# OCCURRENCE OF ANTIRETROVIRAL DRUG RESISTANCE AMONG HUMAN IMMUNODEFICIENCY VIRUS PATIENTS ATTENDING ANTIRETROVIRAL CLINIC IN MUHIMBILI NATIONAL HOSPITAL, DAR-ES-SALAAM TANZANIA, 2007

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A thesis submitted in partial fulfilment for the degree of Master of Science in Laboratory Management and Epidemiology in the Jomo Kenyatta University of Agriculture and Technology

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# DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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# DEDICATION

To my loving children Aimcha, Aisa and Aikunda

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| | |
|---|---|
| 3TC | Lamivudine; 2'3 – dideoxy-3' –thiacytidine |
| ABC | Abacavir |
| AIDS | Acquired Immune Deficiency Syndrome |
| ART | Antiretroviral Therapy |
| ARV | Antiretroviral |
| AZT | Zidovudine |
| Bp | Base pair |
| CA | Capsid Protein |
| CCR5 | CC Chemokine receptor 5 |
| CD | Cluster of differentiation |
| CD4 | Lymphocyte T4 |
| CDC | Center for Disease Control and Prevention |
| cDNA | Complementary deoxyribonucleic acid |
| CRF | Circulating Recombinant Forms |
| CTL | Cytotoxic T-Lymphocyte |
| CXCR4 | CXC Chemokine receptor 4 |
| d4T | Stavudine |
| ddI | Didanosine |
| ddNTP | 2'3-dideoxynucleoside-5 triphospate |
| DNA | Deoxyribonucleic acid |
| dNTP | 2'-deoxyribonucleoside-5 triphosphate |
| ds | Double stranded |
| DTT | Dithiothreitol |

| | |
|---|---|
| EDTA | Ethyleneglycol-bis-(beta-aminoethylether)tetraacetate |
| EFV | Efavirenz |
| *env* | Envelope gene |
| FELTP | Field Epidemiology and Laboratory Training Program |
| *gag* | Group antigen gene |
| gp | Glycoprotein |
| HAART | Highly Active Antiretroviral Treatment |
| HIV | Human Immune Deficiency Virus |
| IDC | Infectious Disease Clinic |
| IDV | Indinavir |
| JKUAT | Jomo Kenyatta University of Agriculture and Technology |
| KEMRI | Kenya Medical Research Institute |
| IL | Interleukin |
| LPV | Lopinavir |
| MNH | Muhimbili National Hospital |
| MOHSW | Ministry of Health and Social Welfare |
| NFV | Nelfinavir |
| NICD | National Institute for Communicable Diseases |
| NIMR | National Institute for Medical Research |
| NNRTI | Non-nucleoside reverse transcriptase inhibitor |
| NRTI | Nucleoside reverse transcriptase inhibitor |
| NVP | Nevirapine |
| PCR | Polymerase Chain Reaction |
| PI | Protease inhibitor |
| PLHA | People living with HIV and AIDS |

| | |
|---|---|
| Pol | Polymerase gene |
| RNA | Ribonucleic acid |
| Rnase | Ribonuclease |
| RT | Reverse transcriptase |
| RTV | Ritonavir |
| SIV | Simian Immunodeficiency Virus |
| SU | Surface envelope protein |
| SQV | Saquinavir |
| TAM | Thymidin analogue mutation |
| TDM | Therapeutic drug monitoring |
| TM | Transmembrane envelope protein |
| tRNA | Transfer ribonucleic acid |
| UNAIDS | United Nations Programme on HIV/AIDS |
| USA | United States of America |
| VL | Viral load |
| WHO | World Health Organization |

# ABSTRACT

Tanzania is among the countries in Sub-Saharan Africa with high prevalence of HIV infection. The country has implemented several interventions for control and prevention, including care and treatment with ARV drugs. The ARV drug treatment programme of patients living with HIV and AIDS started in 2004. Among the major potential challenges inherent in ARV drug treatment programmes is the danger of emergence of ARV drug-resistant strains. This study aimed at determining the occurrence and risk factors of ARV drug resistance among HIV patients receiving ARV treatment at the ART clinic in Muhimbili National Hospital, Dar es Salaam.

A descriptive comparison study selecting cases (HIV patients on ARV treatment for more than six months experiencing treatment failure) and non-cases (HIV patients on ARV treatment for more than six months responding well to treatment) was conducted. Viral load testing and genotypic resistance testing was done on 150 of the patient samples (79 cases and 71 non-cases). Epi Info version 3.3.2 and Microsoft excel were used for general data analysis. Chromas Pro, Bioedit and Mega 2 were used for Phylogenetic analysis. ARV resistant mutations were analysed using Stanford ARV resistance database.

There was no statistically significant difference between cases and non-cases with respect to marital status, education level, gender and mean viral load and development of ARV resistance mutations.

The difference in mean percentage weight gain and the mean percentage CD4 increase between cases and non-cases was statistically significant at a P-value of 0.0001. The clinical

stage of the patients at the initiation of therapy was found to be a significant factor for development of drug failure at a P-value of 0.001.

Only 28 samples were fully sequenced and mostly those with the viral load of more than 400 copies/ml. Subtypes A and C were the most prevalent subtypes followed by subtype D and their recombinants. Overall the occurrence of PI major mutations was 4%, PI minor mutations was 29%, NRTI mutations 64% and NNRTI mutations 71%. The mutations detected were related to the ARV therapy in use, more observed for Lamivudine, Stavudine and Nevirapine. There was no significant association between subtypes and development of resistant mutations. The maintenance of resistance genotypic programs supplied for ARV drug naïve patients and ARV failing patients is important in the management of HIV patients.

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction and literature review

### 1.1.1 Isolation and discovery of Human Immunodeficiency Virus

The Acquired Immune Deficiency Syndrome (AIDS) was first recognized as a clinical entity in 1981 and HIV-1 identified as its causative agent two years later (Gottlieb, 1981, Barre-Sinoussi *et al*, 1983). In 1986, a second HIV type was isolated from patients with AIDS in West Africa and identified as HIV-2 (Clavel *et al*, 1986). Further virological analysis resulted in classification of the HIV viruses in the Lentivirus genus of the family Retroviridae (Barre-Sinoussi *et al*, 1983). The initial epicentre of HIV-1 and HIV-2 infection appears to have been Central Africa and West Africa respectively (Hahn *et al*, 2000).

An estimated 33.2 million people are now infected with HIV, and projections suggest that an additional 45 million people in developing countries will become infected between 2002 and 2010 (UNAIDS, 2007). More than 90% of people living with HIV/AIDS are in the developing world, most of them in Africa, Asia and Latin America. Tanzania is among the countries in sub-Saharan Africa with high prevalence of HIV infection. Data from a population based survey conducted in 2003/2004 revealed a national prevalence of 7%, being slightly higher among females (7.7%) than males, which is 6.3% (National AIDS Control Program, 2005).

The use of antiretroviral drugs to prolong lives of people living with HIV and AIDS (PLHA) and in the prevention of mother-to-child transmission of HIV is now firmly established in Tanzania.

The World Health Organisation (WHO) staging of HIV patients that is used to decide on the level of infections is as follows: Stage I: HIV disease is asymptomatic and not categorized as AIDS, stage II: Includes minor mucocutaneous manifestations and recurrent upper respiratory tract infections, stage III: Includes unexplained chronic diarrhea for longer than a month, severe bacterial infections and pulmonary tuberculosis and stage IV: Includes toxoplasmosis of the brain, candidiasis of the esophagus, trachea, bronchi or lungs and Kaposi's sarcoma; these diseases are used as indicators of AIDS (WHO, 2006).

### 1.1.2 HIV transmission

HIV is present in the blood and genital secretions of virtually all infected individuals regardless of whether or not they have symptoms of AIDS. The spread of HIV can occur when these secretions come in contact with mucocutaneous tissues such as those lining the vagina, anal area, mouth or eyes or when the skin is ruptured by a cut or puncture from a needle or another skin piercing instruments. The most common ways of HIV spread throughout the world include sexual contact, mother-to-child transmission during pregnancy, labor or breastfeeding and sharing of contaminated needles among drug abusers. Transmission through transfusion of contaminated blood and blood products occurs to a lesser extent due to availability of services for screening of donor blood before transfusion (Piot *et al*, 2002).

### 1.1.3 Virology of HIV – 1

#### 1.1.3.1 Virion structure

The HIV-1 virion is enveloped with spherical shape of about 110nm in diameter. It is enveloped by a lipid bilayer that is derived from the membrane of the host cell. The core of HIV particle is made up of the capsid protein (CA), p24 (Figure 1). It contains two copies of the viral RNA and viral proteins such as protease, integrase, and reverse transcriptase. It also contains a wide variety of other macromolecules derived from the cell including $tRNA^{lys}$, which serves as a primer for reverse transcription. The capsid has an icosahedral structure and is encapsidated by a layer of matrix protein (MA), p17. The matrix protein is a continuous shell attached to the envelope of the virus. The envelope contains the lipid and protein constituents of the cell membrane from which it is derived. In addition, it also contains viral proteins forming spikes. These spikes consist of gp120 and gp 41. Gp41 traverses the envelope; gp120 is present on the outer surface and is non-covalently attached to gp41 (Kuiken *et al*, 2007).

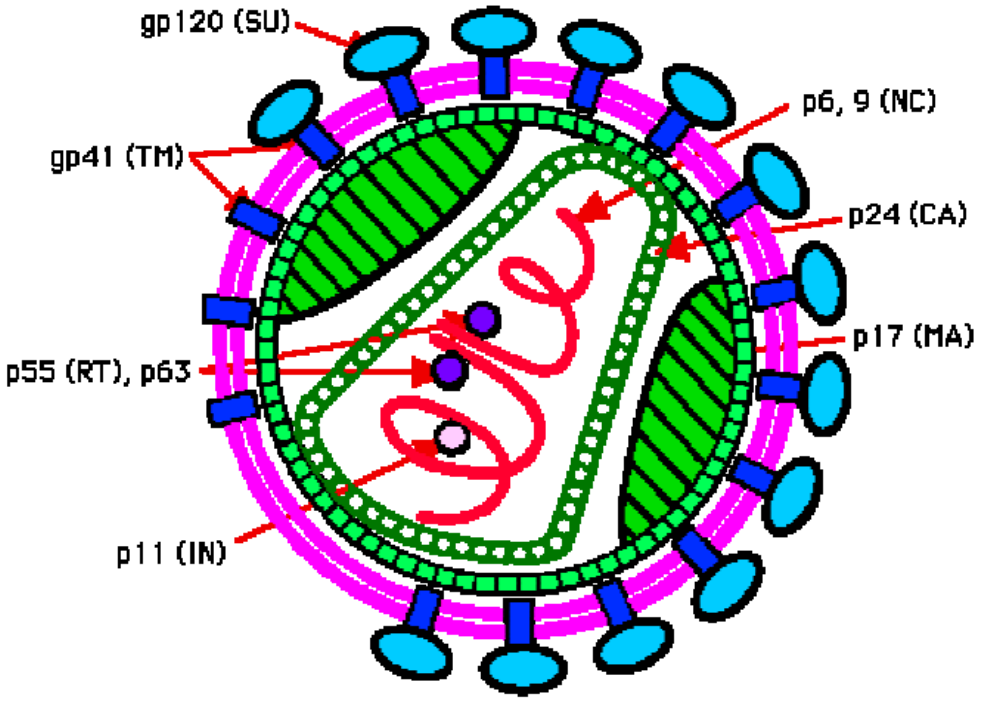


**Figure 1: HIV-1 structure**

### 1.1.3.2 Genome structure

HIV-1 has two genomic structures: single-stranded RNA in virions and double-stranded DNA within the host cell. The virus contains two copies of the same positive sense single stranded RNA about 9,500 nucleotides long. The HIV-1 genome contains nine open reading frames. The *gag* gene encodes a precursor polyprotein $Pr55^{gag}$ which is subsequently cleaved to yield several structural proteins (MA, CA, $P^2$, NC, $P^1$ and $P^6$). The three viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) are encoded by the *pol* gene. The *env* gene encodes for the gp 160 glycoprotein, the precursor of the envelope glycoproteins gp 120 and gp41 (Figure 2). The other six genes encode for the regulatory proteins, Tat and Rev, and the accessory proteins, Vif, Vpr, Vpu and Nef (Turner *et al*, 1999).

