and is affiliated with the Ifakara Health Institute (IHI) and St Francis Referral Hospital (SFRH). The SFRH is an important health facility in Kilombero and ulanga districts in Morogoro, South East Tanzania and serves a population of about 600 000 individuals. The first HIV-1 cases were reported in 1983 (Marcel Stoeckle personal communication) in a period similar to first HIV cases in Tanzania [4]. It is estimated that more than 30 000 individuals have been affected in the area [7]. By July 2008, a total of 2394 patients had been enrolled in the CDCI [7] [8]. As of October 2011, the cumulative number of patients enrolled in the CDCI was 5748 of which 3664 (63.7%) were females and 2084 (36.3%) were males.

Depending on their CD4 counts and HIV-1 WHO clinical staging, participants either received ART (CD4 counts ≤200 cells/μL regardless of WHO stage, CD4 counts ≤350 cells/μL and WHO clinical stage 3, or WHO stage 4 regardless of CD4 cell count) or continued to be regularly followed up every three months. Before ART initiation, patient blood was drawn and plasma prepared and stored at -80°C. Data on clinical, virological, immunological parameters and demographic data of patient samples (i.e. CD4 count, full blood picture, viral load, WHO clinical staging at enrolment, age, sex and home town or village) were collected.

A total of 187 plasma samples from the time period 2005-2007 were randomly selected from all baseline samples collected just before initiation of ART. Of those samples 137 were derived from patients starting ART immediately after this test sample was collected. Fifty additional samples were chosen from HIV-1 infected patients with WHO clinical stage 3 and 4, who were not starting ART immediately. A second survey was conducted with 200 patients enrolled in the cohort in 2009. The 2009 inclusion criteria were: HIV-1 positive patients, age >18 years, with recent CD4 counts >250 cells/µL (WHO clinical stage 1 and 2) or >350 cells/µL (WHO clinical stage 3). All plasma aliquots were sent to Basel, Switzerland, for molecular genotyping.

1.20.2 RNA extraction, RT-PCR, PCR and Sequencing

Viral RNA was extracted from plasma with either the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) or Macherey-Nagel NucleoSpin RNA Virus Kit (Macherey-Nagel GmbH & Co KG, Neumann-Neander, Germany) using the manufacturer's protocol.

Reverse transcription (RT) was performed using specific primer RT2 [32], AffinityScript RT Buffer (500 mM TrisHCl pH 8.3, 750 mM KCl, 30 mM MgCl2), 2 µL of 100 mM DTT (Stratagene, North Torrey Pines Road La Jolla, CA), 0.8 µL dNTP mix (25 mM each dNTP), 1 µL of a RNase Inhibitor, RNase Out (40 U/µL), 1 µL AffinityScript Multiple Temperature Reverse Transcriptase, 1 µL specific Primer RT2 (5'-GATAAGCTTGGGCCTTATCTATTCCAT-3`), (10µM), HPLC purified, and 9.5 µL RNA solution. RT was performed with the following thermal conditions: 42°C for 35 min, 55°C for 25 min, 70°C for 15 min and 5°C for 15 minutes.

Primary PCR (pPCR) was done using Advantage cDNA Polymerase according to the supplier's protocol (Clontech Laboratories, Inc.Mountain, View, USA) with some modifications. Reverse and forward primers RT2 and D1818 (5'-AGAAGAAATGATGACAGCATGTCAGGGAGT-3`) were used. The pPCR mix contained 5 µL 10x Advantage buffer (Clontech), 10 µL dNTP mix (2 mM), 2 µL reverse primer RT2 (10 µM), 2 µL forward primer D1818 (10 µM), 1 µL Advantage Polymerase (5U/µL) and 4 µL of cDNA. Reaction profile was 94˚C for 2 min, 94˚C for 20 sec, 47 ˚C for 20 sec, 68 ˚C for 2 min, 30 cycles and a final elongation step 68˚C for 5 min were performed.

Nested PCR (nPCR) mix for amplification of the HIV-1 reverse transcriptase gene was 5 µL 10x Pfu buffer (Promega Corporation, Woods Hollow Road, Madison, WI USA), 10 µL dNTP mix (2 mM), 2 µL forward primer JG103 5`-AACAATggCCATTgACAgAA[I-Q]-3` (10 µM), 2 µL reverse primer JG202 5`-TCAggATggAgTTCATAICCCA-3` (10 µM), 0.7 µL FIREPol Polymerase (3U/µL), 0.1 µL Pfu Polymerase (3U/µL) and 2 µL pPCR product. Thermocyling conditions were: 94˚C for 2 min, followed by 30 cycles of 94˚C for 15 sec, 47˚C for 15 sec, 72 ˚C for 2 min and a final elongation step 72˚C for 5 min. PCR conditions for amplification of the protease gene were the same as for the RT gene except that a different primer set was used: forward primer D2213A2 (5'-AGCAGGATCCGAAAGACAGGGA-3'), (10 µM), and reverse primer R2598L (5'-CCATCCCGGGCTTTAATTTTACTGG-3'), (10 µM),. The nPCR products were purified with NucleoSpin Extract II kit (Macherey-Nagel) according to the manufacturer's protocol.

Direct sequencing of purified nPCR products was performed either in house or by the commercial supplier, Macrogen, South Korea. The in house protocol used either of the forward primers JG103 or PMF (5'-AACTCAAGACTTTTGGGAAGT-3') or either of the reverse primers JG202 or PMR (5'-TTGTCATGCTACTCTGGAATA-3'). PMF and PMR are centrally located sequencing primers for the RT gene. For nested PCR amplification and sequencing of the protease gene, the reagents and protocol used were the same as above with the exception that forward primer D2213A2 and reverse primer R2598L were used instead of RT-specific nested primers. The sequences obtained for each sample were aligned using the Seqscape Software Programme Version 2.6 (AB, Applied Biosystems, Foster City, CA, USA). The consensus sequences were assessed for drug resistance mutations by using the Stanford University HIV Drug Resistance Database HIVdb programme. The information on the HIV-1 subtype was also obtained from this database.

1.20.3 Viral Load Determination

Viral load was determined with a StepOne Real-Time PCR System (Applied Biosystems) by using a modification of the manufacturer`s instruction. cDNA was synthesized as shown above, but using random primers (0.1µg/µL). The cDNA was then quantified in qRT-PCR. The qRT-PCR reaction contained 12.5 µL TaqMan® Gene Expression Master Mix, 0.125 µL forward primer M2227F, 5'-AGC CTC AAT AAA GCT TGC CTT G-3' (10 µM), 0.125 µL reverse primer M2228R, 5'-CGG GCG CCA CTG CTA G-3' (10µM), 0.5 µL probe HIV-FAM/BHQ, with FAM as a reporter dye located at the 5 prime end and a black hole quencher at the 3 prime end, 5'-TGC CCG TCT GTT GTG TGA CTC TGG TAA-3', (10µM), 5 µL cDNA and RNase free water to a final 25 µL reaction volume. qRT-PCR thermocycling conditions were as follows: Incubation (50^0C, 2min), initial denaturation (95^0C, 10min) and 44 cycles of denaturation (95^0C,

30sec) and annealing and extension (60^0C, 1min). Quantitation of cDNA was done relative to triplicate standard curves generated in each run from serial dilutions of a plasmid containing a viral DNA insert. Three no template controls were included for each run.

1.20.4 CD4+ T-Cell Counts

Single platform technique (SPT) was used to enumerate CD4+ T-helper cells using BD TruCount® tubes (BD Biosciences, San Jose, California, USA). 50 µL of EDTA whole blood was stained using 5 µL monoclonal antibody mixture BD TriTEST™ CD3-FITC/CD4-PE/CD8-PerCP (BD Biosciences) followed by 450 µL 1x BD lysis and fixative solution. Data acquisition and analysis by MultiTEST software was performed using 3 colors BD FACS Calibur (Becton Dickinson Immunocytometry Systems 2350).

1.21 RESULTS

1.21.1 Baseline characteristics

The clinical and demographic characteristics of the 119 patients from 2005-2007 and 120 patients from 2009 were summarised in Table 2-7 and Table 2-8. The median age was 35 years in 2005-2007 and 40 years in 2009. Percentage of female study participants was 68.4 in 2005-2007 and 62.3 in 2009. The median absolute CD4 cell count was 224 cells/µL and median viral load was 53020 copies/ml in 2005-2007 while in 2009 the median absolute CD4 cell count was 420 cells/µL and the median viral load was 39920 copies/mL.

1.21.2 Prevalence of HIV-1 Subtypes

The HIV-1 subtype was determined for each isolate based on the reverse transcriptase (codon 23 to 236) and protease gene (codon 1 to 99) sequences. Subtypes frequencies are shown in Figure 2-10 for both study periods. Within the 119 samples analyzed in the years 2005-2007 HIV-1 subtype C was most prevalent with a frequency of 37.3%. Other subtypes and their frequency of occurrence were A (28. %), D (24.0%) and CRF01_AE (10.7%).

1.21.3 Prevalence of Anti-Retroviral Resistance Mutations

The major and minor drug resistance mutations in 2005-2007 and 2009 are shown in Table 2-5 and Table 2-6 and Figure 2-11. The frequency of major reverse transcriptase inhibitors mutation in 2005-2007 was 8.4% and in 2009 was 3.3%. Whereas the NRTI mutations occurred at frequency of 3.4% and 0.8%, that of NNRTIs occurred at frequency of 7.6% and 3.3% in 2005-2007 and 2009, respectively. No major protease gene mutation was found in these two periods.

1.22 DISCUSSION

The work at CDCI and subsequently KIULARCO cohort in rural Tanzania was initiated at the commencement of ART rollout in these two districts. Close clinical monitoring of patients was installed to guide treatment and switches of drug regimens. The comprehensive data gathered in the course of this rapidly growing cohort also provided a basis for epidemiological studies. A central question was to what extent the deployment of ART will drive the development of drug resistance under the specific conditions of a rural African setting. Like in other areas with limited resources, a number of typical shortfalls can lead to treatment failures, e.g. incomplete adherence to treatment or stock out of drugs at health institutions. Despite little previous availability of

ART in the study area, primary resistance, i.e. new infection by a drug resistant strain, must be expected to occur according to reports from other areas in Tanzania [10] [16] and could be a potential cause of treatment failures. In Tanzania the prevention of mother to child transmission (PMTCT) of HIV-1 policy (2009) recommends combination regimens that include AZT, NVP and 3TC in areas with capacity to offer and monitor ART. For all other areas with no capacity to deliver ART, the policy recommends the use of minimum antiretroviral prophylaxis consisting of a single dose of nevirapine to the mother and the infant [17]. Particularly in Ifakara area, the use of single dose nevirapine for PMTCT was noted since 2004 (Marcel Stoeckle personal communication). This kind of a policy may contribute to development of resistance to nevirapine in the individuals and hence transmission of the resistant strains [18].

In the course of the first years of KIULARCO, treatment failures were observed. This prompted our investigation of transmission of primary resistance in the study area. In an attempt to gather baseline data for molecular epidemiological studies on drug resistant HIV-1 infections among KIULARCO patients, we compiled two molecular data sets, one describing the HIV-1 diversity and subtypes in the study area, the other providing prevalence of DR-mutations in treatment naïve patients. Because prior to ART rollout transmission of resistant strains likely is a rare event, we expected to find very few DR-mutations in treatment naïve individuals.

HIV-1 subtypes C, A, D and CRF01_AE were the most frequent ones among KIULARCO participants. Despite slight differences in the proportion of these subtypes and a presence of a single sample of subtype F, this distribution is in line with other reports from different parts of Tanzania [1]. In studies conducted in the Dar es Salaam, Kilimanjaro, Kagera and Mbeya regions, subtypes A and C were found to be the predominant HIV-1 subtypes [11] [19]. Our finding of a higher proportion of subtype C in Ifakara is plausible, since Ifakara is linked by

railway to Zambia, where subtype C prevails [20]. Subtype B was not detected in the 2005-2007 samples, and was found in six cases (5%) in samples from 2009. Although subtype B is rare in Africa, it has been reported previously from Kilimanjaro, Tanzania [21]. A possible explanation for the detection of subtype B in KIULARCO could be transmissions from individuals with exposure to Western Countries. Subtype F, which was observed in one single sample in 2009, has been reported in Europe, South America and in Central Africa [22] [23]. Therefore this subtype might have been brought to Tanzania from neighbouring West-Central Africa where reports of subtype F occurrence exist [22] [24].

A limitation of our subtype analysis is that it was limited to HIV-1 protease and reverse transcriptase. The sequence data generated for these two regions might not capture the full diversity and may underestimate some of the recombinant forms [11]. The analysis is, however, representative in the context of other studies as the *pol* region has been used previously for subtyping HIV-1 [1] [19]. This approach represents a straight-forward way of identifying circulating HIV-1 subtypes in population studies, especially in resource-poor settings.

Our findings highlight the diversity of HIV-1 subtypes in rural Tanzania and contribute further data points to a countrywide picture of subtype frequencies. The longitudinal comparison indicates stable subtype frequencies over a period of at least four years with minor changes as well as the new appearance of isolates of subtypes B and one single isolate of subtype F in 2009.

We compared the prevalence of DR-mutations in drug naive patients from two surveys, conducted in 2005-2007 and 2009. The rationale for this molecular-epidemiological investigation was to identify potential effects of ART roll out on prevalence of DR-mutations. The two sampling periods represented were chosen at the beginning of the Tanzanian ART

programme (2004) and 5 years after initiation. We assumed that the effects of drug pressure might lead to a first visible increase in transmitted resistance mutations in the population during subsequent years.

In data analysis we first assessed comparability of our sets of samples because the mean time to ART initiation differed between the sets. Prevalence of DR-mutations in 75 samples with immediate ART initiation was compared with 44 samples in 2005-2007 with delayed ART initiation. We also compared WHO stage, age, sex, subtypes, means of CD4 counts and viral load values. Among all the parameters tested, only the higher age was of significance (95% Confidence interval, 0.5-9.5) in samples with delayed start of ART. Since there were no significant differences in DR-mutations and other factors compared between subsets of samples with early versus late treatment start (Table 2-9: Supplementary Table S1 and Table 2-10: Supplementary Table S2), all 2005-2007 samples were pooled for comparative analysis with the 2009 data set. We concluded that variation in time to ART initiation did not lead to a sampling bias.

Our results indicated that the prevalence of major reverse transcriptase mutations was 8.4% in 2005-2007 and 3.3% in 2009. This difference in prevalence was not statistically significant ($p=0.1069$). The prevalence rates of non-nucleoside reverse transcriptase mutations were 3.4% in 2005-2007 and 0.8% in 2009, respectively, ($P=0.2128$). Although the differences in prevalence of RTI mutations were not stastistically significant different, the higher number DR-mutations in 2005/7 might be attributed to a higher number of patients in WHO clinical stage 3 and 4 (Table 2-7) and differences in the CD4 in these two periods (Table 2-8). Patients enrolled in KIULARCO are routinely monitored for CD4 counts and clinical progression. A threshold value of 200 CD4 cells/μL and WHO clinical stages 3 and 4 are currently used by clinicians as criteria

for ART initiation. The observed difference in CD4 counts between the compared sets of patients might be attributed to the fact that people in the community became more aware of the HIV-1/AIDS health care services offered by the cohort, so they attended the clinic at earlier stages of the disease than previously. Differences in the average CD4 counts in the two periods could have influenced the outcome of our comparisons. Individuals in 2005-2007 had lower CD4 count values (275 cells/μL) compared to individuals in 2009 (531 cells/μL), which could indicate that the former group had a weaker immunological control, thus permitting higher viral replication rates and more chances for mutations to arise. But the finding of similar viral load between both sets of patients does not support this explanation. A possible explanation for the lower viral load in 2005-2007 samples might be due to prolonged and poor storage (frequent interruptions in electricity at time of Cohort initiation) condition of samples at Ifakara (Edith Horvath personal communication) which could have led to deterioration of RNA before their shipment to Switzerland for virological count and genotypic assays.