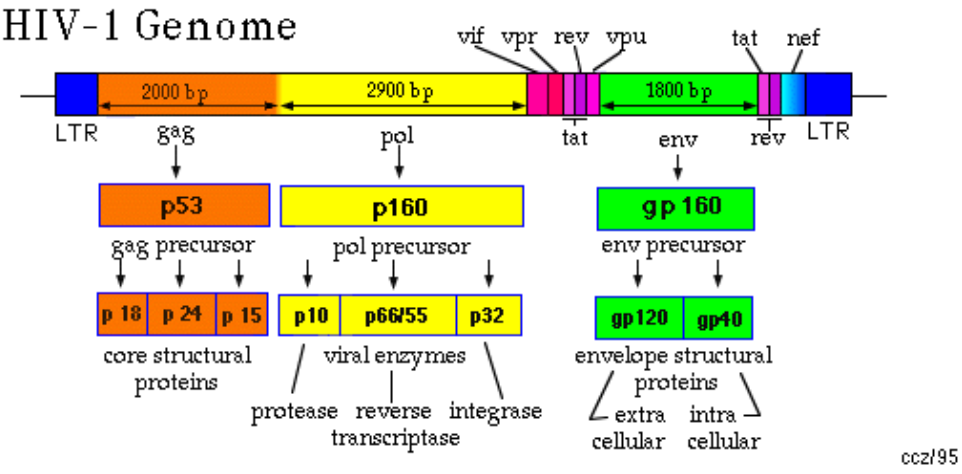


**Figure 2: HIV-1 genome organization**

### 1.1.3.3 Replication cycle

The HIV-1 replication is initiated through specific interactions between the viral glycoprotein gp120 and the amino-terminal immunoglobulin domain of CD4 on the cell surface. HIV-1 infects cells of the immune system, including T lymphocytes, monocytes and macrophages, all of which express the CD4 receptor on the cell surface. However, HIV-1 requires additional cell-surface proteins to promote fusion of the viral and cellular membranes. CCR5 and CXCR4 are co-receptors for HIV-1 entry in vivo. Other additional chemokine receptors have been shown to serve as co-receptors for HIV-1 entry but their relevance *in vivo* remains to be confirmed (Perrin *et al*, 2003).

The HIV-1 surface gp120 attaches to the surface receptors (CD4) of the host cell, and the resulting conformational change in gp120 allows binding of HIV-1 to a second cell surface receptor, CXCR4 or CCR5. The transmembrane gp41 can then interact with a fusion receptor on the cell surface, followed by fusion of virus and cell membrane. Viral RNA enters into the cytoplasm, and reverse transcription by the reverse transcriptase protein is initiated. This event produces a viral cDNA, which is duplicated to form dsDNA. This is then transported to

the cell nucleus and randomly integrated in the chromosomal DNA, through the action of the viral protein integrase. The late phase starts with viral transcription. With the aid of host RNA polymerase II, integrated proviral DNA yields mRNA for synthesis of viral proteins and for incorporation into new virions as viral genomic RNA. The viral regulatory proteins, Rev and Tat regulate these complex steps in viral replication. Env precursor protein (gp160) is synthesized from single and unspliced mRNA is released in the endoplasmic reticulum where it is processed by cellular proteases. Gag and Gag-Pol are synthesized in the cytosol from unspliced viral mRNA, myristoilated and transported to the cell membrane. These polyproteins then assemble with the processed Env proteins and the genomic RNA into budding particles. After budding, the viral particles mature when the viral protease autocleaves the Gag-Pol polyproteins into enzymes and structural proteins which rearrange to form the infectious particles (Tripathi *et al*, 2007).

### 1.1.4 HIV diversity

Human Immunodeficiency Virus (HIV) is the major pathogen responsible for the HIV and AIDS pandemic. HIV is classified in two major groups i.e. HIV-1 and HIV-2.

Globally circulating strains of HIV-1 exhibit an extraordinary degree of genetic diversity, which may influence aspects of their biology such as infectivity, transmissibility, and immunogenicity. Phylogenetic analysis of numerous strains of HIV-1, isolated from diverse geographic origins, have revealed three distinct clades of viruses within HIV-1, which have been termed groups M (Main), N (new or non M, non O) and O (Outlier). The vast majority of strains found worldwide and responsible for the pandemic, belongs to just one of these lineages, group M (Perrin *et al*, 2003). Further analyses of different strains of HIV-1 from diverse geographical area show that isolates can be subdivided into groups, subtypes, sub-

subtypes and Circulating Recombinant Forms (CRFs), based on phylogenetic differences. The subtypes are approximately equidistantly related, exhibiting over 25% amino acid sequence difference in the most variable regions, with up to 20% amino acid difference within a particular subtype and up to 10% amino acid difference within an infected individual (Mosha *et al*, 2003).

The predominant viral forms are those of subtypes A and C, followed by subtype B, which is the virus mainly found in the US and Europe, and the recombinants CRF01_AE and CRF02_AG. The greatest genetic diversity of HIV–1 has been found in Central Africa. This is thought to be due to the presence of numerous co circulating subtypes, with a high frequency and a wide variety of recombinants. Also, in Central Africa, there existed other types of simian lentiviruses for many years even before the introduction of HIV-1 (Peeters, 2000).

The global dispersion of the different HIV-1 subtypes appears to be related to social economic changes, immigration, and international travelling rather than to differences in transmissibility. However, it is reasonable to postulate that the coexistence of different subtypes in a population is also determined by the biological characteristics of subtypes, mainly transmissibility and virulence (Janssens, 1997). Subtype B gained its subtype status because it is the common form of HIV-1 in North America and Europe, and was very heavily represented among the first strains characterized. Diagnostic tests, antiretroviral drugs, and candidate HIV-1 vaccines have so far mainly been developed only for subtype B viruses. However, various other M subtypes, and even group O viruses, have now been reported in the US and in several European countries. This can result in super infection of a single person with two subtypes and consequently in recombination that can affect the biological properties and pathogenesis of HIV strains. The geographic distribution of subtypes is subject to

constant change. Recombinant forms of the virus will continue to appear as long as the different subtypes of HIV-1 continue to circulate between continents (Coffin, 1995).

This extensive genetic diversity of the HIV-1 has direct implications on the development of diagnostics and vaccines as well as on disease prevention and treatment strategies (Perrin, 2003). Efficient targeting of the extensive genetic diversity of HIV-1 constitutes one of the major challenges in present efforts against the pandemic, although the significance of HIV-1 genetic forms for vaccine development and therapy remains to be further investigated. Previous reports suggested that drug efficacy may vary for different subtypes of HIV-1. For example, possible reduced drug susceptibility for subtype G isolates to protease inhibitors was reported to be due to naturally occurring substitutions at three or more positions associated with resistance to protease inhibitors (Holguin *et al*, 2002).

HIV-2 is primarily found in West Africa and West India. HIV-2 isolates have been classified into 5 subtypes (A-E). HIV-2 exhibits a lower sexual and vertical transmission, of around 5-9 fold and 10 –20 fold reduced relative to HIV-1, respectively and a longer incubation period before development of AIDS (Reeves *et al*, 2002).

The extensive genetic variability is the result of four factors. First, it is postulated that there have been multiple introductions of genetically diverse simian viruses into humans, giving rise to different viral groups within HIV-1 and HIV-2 (Reeves *et al*, 2002). Second, primate lentiviruses rapidly accumulated genetic diversity because of the high error rate of reverse transcriptase in the absence of proof reading mechanisms (Coffin, 1995). Third, the virus turnover in HIV infected individuals is very high. In an untreated individual, up to $10^8$ to $10^{10}$ viral particles can be produced per day, which then infect new cells and so each nucleotide

can be mutated per day. Fourth, reverse transcription is known to be highly recombinogenic. This will result into radically different genomic combinations. Retroviral recombination occurs during the reverse transcription step, before integration and is dependent on co-packaging of two different viral genomes (Abecasis *et al*, 2007, Hu *et al*, 1990).

Viral diversity is greatest in sub-Saharan Africa, perhaps reflecting its longer residence in the human population, in which over 30 million of the total estimated worldwide population of HIV-infected individuals lives (Piot *et al*, 2002).

### 1.1.5 HIV-1 inhibitors

Currently, there are four main classes of drugs for the treatment of HIV infected persons including: Nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors and fusion inhibitor. Recently newer ARVs have been licensed for use, including CCR5 inhibitor and integrase inhibitor. All together there are 18 generics and 5 combination products Zidovudine (ZDV) + Lamivudine (3TC), ZDV + abacavir (ABC), ABC + 3TC, Tenofovir (TDF) + emtricitabine (FTC) and Lopinavir (LPV) + ritonavir (RTV) Figure 3. Combination therapy with these drugs may suppress the replication of HIV-1 in infected persons to such an extent that virus become undetectable in plasma, but it does not cure (Richman, 2001). The availability of different drugs and their use in combination therapy have had profound impacts on clinical progression and mortality rates of HIV infected individuals.

The recommendations for initiation of therapy today is to be based on the appearance of symptoms, CD4 + T-cell count, the progression of loss of CD4+ T-cells, viral load (VL), age, social situation, including possible drug abuse. Usually initiation of therapy is considered when the CD4+ T-cell count of the patient is between 200-350X$10^6$/ml (European guidlines,

2003). Out of the available ARV drugs NRTI and NNRTI inhibit the HIV RT enzyme. PI's act upon the HIV enzyme protease and fusion inhibitors blocks the virus entry into the cell. The recently introduced ARV drugs CCR5 and integrase inhibitors interfere with HIV replication by blocking viral binding to CCR5 co-receptor and inhibiting proviral DNA integration with host cell chromosome, respectively.

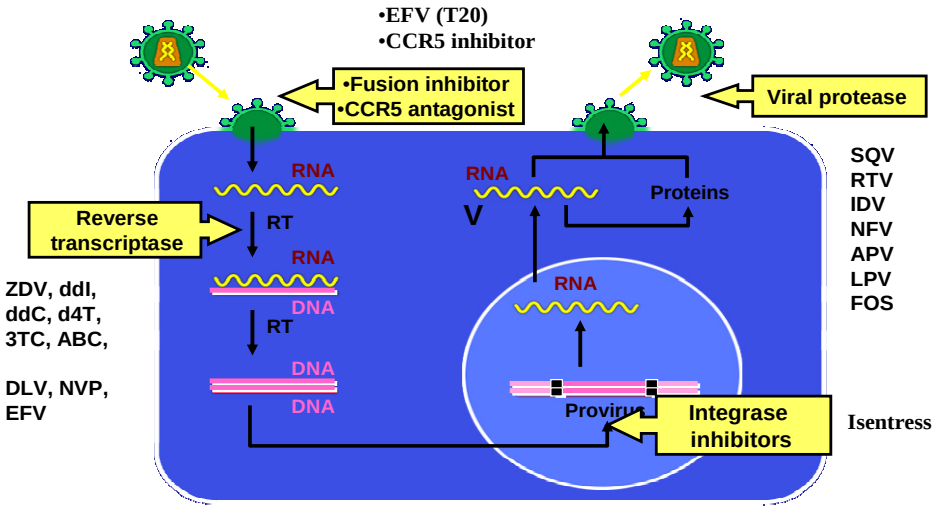


**Figure 3: Current targets of antiretroviral drugs**

Provision of ARV treatment in Tanzania dates back to the mid to late 90's when a few individuals received such medications through the private sector. During this time, ARV treatment was out of reach for the majority of patients because of high cost and most of the programmes in Africa focused mainly on palliative care and treatment of opportunistic infections. Tanzania adopted care and treatment for People Living with HIV and AIDS (PLHA) as one of its key strategies in the health sector response to HIV and AIDS pandemic and developed a care and treatment plan, which was approved by the Government in October 2003. The Plan targets to cover approximately 400,000 HIV infected patients on Anti-Retroviral Therapy (ART) by the end of 2008 (National AIDS Control Program, 2005). In

Tanzania, HIV infected patients with treatment failures attributed to drug resistance have classically been treated empirically by altering the entire drug regimen.