The NNRTI mutations K103N, Y181C and G190A cause high-level resistance to nevirapine, one of the two common NNRTI drugs used in Ifakara and elsewhere in Tanzania [25] [26]. All individuals with DR-mutations in 2009 and 60% of individuals in 2005-2007 carried the K103N mutation. This high prevalence of the K103N mutation may be due to prior use of Nevirapine monotherapy for PMTCT in Tanzania [17]. The prior presence of this mutation in a patient likely influences the outcome of ART by conveying a selective advantage and a head start in the accumulation of DR-mutations and their rapid selection under drug pressure. KIULARCO and other Tanzanian ART programmes can offer only few treatment options [26]. The observed level of primary resistance must be considered as a potential threat to the efficiency of the Tanzanian ART programme [18] [27].

In summary, DR-mutations were present in drug naive patients at both time points compared. Contrary to our expectation, we did not find significant differences in the frequency of resistance mutations between 2005-2007 and 2009, but we identified a trend suggesting higher mutation frequencies in samples collected earlier in the cohort. Minor differences in the repertoire of mutations were observed, most likely reflecting a chance finding owing to the small sample size. The number of KIULARCO patients under ART in the middle of our sampling period was 1,491 (December 2008) [7]. These numbers might be too small to provoke a measurable increase in transmitted resistance mutations within four years.

Overall our findings from both years are in agreement with genotypic results from other studies in Tanzania and elsewhere in Africa, which also indicated that primary drug resistance mutations are present in treatment naive patients even before the scale up of ART [10] [16].

1.23 CONCLUSION

Our molecular typing provides the first baseline information on HIV-1 strains encountered in a rural area in Southern Tanzania. Our data support a relatively stable subtype distribution with limited influx of new strains and a low prevalence of pre-existing drug resistance mutations as low potential threat for the ongoing ART-rollout. Demonstration of the extent and significance of HIV-1 drug resistance mutations in treatment naive individuals is useful for an informed choice of ART and thus can contribute to efforts towards preventing the spread of drug resistance.

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Table 2-5: Major HIV-1 drug resistance mutations in treatment naïve Ifakarara patients in 2005-2007 and 2009.

	Patient ID	Subtype	Major DR-mutations	Sex	Age (yrs)
2005-7	5410033	D	K103N	F	42
	5510060	D	M41L	F	40
	5510135	C	K103N	M	47
	5510184	CRF01_AE	M184V,G190A	F	29
	5510187	C	K103N, Y181C	F	4
	5510259	C	K103N	F	30
	5511494	C	K103N	F	30
	5510599	C	Y181C	F	51
	5510039	A	M184V, G190A	F	31
	5510072	C	K65KR, K70KR, L74LV, M184V, K103N, Y181C	F	45
2009	5514370	A	K103N, M184V, T215F	F	63
	5421041	CRF01_AE	K103N	-	-
	5513670	C	K103N	M	35
	5511139	C	K103N	M	4

DR-SNPs; drug-resistance single nucleotide polymorphisms

Table 2-6: Frequency of observation of HIV-1 DR-Mutations in KIULARCO cohort in 2005-7 and 2009

DR-SNP		2005-7 (n = 119)	2009 (n = 120)
		Frequency (%)	Frequency (%)
Major NRTI Mutations	M41L	1(0.8)	-
	K65KR	1(0.8)	-
	K70KR	1(0.8)	-
	L74LV	1(0.8)	-
	M184V	2(1.6)	1(0.8)
	M184MV	1(0.8)	-
	T215F	-	1(0.8)

Subtotal: major NRTI		7(5.9)	2(1.7)
NNRTI Mutations	K103N	6(5.0)	4(3.3)
	Y181C	3(2.5)	-
	G190A	2(1.7)	-
Subtotal: major NNRTI		11(9.2)	4(3.3)
Total major RT Mutations		18(15.1)	6(5.0)
Minor NRTI Mutations	M41KM	1(0.8)	-
	D67G	-	1(0.8)
	D69S	1(0.8)	-
	V118I	-	3(2.5)
	H221HY	1(0.8)	-
Subtotal: minor NRTI		3(2.5)	4(3.3)
NNRTI Mutations	V90I	-	1(0.8)
	V108I	-	1(0.8)
	V179D	-	1(0.8)
	G190R	1(0.8)	-
Subtotal: minor NNRTI		1(0.8)	3(2.5)
All Minor Mutations in RT		4(3.4)	7(5.8)
Minor Protease Mutations	L33FL	-	1(0.8)
	L10V	9(7.6)	1(0.8)
	L10I	4(3.4)	1(0.8)
	L10IL	-	1(0.8)
	L10IV		1(0.8)
	V11I	2(1.7)	-
	V11IV	-	1(0.8)

	L23F	1(0.8)	-
	A71T	1(0.8)	-
	T74S	1(0.8)	-
	L89V	-	1(0.8)
Total : Minor Protease Mutations		18(15.1)	7(5.8)

DR-SNPs, drug-resistance single nucleotide polymorphisms; RT, Reverse-transcriptase; NRTI, Nucleoside reverse-transcripatse inhibitor; NNRTI, Non-nucleoside reverse-transcripatse inhibitor.

Table 2-7: Comparison of baseline categorical characteristics of KIULARCO patients in 2005-7 and 2009.

	years 2005-7			**year 2009**			**Difference in Proportion**
	Frequency	Proportion	95% CI	Frequency	Proportion	95% CI	95% CI of Difference (p)
WHO Stage	(n = 116)			(n = 118)			
1	39	0.336	± 0.086	60	0.508	±0.090	0.048 - 0.297* (p =0.0084)
2	22	0.190	± 0.071	35	0.297	± 0.082	-0.002 - 0.216 (p =0.0678)
3	35	0.302	± 0.084	21	0.169	± 0.068	0.015 - 0.232* (p= 0.0319)
4	20	0.172	± 0.069	3	0.025	± 0.028	0.073 - 0.221* (p = 0.0001)
Sex	(n = 117)			(n = 118)			
Females	80	0.684	± 0.084	74	0.627	± 0.087	-0.065 - 0.178 (p = 0.4108)
Males	37	0.316	± 0.084	44	0.373	± 0.087	-0.178 - 0.065

							(p = 0.4108)
DR-Mutations	(n = 119)			(n = 120)			
Major RTIs	10	0.084	± 0.050	4	0.033	± 0.032	-0.009 - 0.110 (p =0.1069)
Major NRTIs	4	0.034	± 0.033	1	0.008	± 0.016	-0.011 - 0.062 (p = 0.2128)
Major NNRTIs	9	0.076	± 0.048	4	0.033	± 0.032	-0.015 - 0.100 (p = 0.1666)
Minor RTIs	3	0.025	± 0.028	6	0.050	± 0.039	-0.023 -0.073 (p = 0.4994)
Minor PIs	16	0.134	± 0.061	7	0.058	± 0.042	0.002 - 0.150 (p = 0.0509)
Subtypes	(n = 119)			(n = 120			
A	31	0.280	± 0.079	29	0.242	± 0.077	-0.091- 0.129 (p =0.7670)
C	52	0.373	± 0.089	54	0.450	± 0.089	-0.113 - 0.139 (p =0.8966)
CRF01_AE	11	0.107	± 0.052	9	0.075	± 0.047	-0.053 - 0.088 (p =0.6487)
D	28	0.240	± 0.073	21	0.175	± 0.068	-0.065 - 0.135 (p =0.2658)
B	-	-	-	6	0.050	± 0.039	(p =0.0293)*
F	-	-	-	1	0.008	± 0.016	(p =1.0000)

DR mutation, drug resistance mutation; NRTIs, Nucleoside reverse-transcripatse inhibitors; NNRTIs, Non-nucleoside reverse-transcripatse inhibitors; PIs, Protease inhibitors

*The difference is statistically significant

Table 2-8: Comparison of baseline numeric characteristics in 2005-7 and 2009 in KIULARCO patients

	2005-7		2009		Difference in Means
		95% CI		95% CI	95% CI of Difference (p)
CD4 counts (cells/μL)	(n = 116)		(n = 117)		
Mean	278	278 ± 44	456	456 ± 51	111 – 245* (p = 0.0088)
Viral Load (copies/ml)	(n = 92)		(n = 112)		
Mean	122 599	122 599 ± 43 246	287 217	287 217 ± 95 683	51 967 - 277 269* (p = 0.0046)
Age (Years)	(n = 116)		(n = 118)		
Mean/Median	37	37± 2	42	42 ± 2	2 – 8* (p =0.0006)

* The difference is statistically significant

Table 2-9: Supplementary Table S1: Comparison of baseline characteristics of treatment naive KIULARCO patients with immediate initiation of ART versus patients with ART initiation at a later date in 2005-7.

	Immediate initiation of ART			Initiation of ART at a later date			Difference in Proportion
	Frequency	Proportion	95% CI	Frequency	Proportion	95% CI	95% CI of Difference (p)
WHO Stage	(n = 75)			(n = 41)			
1	25	0.333	± 0.107	14	0.341	± 0.145	-0.172 - 0.188 (p =1.0000)
2	14	0.187	± 0.088	8	0.195	± 0.121	-0.142 - 0.158 (p = 1.0000)
3	23	0.307	± 0.104	12	0.293	± 0.103	-0.160– 0.188 (p = 1.0000)
4	13	0.173	± 0.115	7	0.171	± 0.086	-0.141– 0.146 (p = 1.0000)
Sex	(n = 75)			(n = 42)			
Females	53	0.707	± 0.103	27	0.643	± 0.145	-0.114 - 0.242 (p = 0.5364)
Males	22	0.293	± 0.103	15	0.357	± 0.145	-0.242 - 0.114 (p = 0.5364)
DR-Mutation	(n = 75)			(n = 44)			
Major RTIs	6	0.080	± 0.061	4	0.091	± 0.085	-0.094 - 0.116 (p = 1.0000)
Major NRTIs	3	0.040	± 0.044	1	0.023	± 0.085	-0.045 - 0.080

							(p = 1.0000)
Major NNRTIs	5	0.067	± 0.057	4	0.091	± 0.085	-0.078 - 0.126 (p = 0.7243)
Minor RTIs	2	0.027	± 0.037	-	-	-	-(p = 0.5300)
Minor PRIs	11	0.147	± 0.080	5	0.114	± 0.094	-0.090 - 0.156 (p =0.7825)
Subtype	(n = 75)			(n = 44)			
A	21	0.280	± 0.102	10	0.227	±0.124	-0.107 - 0.213 (p =0.6659)
C	28	0.373	± 0.109	24	0.545	± 0.147	-0.011 - 0.356 (p = 0.0855)
CRF01_AE	8	0.107	± 0.070	3	0.068	± 0.074	-0.064 - 0.141 (p = 0.7444)
D	18	0.240	± 0.097	7	0.159	± 0.108	-0.064 - 0.226 (p = 0.3562)

ART, antiretroviral therapy; DR-mutation, drug resistance mutation; NRTIs, Nucleoside reverse-transcripatse inhibitors; NNRTIs, Non-nucleoside reverse-transcripatse inhibitors; PIs, Protease inhibitors

Table 2-10: Supplementary Table S2: Characteristics of Numeric Variables for Patients with immediate and delayed ART Start in 2005-7 in Ifakara HIV-1 Cohort

	Immediate ART Start		Delayed ART Start		Difference in Means
		95% CI		95% CI	95% CI of Difference (p)

CD4 counts (cells/μL)	(n = 74)		(n = 41)		
Mean	303	303 ± 57	234	234 ± 67	-20 - 158 (p =0.1400)
Viral Load (copies/ml)	(n = 57)		(n = 35)		
Mean	142 447	142 447 ± 59 135	90 276	90 276 ± 62 190	-30 030 - 143 358 (p =0.2483)
Age (Years)	(n = 69)		(n = 36)		
Mean	35	35 ± 13	40	40 ± 10	0.5 - 9.5* (p =0.0396)

ART, antiretroviral therapy

* The difference is statistically significant

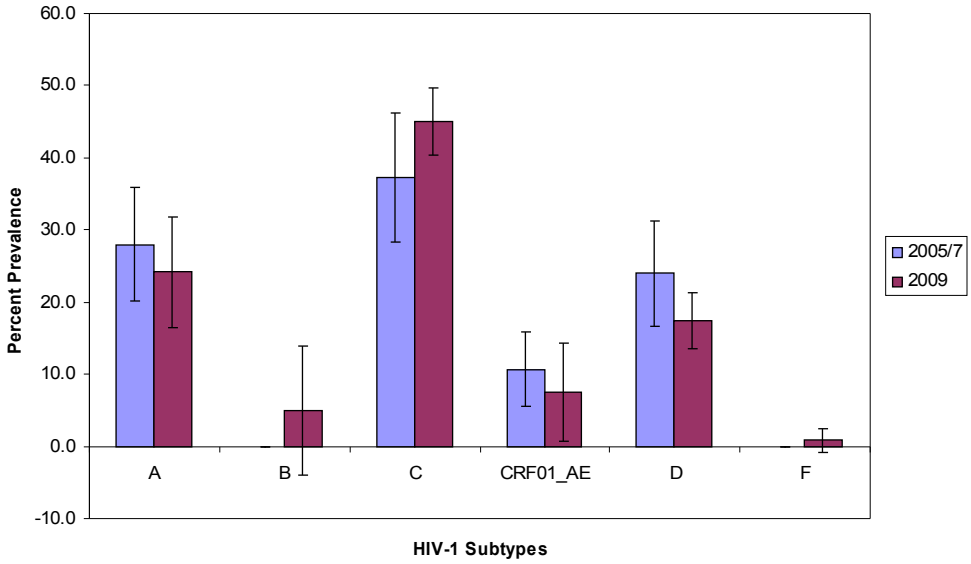

Figure 2-10: Prevalence of HIV-1 Subtypes in Ifakara COHORT in 2005-2007 and 2009. Bar lines represent 95% confidence interval.

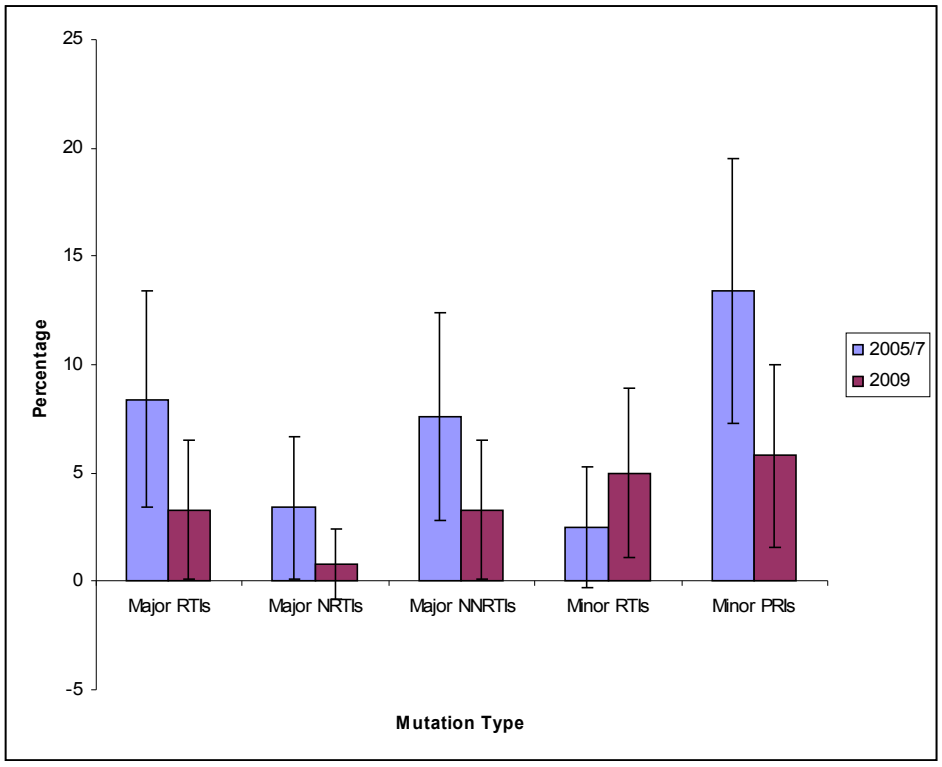

Figure 2-11: Prevalence of HIV-1 DR mutations in 2005-7 and 2009 in Ifakara HIV-1 Cohort. RTIs, reverse-transcriptase inhibitors; NRTIs, nucleoside reverse-transriptase inhibitors; NNRTIs, nonnucleoside reverse-transcriptase inhibitors; PIs, protease inhibitors. Bar lines represent 95% confidence

Chapter 3

HIV-1 drug resistance mutations in patients under treatment from a cohort in rural Tanzania

HIV-1 drug resistance mutations in patients under treatment from a cohort in rural Tanzania

Pax Masimba[1,2], Thomas Klimkait[2,3], Elimsaada Kituma[1,2], Edit Horvath[5], Erik Mossdorf[1], Boniphace Jullu[5], Salim Khamis[4,5] Emmanuel Mwaigomole[4,5], Daniel Nyogea[1,2,5], Marcel Stoeckle[1,2], Christoph Hatz[1,2], Salim Abdulah[5], Marcel Tanner[1,2], Ingrid Felger[1,2]

1 Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

2 University of Basel, CH-4003 Basel, Switzerland

3 Institute for Medical Microbiology, University of Basel, Switzerland

4 St. Francis Referral Hospital, Ifakara, United Republic of Tanzania new name

5 Ifakara Health Institute, Ifakara, United Republic of Tanzania

This manuscript is being prepared for submission

HIV-1 drug resistance mutations in patients under treatment from a cohort in rural Tanzania

1.26 ABSTRACT

1.26.1 Background

In Tanzania antiretroviral therapy (ART) roll out started in 2004. One of the main public health challenges is the emergence and spread of HIV-1 drug resistance. Molecular monitoring of drug resistance mutations in patients under ART, and in particular in cases of suspected clinical resistance, may provide important epidemiological data regarding acquired resistance due to drug pressure or patient adherence to treatment. We investigated the prevalence of resistance mutations in HIV-1 patients under treatment from the KIULARCO cohort in rural Tanzania.