### 1.1.6 ARV resistance

HIV-1 resistance to antiretroviral drugs can be defined as any change that improves viral replication in the presence of an inhibitor. Viral rebound is often associated with the emergence of resistance, which may lead to subsequent immunological decline. Factors associated with an increased rate of viral rebound include pre-HAART use of nucleoside analogue reverse transcriptse inhibitors (NRTI) as single or dual therapy, use of particular antiretroviral drugs and poor adherence to medication. Lower rates of viral rebound have also been reported with increased duration of virological suppression. Today, a significant proportion (20-30%) of HIV-infected patients experience treatment failure despite the effectiveness of multiple drug combination therapy (Boffito *et al*, 2007). Reasons for treatment failure are multiple and include suboptimal adherence to the treatment, suboptimal drug exposure and development of mutants with amino acid substitutions that confer both resistance to the drugs in the current regimen and variable levels of cross resistance to other members of the same drug class (Wainberg *et al*, 1999). However, very few data are available as to how subtype diversity may affect drug susceptibility and resistance.

The evolution of resistance against HIV-1 inhibitors within a patient depends on the generation of genetic variation and on the selection of drug resistance variants during antiretroviral therapy. Substitutions are the most commonly observed genetic change, but insertions and deletions are also often observed for HIV-1. Mutations generated will be lethal to the virus, and many others will have little or no effect on viral function or fitness. How ever some mutations could confer resistance to one or more antiretroviral inhibitors. These

mutations result in alterations to the structure and function of important viral proteins for viral replication, such as Reverse Transcriptase and Protease. Therefore, these variants often have a reduced fitness compared to the wild-type virus and generally exist only as minor variants in the viral population. In the presence of antiretroviral drugs, minor variants with some level of resistance will gradually out compete the wild-type variants. Under the continuous selective pressure of inhibitors and with the presence of residual replication due to the insufficient potency of the treatment, additional mutations will accumulate (Malta *et al*, 2005). They can either increase the level of resistance or they can compensate for the reduced fitness induced by the initial mutations. Thus, resistance development will be slower when an increasing number of mutations are required for resistance to the inhibitors, and when residual virus replication is lower due to more potent antiretroviral therapy (Vandamme *et al*, 1998). Therefore, patient adherence is an important issue affecting the emergence of resistant viruses. These drug resistant mutations can then be transferred to others (Little, 2000). Some drug resistance mutations are present prior to the start of therapy as evidence by the presence of antiretroviral resistance viruses that have been found in drug naïve patients sometimes in early stages of their infections (Yearly *et al*, 1999).

A study in the United States of America (USA) found that over three quarters of patients on HAART with detectable viral load had resistance to at least one class of ARV drugs, and half had resistance to two or three classes (Frenkel *et al*, 1995). In USA, the estimated prevalence of any ARV drug resistant virus in adults under care during the first three years of antiretroviral therapy was found to be 78%. Possible explanations given for the high prevalence of resistance included suboptimal treatment regimens, interruption in supply of antiretroviral drugs, and poor adherence leading to inadequate suppression and selection of

resistance (Little *et al*, 2002). The prevalence of the resistance may vary by drug class; 70% for NRTIs, 31% for NNRTIs, and 42% PIs (Richman, 2001).

### 1.1.7 ARV resistance mutations

During mono-therapy the level of resistance correlates with the number of mutations present depending on the drug, but in combination therapy the picture is more complicated. The thymidine analogue mutations (TAM): 41L, 210W, 70R, 215Y, and 219Q/E/N are selected predominantly by ZDV and d4T but can cause cross-resistance to the other NRTIs if three or more of them are expressed, including the 41L or 210W mutations. The 41L and 215YF are defined as primary RT mutations, and 67N, 70R, 219Q, 210W are secondary mutations. TAM associated mutations: 69D/N, 69_ins, 75T/M/A, 118IQ, 44A/D, and the latter two may also be found as polymorphisms in wild type virus (Havir *et al*, 2000).

Discriminatory mutations 65R, 74VI, 115F, 184V/I, prevent the addition of a NRTI to growing viral DNA chain. The M184V mutation, which is one of the most studied mutations, has a positive effect on HIV-1 RT fidelity, reducing spontaneous HIV mutagenesis. The 74V has been noted to be antagonistic to the 215Y mutation in vitro and decrease the viral fitness, the later effect being additive to 184V when the two mutations are together (Diallo *et al*, 2003). Q151M pathway mutations: 62V, 75I, 77L, 116Y, 151M always accompany the 151M and cause multi-resistance to all NRTI.

The PIs have multiple, overlapping patterns of resistance. High level resistance to most PIs requires the accumulation of multiple mutations. PI-resistant virus exhibits four categories of mutations: The primary, major mutations: 30N, 48V/M, 50V, 50L, 82A/T/F/S, 84V/A/C and 90M. All except the last are situated in the substrate cleft. They cause resistance by

themselves (Havlir *et al*, 2000). The flap mutations are named after their place in the 3-dimential protease, and are second in importance, 46I/L, 47A/V, 53L, 54VTAS, 54ML. 46IL causes resistance to Nelfinavir and together with 84V it contributes to one of the LPV pathways for developing decreased susceptibility. The remaining flap mutations are more often accessory. Polymorphic secondary mutations 10I/V/F/R, 20M/R/I/T, M36I/V, 63P, 71V/T/I, 77I, all except 10I/V/F/R are polymorphic. They contribute to resistance in combination with other mutations. Other non polymorphic mutations include 23I, 24I, 32I, 33F, 73C/S/T/A, 76V, 88S, 88D. They indicate past PI exposure and may cause resistance themselves but are more often accessory (Johnson *et al*, 2005).

The main NNRTI mutations are K103N and Y181C/I. They both confer high-level resistance and there is a substantial cross-resistance between NVP and EFV (Torti *et al*, 2005).

### 1.1.8 Drug resistance assays and their clinical use

Drug resistance can be determined at the genotypic level by the detection of nucleotide and amino acid changes in the gene and protein. Alternatively, it can be determined at the phenotypic level by measuring the ability of an HIV-1 isolate to grow in the presence of an inhibitor or by measuring the HIV-1 RT or PR enzyme activity in the presence of an inhibitor. Viral resistance testing is of importance to clinicians in determining therapy failure and in choice of the appropriate drug combination. Viral resistance testing could also be of benefit for treatment-naïve patients if suspicion of transmission of a resistant virus is high or in settings where the transmission rate of resistant virus is high (Hirsch *et al*, 2000).

#### 1.1.8.1 Genotypic resistance assays

Specific oligonuceotides can be synthesized to allow selective priming and DNA synthesis or to allow selective hybridization after mutations at specific codons conferring resistance have been identified. Selective PCR uses oligonucleotide primers developed to specifically anneal at their 3' ends to either the wild type or mutant codon of interest (Larder *et al*, 1991). Two separate PCRs are performed, one to amplify the wild-type sequence and one to amplify the mutant sequence. After optimisation, selective PCR can give information on the relative proportion of wild type and mutant at the codon being analysed (Van Laethem *et al*, 1999).

Dideoxy chain termination sequencing provides information on all nucleotides of the sequenced region. The target sequence is amplified using PCR and the sequence is determined based on the incorporation of dideoxynucleotides. The various fragments differing in length are separated on a denaturing gel. Most sequencing technologies are semi-automated and make use of fluorescently labelled primers or dideoxynucleotides.

#### 1.1.8.2 Phenotypic resistance assays

The ARV activity in plasma or PBMCs is detected by measuring the ability to produce a DNA copy of a known heteropolymeric RNA template by extending a complementary DNA oligonucleotide primer. The virus is then tested for its ability to grow in the presence of inhibitors by using serial dilutions of the inhibitors and measuring the production of p24 antigen by means of an ELISA test (Japour *et al*, 1995).

#### 1.1.8.3 Genotypic versus phenotypic resistance assays

Both genotypic and phenotypic resistance assays have advantages and disadvantages. They provide complementary information on the resistance of a HIV-1 isolate. Genotypic resistance assays can deliver results within a few days whereas more time is required to obtain phenotypic resistance results. This turn-around time can be an important factor in some

clinical situations where results are needed as soon as possible, for example in guiding decision-making for post exposure prophylaxis and management of primary HIV-1 infection (Lyamuya *et al*, 2000). Both resistance assays have to be performed in highly specialised laboratory facilities. This is especially true for phenotypic resistance assays as they require biosafety level 3 facilities, whereas genotypic resistance assays can be performed in a dedicated molecular biology laboratory (Barlow *et al*, 1995).

The information provided by genotypic resistance assays may be difficult to interpret. Phenotypic resistance assays give a direct measurement of susceptibility towards the inhibitors tested which includes the effect of all mutations and their interactions. The clinical relevance of phenotypic results can, however, also be difficult to interpret (Van Laethem *et al*, 1999).

An important limitation of current available assays is their need of plasma samples with a HIV-1 viral load above 1000 copies/ml. This is required to achieve reliable results. HIV-1 viral load between 50 and 500 copies/ml is associated with a higher risk of resistance than levels below 50 copies/ ml (Raboud *et al*, 1998).

#### 1.1.8.4 Clinical implementation of drug resistance testing

Drug failure, adverse effects or regimen inconvenience that may compromise adherence are considered major reasons for changing therapy. Drug failure has been defined as virological failure (unsatisfactory decreased in viral load), immunological failure (unsatisfactory increase in $CD_4$ cell count) or poor clinical progression. As emergence of viral resistance correlates with rising viral loads, information on viral resistance might predict virological and ultimately clinical response as declines in viral load during antiretroviral therapy are associated with a decreased risk of disease progression (Yerly *et al*, 1995). However, a good virological

response is not always seen when viral resistance is not observed. This may be due to the limitations of the current resistance assays or due to other important factors like adherence and therapy potency (Katzenstein *et al*, 1999).

When drug resistance testing is routinely implemented in the clinical management of HIV-1 infected patients, interpretation of resistance results is a very important factor to be considered. The interpretation of these resistance data can result in advice against a particular drug when an isolate is considered resistant or can result in no restraint against the use of a drug when an isolate is considered sensitive for this drug.

### 1.1.9 Adherence to ARV drugs

Patient's ability and willingness to keep to the prescription seems to be the most important factor for a successful treatment. Adherence is related to several factors, including those related to the patient; such as cognitive, psycho-social factors, fear of side-effects, and the medical regimen; treatment related side-effects, pill burden, food restrictions, as well as the amount of support that is available from relatives and medical advisers, treatment costs, availability of ARV and ability to visit health facility. Self-reported non-adherence from patients on treatment is associated with higher risk of virological rebound (Antinori, 2004).

The level of adherence associated with the highest risk of resistance is not yet known. How ever, it has been suggested that only marginally suboptimal adherence can lead to resistance (Walsh *et al*, 2002).

### 1.2 Rationale and aims of the study

Much of the knowledge about the development of drug resistance to HIV-1 is based on the study of subtype B isolates. Therefore, there is an urgent need to evaluate susceptibility patterns among non-subtype B viruses, which are more prevalent in the world. With growing demand for ARV drugs in resource-limited settings like Tanzania, the susceptibility and resistance patterns of non-subtype B viruses to antiretroviral drugs is an important question.

A better understanding of ARV resistance could be useful in guiding the clinician's decision on which combination therapy to use. Incorporating resistance testing into patient management approach would provide physicians and patients with data to enhance effective use of approved or investigational drugs and may help to avoid the inconvenience, cost and toxicity of drugs in a regimen with little likelihood of conferring benefits. This study assessed the occurrences and risk factors of ARV drug resistance in HIV patients treated with ARV in Muhimbili National Hospital (MNH), Dar es Salaam.

## 1.3 Research question

Is there antiretroviral drug resistance among HIV patients receiving ARV treatment at the ART clinic in Muhimbili National Hospital, Dar-es-Salaam, Tanzania, 2007?

## 1.4 Study hypothesis

**Null hypothesis**

There is no ARV resistance among HIV patients receiving ARV treatment at ART clinic in Muhimbili National Hospital, Dar es Salaam, Tanzania, 2007.

## 1.5 Objectives of the study

### 1.5.1 General objective

To determine the occurrence and risk factors of antiretroviral drug resistance among HIV patients receiving ARV treatment at the ART clinic in Muhimbili National Hospital, Dar es Salaam Tanzania 2007.

### 1.5.2 Specific objectives

1.5.2.1 To determine the occurrence and risk factors of antiretroviral drug resistance mutations among responsive and non-responsive HIV patients on ARV, attending ART clinic in Muhimbili National Hospital.

1.5.2.2 To identify the class of antiretroviral drug that is more likely to induce resistance in Tanzania

1.5.2.3 To determine HIV virus subtype association with ARV resistance.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Study design

A descriptive comparison study of cases and non-cases involving HIV infected patients on ARV treatment attending ART clinic in Muhimbili National Hospital.