1.26.2 Methodology

Plasma samples from 137 randomly selected patients under ART for >6 months were analyzed. In addition we investigated all cases of suspected clinical resistance that were reported in this cohort between 2005 and 2009. Drug resistance (DR) associated mutations were identified by PCR and direct sequencing of the reverse transcriptase (RT) and protease genes.

1.26.3 Results

Amplicons were obtained only from 16/137 samples of randomly selected patients under ART. Major mutations conferring resistance to RT inhibitors were detected in 2/16 (12.5 %) individuals under ART and in 6/17 (35.3%) cases of suspected clinical resistance. Minor mutations were detected in 7/16 (43.8 %) individuals under ART and in 6/17 (35.3%) cases of suspected resistance. In protease gene no major mutations were found in any group, but minor mutations occurred in 2/17 (11.8%) suspected resistance cases.

1.26.4 Conclusion

The detected mutations among individuals under treatment pose a threat to ART outcomes in KIULARCO cohort. If these mutations are transmitted in the population, they may lead to an increase in prevalence of resistant viral strains in this area. Because only 35% of the suspected clinical resistance cases carried DR mutations, other factors must have contributed to the compromised ART response, e.g. non-adherence. Further studies are required to determine other major reasons for ART failure in rural Tanzania.

1.27 INTRODUCTION

About 2.1 million people with HIV-1/AIDS were recorded in Tanzania resulting in a HIV-1 prevalence of 6.5% by 2009 [1]. The Tanzanian antiretroviral treatment (ART) program started officially in 2004 [2]. By 2009, 235 092 (55.6%) out of the 422 632 eligible ART recipients received treatment. ART in Tanzania is provided by 563 care and treatment clinics (CTC) present throughout the country [1]. An HIV-1 treatment cohort was established in Ifakara, rural Tanzania, in late 2004. This cohort, named Kilombero-Ulanga Cohort (KIULARCO), was among the first centers in Tanzania to implement this program [3]. By October, 2011, the cumulative number of patients enrolled in KIULARCO was 5748, and 3940 patients were on ART.

Several studies in developing as well as developed countries have reported some resistance in patients receiving ART [4] [5] [6]. For example, Bannister et al., 2011, observed a prevalence of 35% drug resistance mutations when estimating the prevalence of accumulated HIV drug resistance in patients receiving antiretroviral therapy in a group of 6498 EuroSIDA patients who were under follow-up on ART in 2008 [4] .

Roll out of ART in Africa can be expected to also result in development and spread of drug resistance. Major reasons could be lack of funds, infrastructure and technical personnel in these countries threatening regular and uninterrupted procurement, distribution, and safe storage of antiretroviral drugs [6]. In view of an expected emergence of resistance to ART, surveillance of the frequency of resistant strains appearing in patients under ART can provide useful epidemiological information, e.g. on the risk of transmission of acquired resistance mutations or on mutation frequencies that will guide the choice of locally suitable first and second line ART regimen.

In Tanzania, major demographic and socio-economic differences exist not only between urban and rural areas but also among different urban areas. A few studies conducted in urban areas of Tanzania reported varying prevalence rates of HIV-1 drug resistance (DR) mutations in treatment naïve individuals [7] [8] [9]. Reports on DR mutations in treatment-experienced individuals from Tanzania are rare. In a study by Nyombi and colleagues, carried out among women who had received a single dose nevirapine (sdNVP) to prevent mother to child transmission (PMTCT) of HIV-1 in Kagera and Kilimanjaro regions in 2005, the prevalence of NNRTIs mutations was 1.6% and 11.5% in Kagera and Kilimanjaro, respectively [7]. Only one study so far has been conducted in individuals under treatment from Hydom in rural Tanzania, which showed a trend towards increasing prevalence of DR mutations following ART use [10].

In view of substantial differences in frequencies of DR mutations detected in different settings in Tanzania, further investigations, particularly in rural areas, could reveal an even more severe picture of DR due to infrastructural problems outside the reach of urban health facilities. Restricted availability of ART could enhance emergence of drug resistance in these areas. Substantial differences in both, frequencies of DR mutations and haplotypes, may be found when

comparing urban and rural settings. We hypothesized that shortfalls in the distribution of ART and non-adherence to the prescribed treatment are more frequent in rural than in urban settings in Tanzania and, as a consequence, that the prevalence of acquired DR mutations would be higher in remote areas.

To test this hypothesis we carried out a genotypic analysis of HIV-1 virus from 137 randomly selected KIULARCO patients who had been on ART for at least six months in order to describe the prevalence of resistance mutations in the reverse transcriptase and protease genes. During the first 5 years of treatment and care for KIULARCO patients, 17 cases of suspected drug resistance were recorded. Viral isolates from these patients were also analyzed in order to identify DR mutations as possible causes of treatment failure.

1.28 MATERIAL AND METHODS

1.28.1 Study Site and Subjects

The KIULARCO HIV cohort was established in 2004 in Kilombero and Ulanga districts, in Morogoro region, southern Tanzania, for the dual purpose of implementing care and treatment of HIV/AIDS patients according to Tanzania National AIDS Control Care Programme (NACP) and to conduct research on essential minimal care for HIV patients in rural resource poor settings. Patients were enrolled at the Chronic Disease Center (CDC) affiliated with Ifakara Health Institute and St. Francis Referral Hospital (SFRH), the latter being the main district hospital in the two districts providing treatment and care for a population of > 600,000. In this area 30,000 people are leaving with HIV/AIDS [3].

137 patients under treatment were randomly selected. They started ART after meeting CD4 and WHO stage criteria adopted by the Tanzanian NACP. The following criteria for ART initiation were applied: WHO clinical stage 4, CD4 count of <200 cells/µl, or WHO stage 3 with CD4 count <350 cells/µl. The first line drug combination contained Stavudine/Zidovudine + Lamivudine + Nevirapine/Efavirenz, the second line consisted of Abacavir + Didanosine + Ritonavir-boosted Lopinavir. Patients were monitored for their CD4 counts and HIV-1 WHO staging just before and 3 months after initiation of ART. Thereafter routine visits occurred every six months. Data on clinical, virological or immunological parameters of patient samples were collected and recorded. The data included: CD4 count and full blood picture, viral load, WHO stage, ART regimen, reason for ART initiation, changes of ART regimen and reasons for change, ART failure and reason for failure, adherence grade, and demographic data, such as sex, place of origin, marital status, and referral sites. This study was approved by ethics review bodies of Tanzania, Ifakara Health Institute (IHI) institutional review board, National Institute for Medical Research (NIMR), and the Ethics Committee of Canton Basel (EKBB). Patients taking part in this study have given their informed consent.

Plasma samples from 137 randomly selected individuals on ART were collected between 2005 and 2007. Samples from the 17 patients with suspected resistance were obtained between 2005 and 2009.

1.28.2 RNA extraction, RT-PCR, PCR and Sequencing

Viral RNA was extracted from plasma with either the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) or Macherey-Nagel NucleoSpin RNA Virus Kit (Macherey-Nagel GmbH & Co KG, Neumann-Neander, Germany) using the manufacturer's protocol.

Reverse transcription (RT) was performed using specific primer RT2, AffinityScript RT Buffer (500 mM TrisHCl pH 8.3, 750 mM KCl, 30 mM MgCl2), 2 µl of 100 mM DTT (Stratagene, North Torrey Pines Road La Jolla, CA), 0.8 µl dNTP mix (25 mM each dNTP), 1 µl of a RNase Inhibitor, RNase Out (40 U/µl), 1 µl AffinityScript Multiple Temperature Reverse Transcriptase, 1 µl specific Primer RT2 (5'-GATAAGCTTGGGCCTTATCTATTCCAT-3`), (10 µM), HPLC purified, and 9.5 µl RNA solution. RT was performed with the following thermal conditions: 42°C for 35 min, 55°C for 25 min, 70°C for 15 min and 5°C for 15 min.

All primers used in this work were synthesized by Eurofins (Eurofins MWG Operon, Huntsville, Alabama 35805, USA.

Primary PCR (pPCR) was done using Advantage cDNA Polymerase according to the supplier's protocol (Clontech Laboratories, Inc.Mountain, View, USA) with some modifications. Reverse and forward primers RT2 and D1818 (5'-AGAAGAAATGATGACAGCATGTCAGGGAGT-3`) were used. The pPCR mix contained 5 µl 10x Advantage buffer (Clontech), 10 µl dNTP mix (2 mM), 2 µl reverse primer RT2 (10 µM), 2 µl forward primer D1818 (10 µM), 1 µl Advantage Polymerase (5U/µl) and 4 µl of cDNA. Reaction profile was 94˚C for 2 min, 94˚C for 20 sec, 47˚C for 20 sec, 68˚C for 2 min, 30 cycles followed by a final elongation step at 68˚C for 5 min.

The nested PCR (nPCR) mix for amplification of the HIV-1 reverse transcriptase gene contained 5 µl 10x Pfu buffer (Promega Corporation, Woods Hollow Road, Madison, WI USA), 10 µl dNTP mix (2 mM), 2 µl forward primer JG103 5`-AACAATGGCCATTGACAGAA[I-Q]-3` (10 µM), 2 µl reverse primer JG202 5`-TCAGGATGGAGTTCATAICCCA-3` (10 µM), 0.7 µl FIREPol Polymerase (3U/µl), 0.1 µl Pfu Polymerase (3U/µl) and 2 µl pPCR product.

Thermocyling conditions were: 94°C for 2 min, followed by 30 cycles of 94°C for 15 sec, 47°C for 15 sec, 72 °C for 2 min and a final elongation step at 72°C for 5 min. PCR conditions for amplification of the protease gene were the same as for the RT gene except that a different primer set was used: forward primer D2213A2 (5'-AGCAGGATCCGAAAGACAGGGA-3'), (10 μM), and reverse primer R2598L (5'-CCATCCCGGGCTTTAATTTTACTGG-3'), (10 μM). nPCR products were purified with NucleoSpin Extract II kit (Macherey-Nagel GmbH & Co KG, Neumann-Neander, Germany) according to the manufacturer's protocol.

Direct sequencing of purified nPCR products was performed either in house using a Genetic analyser 3130 (Applied Bisosystems, Switzerland) or by a commercial supplier, Macrogen, South Korea. The in house protocol used either one of the two forward primers JG103 or PMF (5'-AACTCAAGACTTTTGGGAAGT-3') or one of the two reverse primers JG202 or PMR (5'-TTGTCATGCTACTCTGGAATA-3'). PMF and PMR were centrally located sequencing primers for the RT gene. For nested PCR amplification and sequencing of the protease gene, the reagents and protocol used were the same as above with the exception that forward primer D2213A2 and reverse primer R2598L were used instead of RT specific nested primers. The sequences obtained for each sample were aligned using Seqscape Software Program Version 2.6 (Applied Biosystems). The consensus sequences were assessed for drug resistance mutations by using the Stanford University HIV Drug Resistance Database HIVdb program. The information on the HIV-1 sub-type was also obtained from this data base.

1.28.3 Viral Load Determination

Viral load was determined with a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a modification of the manufacturer`s instruction. cDNA was synthesized

as shown above, but using random primers (Eurofins MWG Operon) (0.1µg/µl). The cDNA was quantified by qRT-PCR. The qRT-PCR reaction contained 12.5 µl TaqMan® Gene Expression Master Mix, 0.125 µl forward primer M2227F, 5'-AGC CTC AAT AAA GCT TGC CTT G-3' (10 µM), 0.125 µl reverse primer M2228R, 5'-CGG GCG CCA CTG CTA G-3' (10µM), 0.5 µl probe HIV-FAM/BHQ, with FAM as a reporter dye located at the 5' end and a black hole quencher at the 3' end (5'-TGC CCG TCT GTT GTG TGA CTC TGG TAA-3'; 10µM), 5 µl cDNA and RNase free water to a final 25 µl reaction volume. The qRT-PCR thermocycling conditions were as follows: incubation (50°C, 2 min), initial denaturation (95°C, 10min) and 44 cycles of denaturation (95°C, 30 sec) and annealing and extension (60°C, 1min). Quantitation of cDNA was done relative to triplicate standard curves generated in each run from serial dilutions of a plasmid containing a viral DNA insert. Three no template controls were included in each run.

1.28.4 CD4 Counts

A single platform technique was used to enumerate CD4+ T-helper cells using BD TruCount® tubes (Becton Dickinson Biosciences, San Jose, California, USA). 50 µl of EDTA whole blood was stained using 5 µl monoclonal antibody mixture BD TriTEST™ CD3-FITC/CD4-PE/CD8-PerCP followed by 450 µl 1x BD lysis and fixative solution. Data acquisition and analysis by MultiTEST software was performed using 3 colors BD FACS Calibur.

1.29 RESULTS

1.29.1 Characteristics of the Patients

Plasma samples from randomly selected 137 individuals on ART were analyzed, but only 16/137 (11.7 %) samples could be amplified and sequenced. In contrast, all samples from 17 patients with suspected drug resistance were successfully amplified and sequenced. The baseline characteristics of the randomly selected group are shown in Table 3-11 while the characteristics of all patients with positive results (with positive viral PCR product and a viral sequence) are shown in Table 3-12. Among the patients randomly selected on ART 50% were female, whereas in the suspected resistance group 64% were women. The mean CD4 count for patients under ART was 247 cells/µl after six months of ART usage, indicating that most of patients had CD4 counts that were only slightly higher than recommended CD4 level requiring initiation of ART. We also measured CD4 counts in patients with suspected resistance at the time point of the first change of ART regimen. Mean CD4 count in this group was 76 cells/µl. Viral loads (VL) were determined for both groups of patients. Mean VL in the group of randomly selected ART patients was 124 022 copies/ml, in the suspected resistance group mean VL was 317 955 copies/ml. As expected, VL was higher in the cases of suspected resistance than in randomly selected ART patients.

In the selected group of ART patients, 87.4% received starvudine, lamivudine and nevirapine as a first line regimen. The remaining patients in this group received zidovudine, lamivudine and nevirapine (6.3%) or efavirenz + emtricitabine + tenofovir disoproxil fumarate (6.3%). Patients in the suspected resistance group had received starvudine, lamivudine and nevirapine (76.9%), efavirenz + emtricitabine + tenofovir disoproxil fumarate (15.4%) or zidovudine, lamivudine and

nevirapine (7.7%) as their first line regimen. A second line ART combination containing abacavir, didanosine and ritonavir-boosted lopinavir was given to 75% of patients in the suspected resistance group. Other patients in this group changed their regimen to zidovudine, lamivudine and efevirenz from starvudine, lamivudine and nevirapine (16.7%) or starvudine, lamivudine and nevirapine from zidovudine, lamivudine and efavirenz (8.3%). The randomly selected patients were under treatment for an average of 11.6 months, those in the suspected resistance group had been on ART for 19 months at the time of sampling.

1.29.2 HIV-1 Drug Resistance Mutations

Table 3-13 and Table 3-14 summarize resistance mutations detected in both groups, the randomly selected patients on ART and the suspected resistance group. In the first group, 2 of the 16 (12.5%) patients from whom sequences could be obtained, harboured mutations conferring resistance to reverse transcriptase inhibitors. One of these patients carried virus with mutations conferring resistance to both NRTIs (M184V) and NNRTIs (G190A), while in the other patient only a NNRTI mutation (Y181C) was found. Among patients with suspected resistance, 6/17 (35.3%) harboured major mutations to NRTIs and 6/17 (35.3%) major mutations to NNRTIs and 7/17 (41.2%) harboured minor RTI mutations.

Out of the 17 individuals with suspected drug resistance, 7 harboured mutated viral strains. In 6 of these 7 patients multiple mutations to both NRTIs and NNRTIs were detected. Two individuals (11.8%) in this group also carried minor mutations to protease inhibitors (I54AV, I84V and L10F for the first individual and L10I for the second individual).

1.30 DISCUSSION

Our attempts to genotype viral isolates from randomly selected patients under treatment highlight the limited sensitivity of detection in such samples. This argues for an overall efficacious treatment in most KIULARCO patients, because under optimal ART, VL might well remain under the detection limit [11]. As a consequence, the number of viral sequences deriving from treated patients was very small in our study. Nevertheless, our findings provide a first and preliminary indication of a rather low frequency of DR-mutations among KIULARCO patients. The fact that we were successful in amplifying viral sequences from all cases with suspected resistance, argues against a technical problem, but indicates that the most probable cause of amplification failure was indeed a very low viral load in most of the 137 samples analyzed. The 16 successful amplifications probably detected viral RNA only in samples with elevated or temporarily raised VL.

However, the 137 randomly selected plasma samples used in our investigation had been stored for a considerable period of time prior to our molecular analyses. This might have impaired the quality of the viral RNA and thus amplification success. In this case our low sensitivity of RT-PCR was rather due to frequent power cuts and suboptimal storage. Previously we have analyzed earlier samples collected from these 137 individuals prior to start of ART. Most of these earlier samples yielded a PCR product (Masimba et al. submitted). Considering the low efficacy of sequencing viral isolates from patients under ART, the analysis of samples of cohort patients not yet under treatment could provide DR-mutation prevalence data much faster. Such data would be equally useful for monitoring the spread of DR mutations in the study area.

Out of the 16 randomly selected individuals on ART, from whom a viral sequence could be obtained, two (12.5%) harboured major mutations conferring resistance to reverse transcriptase inhibitors. The three mutations detected in this group were Y181C and G190A, both implying resistance to NNRTIs, and M184V, conferring resistance to NRTIs. Patients were receiving combination therapy containing starvudine lamivudine and nevirapine for a mean duration of almost 1 year. The M184V mutation has been reported to be selected in patients receiving a lamivudine containing regimen [12] [13].

Several studies conducted in different parts of Africa have reported varying prevalence rates of DR mutations for patients receiving ART. Dagnra et al. (2011) reported a prevalence of 24.5% for patients receiving the same combination of ART as used in our study for 12 months in Lome, Togo [14]. This higher prevalence was explained by the fact that half of the patients in the Togo study had interrupted their treatment for >1 month. Among 16 patients, only one was reported to be non-adherent. Because no adherence problems were evident in case report forms of our 16 ART recipients with PCR amplified viral sequences, the discrepancy in adherence between our patients and those in Togo could explain the observed difference in DR mutation prevalence. Several other factors, such as suboptimal drug levels due to differences in host drug metabolism, presence of other diseases interfering with drug absorption, drug toxicity, use of low potent drugs, non-adherence and long duration of ART are known to contribute to development of resistance in HIV-1 treated patients [7] [8]. None of these factors have been investigated so far in KIULARCO patients. In general, adherence in the studied subset of KIULARCO patients was good. Adherence information was extracted from medical reports, but this can not rule out the possibility of missed doses. In a study conducted in 2003 in Kampala, Uganda, a mutation prevalence of 19% was observed for major NNRTIs mutations and 16.8% for major NRTI

mutations in patients receiving a nevirapine-based or efavirenz-based combination therapy (74% and 26% of patients, respectively) for >9.5 months. In the Ugandan study, 23% of patients had reported treatment interruption for >4 days and these treatment interruptions were strongly associated with virological failure [15]. In Haydom, rural Tanzania, where ART had been provided since 2003, major DR mutations were detected in samples from 2007 and 2008 at a prevalence of 3.9% for patients who had been on ART for 1 year [10]. As reasons for the good ART response to treatment in this study the following concomitant factors were given: adherence counselling, presence of home-based carers, regular peer-support meetings, and continuous uninterrupted drug supply.

The 12.5% prevalence of major mutations detected in KIULARCO patients under ART is three times higher than results from the Hydom study, which was conducted also in a rural area in Northen-East, Tanzania [10]. It is also higher than the results obtained from Kagera and Kilimanjaro studies [7]. The observed relatively high prevalence of DR mutations among KIULARCO treatment experienced patients supports our hypothesis on increased numbers of mutations in rural settings. The possible explanation for this still moderately high level of genetic resistance could be due to drug stock outs or drug adherence problems in some patients. These findings emphasize that it is only possible to achieve high ART efficiency in a rural setting when ART programs are well organized and coordinated to ensure optimal ART delivery and usage, and that this programmes should be carefully monitored.

Among the patients with suspected DR, 6/17 patients (35.3%) harboured major DR mutations affecting both, NRTIs and NNRTIs. These patients had started ART at a mean CD4 count of 188 cells/ml and had been on an either nevirapine-based combination (84.6%) or efavirenz-based combination (15.4%) for a mean period of 19 months. The average CD4 counts at the time of

first ART change was 76 cells/µl. Such a low CD4 count was one of two criteria used for defining a suspected case of resistance. The second clinical criterion was based on WHO clinical staging. In this group of suspected DR cases, the mean duration of ART was 19 months, which exceeded that in our randomly selected ART patients by 7 months. Mutations associated with DR have been shown to increase with the time elapsed since ART initiation [10]. It possible that the longer lasting ART in suspected DR cases could have contributed to the emergence of the observed mutations, but primary resistance in these patients cannot be ruled out.

The most common mutations in cases of suspected DR were Y181C, G190A, and K103N (NNRTIs) and M41L, M184V, D67N and T215F/Y (NRTIs). These mutations were also reported from patients failing their first ART regimen in other studies [16] [17] [18] [19]. Some of the minor RT mutations observed in this group have some clinical impact when occurring in association with major RT mutations. For example, E44D when occurs with M41L, as seen in patient (ID 7150047, increases resistance to NRTI, in particular to lamividune [20]. F116Y belongs to the Q151M cluster that is associated with resistance to multiple NRTIs. Both F116Y and Q151 were observed in patient ID 7121093. In 2002, Deval and coworkers found that this haplotype exerts its effects through substrate discrimination at the level of nucleotide incorporation [21]. All patients with suspected DR, who harboured major mutations in RT, carried at least one minor mutation. It is likely that this combination of mutations contributed to the reduced ART efficacy in these patients.

The presence of only few minor mutations PR and the absence of major mutations in PR in our study are consistent with the rare use of protease inhibitors in KIULARCO [3]. PR inhibitors are used only as second line drugs in Tanzanian and Sub-Saharan Africa [2] [6]. Similar low frequency of minor PR mutations or absence of major PR mutations have been reported from other sites with no or very restricted use of PR inhibitors [22].

1.31 CONCLUSION

Our pilot data has shown for KIULARCO patients under ART that a prevalence of DR mutations is lower than in other parts of Africa, but 3-fold higher than reports from another rural site in Tanzania. This suggests that the KIULARCO ART program has some limitations in its effectiveness. In 17 patients with suspected drug resistance, major DR mutations were observed at a prevalence of 35%. Yet, the majority of these 17 treatment failures seemed to be due to causes other than the genetic background of the virus, e.g. compliance with treatment, and hence further studies are needed to investigate these factors. Continuous monitoring should be instituted to further improve the outcome of the ART programme in this cohort.

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Table 3-11: Baseline characteristics of studied subset of KIULARCO patients

Variable	Value	%
WHO Stage	(n = 116)	

1	39	33.6
2	22	19.0
3	35	30.2
4	20	17.2
Sex	(n = 117)	
Females	80	68.4
Males	37	31.6
HIV-1 Subtype	(n = 119)	
A	31	28.0
C	52	37.3
CRF01_AE	11	10.7
D	28	24.0
CD4 counts (cells/µl)	(n = 119)	
Mean	278	na
Viral Load (copies/ml)	(n = 90)	
Mean	124 904	na
Age (Years)	(n = 116)	
Mean	37	na
First ART Regimen	(n = 97)	
1a	67	69.1
1b	1	1.0
1c	24	24.7
1d	2	2.1
1e	3	3.1

1a, starvudine + lamivudine + nevirapine; 1b, zidovudine + lamivudine + nevirapine; 1c, zidovudine + lamivudine + efavirenz, Adults and Paediatrics; 1d, starvudine + lamivudine + efavirenz; 1e, efavirenz + emtricitabine + tenofovir disoproxil fumarate. na, not applicable.

Table 3-12: Characteristics of study participants with detectable viral RNA

Characteristic	**Patients on ART (n= 16)**	**Suspected Resistance Cases (n= 17)**
Age (years)		
Mean	36	31
Sex (percent)		
Female	50	64.3
Male	50	35.7
CD4 Counts at baseline(Cells/µl)		
Mean	247 ± 187	188 ± 78
CD4 Counts at First ART change (Cells/µl)		
Mean	na	76 ± 60
Viral load (Copies/mL)		
Mean	171 443 ± 297 083	317 955 ± 636 925
WHO Stage (percent)		
WHO 1	25	41.7
WHO 2	0	25.0
WHO 3	75	8.3
WHO 4	0	25.0
Duration on 1st ART (Months)		
Mean	11.6	19
1st Regimen (percent)		
1a(30)	87.5	76.9

1b	6.3	7.7
1c	0	15.4
1e	6.3	0
Second ART (percent)	(n = 1)	(n = 12)
1c	0	16.7
1a(30)	0	8.3
2a	0	75.0

ART, antiretroviral therapy; NRTIs, Nucleoside reverse-transcripatse inhibitors; NNRTIs, Non-nucleoside reverse-transcripatse inhibitors; PIs, Protease inhibitors; 1a(30), starvudine (30) + lamivudine + nevirapine, adults; 1b, zidovudine + lamivudine + nevirapine, adults and paediatrics; 1c, zidovudine + lamivudine + efavirenz, adults and paediatrics ; 1e, efavirenz + emtricitabine + tenofovir disoproxil fumarate; na, not applicable (not done).

Table 3-13: Drug resistance mutations in randomly selected patients on ART and in suspected resistance cases

	Patient ID	HIV-1 Subtype	Sex	Age	Mutation Drug Class			Regimen Used
					NRTIs	NNRTIs	PIs	
Randomly selected patients under ART	5510425	C	F	38	-	K101Na	-	1a (30)
	5510599	C	F	52	-	Y181C G190Ra	-	1a (30)
	5510817	C	M	55	-	A98Ga	-	1a(30)
	5510423	C	M	35	-	K103Ra	-	1a(30)
	5510255	A	F	13	M184V	G190A E138Qa	-	1b
	5510270	C	F	8	M41Ia K103Ra	-	-	1a(30)
Suspected resistance cases	7150047	CRFAE_01	M	60	M41L E44Da M184V L210Sa T215Y	G190A A98Ga K101Ea V179Ta	I54AVa I84Va L10Fa	1a(30)
	5510582	C	F	12	-	V106Na	-	1b
	5510946	A	M	42	M41L D67N V75M L210W T215F	L100ILVaK103Na V108ILVa E138Ra V179Ta G190S H221Ya	L10Ia	1c
	5510628	C	M	32	M41L D67N	Y181YC	-	1c

					K70R V75M M184V T215F K219E	G190S		
	5510255	CRF01_ AE	F	16	M184V	G190A K101Ea E138Qa	-	1a(30)
	5511110	C	F	24	D67N D67Ta Q151M M184I K219Q	V90Ia K101aQ K103N Y181C G190A	-	1a(30)
	7121093	C	F	37	M184V F116Y Q151M T215Y	K103N	-	1a(30)

ART, Antiretroviral therapy; NRTIs, Nucleoside reverse-transcripatse inhibitors; NNRTIs, Non-nucleoside reverse-transcripatse inhibitors; PIs, Protease inhibitors; 1a(30), Starvudine (30) + Lamivudine + Nevirapine, Adults; 1b, Zidovudine + Lamivudine + Nevirapine, Adults and Paediatrics; 1c, Zidovudine + Lamivudine + Efavirenz, Adults and Paediatrics ; Atripla, Efavirenz + Emtricitabine + Tenofovir disoproxil fumarate.

[a]Minor HIV-1 drug resistance mutations

Table 3-14: Frequencies of DR mutations in randomly selected patients on ART and in suspected drug resistance patients

Type of Mutation	**Randomly selected patients on ART (n = 16)**		**Suspected resistance cases (n = 17)**	
	Frequency	Percentage	Frequency	Percentage
Major NRTI Mutations				

M41L	0	0	3	17.6
D67N	0	0	3	17.6
K70R	0	0	1	5.9
V75M	0	0	2	11.8
Q151M	0	0	2	11.8
M184V	1	6.3	4	23.5
L210W	0	0	1	5.9
T215Y	0	0	2	11.8
T215F	0	0	2	11.8
K219E	0	0	1	5.9
K219Q	0	0	1	5.9
Individuals with major NRTI mutations	1	6.3	6	35.3
Major NNRTI Mutations				
L100I	0	0	1	5.9
K103N	0	0	3	17.6
Y181C	1	6.3	2	11.8
G190A	1	6.3	3	17.6
G190S	0	0	2	11.8
Individuals with major NNRTI mutations	2	12.5	6	35.3
Individuals with major RTI mutations	2	12.5	6	35.3
Minor RTI mutations				
M41I	1	6.3	0	0
E44D	0	0	1	5.9
D67T	0	0	1	5.9
V90I	0	0	1	5.9
A98G	1	6.3	1	5.9
L100V	0	0	1	5.9

K101E	0	0	2	11.8
K101N	1	6.3	0	0
K101Q	0	0	1	5.9
K103R	1	6.3	0	0
V106N	0	0	1	5.9
V108IL	0	0	1	5.9
F116Y	0	0	1	5.9
E138R	0	0	1	5.9
E138Q	1	6.3	1	5.9
V179T	0	0	2	11.8
G190R	1	6.3	0	0
L210S	0	0	1	5.9
L221Y	0	0	1	5.9
L234S	0	0	1	5.9
Individuals with Minor RTI mutations	6	37.5	7	41.2
Minor PI Mutations				
L10I	0	0	1	5.9
L10F	0	0	1	5.9
I54AV	0	0	1	5.9
I84V	0	0	1	5.9
Individuals with minor PRI mutations	0	0	2	11.8

ART, antiretroviral therapy; DR-mutations, drug resistance mutations; NRTI, nucleoside reverse-transcriptase inhibitor; NNRTI, non-nucleoside reverse-transcriptase inhibitors; PI, protease inhibitor

Chapter 4

Development of a Microarray for Genotyping HIV-1 Drug Resistance Mutations in the Reverse Transcriptase Gene

Development of a Microarray for Genotyping HIV-1 Drug Resistance Mutations in the Reverse Transcriptase Gene

Pax Masimba[1,2*], Janet Gare[1,2*&], Thomas Klimkait[2,3], Marcel Tanner[1,2], Ingrid Felger[1,2]

*Shared first authorship

1 Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

2 University of Basel, CH-4003 Basel, Switzerland

3 Institute for Medical Microbiology, University of Basel, Switzerland

4 St. Francis Referral Hospital, Ifakara, United Republic of Tanzania

5 Ifakara Health Institute, Ifakara, United Republic of Tanzania

& Current affiliation: Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea

This manuscript is being prepared for submission

1.33 ABSTRACT

1.33.1 BACKGROUND

The success of antiretroviral therapy (ART) to HIV-1 is compromised by development of drug resistance (DR) due to mutations in viral genes, the product of which is targeted by ART drugs. For ART deployed in resource poor settings, the viral reverse transcriptase (RT) is the main drug target, and to a lesser extend also protease (PR). Monitoring of these DR mutations in both drug naïve and drug experienced patients can contribute to optimization of ART at individual and population level. In Tanzania molecular analyses of DR are limited in their availability owing to high costs. Therefore, a simple, cheap and robust tool for DR genotyping was developed based on microarray technology. Detection of single nucleotide polymorphism (SNP) was targeted specifically at local strains prevailing in the HIV-1 cohort in Ifakara, rural Tanzania, and was restricted to those 25 DR mutations most relevant for the locally available ART regimen.