### 2.1.1 Case

A case was defined as any HIV-1 infected patient attending ART clinic in MNH who had been on ARV treatment for more than six months and had not been clinically and/ or immunologically responding to treatment from January to September 2007.

### 2.1.2 Non-case

A non-case was any HIV-1 infected patient attending ART clinic in MNH who had been on ARV treatment for more than six months and had been clinically and/ or immunologically responding well to treatment from January to September 2007.

### 2.1.3 Definition of treatment failure

**2.1.3.1 Clinical failure:** Clinical disease progression with the development of opportunistic infections or malignancy when the drugs have been given sufficient time to induce a protective degree of immune restoration.

**2.1.3.2 Immunological failure:** Fall of over 30% in CD4 counts from the peak value or a return to or below the pre-therapy baseline (Dybul *et al*, 2002).

## 2.2 Study area

In Tanzania, free ARV treatment is offered to individuals with CD4 counts below 200 cells/ ul, and the first-line line therapy is mainly Triomune (a combination of Stavudine, Lamivudine and Nevirapine). In cases of therapeutic failure, second line regimens are being provided.

MNH is a National Hospital located at Dar es Salaam, eastern zone of Tanzania (Figure 4). The ART Clinic in the hospital is run daily and since its inception in 2003, it has enrolled an estimated number of 6000 people living with HIV and AIDS. MNH was selected for this study because it is the only national hospital in Tanzania and was the first to establish a clinic for the care of PLHA in 2000 and the first to enrol patients on ARV in the country in 2004.


**Figure 4: A map of Tanzania**

### 2.3 Study population

The study population included people living with HIV and AIDS, males and females of all age groups who were on ARV therapy and attending ART clinic at MNH.

#### 2.3.1 Inclusion criteria

2.3.1.1 People with HIV/AIDS who have been on treatment for more than six months and are either responding well to treatment (non-cases) or experiencing drug failure (cases).

2.3.1.2 All patients who gave informed consent to participate in the study before enrolment.

### 2.3.2 Exclusion criteria

All patients who did not meet the inclusion criteria.

## 2.4 Sampling procedures

A total of five health personnel involved in patient recruitment were trained for three days prior to the beginning of the study. This involved two clinicians and three nurses. A systematic random sampling method was used in which every third patient was selected and assessed whether they met criteria for being a case or non-case. The clinician assigned each randomly selected patient disease stage using the World Health Organisation (WHO) classification and the patients grouped in Stages I, II, III and IV accordingly. All patients who met selection criteria were requested to voluntarily sign the informed consent before enrolment into the study.

## 2.5 Sample size

The Epi Info stat cal (comparison of ill and not ill) was used to calculate the sample size. The prevalence of ARV resistance used in this calculation was 57.40 % (Adje *et al*, 2001). The sample Size obtained was 144, (72 cases and 72 non-cases).

## 2.6 Data collection

Tools for data collection included standardised questionnaires, patient records and patient's blood sample.

### 2.6.1 Epidemiological data collection

Patients were provided with questionnaires to document their social demographic characteristics and drug/ clinical history. More patients' information was also obtained through desk review of the clinical and laboratory records from the patient's files.

### 2.6.2 Laboratory data collection

At the clinic, a blood sample for viral load testing and viral DNA sequencing was obtained by the clinician attending the patients.

## 2.7 Laboratory methods

### 2.7.1 Blood collection

At the clinic, 10 mls of blood from the patients was collected into EDTA tubes. The specimen was given a new study number and all the patient's identifiers were removed. After the clinic, samples were taken to the MNH laboratory.

### 2.7.2 Separation of plasma

The whole blood collected in the EDTA tubes was centrifuged for 10 minutes at -15$^{o}$C to separate plasma from cells. The plasma was then aliquoted into 3 cryovial tubes of 2 ml each and stored immediately at -80$^{o}$C. Under cold chain the plasma samples were transported to Kenya Medical Research Institute (KEMRI) in Nairobi Kenya and National Institute for Communicable Diseases (NICD) in Johannesburg South Africa for viral load testing, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and sequencing. Another set of samples was stored at the MNH Laboratory.

### 2.7.3 RNA extraction

The viral RNA extraction was performed using QIA amp viral RNA kit (Qiagen, Hilden, Germany) and 96% ethanol, according to the manufacturer's instructions. 140 µl of the plasma sample was first lysed under highly denaturing conditions with 560 µl of buffer AVL to inactivate RNase and to ensure isolation of intact viral RNA. This was incubated at 20$^{o}$C for 10 minutes and then 560 µl of 96% ethanol was added. Buffering conditions were adjusted to provide optimum binding of the RNA to the QIA amp membrane, and the sample was loaded onto the QIA amp spin column. The viral RNA was adsorbed onto QIA amp silica gel membrane, and the contaminants were efficiently washed in two steps with 500 µl of buffer AW1 and 500 µl of buffer AW2 respectively. The RNA was then eluted with 60 µl of buffer AVE in special RNase free tubes and then stored at –80°C for use subsequently during cDNA synthesis.

### 2.7.4 cDNA synthesis

In the presence of primer IN3 and Super Script II RT system (Invitrogen™ life technologies), RNA was copied by reverse transcriptase to give single stranded cDNA. 7 µl of master mix 1 (Table 1) was added to each of the 0.2 ml PCR reaction tubes together with 5 µl of RNA and incubated at 65°C for 5 minutes.

**Table 1: cDNA synthesis master mix 1**

| **Component** | **1X** |
|---|---|
| Primer IN3 (50pmol/ ul) | 0.5 µl |
| dNTP mix (10mM) | 1.0 µl |
| Rnase Free water | 5.5 µl |
| **Total** | **7.0 µl** |

After the incubation, 7 µl of master mix 2 (Table 2) was added to the PCR reaction tube giving a total volume of 19 µl and incubated at 42°C for 2 minutes.

**Table 2: cDNA synthesis master mix 2**

| **Component** | **1X** |
|---|---|
| 5 X First strand Buffer | 4.0 µl |
| 0.1 M DTT | 2.0 µl |
| RNase Out (40 U/ µl) | 1.0 µl |
| **Total** | **7.0 µl** |

After the incubation, 1 µl of Super Script II RT (15U/µl) enzyme was added to the PCR reaction tube giving a total volume of 20 µl. This was then incubated at 42°C for 2 hours and then the RT enzyme was inactivated by heating at 70°C for 15 minutes. This was then followed by addition of 1 µl of RNase H (2U/µl) and incubated at 37°C for 20

minutes. The cDNA produced was used directly for nested PCR or stored at –20°C until use.

### 2.7.5 Polymerase Chain Reaction (PCR)

A nested PCR was performed involving an outer and inner cycles with Long Template PCR system (Roche Biosystem, Germany) and 20mM outer primers (G25REV and IN3) and 20mM inner primers (AV150 and PolM4) using an in house protocol. The purpose of nested PCR was to obtain more specific DNA products. The two outer and inner primers were designed to bind in opposite directions of complimentary strands of DNA (template molecule). These primers were duplicates of nucleotide sequences on either side of the piece of DNA. The sequence between the two primer binding sites was amplified exponentially with each PCR cycle. Amplification was performed with an automated DNA thermal cycle (Gene Amp PCR system 9600 and 9700) with a heated coverlid which consists of three steps: The first step was denaturation by heat at $94^{o}$C for 2 minutes then $94^{o}$C for 10 seconds, to separate double-stranded DNA into two single strands. The second step was the annealing step in which the primers bind to the target site. Annealing took place at 50°C for 30 seconds. In the final step, the reaction was heated to $68^{o}$C for 2 minutes optimal for DNA polymerase, which synthesized copies of the target sequences by extending the primers. A PCR assay consisted of 35 cycles that were performed automatically by a programmable heating block with an increment of elongation time of 20 seconds after every cycle. Every circle was finalized by an elongation temperature of $68^{o}$C for 7 minutes. All the preparations were performed on ice.

In the outer PCR, we used outer primers to amplify the viral DNA produced from cDNA synthesis producing a fragment of 2400 bp. The outer PCR master mix (Table 3) was prepared for all the samples together with addition of one sample volume. The mix was then distributed into 0.2 ml tubes and then mixed with proviral DNA.

**Table 3: Outer PCR master mix**

| **Component** | **1X reaction volume** |
|---|---|
| 10X Buffer 1 + Mgcl2 (17.5 mM) | 5 µl |
| 10 mM dNTP mix | 7 µl |
| Primer G25Rev (50 pmol/µl ) | 0.5 µl |
| Primer IN3 (50 pmol/µl) | 0.5 µl |
| Expand long template enzyme (5U/µl) | 0.75 µl |
| Distilled water | 31.25 µl |
| **Total** | **45 µl** |
| cDNA template | 5 µl |
| **Total Volume** | **50 µl** |

The inner PCR was performed with inner primers and amplified the outer PCR product producing a fragment of 1770 bp. Inner PCR master mix (Table 4) was prepared for all the samples together with addition of one sample volume. The mix was then distributed into 0.2 ml tubes and then mixed with outer PCR product making a total volume of 50 µl.

**Table 4: Inner PCR master mix**

| **Component** | **1X reaction volume** |
|---|---|
| 10X Buffer 1 + Mgcl2 (17.5 mM) | 5 µl |
| 10 mM dNTP mix | 7 µl |
| Primer G25Rev (50 pmol/µl) | 0.5 µl |
| Primer Pol M4 (50 pmol/µl) | 0.5 µl |
| Expand long template enzyme (5U/µl) | 0.75 µl |
| Distilled water | 31.25 µl |
| **Total** | **45 µl** |
| First Round PCR product | 5 µl |
| **Total Volume** | **50 µl** |

### 2.7.6 Detection of PCR product

Agarose gel Electrophoresis was used to detect the PCR products. The 1 % agarose gel was prepared by dissolving 1gm of gel to 100 mls of TAE buffer, heated in the microwave at 100$^{o}$C. The gel was left to cool then 5 µl of Ethidium Bromide was added to the dissolved gel. The gel was then poured in the gel chamber and left to polymerase for 30 minutes, and then the chamber was filled with TAE buffer. The 5 µl of samples mixed with 2 µl of 10X loading dye were loaded to the gel followed by molecular marker in a separate chamber. This was then electrophoresed at 100 Volts and 500 Amp for 1 hour and read under the ultraviolet light. During electrophoresis, the molecules migrated at a speed that reflected their size producing different bands for different sizes of PCR product.

### 2.7.7 Purification of PCR product

Purification of inner PCR product was done to obtain a pure DNA for sequencing. This was done through loading the 45 µl of inner PCR product mixed with 3 µl of 10X loading buffer solution to 1 % agarose gel in a gel chamber. The gel was also loaded with 10 µl of 1 kb molecular marker mixed with 3 µl of 10X loading buffer solution. This was electrophoresed at 100 Volts and 500 Amps for 1 hour and examined under UV light. The required DNA fragment was excised from the agarose gel with a clean, sharp scalpel and weighed in colourless tubes.

The recovery of the DNA from the excised gel fragment was done by QIA Quick gel extraction kit according to manufactures instructions. This kit is designed to extract and purify DNA of 70 bp to 10 kb from standard or low melting agarose gel in TAE or TBE buffer. The recovery was done by dissolving the gel with PCR product in 3 gel volume of buffer QG and 1 gel volume of isopropanol. The mix was then added to the QIA quick columns and washed with 0.5 ml of buffer QG and 0.75 ml of buffer PE in a two step washing process at a centrifugation speed of 13,000 rpm for 1 minute each. The elution of the PCR product was done by adding 40 µl of distilled water to the centre of the QIA quick membranes columns, incubated for 1 minute at room temperature then centrifuged for 1 minute at a speed of 13, 000 rpm. The product was stored at –20°C for sequencing.

### 2.7.8 HIV viral load testing

HIV-1 RNA in plasma was detected by nucleic acid amplification PCR technology using the COBAS AmpliScreen HIV-1 test, version 1.5 and primers (upstream primer, downstream primer and probe primer). The COBAS AmpliScreen HIV-1 test was conducted in five major processes: Sample processing, reverse transcription of target RNA to generate complementary DNA (cDNA) through cDNA synthesis, PCR amplification of target cDNA using primers (nested PCR) and hybridization of the amplified products to oligonucleotide probes specific to the target(s) in a thermal cycler segment of the COBAS AMPLICOR analyser which automatically performed the reverse transcription, amplification and detection. Detection of the probe-bound amplified products was done by calorimetric determination in which results were expressed as absorbance values at 660 nm with a detection level above 400 copies/ ml.