1.33.2 METHODS

A 645 bp fragment of the RT gene was reverse transcribed and amplified by PCR. Primers for mini-sequencing were designed based on alignments of local HIV-1 variants. Tagged primers, positioned directly adjacent to the SNP, were extended by 1 fluorochrome-labeled dideoxynuclotide triphosphate (ddNTPs), whereby this nucleotide, enzymatically incorporated according to base pairing rules, indicated the SNP allele of the sample tested. Extension products were hybridized to anti-tags spotted on microarray slides. Images on the slide were analyzed with a laser scanner and Genepix software. Genotype calling was performed with in-house

developed software. This HIV-1 typing tool was validated with 102 HIV-1 directly sequenced samples deriving from the Tanzanian target population.

1.33.3 RESULTS AND CONCLUSION

Microarray derived genotypes were concordant with the corresponding sequence-based SNP in 92.7% of the 2550 compared. Once the locally adapted microarray had been established, genotyping was easy to perform and completed within 4 hours. Major problems were missing data, as some SNPs could not be detected in some samples, possibly due to excessive mismatches between PCR product and extension primer. Nine-teen SNPs were genotyped reliably, while performance was suboptimal for six SNPs. Due to missing data this SNP array should be considered preferentially for population studies on prevalence of SNPs rather than for individual diagnostics and represents a fast and cheap alternative to sequencing.

1.34 INTRODUCTION

Development of HIV-1 drug resistance has been one of the major obstacles in the success of Antiretroviral Therapy (ART) to HIV-1 patients [1]. One of the major factors which accounts for the development of resistance to these drugs is the emergence of mutations in the reverse transcriptase (RT) gene which is among the HIV-1 drug targets (2).

A number of phenotypic and genotypic assays have been used to detect HIV-1 drug resistance (DR) mutations (3). Phenotypic assays measure directly the extent to which an antiretroviral drug inhibits HIV-1 virus replication *in vitro*, and is performed by measuring an increase in the inhibitory concentration (IC) that is required to inhibit in-vitro growth by 50 percent (IC50) compared with virus replication in the absence of drug. Results are reported as a fold-change in

drug susceptibility of the patient sample compared with a laboratory reference strain (4). Phenotypic testing reflects the net effect of HIV-1 mutations on susceptibility to each tested drug and is more useful in patients with complex mutation patterns (5). Genotypic assays identify DR-associated mutations in viral RNA isolated from HIV-infected individuals. In comparison to phenotypic assays, genotypic testing is considered to have the advantages of rapid turn-around time of about 1-2 weeks, lower cost, and enhanced detection of resistance-associated mutations in mixed virus populations (5).

In developed countries, both genotypic and phenotypic assays are used for monitoring HIV-1 patients at the beginning of ART, and in case of suspected drug resistance. Due to their prohibitive costs, these assays are not available in developing countries like Tanzania, where the number of patients under ART has increased massively since the start of the National programme (NACP) in 2004 (6). In view of the rapidly increasing need for molecular monitoring of the prevalence and spread of DR also in resource-poor settings, an additional molecular tool for robust and cheap DR genotyping is warranted. While nucleotide sequencing certainly remains the gold standard for molecular detection of DR-SNPs, surveillance of population samples and molecular epidemiological research project could greatly benefit from a highly parallel and fast tool to determine only a small number of DR-SNPs that are most essential for a specific location and available drugs. We therefore investigated the option to genotype multiple SNPs using a microarray platform.

A microarray platform had been developed previously in our laboratory for detection of SNPs in drug resistance marker genes of malaria parasites (7). For this application the mini-sequencing principle provided highly specific base calling and parallel genotyping of many SNPs at a time (8). We intended to adopt this platform for genotyping local HIV-1 variant in Ifakara. For proof-

of-concept this development of an HIV-1 microarray was restricted to genotyping only mutations associated with resistance to reverse transcriptase inhibitors (RTI), in particular those that were used in the Ifakara HIV-1 cohort, i.e. starvudine, lamivudine, zidovudine, abacavir, didanosine, nevirapine and efavirenz.

Due to the high error rate of HIV-1 reverse transcriptase, a very extensive polymorphism is observed among the HIV-1 strains. Therefore primers needed to be designed based on local subtypes and strains occurring in the study area. Our intention was to provide an affordable option for monitoring HIV-1 drug resistance in Tanzania by genotyping on microarray, which permits highly multiplexed SNP analysis in a single run and also requires less hand-on-time and resources (7).

1.35 MATERIALS AND METHODS

Viral RNA was extracted and reverse transcribed from 140 µl plasma collected from 102 Tanzanian HIV-1 patients attending the Chronic Disease Centre of Ifakara (CDCI) at St. Francis Referral Hospital (SFRH). All procedures have been described previously (Masimba et al. submitted manuscript). A fragment of 645bp spanning positions 23 – 236 in the RT gene was amplified by nested PCR as described.

1.35.1 Design of Extension Primers, Tags and Anti-Tags

Extension primers were designed for 25 prioritized DR-SNPs in the RT gene. Per SNP one or more extension primers were designed using a Clustalw1 alignment of 126 sequences from HIV-1 samples of patients from the KIULARCO cohort in Ifakara, Tanzania. These sequences derived from a molecular epidemiological study presented elsewhere (Masimba et al. submitted

manuscript). Extension primers were designed in either the forward or reverse direction to maximize sequence conservation between the designed primer and the variety of template sequences. Single base extension (SBE) software (9) was used to design a set of 100 tags and anti-tags under the following parameters: length 17-25 bp, melting temperature (Tm) 53-62°C, homodimer temperature 40^0C. One individual tag was added to the 5` end of a single extension primer, whereby the SBE program was used to select the optimal tag/extension primer pairs by assessing the Tm and the potential for hair-pin formation, homodimer and heterodimer formation. For spotting on microarrays 55 anti-tags, i.e. reverse complement of the tags selected for the extension primers, were synthesized via a C7 aminolinker for covalent coupling to the aldehydeglass slide. Oligonucleotides used as extension primers, anti-tags, as well as Cy5- and Cy3-prelabeled oligonucleotides used as spotting controls and one additional Cy5-labelled hybrization control were all purchased from Eurofins MWG/Operon GmbH (Ebersberg, München, German). Labeled ddNTPs were purchased from Perkin Elmer, Schwerzenbach, Switzerland.

1.35.2 Array design and production

55 anti-tags plus 2 Cy3- and Cy5-prelabeled spotting controls were spotted at the Center of Integrative Genomics, University of Lausanne, Switzerland, on Arrayit aldehyde-coated slides with a 12 well mask (Supermask 12 Super Aldehyde Slides purchased from Anopoli, Eichgraben, Austria). Oligonucleotides were dissolved as a 10x stock (500µM) in 180 mM phosphate buffer pH 8.0 and spotted after dilution in spotting buffer (3xSSC buffer with 1.5 M betaine) at a concentration of 50 µM (labeled spotting controls at a concentration of 0.5 µM). The array design featured 14X14 spots with a spot-to-spot distance of 250 µm and was printed in

triplicate per masked well. After printing, slides were kept on a chamber at 50% relative humidity and baked the next day at 80°C for 90 min.

Prior to hybridization slides were pre-treated by an incubation at 80°C for 90 min, followed by washing steps, 2x2 min in 0.2% SDS and 3x2 min in distilled water and was dried by centrifugation at 800 rpm for 5 min, reduced in 50mM triethanolamine titrated with boric acid to pH 8.0 at 50^0C for 30 minutes, washed three times with 0.2% SDS for 1 minute, washed twice in distilled water for 1 minute and finally dried by centrifugation for 5 minutes at 800 rpm. Slides were then kept at room temperature in a dry, clean and dark place until used in hybridization experiments.

1.35.3 Primer Extension and hybridization

Prior to the primer extension reaction, nested RT-PCR products were subjected to a Shrimp Alkaline Phosphatase (SAP) digest (Amersham Biosciences, Freiburg, Germany) to eliminate all non incorporated nucleotides. This reaction was carried out as previously described (1). Primer extension with Cyanine-3 and Cyanine-5 labeled dideoxynucleotide Triphosphates (Cy3-ddNTPs and Cy5-ddNTPs from Perkin Elmer, Schwerzenbach, Switzerland) was carried out as described previously (7). Because the scanner used supported only dual fluorescence measures, two extension reactions were performed with different permutations of Cy3 and Cy5 labelled ddNTPs. Table 4-15 shows the composition of both reaction mixes and indicates the required reaction mix for each extension primer. These two combinations of differentially labeled ddNTP were sufficient to differentiate all wild-type from mutant alleles. Extension products from both reactions were combined before denaturation and hybridization performed as

described previously (7) with one modification in that hybridization was performed at 55^0C for two hours.

1.35.4 Washing

After hybridization, the slide washed at room temperature (20^0C) in 3 consecutive buffers (temperature of the washing buffer was set at around 25^0C). One wash round consisted of 2X SSC + 0.2% SDS for 3 minutes, followed by 2X SSC for 2 min and finally 2X SSC + 2% Ethanol for 1 min. The number of rounds depended on the background fluorescence and fluorescence intensity of spots. To adjust washing conditions to the background intensity, a slide was quickly dried with compressed air after each round and then pre-scanned. This was followed by additional rounds until the background fluorescence was satisfactorily removed without compromising signal intensity yet. Usually three rounds were necessary.

1.35.5 Image and Genotype Scoring

After drying a slide was scanned in a GenePix® microarray scanner 4100A (Axon Instruments, Genepix, USA) and images were stored as Tagged Image File Format (TIFF) file. Images were interpreted by running the Genepix software in combination with a file containing the array layout. All spots with pre-labeled tags or anti-tags gave strong signals at their defined locations and could thus be used to position the array. The data retrieved was stored in a GenePix Result (GPR) file which was transferred to an in house generated receiver operating characteristic (ROC) Classifier program for SNP calling. This program evaluated and scored the triplicate hybridization signals for each SNP into wildtype or mutant based on threshold values from a set of positive (triplicate spotting and hybridization controls and negative (unused anti-tags) controls present on each slide.

1.35.6 Cloned HIV plasmids

Cloned fragments of the HIV-1 genome were used for microarray validation. These fragments, derived from anonymized Swiss HIV-1 Cohort samples and cloned in puc18 plasmids were made available from the Institute for Medical Microbiology, University of Basel (10). Individual bacterial colonies were picked and plasmid DNA was extracted using QIAprep Miniprep Spin colums (Qiagen, Germany) according to the manufacturer's instructions. Therefore, each of these cloned fragments represented an individual HIV-1 RT gene, thus suitable for test validation and assessing background hybridization. Plasmid inserts were of HIV-1 subtype A, AE/A or C.

1.36 RESULTS

1.36.1 Design of extension primers, tags and anti-tags

For 25 SNPs in the RT gene a total of 51 extension primers (1 to 8 extension primers per SNP) were designed to compensate genetic diversity in the targeted sequence. Extension primers, tags and anti-tags are listed in **Table 4-16**. Prior to hybridization experiments, all extension primers were tested by individual PCRs involving one of the extension primers plus either the forward or reverse primers normally used in nPCR. As templates 4 cloned RT gene fragments (subtypes A, AE2 and C) were used as well as 102 RT-PCR products from Tanzanian HIV patients. All extension primers yielded DNA fragments of the expected size, indicating a sufficient degree of sequence conservation between these primers and the different templates tested (Figure 4-13).

1.36.2 Array design and spotting

SBE software was used to select 55 oligonuclotide anti-tags and 2 spotting controls, the latter being produced with Cy3 or Cy5 fluorescent label at their 3'

end. One anti-tag was reserved for a hybridization control, for which the Cy5 pre-labeled tag was added to the extended primers prior to hybridization. Unused tags and printed buffer spots were used as negative controls. The array printed on Arrayit slides consisted of triplicates of the 55 anti-tags and controls in a 14x14 spot lay out (A B), thus generating 3 data points for each position per sample. The separation of each slide by a mask permitted to test in 12 patient samples. The Cy5-labelled spotting control was found to decay fast during storage of slides, in contrast to the Cy3-control. Therefore the hybridization control also carrying a Cy5 label was used to control hybridization success and to support correct positioning of the array for which fixed and strong signals were required. Quantitative results obtained from all pre-labeled controls suggested a good reproducibility of the hybridization on microarray.

1.36.3 Optimization of washing

Despite systemic tag/anti-tag design, establishing of optimal wash conditions for hybridized slides was a challenge in generating optimal signal intensities for all spots of an array. The number of wash steps depended on the background fluorescence and the spot intensity on each particular slide and had to be adapted after a pre-scan after each washing round. Most slides were washed three times, with each round consisting of 2X SSC + 0.2% SDS for 3 minutes, followed by 2X SSC for 2 min and finally 2X SSC + 2% Ethanol for 1 min. Minor differences in slide pretreatment conditions and duration of storage could have contributed to these differences.

1.36.4 Comparison of microarray-based SNP typing and direct sequencing using cloned RT fragments and 102 field samples from Tanzania

In contrast to PCR fragments from field samples, which likely represent populations of sequences, cloned HIV-1 genome fragments harbor a single sequence and were therefore ideal for validating the specificity of hybridization on microarray and for determining individual anti-tags that give rise to hybridization background. Four cloned fragments, generated previously from Swiss HIV-1 cohort samples, were used, which represented sequences of subtypes C, AE2 and A2. For these cloned RT genes, the agreement between microarray results and direct sequencing varied for the different cloned fragments. With 2 plasmids containing C1 and A2 subtype sequences (0720235-C1 and 070510-A2), perfect agreement between sequence and microarray genotype was obtained, whereas only 88% agreement was reached for a plasmid with a AE2 subtype insert (6017225-AE2) and 72% for a subtype C1 insert (072073-C1). Because these plasmid inserts derived from patients of a Swiss HIV cohort, they likely represented variants with substantial sequence deviation from our extension primer sequences, which had been optimized for Tanzanian subtypes. Overall the concordance between both typing methods was 90%, the data for each SNP tested is shown in Table 4-17.

Next our HIV-1 SNP array was validated by typing 102 field samples from HIV-1 patients from Ifakara, Tanzania. The agreement between microarray and direct sequencing was 92.7% (Table 4-17). Extension primers of 5 SNPs had concordance of 100%. 14 SNPs had a concordance of more than 90% and only one SNP had concordance of less than 70% compared to direct sequencing. The SNPs with 100% agreement were: M41L1, L74V, V75I, T215FY and K219E. The SNPs with between 90% - 99% agreement were: M41L, D67E, K70R, T215FY1, T215FY2 and L74I (99%), K219Q (98%), L100I, Y181C and M184V (96.1%), M184I (95.1%), G190A

and K103N (93.1%). The SNPs with <90% agreement were: D67N (87.3%), Y188L1 (86.3%), K219N (78.4%), Y188l2 (71.6%) and L210W (62.7%). The L210W SNP performed poorly compared to all other SNPs. The sequence alignment of all 102 Tanzanian samples tested sequences revealed in samples which failed to produce a signal a number of mismatches with the designed extension primer. Thus, the extension primer for SNP L210W was located in a region of considerable polymorphism and we failed to design a primer which would harbour less than three mismatches with any of the sequences. To compensate the polymorphism around SNP L210W, a total of 8 extension primers were designed for this SNP alone, but still only 60% of samples produced a signal by all these primers together. Primer mismatches seemed to be the main reason for missing data and the wrong signals. Another SNP, Y181C, initially also suffered from missing data, but after re-designing a set of 5 extension primers for this SNP, correct signals were recovered and in 96% of samples.