### 2.7.9 Sequencing PCR product

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. Sequencing was performed by using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Brussels, Belgium), Centri-Sep columns (Princeton Separations) and sequencing primers (G25Rev, Pol Mo, Pol M1, Pol Mg, Pol M8, Pol M4 and IN5.

The big dye terminator contained thermostable DNA polymerase and fluorescently labelled dideoxynucleotide (ddNTPs) chain terminators. The ddNTPs differ from normal dNTPs in that they lacked a hydroxyl group at the 3'-carbon position as well as at the 2'-

carbon position thus caused termination of chain synthesis. The thermostable DNA polymerase was used together with a temperature cycling format of denaturation, annealing and DNA synthesis, as in PCR. The difference from conventional PCR was that only one primer was used and ddNTPs were present in the reaction mixture instead of dNTP. The product accumulated linearly rather than exponentially due to the presence of only one primer.

The sequencing master mix (Table 5) was distributed to 0.2 ml PCR tubes together with 5 µl of PCR product and 1.5 µl of primer to each tube making a total volume of 20 µl. The mix was then placed in the thermo cycler with the following conditions:

$96^{o}C$ – 1 minute

$96^{o}C$ – 10 seconds
$50^{o}C$ – 5 seconds
$64^{o}C$ – 4 minutes
} 25 cycles

$4^{o}C$ – infinity

**Table 5: Cycle sequencing master mix**

| **Component** | **1X reaction volume** |
|---|---|
| 5 X Buffer | 2.0 µl |
| Big Dye Terminator | 4.0 µl |
| Water | 7.5 µl |
| **Total** | **13.5 µl** |

The sequencing reaction was then purified by using Centri-sep columns to remove unincorporated fluorescent nucleotides from the sequencing reaction. This was done by first hydrating the dry gel for 30 minutes and ensuring that all the air bubbles were removed from slurry by gently tapping the columns. The columns were then allowed to drain and then placed on the centrifuge with column tab facing directly out from centre and centrifuged at 13,000 rpm for two minutes to remove interstitial fluid. 20 µl of sequencing product was transferred directly to the top of the gel bed centrifuged with the column tab facing out for 2 minutes at 13,000 rpm. The purified samples (about 20µl) collected in the bottom of the collection tube were prepared for sequencing by first denaturing the enzyme at 96°C for 2 minutes then cooled at 4°C for 2 minutes. The samples were then loaded to the ABI prism 3100 automated sequencer. This is a multi-colour fluorescence-based DNA analysis system using the proven technology of capillary electrophoresis with 16 capillaries operating in parallel.

## 2.7 Data analysis

Data was coded, double entered, cleaned and analysed in the computer. Epi Info Version 3.3.2 and Microsoft Excel were used for general data analysis. Sequences were aligned using Chromas pro and edited manually using Bioedit. Positions where most of the sequences had gaps and regions that could not be aligned unambiguously were omitted from the analysis. Generation of NJ Phylogenetic tree was done using Mega 2. Genotypic analysis was done using Stanford ARV Resistance database where by mutations conferring resistance to Anti retroviral drugs were determined. In this database, pol sequences were loaded in FASTA format. The results provided were current sequence

subtype, the available ARV resistance mutations categorised as Protease major and minor mutations, Nucleoside and Non nucleoside reverse transcriptase mutations. The level of resistance to ARV drugs was provided as high, intermediate and low for each individual ARV drug. Yates Corrected $\chi 2$ test analysis was used to determine the risk factors difference among cases and non-cases.

# CHAPTER 3

# RESULTS

A total of 150 HIV infected patients on ARV treatment (79 cases and 71 non-cases) attending ARV clinic in MNH were recruited and consented to participate in the study.

## 3.1 Descriptive analysis of the study subjects

### 3.1.1 Social and demographic characteristics

There were a total of 97 (65%) females and 53 (35%) males, among whom 51 (34%) females and 28 (19%) males were cases and 46 (31%) females and 25 (17%) males were non-cases (Table 6).

In this study population 69 (46%) patients were in marriage among whom 35 (51%) were cases. A total of 81 (54%) patients were not in marriage (single, divorced and widowed) among whom 44 (54%) were cases and 37 (46%) were non-cases (Table 6).

The education level of 88 (59%) patients was primary school and below, among whom 46 (52%) were cases and 42 (48%) were non-cases. The education level of 37 (25%) patients was secondary school and above among whom 18 (49%) were cases and 19 (51%) were non-cases. The education level of 25 (17%) of the patients could not be ascertained (Table 6).

**Table 6: Social demographic characteristics of patients**

| Variable | Cases | | Non-cases | | Total |
|---|---|---|---|---|---|
| | **Frequency** | **Percentage (%)** | **Frequency** | **Percentage (%)** | |
| **Gender** | | | | | |
| Female | 51 | 65% | 46 | 65% | 97 (65%) |
| Male | 28 | 35% | 25 | 35% | 53 (35%) |
| **Total** | **79** | **100%** | **71** | **100** | **150 (100%)** |
| **Marital status** | | | | | |
| In marriage | 35 | 44% | 34 | 48% | 69 (46%) |
| Not in marriage | 44 | 56% | 37 | 52% | 81 (54%) |
| **Total** | **79** | **100%** | **71** | **100** | **150 (100%)** |
| **Education Level** | | | | | |
| Primary school and below | 46 | 72% | 42 | 69% | 59 (59%) |
| Secondary school and above | 18 | 28% | 19 | 31% | 37 (25%) |
| **Total** | **64** | **100%** | **61** | **100%** | **125 (84%)** |

The mean age for the cases was 41 years (SD = 11: Median = 40: Range = 6-66 years), and the mean age for non-cases was 38 years (SD = 12: Median = 39: Range 6-66 years). This difference was not statistically significant (P-value = 0.1276).

### 3.1.2 Clinical characteristics of patients

The mean viral load for the cases was 27109 copies /ml (SD = 86732: Median = 400), while for non-cases was 21631 copies /ml (SD = 120514: Median = 400). This difference was not statistically significant (P-value = 0.1595).

There were statistically significant differences between the mean percentage weight gain [cases (6%) and non-cases (20%)] and the mean CD4 increase [cases (8%) and non-cases (35%)] at a P-value of 0.0001.

The ARV drug Triomune was given to 76 (96%) cases and 67 (94%) non-cases. This difference was statistically not significant with an Odds Ratio of 0.56 (0.05 – 6.53) and a P-value of 0.90. Other ARV drugs used were as shown in Figure 5. Most of the patients continued with their initial therapy despite of immunological and clinical failure. A total of 18 (23%) cases and 7 (10%) non-cases reported change of therapy during the course of treatment with ARV drugs. There were several reasons for changing therapy as mentioned by cases and non- cases. Among cases, the reasons for changing therapy were: Adverse reactions 8(43%), treatment failure 7 (38%), poor adherence 1 (6%), pregnancy 1(6%) and drug out of stock 1 (6%). Among non-cases, the reported reasons for changing therapy were: Adverse reactions 6 (86%) and poor adherence 1 (14%).

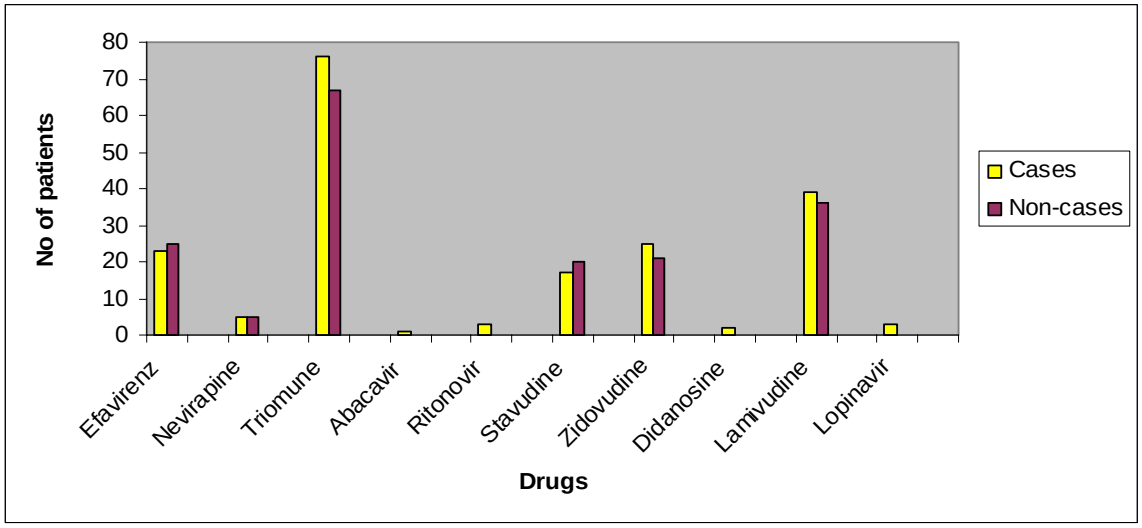


**Figure 5: ARV drugs given to the study population at MNH, 2007**

Both cases and non-cases reported to have opportunistic infections during the period of treatment; however, more opportunistic infections were reported by cases than non-cases as shown in Figure 6.

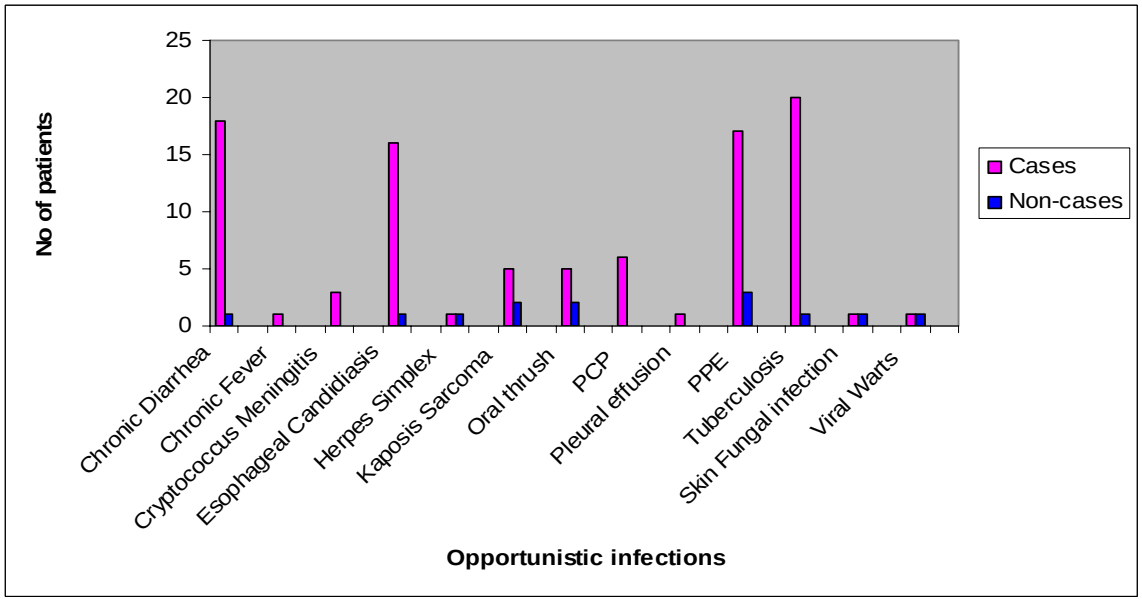


**Figure 6: Opportunistic infections as reported by the study group at MNH, 2007**

All patients were using cotrimoxazole, 11 (14%) of cases were using anti tuberculosis drugs within a period of 6 months, 33 (42%) of cases and 18 (25%) of non-cases were using fluconazole. None of the non-cases were on anti tuberculosis drugs.

### 3.2 Bivariate analysis

**Table 7: Bivariate analysis of social and clinical characteristics of study group**

| **Variable** | **Cases** | **Non-cases** | **OR (95% CI)** | **P-value** |
|---|---|---|---|---|
| **Sex** | | | | **Yates Corrected, 2-tailed** |
| Female | 51 | 46 | | |
| Male | 28 | 25 | 0.98 (0.51-1.94) | 0.89 |
| **Marital Status** | | | | **Yates Corrected, 2-tailed** |
| In marriage | 35 | 34 | | |
| Not in marriage | 44 | 37 | 0.87 (0.45-1.65) | 0.78 |
| **Education Level** | | | | **Yates Corrected, 2-tailed** |
| Primary Level and Below | 46 | 42 | | |
| Secondary Level and Above | 18 | 19 | 1.16 (0.54-2.49) | 0.86 |
| **WHO staging of the disease at initiation of therapy** | | | | **Yates Corrected, 2-tailed** |
| Stage II | 44 | 58 | 0.28 (0.13 – 0.60) | 0.001 |
| Stage III and IV | 35 | 13 | | |

There was no significant statistical difference between cases and non-cases with respect to gender (P-value = 0.89), marital status (P-value = 0.78) and education level (P-value = 0.86) (Table 7).