1.37 DISCUSSION

The microarray-based SNP typing approach, originally developed and in use in our laboratory for several years for genotypng DR resistance marker of the malaria parasite *Plasmodium falciparum*, (7) was applied for genotyping DR-SNPs in the RT gene of HIV-1. The high mutation rate and genetic diversity questioned a solely hybridization-based approach. Our approach tried to overcome this challenge by (i) performing allelic discrimination in an unequivocal enzymatic reaction by applying the mini-sequencing principle, and (ii) adapting the extension primers to the locally prevailing viral strains, i.e. producing a genotyping tool dedicated for our specific population and country. In our experiments hybridization on micrarray was limited to perfectly base-paired tags and anti-tags, which were selected bioinformatically to prevent unspecific hybridization. The critical step in our approach was adequate binding of the

extension primers to diverse field isolates. The efficiency of the extension reaction depends on the number and positions of mismatches within an extension primer. An increasing number of mismatches will lead to decreasing signal strength or finally to a missing data point. Facing these problems of homology were the major challenges in developing this genotyping chip. The overall aim of this development was to provide a cheaper alternative to classical sequencing for resource poor setting, by typing only the minimal essential SNPs.

HIV-1 samples from Tanzania were used to design extension primers for 25 SNPs with the aim to reduce mismatches between field isolates and primers. We were able to correctly identify 92% of all data points. Because hybridization on microarray between perfectly matching and optimized sequences likely does not cause any sensitivity problem, we assumed suboptimal primer annealing due to mismatches to be responsible for the missing data and we investigated the number of mismatches between primers and in the corresponding sequences for some of the SNPs that have failed. We found that the occurrence of more than 3 mismatches within an extension primer was detrimental to the PCR efficiency, while less than three primer mismatches located in the central position of the primer did not greatly affect PCR efficiency. Also primer mismatches located as close as 3 nucleotide from the 3` end compromised PCR efficiency.

When omitting results from extension primers, which never produces signals in the microarray (corresponding to 187/2550 SNPs), the concordance of microarray and sequencing increased to 98%. To overcome excessive mismatches, we designed additional primers specific for individual lineages of similar sequences. For SNP K210W, 8 different extension primers were added to the multiplexed extension reaction, but we failed to reliably produce typing results for this SNP in a hypervariable region. However, for other SNPs our approach of using >1 extension primers and to incorporate wobbles at polymorphic positions resulted in an increase in signal production, e.g.

designing 5 primers for Y181C led to 96.1% congruence, and 4 primers for K103N led to 93.1% congruence.

The major task in further development of this microarray will be to reduce missing data. This may be achieved by designing additional extension primers or by optimizing primer annealing conditions, i.e. annealing temperature and duration, or salt concentration in the multiplex extension reaction. The advantage of a microarray with spotted anti-tags is that it is flexible to permit the addition of more tagged extension primers into the reaction, in case one single primer cannot cover all sequence diversity in this particular region. Similarly, additional extension primers for new SNPs can be easily added, provided suitable free anti-tags are available on the array. On the other hand, the development of this microarray depended greatly on the availability of a large number of nucleotide sequences. Such comprehensive sequencing data to inform about polymorphism up and downstream of a targeted SNP is critical for the design of extension primers.

This SNP-chip was developed primarily for population studies, e.g. for determining the prevalence of transmitted DR-SNPs, or for identifying reasons for treatment failure. For such questions, the restricted data obtained by this method is adequate. For other research question or for individual diagnosis direct sequencing is more advantageous, because much additional information is gained. The obvious advantages of genotyping by microarray over direct sequencing are its simplicity, once it is set up; slides are easy to store and consumables (slides and reagents) are cheaper than sequencing materials; in particular the equipment required is less pricy. Moreover, the microarray typing protocol is faster than sequencing, with about 2 hours per 12 samples from the completed PCR purification step to obtaining results.

1.38 CONCLUSION

Genotyping by microarray has shown good agreement with sequencing in >100 field samples. To reliably generate complete DR haplotypes, further optimization is required. Given the simplicity of its use, low running costs and short processing time to results, this microarray platform has potential as alternative tool for monitoring resistance mutations in population-wide studies. Provided sequence information is available from other regions or other countries, the required extension primers can be quickly adapted to local HIV-1 variants.

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Table 4-15: Composition of Extension Primer and ddNTP reaction mixes

Extension Primer Mix 1	Extension Primer Mix 2
T-2-A-M41L ATTTTTGAAATTTTTCCTTCCTTTTCCA	T-1-A_K103N CCCACATCCAGTACTGTCACTGATTT
T-3-S_K65R TATAACACTCCAGTATTTGCCATAAAAA	T-5-A_D67E AAATCTACTAATTTTCTCCACTTAGTACT
T-4-A_D67N ATCTACTAATTTTCTCCACTTAGTACTGT	T-8-A_L74V TCTTTTATTGAGTTCTCTGAAATCTACTA
T-6-A_K70R TCCCTGAAATCTACTAATTTTCTCCACT	T-10-A_L100I AGTACTGTCACTGATTTTTTCTTTTTTA
T-7-S_K70R ATTTGCCATAAAAAAGAAGGACAGTACTA	T-13-S_Y188L2 ATCTATCAATACATGGATGACTTGT
T-9-A_V75I AGTTCTTTTATTGAGTTCTCTGAAATCTA	T-15-S_G190A TCTATCAATACATGGATGACTTGTATGTA
T-11-S_Y181C TAGAGCACAAAATCCAGAAATAGTTATCT	T-16-S_L210W AGAGGAGTTAAGAGCACATCTAT
T-12-A_Y188L1 TGCCCTATTTCTAAGTCAGATCCTAC	T-18-S_T215FY1 TAAGAGGACATCTATTGAGGTGGGGATTT
T-14-A-G190A CTCTATGCTGCCCTATTTCTAAGTCAGAT	T-19-A-K219Q ATGGAGGTTCTTTCTGATGTTTYT
T-17-A_T215FY1 TTCTGATGTTTCTTGTCTGGTGTG	T-20-S_M41L AGTGACAGTACTGGATGTGGGGG
T-23-A_T215FY2 TTCTTTCTGATGTTTCTTGTCTGGTGTG	T-21-S_T215FY AGAGGACATCTATTGAGGTGGGGATTTA
T-24-A_L74I CTTTTATTGAGTTCTCTGAAATCTACTA	T-22-A_T215FY TTCTGATGTTTTTTGTCTGGTGTG
T-28-S_184I AACCCAGAAATAGTTATCTATCAATATAT	T-27-A_K219N GAAATGGAGGTTCTTTCTGATGTTT
T-29-A_184V TAAATCAGATCCTACATACAAGTCATCCA	T-31-S_L74V AAGGACAGTACTAAGTGGAGAAAA
T-30-S_L74I AAGAAGGACAGTACTAAGTGGAGAAAA	T-33-S_L100I GGATACCACACCCAGCAGGG
T-32-S_L75I AAGGACAGTACTAAGTGGAGAAAATTA	T-33.2-S-L100I GGATACCACACCCAGCGGGG
160L74I1.1 ATTGAGTTCCCTGAAATCTACTA	T-34-S-K103N ACACCCAGCAGGGTTGAAAAAGAA
78M184V1.1 AGATCCTACATACAAATCATCCA	17M41L1.1 TTTGTAATTTTTCCTTCCTTTTCCA
144K219E1.1 TGGGGATTTACCACACCAGAC	02K103N1.1 ACATCCAGTACTGTCACTGATTT
T-02.1-A-Y181C ACATACAAGTCATCCATATATTGA	130L210W1.1 TCTGGTGTGGTAAATCCCCATTTI
T-38-A-Y181C ACATACAAGTCATCCATGTATTGA	T-39-S-L210W AGAGGAGTTAAGAGCACAYTTAT

T-40-A-Y181C ACATACAAGTCATCCACATATTGA	T-38-S_L210W AGAGGAGTTAAGAGCACATCTsT
T-45.1-A-Y181C ACATACAAGTCATCCACATATTGA	T-41-A-L210W TCTCGTCTGGAGTGAAAAATCCCCATTTT
T-45-A-Y181C CATACAAGTCATCCACATATTGG	T-42-S-L210W AGAGGAGTTAAGAGCWCACCTAT
T-50-S-L100I GGATACCACACCCAGCAGGI	T-43-S-L210W AGAGGAGTTAAGAGCWCATCTAT
	T-44-A-L210W TCTTGTCTGGTGTGGTAAATCCCCATTTC
	T-46-S-K219Q TGGGGATTTACCACACCAGAI
	T-47-A-L210W TTTTGTCTGGTGTGGTAAACCCCCACTTC
	T-49-A-L210W TCTTGTCTGGTGTGGTAAATCCCCACCTT
	T-51-S- K219Q TGGGGATTTACCACACCAGAC
Dideoxy-dNTP Mix 1	dideoxy-dNTP Mix 2
ddATP-CY3	ddUTP-CY3
ddCTP-CY3	ddCTP-CY3
ddGTP-CY5	ddATP-CY5
ddUTP-CY5	ddGTP-CY5

Table 4-16: Supplementary Table 1: List of extension primers, tags and anti-tags and spotting and hybridization controls.

Extension Primer (5´→3´)	Tag (5´→3´)	Anti-Tag (5´→3´)
T-1-A_K103N CCCACATCCAGTACTGTCACTGATTT	T-1 GGTTCCCGATTTATCGATCCC	AT-1 GGGATCGATAAATCGGGAACC
T-2-A-M41L ATTTTTGAAATTTTTCCTTCCTTTTCCA	T-2 CATGTGGTACAATGGAACAGCTA CT	AT-2 AGTAGCTGTTCCATTGTACCA CATG
T-3-S_K65R TATAACACTCCAGTATTTGCCATAAAAA	T-3 TCAGGGAACTTCGATGCTGC	AT-3 GCAGCATCGAAGTTCCCTGA
T-4-A_D67N	T-4 GACTGACCCGCTTGAGTTAGT	AT-4

ATCTACTAATTTTCTCCACTTAGTACTGT		ACTAACTCAAGCGGGTCAGTC
T-5-A_D67E AAATCTACTAATTTTCTCCACTTAGTACT	T-5 GTTCAATCAGAAAACACCTGCGG	AT-5 CCGCAGGTGTTTTCTGATTGAAC
T-6-A_K70R TCCCTGAAATCTACTAATTTTCTCCACT	T-6 CTGCAAGCAGGTTGTGCTCT	AT-6 AGAGCACAACCTGCTTGCAG
T-7-S_K70R ATTTGCCATAAAAAAGAAGGACAGTACTA	T-7 GGCGGTTCATGGAATTCCC	AT-7 GGGAATTCCATGAACCGCC
T-8-A_L74V TCTTTTATTGAGTTCTCTGAAATCTACTA	T-8 GTCCTACGTCGAGTAGAGAAAGTC	AT-8 GACTTTCTCTACTCGACGTAGGAC
T-9-A_V75I AGTTCTTTTATTGAGTTCTCTGAAATCTA	T-9 CATTTGCGTTTCTCTGGGTAATGC	AT-9 GCATTACCCAGAGAAACGCAAATG
T-10-A_L100I AGTACTGTCACTGATTTTTTCTTTTTTR	T-10 CCTGTCGGGAGCAGTACA	AT-10 TGTACTGCTCCCGACAGG
T-11-S_Y181C TAGAGCACAAAATCCAGAAATAGTTATCT	T-11 ATCTACTACCACCTCCAACGG	AT-11 CCGTTGGAGGTGGTAGTAGAT
T-12-A_Y188L1 TGCCCTATTTCTAAGTCAGATCCTAC	T-12 GGGCGGACTACATCGAAATTACC	AT-12 GGTAATTTCGATGTAGTCCGCCC
T-13-S_Y188L2 ATCTATCAATACATGGATGACTTGT	T-13 CCGAAACAACGCAGAACTCAC	AT-13 GTGAGTTCTGCGTTGTTTCGG
T-14-A-G190A CTCTATGCTGCCCTATTTCTAAGTCAGAT	T-14 CTCTCCACAGTGCAGCGA	AT-14 TCGCTGCACTGTGGAGAG
T-15-S_G190A TCTATCAATACATGGATGACTTGTATGTA	T-15 TGGCCTTGTGAATCCACCC	AT-15 GGGTGGATTCACAAGGCCA

T-16-S_L210W AGAGGAGTTAAGAGCACATCTAT[1]	T-16 CGAAAAACCACGCCGTATTTCA	AT-16 TGAAATACGGCGTGGTTTTTC G
T-17-A_T215FY1 TTCTGATGTTTCTTGTCTGGTGTG	T-17 TCACTTACGACCGTTTTGTCTACA	AT-17 TGTAGACAAAACGGTCGTAA GTGA
T-18-S_T215FY1 TAAGAGGACATCTATTGAGGTGGGGAY TT	T-18 GAGAGGCATGCGTTTCACG	AT-18 CGTGAAACGCATGCCTCTC
T-19-A-K219Q ATGGAGGTTCTTTCTGATGTTTYT	T-19 GACCGGCAATTCGTTATCCAC	AT-19 GTGGATAACGAATTGCCGGT C
T-20-S_M41L AGTGACAGTACTGGATGTGGGGG	T-20 GTCAAATTCGACAGCTGGAAGG	AT-20 CCTTCCAGCTGTCGAATTTGA C
T-21-S_T215FY AGAGGACATCTATTGAGGTGGGGATTT A	T-21 GAAGCCGTCTCTGTTGTTTTCC	AT-21 GGAAAACAACAGAGACGGCT TC
T-22-A_T215FY TTCTGATGTTTTTTGTCTGGTGTI	T-22 CAGAGATCCATTGGCGCGT	AT-22 ACGCGCCAATGGATCTCTG
T-23-A_T215FY2 TTCTTTCTGATGTTTCTTGTCTGGTGTY	T-23 CGCATAATGACCCAACTTCGAG	AT-23 CTCGAAGTTGGGTCATTATG CG
T-24-A_L74I CTTTTATTGAGTTCTCTGAAATCTACTA	T-24 GCTGCCGGCTATTTTTGGAG	AT-24 CTCCAAAAATAGCCGGCAGC
T-27-A_K219N GAAATGGAGGTTCTTTCTGATGTTT	T-27 CCCCCGAGAAGGTTTATGTTTAAC	AT-27 GTTAAACATAAACCTTCTCGG GGG
T-28-S_184I AACCCAGAAATAGTTATCTATCAATATA T	T-28 AGCCTCGGGTCTACATCGT	AT-28 ACGATGTAGACCCGAGGCT
T-29-A_184V TAAATCAGATCCTACATACAAGTCATC	T-29 CAGCAGTCCGATGCCTGG	AT-29

CA		CCAGGCATCGGACTGCTG
T-30-S_L74I AAGAAGGACAGTACTAAGTGGAGAAAA	T-30 CGCCTAGACCTTTTAGCTAGCC	AT-30 GGCTAGCTAAAAGGTCTAGG CG
T-31-S_L74V AAGGACAGTACTAAGTGGAGAAAA	T-31 GGAGCTTTTGCTGTTCGGTC	AT-31 GACCGAACAGCAAAAGCTCC
T-32-S_L75I AAGGACAGTACTAAGTGGAGAAAATTA	T-32 CGGGGTATGACATACTATTGACCA	AT-32 TGGTCAATAGTATGTCATACC CCG
T-33-S_L100I GGATACCACACCCAGCAGGG	T-33 GTTGGCGGGTTATTACAGGG	AT-33 CCCTGTAATAACCCGCCAAC
T-34-S-K103N ACACCCAGCAGGGTTGAAAAAGAA	T-34 TGCGATTGTATACCCGCTCC	AT-34 GGAGCGGGTATACAATCGCA
		AT-35 GCGTAAATCATACGCCTGGG TC[2]
		AT-36 ACGCGTTACGTTAGAGATAA GGCTA[2]
		AT-37 GCCTCCACCCTTCTCAAGAAT A[2]
T-38-S_L210W AGAGGAGTTAAGAGCACATCTsT[1]	T-38 TTTCCGGATTCACCCGTACC	AT-38 GGTACGGGTGAATCCGGAAA