The clinical stage of the patients at the initiation of therapy was found to be a significant factor for development of drug failure at a p-value of 0.001 (Table 7). This is because both cases and non-cases that started therapy at clinical stage II were more likely to respond to treatment than those who started therapy at clinical stage III and IV.

### 3.3 Laboratory results

It was possible to sequence 28 samples out of the 150 samples collected due to low viral load of < 400 copies/ml. Twenty three out of twenty four samples (96%) with viral load of more than 400 copies/ ml were successfully sequenced at the National Institute for Communicable Diseases in South Africa. Five samples out of 126 with viral load of less than 400 copies /ml were successfully sequenced at the Kenya Medical Research Institute Laboratory in Kenya.

#### 3.3.1 Descriptive analysis of the sequenced population

There were 19 (68%) cases and 9 (32%) non-cases in which 16 (57%) were females and 12 (43%) were males. Thirteen (46%) were married, 12 (43%) were single and 3 (11%) were widowed. The majority (43%) were on Triomune alone and the rest (57%) were on more than one therapy, including triomune. Eight (29%) of the patients reported to have change therapy while 20 (71%) have no history of changing therapy.


**Figure 7: Phylogenetic tree of the sequenced samples from MNH, 2007**

The most prevalent subtypes were A (35%) and C (35%) followed by subtype D (14%) and their recombinants BD (4%), CD (4%), CRF01_AE (4%) and CRF01_AG (4%) as shown in Figure 7 and Table 8.

**Table 8: Subtype distribution among cases and non-cases**

| **Subtype** | **Cases** | **Non-cases** | **Total** | |
|---|---|---|---|---|
| | | | Number | Percentage (%) |
| A | 7 | 3 | 10 | 35 |
| C | 7 | 3 | 10 | 35 |
| D | 3 | 1 | 4 | 14 |
| BD | 0 | 1 | 1 | 4 |
| CD | 0 | 1 | 1 | 4 |
| CRF01_AE | 1 | 0 | 1 | 4 |
| CRF02_AG | 1 | 0 | 1 | 4 |
| **Total** | **19** | **9** | **28** | **100** |

### 3.3.2 Antiretroviral (ARV) mutations

The ARV mutations were grouped as mutations conferring resistance to Protease Inhibitors (major and minor mutations), Nucleoside Reverse Transcriptase Inhibitors and Non Nucleoside Reverse Transcriptase Inhibitors.

Overall, the prevalence of samples with one or more of PI major mutations was 1 (4%), PI minor mutations were 8 (29%), NRTI mutations were 18 (64%) and NNRTI mutations were 20 (71%) Table 9. M184V was the most prevalent mutation among NRTI (Figure

8) and G190A among NNRTI (Figure 9). There was only one PI major mutation M46V and two Protease minor mutations (L10I and L10V). The least prevalent mutations among the NRTI were K70E, Q151H, T69S, E44D, V75T, D67S, T215I, V75I, T69I, L74I, V75A, V118I, L210W and T215Y (Figure 8). The list prevalent mutation among the NNRTI was G190S (Figure 9).

**Table 9: Antiretroviral (ARV) resistance mutations**

| **Type of mutation** | **Cases** | | **Non-cases** | | **Total** | |
|---|---|---|---|---|---|---|
| | Number | Percentage (%) | Number | Percentage (%) | Number | Percentage (%) |
| PI major mutations | 1 | 11 | 0 | 0 | 1 | 4 |
| PI minor mutations | 6 | 32 | 2 | 22 | 8 | 29 |
| NTRI resistance mutations | 12 | 63 | 6 | 67 | 18 | 64 |
| NNRTI resistance mutations | 13 | 68 | 7 | 78 | 20 | 71 |

Among cases, 13 (68%) had resistance mutations to at least one of the groups of ARVs while 7 (78%) of non-cases had resistance mutations to at least one of the groups of ARVs. There were no non-cases with any PI major mutations, 6 (32%) of cases and 2 (22%) of non-cases had PI minor mutations, 12 (63%) cases and 6 (67%) non-cases had mutations conferring resistance to NRTI and 13 (68%) cases and 7 (78%) non-cases had mutations conferring resistance to NNRTI (Table 9).

The difference in observed mutations among cases and non-cases could not be statistically measured due to the low number of sequenced samples, however more mutations were observed among cases than in non-cases.

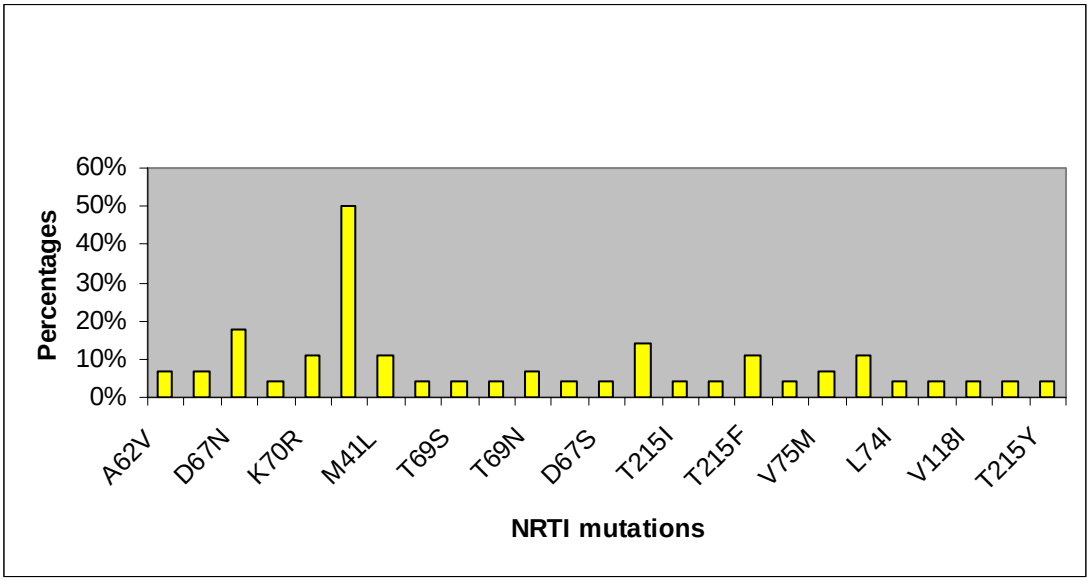


**Figure 8: NRTI mutations among the sequenced samples from MNH, 2007**

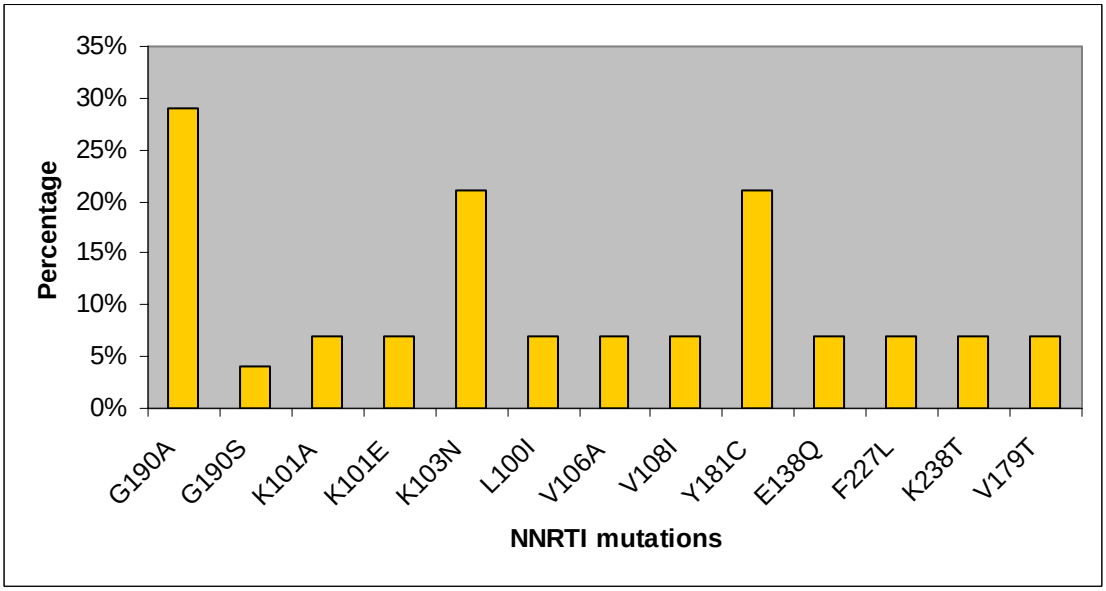


**Figure 9: NNRTI mutations among the sequenced samples from MNH, 2007**

### 3.3.3 ARV resistance pattern

**Table 10: ARV drugs resistance pattern**

| Type of Inhibitor | Cases | Non-cases | Total |
|---|---|---|---|
| High level resistance to PI | 0 | 0 | 0 |
| Intermediate resistance to PI | 0 | 0 | 0 |
| Low level resistance to PI | 2 | 0 | 2 |
| High level resistance to NRTI | 12 | 6 | 18 |
| Intermediate resistance to NRTI | 9 | 2 | 11 |
| Low level resistance to NRTI | 9 | 6 | 15 |
| High level resistance to NNRTI | 13 | 7 | 20 |
| Intermediate resistance to NNRTI | 13 | 7 | 20 |
| Low level resistance to NNRTI | 10 | 7 | 17 |

#### 3.3.3.1 Protease Inhibitors (PI)

##### 3.3.3.1.1 High level resistance to Protease Inhibitors

None of the sequences showed significant high-level resistance to the PIs (Table 10).

##### 3.3.3.1.2 Intermediate level resistance to Protease Inhibitors

There were no sequences with significant mutations that confer intermediate level resistance to any of the Protease Inhibitors.

##### 3.3.3.1.3 Low level resistance to Protease Inhibitors

One sequence (4%) had mutations that conferred low-level resistance to Fosamprenavir, Amprenavir, Nelfinavir and Lopinavir. One sequence (4%) had mutations that conferred

low-level resistance to Nelfinavir alone. Both these two sequences were from cases and there was no any non-case with low-level resistance to Protease Inhibitors (Table 10).

#### 3.3.3.2 Nucleoside Reverse Transcriptase Inhibitors (NRTI)

##### 3.3.3.2.1 High level resistance to Nucleoside Reverse Transcriptase Inhibitors

Overall there were 12 (63%) cases and 6 (67%) non-cases sequenced which showed high level resistance to NRTI. Two sequences (7%) had mutations that conferred high level resistance to Lamivudine, 11 sequences (39%) to Lamivudine and Emtricitabine, 2 sequences (7%) to Lamivudine, Emtricitabine and Zidovudine, 2 sequence (7%) to Lamivudine, Emtricitabine, Zidovudine, Abacavir, Stavudine and Didanosine and 1 sequence (4%) to Lamivudine, Emtricitabine, Zidovudine and Stavudine (Table 10).

##### 3.3.3.2.2 Intermediate level resistance to Nucleoside Reverse Transcriptase Inhibitors

Overall 9 (47%) of cases and 2 (22%) of non-cases sequences had intermediate level resistance to NRTI. One sequence (4%) had Intermediate resistance mutations to each of the following single ARVs: Didanosine, Tenofovir, Emtricitabine and Zidovudine. One sequence (4%) had Intermediate resistance mutations to each of the two ARVs Abacavir and Didanosine, Abacavir and Stavudine, Stavudine and Didanosine and Zidovudine and Stavudine. There was no significant difference in the development of intermediate level resistance towards NRTI among cases and non-cases (Table 10)

##### 3.3.3.2.3 Low level resistance to Nucleoside Reverse Transcriptase Inhibitors

Overall 9 (47%) of cases and 6 (67%) of non-cases sequences had low-level resistance to NRTI. One sequence (4%) had Low Resistance mutations to Stavudine and 9 (32%)

sequences had Low Resistance mutations to Abacavir. Three sequences (11%) had mutations conferring resistance to Abacavir and Didanosine, 1 sequence (4%) to Didanosine and Tenofovir and 1 sequence (4%) to Abacavir, Stavudine and Didanosine (Table 10).

#### 3.3.3.3 Non Nucleoside Reverse Transcriptase Inhibitors (NNRTI)

##### 3.3.3.3.1 High level resistance to Non Nucleoside Reverse Transcriptase Inhibitors

Overall, 13 (68%) of cases and 7 (78%) of non-cases sequences had high-level resistance towards NNRTI. There were 6 sequences (21%) that had mutations conferring High Level resistance to Nevirapine, 6 sequences (21%) to Nevirapine and Efavirenz, 6 sequences (21%) to Nevirapine and Delcitavir and 2 (7%) to Nevirapine and Efavirenz. There was no significant difference in the development of high-level resistance towards NNRTI among cases and non-cases (Table 10).

##### 3.3.3.3.2 Intermediate level resistance to Non Nucleoside Reverse Transcriptase Inhibitors

Overall, 13 (68%) of cases and 7 (78%) of non-cases sequences had intermediate level resistance towards NNRTI. There were 8 sequences (29%) conferring Intermediate Level resistance to Delcitavir, 9 sequences (32%) to Efavirenz and 3 sequences (11%) to Efavirenz and Delcitavir. There was no significant difference in the development of intermediate level resistance towards NNRTI among cases and non-cases (Table 10).

#### 3.3.3.3.3 Low level resistance to Non Nucleoside Reverse Transcriptase Inhibitors

Overall, 10 (53%) of cases and 7 (78%) of non-cases sequences had low-level resistance towards NNRTI. There were 17 sequences (61%) with mutations conferring Low Level Resistance to Efavirenz. There was no significant difference in the development of low-level resistance towards NNRTI among cases and non-cases (Table 10).

### 3.3.4 Subtype association ARV resistance mutation development

Overall the ARV resistance mutations were distributed through out all subtypes; however there were more in Subtypes A and C as these were also the most prevalent subtypes sequenced (Table 11). There was no significant association between subtypes and development of ARV resistance mutations.

**Table 11: Subtypes and associated ARV resistance mutations occurrences**

| **Variable** | **Subtypes** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | C | D | BD | CD | CRF01 | CRF02 | Total |
| PI major mutations | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| PI minor mutations | 4 | 1 | 2 | 0 | 1 | 0 | 1 | 8 |
| NRTI mutations | 7 | 4 | 3 | 1 | 1 | 1 | 1 | 18 |
| NNRTI mutations | 7 | 6 | 3 | 1 | 1 | 1 | 1 | 20 |
| Total | 10 | 10 | 4 | 1 | 1 | 1 | 1 | 28 |

### 3.4 Study limitation

Most of the patients had low viral load < 400 copies/ml, thus could not be successfully sequenced.

# CHAPTER 4

# DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

## 4.1 Discussion

In this study, 150 samples from HIV infected patients who were on treatment for more than six months were evaluated through viral load testing and 28 of them sequenced at their pol region. This study specifically assessed the effect of long-term (more than 6 months) single or multiple therapies on genotypic outcomes in HIV-infected patients. As NRTI are an important component of combination ARV therapy, detecting cross-resistance after failure to a first-line regimen is an important issue in the management of subsequent treatments. In this molecular epidemiological study performed in samples obtained from patients attending a care and treatment clinic, a high prevalence of mutations related to drug resistance in RT genes was detected and the prevalence of different genetic subtypes estimated.

This study demonstrated that the mean percentage weight gain and CD4 increase were lower among cases as compared to non-cases, an indication of the treatment failure. This could also be supported by the fact that more opportunistic infections were reported in cases than non-cases suggesting that in the event of ART failure, patients remain vulnerable to opportunistic infections due to resurgence of viral load and deterioration of immunological status.

Importantly, the study demonstrated that initiation of therapy late in the stage of disease resulted in increased prevalence of treatment failure, a finding also observed in another study on effect of stage of disease and drug dose (Richmann *et al*, 1990). This could also be due to the high number of viral population that multiplies in the presence of drug pressure allowing generation of more resistant viruses.

Overall, the highest percentage of samples clustered with subtype A and C in both cases and non-cases. These findings complement and are consistence with existing HIV molecular epidemiological data in Tanzania, which indicated presence of subtypes A, C, D and their recombinants (Lyamuya *et al*, 2000; Hoelscher *et al* 2002). It is important to note that in spite of the methodological differences in subtyping, the different scientists detected the same subtypes, though at variable proportional distribution.

In this study, there was no significant association between the observed pattern of amino acid changes and the different HIV-1 subtypes in both cases and non-cases. This is in agreement with another study that has shown that the clinical response to ARV therapy is independent of HIV-1 subtype and baseline polymorphisms of the protease gene (Holguin *et al*, 2004). Another study has also shown that the mutations that cause drug resistance in subtype B viruses appear to be the main mutations that cause drug resistance in non-B viruses (Kantor *et al*, 2005). It is still unclear whether there are differences in the evolution of disease or in response to ARV therapy among distinct HIV-1 subtypes. Although some studies addressing different clades have failed to show differences in virological response after treatment or in the prevalence of resistance mutation in patients

failing therapy (Kantor *et al*, 2004), subtle differences are starting to emerge. In contrast, some recent reports have shown an increased prevalence of mutations associated with NVP and a faster progression to disease in patients with subtype D (Eshleman *et al*, 2004).

The occurrence of resistance mutations to at least one of ARV drug was high in both cases and non-cases, 4% (PI major mutations), 29% (PI minor mutations), 64% (NRTI mutations) and 71% (NNRTI mutations). However, it is important to note that not all genotypic drug-resistance algorithms give similar interpretations. The high occurrence of ARV drug resistance mutations present in this study is in contrast to low (<5%) prevalence noted among ARV naïve antenatal clinic attendees in Tanzania (Somi *et al*, 2007).

In the RT gene, there were multiple polymorphisms detected whose significance to future ARV susceptibility is unknown. This is because, understanding the roles of these polymorphic codons, alone or in combination, is limited. During analysis of the resistance-associated mutations to RT inhibitors in all sequence samples, it was observed that the M184V mutation was the most prevalent among sequenced cases and non-cases. The second group of more prevalent RT mutations were the TAMs that lead to strong resistance against the majority of RTI, including Emtricitabine which has not been in use in Tanzania which signifies also a possibility of cross resistance mutations. This has previously been demonstrated in other studies in which subjects experiencing virological failure on stavudine (d4T)-containing regimens often contains TAMs (Ross *et al*, 2001).

This is in contrast to another study which found that the emergence of TAMs may be affected by the presence of M184V as this diminishes viral replicative capacity and ameliorates TAM-mediated resistance (Ho *et al*, 2003). The high level of resistance observed towards Lamivudine and Stavudine could be explained by the high level of usage of these drugs.

Among the NNRTI associated mutations, G190A was the most prevalent in both cases and non-cases. Probably this is a consequence of the drug selective pressure in treated patients and also due to the large application of these classes of drugs in the Tanzania therapeutic protocols during the last years. The high level of resistance towards Nevirapine could be attributed to the high usage of this medication and this calls for more attention on the use of Nevirapine in the country. .

The PI associated mutations identified in this study are at highly polymorphic sites, however associated with low level resistance to PI. Interestingly, resistant mutation in the PI genes was also observed in the study on ARV naïve antenatal clinic attendees (Somi *et al*, 2007). Other studies have suggested that the presence of minor protease mutations is associated with the development of resistance to PIs and subsequently reduced efficacy to PI-based therapy (Muzammil *et al*, 2003).

The genetic sequencing of HIV was not possible from a large number of samples, probably due to genetic variation in the primer binding sites and/or low viral load (below 400 copies/ ml). This has previously observed in other studies (Barlow *et al*, 1995). This

is because most of the assays require plasma samples with HIV viral load above 1000 copies/ml in order to achieve reliable results. The in house method used for sequencing in this study requires plasma viral load of more than 400 copies/ ml.

For the clinician, there are several difficulties when the results of a genotypic resistance test are to be applied, thus predicting which ARV combination that is going to be the best for a certain patient is an even greater challenge. This is because not all facts are known and humans are even more unpredictable than the virus. Additionally, there is a lack of knowledge about what is going on in minor virus populations. Thus, monitoring of the emergence of drug-resistant HIV in populations starting and using ARV therapy would be a useful approach for planning effective treatment programs in Tanzania.

## 4.2 Conclusions

The occurrence of ARV drug resistance mutations observed in this study was high in both cases and non-cases with no statistical significant difference between cases and non-cases. Mutations detected by resistance genotyping analysis were related to the ARV drugs prescribed, mainly NNRTI (Nevirapine) and NRTI (Lamivudine and Stavudine) than to any other ARV drug.

The risk factors observed in this study were similar in both cases and non-cases with exception of percentage weight gain and percentage CD4 increase which was higher in non-cases than in cases. More opportunistic infections were observed in cases than in non-cases. In both cases and non-cases, the clinical stage at initiation of therapy was an important factor towards disease progression.

The most prevalent HIV-1 subtypes were A (35%) and C (35%), however genetic sequencing of HIV was not possible from a large number of samples due to low viral load. There was no evidence that non-B viruses develop resistance by mutations at positions that are not associated with resistance in subtype B viruses.

### 4.3 Recommendations

The National Aids Control Program need to establish and maintain a national surveillance for ARV resistant HIV in the country to Drug naïve HIV patients and ARV treated patients. This will help to track the trend of emergence of new ARV resistant HIV variants and the information obtained will be used to guide development of treatment policy. Issues related to HIV drug resistance to be also factored into decisions regarding prophylaxis of HIV exposed heath care workers and treatment of HIV-infected patients in the foreseeable future.

There is a need to use viral load in monitoring patient's progression and response to ARV and the use of ARV resistance testing before and subsequently during treatment especially when experiencing drug failure.

There is a need to further study a large population of patients who are on ARV at MNH to obtain the actual prevalence and associated risk factors of ARV resistance. This is because a good virological response is not always seen when viral resistance is not observed.

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# APPENDICES

## APPENDIX 1: RESEARCH ETHICAL CLEARANCE

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**CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA**

This is to certify that the research entitled: Antiretroviral (ARV) drug resistance in Human Immunodeficiency Virus (HIV) Patients attending Muhimbili National Hospital, Dar es Salaam Tanzania, Mosha F *et al*), whose Principal Investigator is Fausta Mosha, has been granted ethics clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is made available to the Ministry of Health and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine.

**Name: Dr Andrew Y Kitua**

**Signature**

**CHAIRMAN**
**MEDICAL RESEARCH**
**COORDINATING COMMITTEE**

**Name: Dr Deo M Mtasiwa**

**Signature**

**CHIEF MEDICA L OFFICER**
**MINISTRY OF HEALTH, SOCIAL WELFARE**

**CC: RMO**
**DMO**

## APPENDIX 2: SWAHILI TRANSLATED LEAFLET GIVEN TO THE PATIENTS

**Virusi sugu dhidi ya dawa za kurefusha maisha kwa wagonjwa wanaoishi na virusi vya UKIMWI.**

Kukosekana kwa dawa za kutibu virusi vya UKIMWI ni changamoto kwa dunia, aidha kusambaa kwa virusi vya UKIMWI ambavyo ni sugu dhidi ya dawa za kurefusha maisha kwa wagonjwa wanaoishi na virusi vya UKIMWI in janga la dunia.

Baadhi ya sababu zinazochangia usugu wa virusi vya UKIMWI dhidi ya dawa zinazotumiwa kurefusha maisha ya wagonjwa wanaoishi na virusi ni :

- Kukatisha dawa baada ya kuanza kuzitumia
- Kuambukizwa na virusi sugu
- Upungufu wa kinga mwilini
- Udhaifu wa dawa kiasi cha kushindwa kudhibiti mazalio ya virusi mwilini.

**Kwa nini uchunguzi huu ni muhimu?**

- Kujua ukubwa wa tatizo la virusi sugu dhidi ya dawa za kurefusha maisha nchini
- Kuainisha sababu zinaochangia virusi vya UKIMWI kuwa sugu dhidi ya dawa husiki
- Kutambua aina ya dawa ambayo inafaa kutumika dhidi ya virusi vinavyopatikana Tanzania
- Kutambua aina ya virus vya UKIMWI vinavyopatikana Tanzania.