T-39-S-Y181C TAGAGCACAAAATCCAGAAATAGTTAT wT	T-39 GATCGGACGACGCTTGGG	AT-39 CCCAAGCGTCGTCCGATC
T-40-A-Y181C ACATACAAGTCATCCACATATTGA	T-40 TAGAGGAGGCGGGAGTTTTT	AT-40 AAAAACTCCCGCCTCCTCTA
T-41-A-L210W TCTCGTCTGGAGTGAAAAATCCCCATT TT[1]	T-41 AGCCAATGAATGACAATTCGTGCA	AT-41 TGCACGAATTGTCATTCATTG GCT
	T-42 GCACCACAGTCCGGTATTGC	AT-42 GCAATACCGGACTGTGGTGC
T-43-A-K103N CC CAC ATC CAA TAC TGT TAC TGA CTT	T-43 TTTCACACACGGCCACTTTTC	AT-43 GAAAAGTGGCCGTGTGTGAA A
T-44-A-L210W TCTTGTCTGGTGTGGTAAATCCCCATT TC[1]	T-44 TGTTTGAACTAGTGGCGTCACG	AT-44 CGTGACGCCACTAGTTCAAA CA
T-45-A-Y181C CATACAAGTCATCCACATATTGG	T-45 GGTGATAGGCAACGAGGTCT	AT-45 AGACCTCGTTGCCTATCACC
T-46-S-K219Q TGGGGATTTACCACACCAGAI	T-46 GGGGATCCTAGACTTTGATGCT	AT-46 AGCATCAAAGTCTAGGATCC CC
T-47-A-L210W TTTTGTCTGGTGTGGTAAACCCCCACT TC[1]	T-47 CTTAGTCCTCTGACTGTCTCTGTC	AT-47 GACAGAGACAGTCAGAGGAC TAAG
		AT-48 GACACACTTGTTGGACGCAA

		G[2]
T-49-A-L210W TCTTGTCTGGTGTGGTAAATCCCCACCTT[1]	T-49 GTGTTTGTCTACTTCGTGTGTGC	AT-49 GCACACACGAAGTAGACAAACAC
T-50-S-L100I GGATACCACACCCAGCAGGI	T-50 ATGGAACCTATAATCTAGGATGGCG	AT-50 CGCCATCCTAGATTATAGGTTCCAT
T-51-A-L210W TTTTGTCTGGGGTAGTCAATCCCCAGCTC[1]	T-51 TCGTATAAGTCACGTTCTCCTTGG	AT-51 CCAAGGAGAACGTGACTTATACGA
		AT-52 CATTACTCCCTCCCGTCATGT[2]
17M41L1.1 TTTGTAATTTTTCCTTCCTTTTCCA	17.1 CAACATCATCACGCAGAGCATCATT	17.1 AATGATGCTCTGCGTGATGATGTTG
160L74I1.1 ATTGAGTTCCCTGAAATCTACTA	160.1 CCACGTACTGTCCGGAATACACGAC	160.1 GTCGTGTATTCCGGACAGTACGTGG
02K103N1.1 ACATCCAGTACTGTCACTGATTT	02.1 TGCCCCGTTGCCCCGTTGCCCCGT	02.1 ACGGGGCAACGGGGCAACGGGGCA
75Y181C1.1 CATACAAGTCATCCATATATTGA	75.1 TAACACAAGAGCAGCTTGAGGACG	75.1 CGTCCTCAAGCTGCTCTTGTGTTA
78M184V1.1 AGATCCTACATACAAATCATCCA	78.1 ACAGCCTCGCAGATGACGAATCATT	78.1 AATGATTCGTCATCTGCGAGGCTGT

130L210W1.1 TCTGGTGTGGTAAATCCCCATTTI[1]	130.1.1 TACCAACTGTATGCGCATGTGCAC C	130.1.1 GGTGCACATGCGCATACAGT TGGTA
144K219E1.1 TGGGGATTTACCACACCAGAC	144.1.1 TTCAGTGTATGACGACCAGAGCG TT	144.1.1 AACGCTCTGGTCGTCATACA CTGAA
	[Cy3]AGAAGATGCCTAGTATATG	AT-61 CATATACTAGGCATCTTCT
		[Cy5]ATGCAACCATCAAGT- [AmC7~Q]
		[Cy3] GCTCAGCTGTATTAGAA- [AmC7~Q]

[1] Extension primers never giving a signal or with inconsistent performance

[2] 5 additional anti-tags were designed and spotted to permit future use; these were utilized for quantification of background hybridization)

Table 4-17: Supplimentary Table 2: Comparison of microarray with direct sequencing using field samples and cloned DNA fragments.

SNP	M41L	K65R	D67N	D67E	K70R	L74V	V75I	L100I	Y181C	Y188L1	Y188L2	G190A	G190A	L210W	T215FY1	T215FY1	K219Q	T215FY	L74I	K219N	M184I	M184V	K103N	M41L1	K219E	Agreement
Field Samples																										
ET33	1	0	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	22
ET34	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	23
ET36	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	24
ET37	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	22

ET39	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	22
ET44	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	24
ET48	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	22
ET50	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	24
ET53	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	23
ET56	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
ET59	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	23
5510266	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
5510423	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	22
5510599	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	21
5510305	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	22
5510377	1	0	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	20
5510376	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
5510388	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	23
5511611	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
5410055	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
5510060	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	23
5510184	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	21
5510603	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	23
FTM	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	22
5510508	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0	1	1	1	1	1	22
5510026	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	24
5510817	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
5510236	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	24
5510718	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	22
5510504	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	22
5510075	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
5510270	1	0	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0	1	1	1	1	1	21
5510135	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	23
5514370	1	1	1	1	1	1	1	1	1	0	0	0	1	0	0	1	1	1	1	0	1	1	0	1	1	18

HLM5	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	23
HLM 6	1	0	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0	1	1	1	1	21
HLM 7	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	0	1	1	21
HLM 13	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	23
HLM 17	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	23
HLM 23	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	21
HLM 25	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM 27	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM29	1	0	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	22
ET 2	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	1	1	1	20
HLM 32	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM 35	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM 36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
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HLM 41	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM 69	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM 71	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	24
HLM 73	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM 74	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM76	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	23
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HLM 78	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	24
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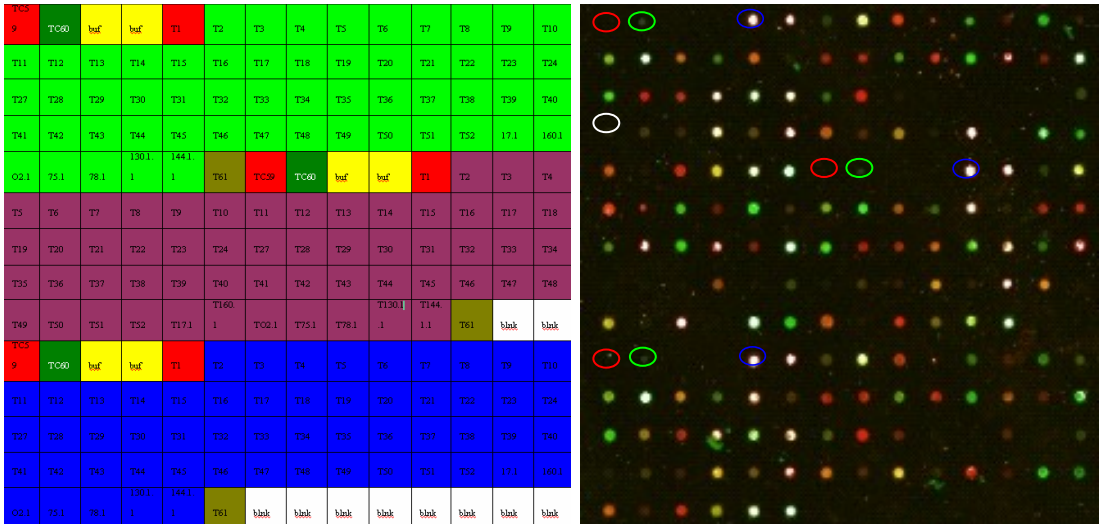
HLM 80	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM 83	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	23
HLM 86	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM 87	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	24
HLM 88	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	24
ET 7	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	1	0	1	19
ET 7	1	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	0	1	1	1	1	1	0	1	19

5514352	1	0	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	20
5510255	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	22
5511110	1	1	0	1	1	1	1	0	1	1	0	1	0	1	1	1	0	1	1	1	0	1	0	1	1	18
5514391	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	23
HLM-89	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM-91	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM-92	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM-93	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	23
HLM-172	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM-173	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM-175	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM-176	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	24
HLM-177	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM-178	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM-180	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	23
HLM-181	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM-182	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM-185	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM-258	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
HLM-259	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	22
HLM-260	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	22
HLM-261	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	0	1	1	22
HLM-262	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	23
HLM-263	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM-264	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	23
HLM-265	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	24
HLM-267	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM-182	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM-271	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	23
HLM-267	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25

HLM-182	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
HLM-271	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	23
05510783-A	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	24
5510075	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	23
ET 7	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	1	0	1	19
HLM 88	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	24
HLM 80	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
07510783-B	1	1	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	22
% agreement	99.0	75.5	87.3	99.0	99.0	10 0.	10 0	96 .1	96 .1	86 .3	71 .6	93 .1	93 .1	62 .7	99 .0	99 .0	98 .0	10 0	99 .0	78 .4	95 .1	96 .1	93 .1	10 0	10 0	92.7
agreement	10 1	77	89	10 1	10 1	10 2	10 2	98	98	88	73	95	95	64	10 1	10 1	10 0	10 2	10 1	80	97	98	95	10 2	10 2	2363
Cloned RT Fragments																										
0720235-C1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
6017225-AE2	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	1	1	1	22
072073-C1	1	0	1	1	1	1	1	0	0	0	1	0	1	0	1	1	1	1	1	0	1	1	1	1	18
070510-A2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
agreement	4	3	4	4	4	4	4	3	3	3	3	3	4	3	4	4	4	4	3	3	3	4	4	4	4	90



A **B**

Figure 4-12: HIV-1 SNP typing microarray (Figure A and B)

HIV-1 SNP typing microarray. (A) Design and layout of microarray. Triplicates are depicted in different colours. T1, TC59, TC60, T61 and blnk denote Cy5 spotting control-2, Cy5 spotting control-1, Cy3 spotting control, Cy3 pre-labeled hybridization control and printed buffer spots (blank), respectively. Some of the anti-tags were not in use in this experiment (T35, T36, T37, T48 and T52) and therefore utilized as negative control for calculating the cut-off. (B) Image of a HIV-1 SNP typing microarray after hybridization with a Tanzanian field sample. Each array consists of 14x14 spots. Cy5 spotting control (degenerated, indicated by red circles); Cy3 spotting control (green circles); Cy5-prelabeled hybridization control (blue circle); example of missing data (white circle)).

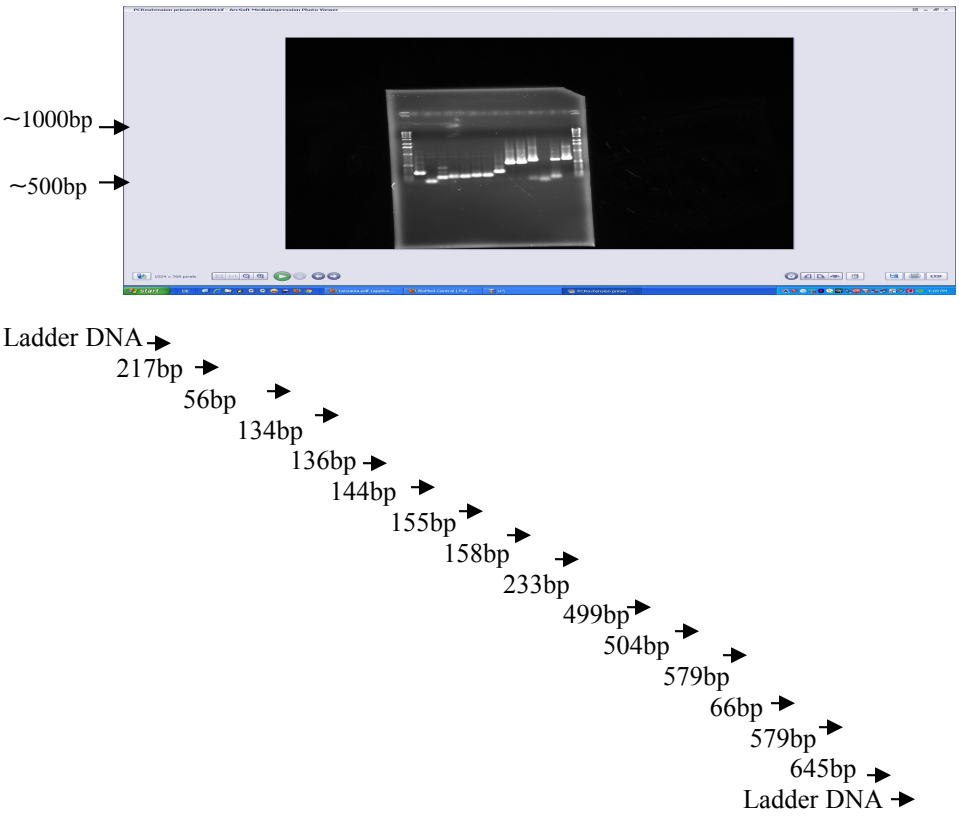


Figure 4-13: Supplementary Figure 1: Validation of extension primers by PCR on cloned RT fragments and viral cDNA from Tanzania. Expected fragment sizes range from 56- 645 bp.

Chapter 5

Discussion and Conclusion

Discussion and Conclusion

1.41 DISCUSSION

The WHO recommends monitoring of HIV-1 drug resistance for the effectiveness of ART programmes. Monitoring of HIV-1 drug resistance allows optimization of the available therapeutic options and provides epidemiological data to describe the magnitude of HIV-1 resistance in the population. These epidemiological data can also be used to develop appropriate strategies that can limit the spread of the resistant strains. In view of the above it was intended in this study to monitor drug resistance in an established HIV-1 cohort in a rural setting of Tanzania.

In the course of this PhD project three studies were conducted: (i) sequencing the RT and PR genes from a total of 387 samples from drug naïve patients so as to establish the prevalence of HIV-1 DR-SNPS (primary or transmitted drug resistance) and HIV-1 subtypes in these two time period (2005-7 and 2009) (ii) genotyping follow up samples from patients under ART, including presumptive cases of drug resistance, to establish the prevalence of DR-SNPs after initiation of ART (aquired resistance), and (iii) using an alternative, microarray-based approach to genotype samples from drug naïve patients.

The first paper as indicated in chapter 2 of the thesis, describes the prevalence of HIV-1 subtypes and drug resistance mutation in drug naïve patients. In 2005-2007 a total of 187 patient samples were used, out of which 119 (63.6%) could yield final sequences for subsequent analysis. In 2009, 200 patient samples were used and 120 samples (60%) were successfully sequenced for data analysis. A high level of PCR negativity was encountered in drug naïve patients and was

equally high at both time points. This could be caused by problems in storage of these samples in Ifakara. In KIULARCO activities, frequent interruptions of power supply were reported from the IHI laboratory (Edit Horvath personal communication). This could have led to degradation of RNA in some of the samples. In addition, plasma separations from the patient blood samples were suboptimal, because quite a number of plasma samples were red in colour suggesting haemolysis. This indicates that blood samples might have been left on the bench for too long time, despite the recommendation that the processing time should not greatly exceed 1-2 hours. It is likely that such suboptimal sample processing together with repeated cycles of freeze and thaw due to power cuts, had a negative impact on the outcomes of our PCR experiments.

Our analysis of subtypes in KIULRCO showed agreement with other reports from Tanzania. Prevalence of HIV-1 subtypes did not vary significantly between the two periods compared, with the exception of the occurrence of HIV-1 subtpe B and F in 2010. Our results are in agreement with other studies in Tanzania which have shown that subtypes C, A and D are the most prevalent subtypes in Tanzania [108] [109]. The minor differences between our HIV subtype distribution and that from other studies conducted in Tanzania could be explained by the sequenced fragment of the viral genome used by us for subtype determination. We have based our subtype analysis on the *pol* region only. This approach is prone to underestimate recombinant forms [110] [112]. But the *pol* region has been used for this purpose also by other researchers, because it has the advantage of providing information on drug resistance mutations as well as subtype information, both based on the same sequence [112] [113].

DR mutations were observed at both time intervals analysed. The prevalence of major NRT mutations was 5.9% in 2005-2007 and 1.7% in 2009. This difference in prevalence was not statistically significant (p=0.102). Similarly, prevalence of NNRT mutations was 9.2% in 2005-

2007 and 3.3% in 2009, again this difference did not reach statistical significance ($p = 0.0671$). A higher number of patients in the clinically more severe WHO stages 3 and 4 could be responsible for the higher number of DR-SNPs. This suggests that right at the start of KIULARCO activities a specific group of HIV patients might have entered the CDCI. This is supported by observed differences in mean CD4 counts between the two groups of patients compared. In contrast, the finding of similar viral load between both sets of patients disagrees with the assumption of two distinct groups of patients. An explanation for low viral loads at the earlier time point despite lower CD4 counts could be the earlier mentioned problems in long term storage of plasma.

All individuals with DR-SNPs in 2009 and 60% of individuals in 2005-2007 carried the K103N mutation. We do not know the reasons for the high prevalence of this K103N mutation but this could be due to prior use of nevirapine monotherapy for PMTCT in Tanzania [92] and in the area (Marcel Stoeckle personal communication). It has been observed in other studies that the use of nevirapine monotherapy for PMCTC could lead to the selection of this K103N mutation [96]. The observed level of primary resistance must be considered as a potential threat to the efficiency of the KIULARCO and Tanzania ART programmes.

The second paper (Thesis chapter 3) describes prevalence of DR-SNPs in 137 follow up samples from 2005-2007 patients. The average time of ART intake at the time of collecting the follow up sample was 11.6 months. These follow up patients had been on first line regimen containing starvudine + lamivudine + nevirapine (87.4%), zidovudine + lamivudine + nevirapine (6.3%) or emtricitabine + tenofovir disoproxil fumarate + effavirenz (6.3%). The rationale for this study was to investigate a potential development of acquired resistance following ART usage in the area. Such information could contribute to an assessment of the effectiveness of drug regimens supplied in KIULARCO.

The PR and RT genes were successfully PCR amplified and sequenced in 16 out of 137 (11.7%) patient samples. In those 16 sequenced samples, 2 (12.5%) sequences harboured major DR-SNPs to RTIs, while six other sequences harboured minor DR-SNPs to RTIs. All the six (2 patients with major and 6 with minor mutations) patients with RT mutations had been on ART regimen containing nevirapine-based combination for a mean period of 19 months. Studies conducted in other parts of Tanzania and East Africa had revealed variable levels of resistance in ART experienced patients. For example, one study conducted in Haydom Lutheran hospital, in Manyara, North-East rural Tanzania [96] detected HIV-1 resistance mutations at a rate of 3.9% after 1 year of ART usage. This low level of resistance in Hydom was due to adherence counseling, presence of home-based carers, regular peer-support meetings, and continuous uninterrupted drug supply. In another study conducted in Kampala urban in Uganda [115] revealed a prevalence of 72% of PCR and sequencing positive patients on ART for average period of 37.7 weeks. This variability can be explained by the fact that in Uganda study 23.4% of participants had interrupted their doses for duration of more than four days and this was shown to be strongly associated with treatment failure. Our resistance findings were higher than that

obtained in Hydom but lower than that observed in Uganda study, suggesting that the data we obtained was in the range of values obtained from these two studies.

Following observation of clinical resistance in KIULARCO patients, samples collected at the time of suspected DR were genotyped for DR-SNPs. Six out of 17 patient samples harboured major DR-SNPs to RTIs and at least one minor RTI mutation. In addition, viral sequences from 2 patient samples (11.8%) carried minor DR-SNPs to PRIs. The majority of the 17 patients (11 patients) did not harbour any DR-SNPs suggesting that the clinical failure observed in these patients was not due to acquisition of viral drug resistance. Several factors such as drug toxicity, use of low potent drugs, non-adherence and long duration of ART are known to risk factors contributing to development of resistance in HIV-1 treated patients [116]. Of these factors, only adherence was recorded in the subset of KIULARCO patients studied and in general the adherence reported was good. Adherence information was extracted from medical reports, but this cannot rule out the possibility of missed doses. Other factors associated with resistance were not evaluated in this study.

Thesis chapter four presents a microarray-based genotyping approach as an alternative to sequencing. A hallmark of this approach was to adapt all hybridization probes to locally prevalent HIV variants. Sequence alignments of the 126 viral isolates from drug naïve patients were used to design extension primers for microarray optimization. Using sequence information from KIULARCO samples was a prerequisite in the optimization process, since marked sequence variation could affect the performance of the primer extension reaction. By minisequencing at the 3’ end of the designed oligonucleotides mutations in the RT gene were detected. The direction of these extension primers was either directly the 5' or directly the 3' of the targeted SNP. The choice of either of the two possible primers was based on optimal

sequence conservation in the alignment of the 126 sequences. The length of extension primers varied to minimize variation in melting temperatures of all extension primers. This was required because all extension primers were used in a multiplex extension reaction. A total of 25 SNPs in the RT gene representing the most important mutations for drugs used in KIULARCO were targeted. To maximize sensitivity, up to six different extension primers were designed to target a single SNP. This number depended on the number of mismatches observed in sequence alignment. The aim was to provide per SNP confirmatory results from 2 extension primers placed in opposite directions of the targeted position

The microarray was validated by using 4 cloned fragments which had been generated previously in the course of a phenotyping assay established for samples from the Swiss HIV-1 cohort. Because cloned inserts represent a single template, this material was considered highly suitable to validate the specificity of hybridization on microarray and to determine individual anti-tags that give rise to hybridization background. When using the cloned material the agreement between the microarray and direct sequencing was 90%. The result varied for the different cloned fragments. With 2 plasmids containing a RT fragment of C1 and A2 subtype (0720235-C1 and 070510-A2) perfect agreement between sequence and microarray genotype was obtained, whereas only 88% concordance was reached for a plasmid with a AE2 subtype insert (6017225-AE2) and 72% for a subtype C1 insert (072073-C1). Because these plasmid inserts derived from patients in a Swiss HIV cohort, they likely represented variants with substantial genetical distance to the extension primer sequence that had been optimized for Tanzanian subtypes.

The performance of the HIV microarray was principally validated by genotyping 102 field samples from HIV-1 patients from a HIV cohort in Ifakara, Tanzania. The agreement between microarray and direct sequencing was 92.7%. Five extension primers (i.e. SNPs) had

concordance of 100%, fourteen SNPs had concordance of more than 90% and only one SNP had concordance of less than 70%. Extension primers that were designed for K210W, Y188L, K65R and K219N performed sub-optimally in this microarray with an efficiency of 62.7% 71.6%, 75.5%, and 78.4%, respectively.

Several difficulties were encountered in the micorarray development and optimization process. One of the challenges was missing data, likely due to an exceeding number of mismatches between isolate and sequence in case of an extension primer positioned at a region of imperfect sequence conservation. This problem is entirely due to the well-known extreme diversity among HIV genomes. This seems to apply even for HIV variants from a restricted population. Similarly, it was challenging to generate uniform signal intensities for all spots of the microarray. Differences in slide pretreatment conditions and duration of slide storage could have contributed to these difficulties.

In summary, the viral sequence diversity will inevitably lead to missing data in SNP calling due to inefficient hybridization during the primer extension step. Depending on the sequence of a particular isolate, it is likely that one or more data points might be missing. HIV genotyping on our microarray in its current form must be considered, due to biological reasons of sequence variation, an imperfect tool. Yet, the agreement of >90% with sequencing results suggests a possible role of this tool in molecular epidemiological studies, where missing data, if it is not systematic, causes less problems. The further use of this tool greatly depends on the genotyping techniques available and on costs of the microarray technique versus costs of sequencing. Because the latter costs have decreased substantially over the past years, it seems likely that sequencing and next generation sequencing will be widely available and affordable in future.

1.42 CONCLUSION

The molecular work in this study has provided the first baseline information on HIV-1 strains encountered in Ifakara, a rural area in Southern Tanzania. The study has shown a relatively stable subtype distribution (C, A, D and CRF01_AE) within 5 year period in the area. In 2009, however, there was introduction of new subtypes B and F at frequencies lower < 5% and these subtypes are very rare in Tanzania. The generated subtype distribution information adds and contributes to the already existing data of HIV-1 distribution in Tanzania, especially so, in rural parts of Tanzania.

There was a low prevalence of primary drug resistance mutations and these mutations create a low potential threat for the ART programme in the area. The demonstration of the extent and significance of HIV-1 drug resistance mutations in treatment naive individuals is useful for an informed choice of ART and thus can contribute to efforts towards preventing the spread of drug resistance.

The detection of very low frequencies of DR-SNPs in randomly selected patients who had been on ART suggests that the drug combinations deployed by KIULARCO are effective and that no major adherence problems prevail. In addition, one-third of patients with suspected drug resistance harboured DR-SNPs. Since majority of suspected resistance cases were not due to the known DR-SNPs, further studies are suggested to investigate and address factors that might have contributed to the treatment failures in these patients

The microarray that was developed showed a good sensitivity. Due to the technical simplicity in the development, low running cost, the easiness of running the experiment, the shorter time required to get results and the flexibility to allow incorporation of new mutations, this microarray

platform has a potential to be used as an alternative genotyping tool for monitoring resistance mutations at a population level in Ifakara cohort, Tanzania and also in other developing countries. However the main challenge encountered was missing data and problems with background signals.

The focus of this work was on primary and acquired resistance that was done in a cross sectional study. Next studies should also include longitudinal studies that can provide the dynamics of resistance mutations as well as subtype distribution in the area.

Our results highlight the need to conduct studies in KIULARCO on patient adherence to ART so as to investigate the possible cause of treatment failure observed in some of our patients.

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Appendix

Appendix 1 – Curriculum Vitae

Pax Masimba

Missionstrasse 59

4055 Basel

Switzerland

Phone office: +41 61 284 82 86

Fax: +41 61 271 86 54

Mobile: +41 783075460

Email: Pax.Masimba@unibas.ch

PERMANENT ADDRESS:

Institute of Traditional Medicine

Muhimbili University of Health and

Allied Sciences

P.O. Box 65001

Dar es Salaam

Tanzania

Tel.: +255 22 2150096

Fax: +255 22 2151596

Mobile number:+255 787 526772

Email: pjessey@muhas.ac.tz, paxjmasimba@yahoo.co.uk

PERSONAL INFORMATION

Date of birth: 30.10.1972

Place of birth: Tanzania

Nationality: Tanzanian

Marital status: married, 3 children

Educational Qualifications: Master of Veterinary Medicine

Appendix

WORK EXPERIENCE

08/2008 — **PhD thesis**, Swiss Tropical & P H Institute, University of Basel, Switzerland

Supervisor: PD Dr. Ingrid Felger

Thesis title: Molecular Monitoring of HIV-1 Drug Resistance in Ifakara HIV-1 Cohort, Tanzania

- To develop a microarray for monitoring HIV-1 drug resistance in the reverse transcriptase in Ifakara HIV-1 Cohort, Tanzania
- Genotyping by direct sequencing to establish prevalence of reverse transcriptase and protease gene drug resistance mutations in the cohort
- Establish Diversity of HIV-1 subtypes in the cohort

2005-2008: Assistant Research Fellow at the Institute of Traditional Medicine, Department of Biological and Pre-Clinical Studies, Muhimbili University College of Health and Allied Sciences, Dar es Salaam, Tanzania

2003-2005: Research Officer/Veterinary Tutor at Animal Diseases Research Institute, Dar es Salaam, Tanzania

10/ 2000 – 11/2003 — **Master thesis**, Sokoine University of Agriculure, Morogoro, Tanzania

Dissertation Title: The Role of Rodents as Reservoir Hosts for *Borrelia duttoni*, an aetiological agent of East African Tick-borne Relapsing Fever

- Detection of *Borrelia duttoni* from blood of field rats, soft ticks haemolymph and patient blood by light and bright field microscopy, culture and by Polymerase Chain reaction (PCR)

Appendix

UNDERGRADUATE STUDIES

08/ 1994 – 11/1999	**Bachelor of Veterinary Medicine**, Sokoine University of Agriculture, Morogoro, Tanzania

LANGUAGES

English	good written and oral skills
Kiswahili	Mother Tongue

PUBLICATIONS

- Esther Innocent, Mainen J. Moshi**, Pax J. Masimba,** Zakaria H. Mbwambo, Modest C. Kapingu, Appolinary Kamuhabwa**.** Screening of Traditionally used Plants For *In Vivo* Antimalarial Activity In Mice. *Afr. J. Traditional*, Complementary and Alternative Medicines. (2009) 6 (2): 163 - 167.

- Mainen, J. Moshi, **Pax J. Masimba**, Ramadhani S.O. Nondo, Zakaria H. Mbwambo, Modest C. Kapingu, Makuru Mohamed, and Fikira Kimbokota. *Anticonvulsant Activity of Extrats of Diospyros Fischeri Stem Bark.* Afr. J. Traditional, Complimentary and Alternative Medicines,(2007) 4 (1): 94-98.

- Zakaria H. Mbwambo, Mainen J. Moshi, **Pax J Masimba**, Modest C. Kapingu, Ramadhani S.O. Nondo. Antimicrobial Activity and Brineshrimp Toxicity of Extracts of *Terminalia brownii* Roots and Stem. African Journal of Traditional, Complementary and Alternative Medicines (2007) 7:9

- Mainen, J.Moshi, Zakaria, H. Mbwambo, Ramadhani, S.O. Nondo, **Pax J. Masimba,** Appolinary Kamuhabwa, Modest C.Kapingu, Pascal Thomas, Marco Richard. Evaluation of ethnomedical claims and Brine shrimp toxicity of some plants used in Tanzania as traditional medicines. African Journal of Traditional, Complementary and Alternative Medicines (2006) 3:3

- Mainen J.Moshi, Zakaria H. Mbwambo, Ramadhani S.O. Nondo, **Pax J. Masimba**, Modest C. Kapingu and Edward S. Magelewanya. Anticonvulsant Activity of Diospyros Fischeri Root Extracts. African Journal of Traditional, Complementary and Alternative Medicines, 2007 4 (2): 226-230.

- Mainen J. Moshi, Carolien J.P. van den Beukel, Omar J.M. Hamza, Zakaria H. Mbwambo, Ramadhani O.S. Nondo, **Pax J. Masimba**, Mecky I.N. Matee, Modest C.Kapingu, Frans Mikx, Paul E. Verweij, and André J.A.M. van der Ven**.** Brine Shrimp Toxicity Evaluation

of Some Tanzanian Plants Used Traditionally for the Treatment of Fungal Infections. *Afr. J. Trad. CAM* (2007) 4 (2): 219 – 225

MANUSCRIPT IN PREPARATION

Pax Masimba, Elimsaada Kituma, Thomas Klimkait, Edit Horvath, Marcel Stoeckle, Christoph Hatz, Emmanuel Mwaigomole, Salim Hamis, Boniphace Jullu, Salim Abdulla, Marcel Tanner, Ingrid Felger. Prevalence of Drug-Resistance Mutations and HIV-1 Subtypes in an HIV-1 COHORT in rural Tanzania (Submitted to AIDS Research and Human Retroviruses, November 2011)

Pax Masimba, Thomas Klimkait, Elimsaada Kituma, Edit Horvath, Boniphace Jullu, Salim Hamis, Emmanuel Mwaigomole, Daniel Nyogea, Marcel Stoeckle, Christoph Hatz, Salim Abdulah, Marcel Tanner, Ingrid Felger. HIV-1 drug resistance mutations in antiretroviral treated individuals from KIULARCO cohort in rural Tanzania

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