**Ni wakati gani uchunguzi huu unafanyika?**

- Kabla ya kumuanzishia mgonjwa dawa
- Dawa kushindwa kumsaidia mgonjwa baada ya kuzitumia kwa muda
- Kujua kama mgonjwa aliambukizwa na virusi sugu au amevipata baada ya kutumia dawa
- Kujua ukubwa wa tatizo katika nchi au kituo kinachotoa huduma za dawa za kurefusha maisha kwa wagonjwa wanaoishi na virusi vya UKIMWI

**Jinsi ya kufanya ushunguzi wa usugu wa virusi dhidi ya dawa za kurefusha maisha**

Mpaka sasa Tanzania hatujaweza kuwa na vifaa vya kuweza kufanya uchunguzi huu, hivyo damu kwa ajili ya kufanya ushunguzi huu itapelekwa nchini Kenya.

**Ni wagonjwa gani watahusishwa kwenye uchunguzi huu?**

Wagonjwa wanaoshi na virusi vya UKIMWI waliokwisha tumia dawa kwa zaidi ya miezi sita na wanahudhuria kliniki ya kutoa huduma ya matibabu ya dawa za kurefusha maisha katika hospitali ya Muhimbili.

## APPENDIX 3: CURRENT CLASSES OF ANTIRETROVIRAL DRUGS

| **RT inhibitors** | **Protease inhibitors** | **Fusion inhibitors** | **Novel ARVs** |
|---|---|---|---|
| **Nucleoside analogues** | Amprenavir AMP | Tenofovir | CCR5 inhibitors |
| Abacavir ABC | Indinavir IND | | Integrase inhibitors |
| Didanosine ddI | Lopinavir LOP | | |
| Lamivudine 3TC | Nelfinavir NFV | | |
| Stavudine d4T | Ritonavir RIT | | |
| Zidovudine AZT | Saquinavir SAQ | | |
| Zalcitabine ddC | | | |
| Trizivir TRZ | | | |
| **Non Nucleoside analogues** | | | |
| Delavirdine DLV | | | |
| Efavirenz EFV | | | |
| Nevirapine NVP | | | |

**APPENDIX 4: DATE COLLECTION TOOL AT THE CLINIC**

Questionnaire number: …… Date of specimen collection:……...

Hospital number:……. Study Number: ……..

Study Group:……. District:……...

Age: ……. Gender:……....

Occupation:…….. Education Level:…….

Below Primary level ☐

Primary level ☐

Secondary level ☐

Post secondary level ☐

Marital Status:……. Spouse HIV status:……..

Date of HIV Diagnosis:…… Date of Starting treatment: ……...

ARV Treatment used:

…….

Any history of therapy change: **Yes** ☐ **No** ☐

Reason for therapy change: .....

Any other treatment that you have been using for the past 6 months:

……

Number of Hospitalization in the past 6 months: ……..

Percentage weight gain in the past 6 months:……

Percentage CD4 increase in the past 6 months: …….

Opportunistic infections experienced in the past 6 months:

……

…….

**Laboratory Results**:

Viral Load: …….

PI Major Mutations: …….....

PI Minor Mutations:……....

NRTI Mutations: ……....

NNRTI Mutations:……..

High Level Resistance To:

| | |
|---|---|
| Abacavir (ABC) | ☐ |
| Didanosine (ddI) | ☐ |
| Lamivudine (3TC) | ☐ |
| Stavudine (d4T) | ☐ |
| Zidovudine (AZT) | ☐ |
| Zalcitabine (ddC) | ☐ |
| Trizivir (TRZ) | ☐ |
| Delavirdine (DLV) | ☐ |
| Efavirenz (EFV) | ☐ |
| Nevirapine (NVP) | ☐ |
| Amprenavir (AMP) | ☐ |
| Indinavir (IND) | ☐ |
| Lopinavir (LOP) | ☐ |
| Nelfinavir (NFV) | ☐ |
| Ritonavir (RIT) | ☐ |
| Saquinavir (SAQ) | ☐ |
| Tenofovir | ☐ |

Intermediate Level Resistance To:

- Abacavir (ABC) ☐
- Didanosine (ddI) ☐
- Lamivudine (3TC) ☐
- Stavudine (d4T) ☐
- Zidovudine (AZT) ☐
- Zalcitabine (ddC) ☐
- Trizivir (TRZ) ☐
- Delavirdine (DLV) ☐
- Efavirenz (EFV) ☐
- Nevirapine (NVP) ☐
- Amprenavir (AMP) ☐
- Indinavir (IND) ☐
- Lopinavir (LOP) ☐
- Nelfinavir (NFV) ☐
- Ritonavir (RIT) ☐
- Saquinavir (SAQ) ☐
- Tenofovir ☐

Low Level Resistance To:

- Abacavir (ABC) ☐
- Didanosine (ddI) ☐
- Lamivudine (3TC) ☐
- Stavudine (d4T) ☐
- Zidovudine (AZT) ☐
- Zalcitabine (ddC) ☐

| | |
|---|---|
| Trizivir (TRZ) | ☐ |
| Delavirdine (DLV) | ☐ |
| Efavirenz (EFV) | ☐ |
| Nevirapine (NVP) | ☐ |
| Amprenavir (AMP) | ☐ |
| Indinavir (IND) | ☐ |
| Lopinavir (LOP) | ☐ |
| Nelfinavir (NFV) | ☐ |
| Ritonavir (RIT) | ☐ |
| Saquinavir (SAQ) | ☐ |
| Tenofovir | ☐ |

## APPENDIX 5: ENGLISH CONSENT FORM

We have seen you in the clinic for HIV counselling, testing and treatment. We are asking for your consent to use your blood for Antiretroviral Resistance Testing (ARV), so as to know if the drugs you taking are working. The results of this study will help the Government to know the extent of ARV resistance and also modify your treatment when ever required.

In 2003 the Government of Tanzania endorsed the National Care and Treatment Plan for people infected by HIV and AIDS. The use of ARV to people living with HIV/AIDS is becoming very popular. However, little is known about ARV resistance in the country.

We will collect your blood in EDTA tube from you, which will be used for CD4 testing and ARV resistance testing. The tubes will bear no client identifier; the unlabeled specimens will be collected and delivered to the reception, National HIV Reference Laboratory, Department of Microbiology and Immunology, MUCHS. Each sample will be assigned a laboratory number. We also request you to feel in the given questionnaire.

Your blood will make this study very successful and will widen our knowledge on the current problem of ARV resistance.

If you don't feel comfortable to let us use your blood, this will in no way influence other necessary tests or treatments that are required. You don't have to give a reason for not agreeing.

I have understood the purpose of the study and allow you to use my blood for ARV resistance testing.

Name

……..

Signature or right thumb print

……

Witness

……

Signature

……

Date

……..

## APPENDIX 6: SWAHILI CONSENT FORM

**Fomu ya makubaliano**

Tumekuona leo kwenye klini yetu ya ushauri nasaha na huduma za matibabu ya dawa za kurefusha maisha kwa wagonjwa wanaoishi na virusi vinavyosababisha UKIMWI. Tunaomba uturuhusu kutumia damu yako kwa ajili ya kuchunguza kuwepo kwa virusi sugu dhidi ya dawa zinazotumiwa kurefusha maisha kwa wagonjwa wanaoishi na virusi vya UKIMWI. Hii itatusaidia kujua kama dawa unazotumia zinafanya kazi yake ya kupunguza makali ya virusi vya UKIMWI. Uchunguzi huu utaisidia serikali kufanya maamuzi sahihi inapoamua aina ya dawa ya kutumiwa nchini kwa wagonjwa wanaoishi na virusi vinavyosababisha UKIMWI.

Mnamo mwaka 2003, Serikali ya Tanzania ilianzisha mpango wa Huduma za matibabu kwa wagonjwa walioambukizwa virusi vinavyosababisha UKIMWI. Mpango huu umeongeza upatikanaji na utumiaji wa dawa hizo hapa nchini. Hata hivyo, ufahamu wa dawa hizo kwa wataalamu wetu walio wengi hapa nchini bado ni mdogo.

Tutachukua damu yako kiasi cha ujazo wa mililita tano na kuweka kwenye chupa hii ndogo yenye dawa inayoitwa EDTA kwa ajili ya kuzuia damu isigande. Damu hii itatumika kupima usugu wa virusi kwenye mwili wako dhidi ya dawa zinazotumika kupunguza makali ya virusi vya VVU. Chupa hi haitakuwa na jina lako wala utambulisho wako wowote, aidha itapewa namba maalumu.

Damu hii itapelekwa kwenye maabara ya Muhimbili ambapo itahifadhiwa kabla ya kupelekwa nje ya nchi kwa ajili ya vipimo hivyo. Tunakuomba utusaidie kujaza dodosa hii itakayoweza kutupa mambo muhimu yanayochangia usugu wa dawa za kupunguza makali ya virusi vinavyosababisha UKIMWI.

Damu yako itasaidia uchunguzi huu na pia kuongeza ujuzi wa wataalamu wetu hapa Tanzania kuhusu usugu wa dawa za kurefusha maisha kwa wagonjwa wanaoishi na virusi vya UKIMWI.

Kushiriki kwako kwenye uchunguzi huu ni kwa hiari yako na hata kama hautatoa idhini ya kutumia damu yako kwenye uchunguzi huu, haitaathiri huduma mbali mbali tunazokupa kwenye klini hii. Aidha haulazimiki kutoa maelezo yoyote kama hotopenda kushiriki kwenye uchunguzi huu.

Nimeelewa vizuri nia na madhumuni ya uchunguzi huu, natoa idhini ya matumizi ya damu yangu kwa ajili ya kuchunguza usugu wa virusi dhidi ya dawa ya kurefusha maisha kwa wagonjwa wanaoishi na virusi vinavyosababisha UKIMWI.

Jina Kamili

……..

Sahihi

……

Tarehe

……..

## APPENDIX 7: SEQUENCES USED FOR ALIGNMENT AND PHYLOGENETIC ANALYSIS

MD10618/07, MD10621/07, MD10633/07, MD10635/07, MD10644/07, MD10682/07

MD10684/07, MD10690/07, MD10706/07, MD10715/07, MD10733/07, MD10734/07

MD10740/07, MD10743/07, MD10744/07, MD10748/07, MD10749/07, MD10754/07

MD10761/07, MD10766/07, MD10770/07, MD10771/07, MD10620/07, MD10672/07

Sample 4, A.IN.01.15, A1.KE.00.N, A1.RU.03.0, A1.TZ.01.A, A1.TZ.97 A1.UA.00.9,

A1.UG.98.9, A1.UZ.02.0, A2.CD.97.9, B.CN.02.02, B.CO.01. B.DE.86.D3, B.KR.04.04,

B.NL.86.32, B.RU.04.04, B.TH.90.BK, B.TW.94.TW B.US.x.AC, B.UY.01.01,

B.YE.02.02, C.AR.01.AR, C.BR.98.98, C.BW.00.00 C.BW.98.98, C.ET.02.02, C.GE.03.03,

C.IL.99.99, C.KE.00.KE, C.TZ.01.BD C.TZ.01.BD, C.US.98.98, C.UY.01.TR, C.ZA.03.03,

C.ZA.98.TV, C.ZA.99.99 C.ZM.96.96, D.CM.01.01, D.KE.01.NK, D.KE.97.ML,

D.TD.99.MN, D.TZ.01.A2 D.UG.98.98, D.UG.99.99, D.YE.02.02, D.ZA.86.R4,

F1.BE.93.V, F1.BR.93.9 F1.FI.93.F, F1.FR.96.M, F2.CM.02.0, G.CM.01.01, G.ES.00.X5,

G.KE.93.HH G.NG.01.01, G.SE.93.SE, H.BE.93.VI, H.CF.90.05, J.SE.93.SE, J.SE.94.SE

K.CD.97.EQ, K.CM.96.MP, U.CD.90.90, U.GR.99.GR, 01_AE.JP.9, 01_AE.TH.9

01_AE.US.0, 01_AE.US.9, 03_AB.RU.9, 04_cpx.CY, 05_DF.BE.9, 07_BC.CN.9

07_BC.CN.x, 08_BC.CN.9, 10_CD.TZ.9, 14_BG.DE.0, 14_BG.ES.0, 19_cpx.CU

25_cpx.CM, 29_BF.BR.9, 31_BC.BR.0, A1C.KE.00, A1C.RW.92, A1C.TZ.01

A1C.TZ.01, A1C.TZ.97, A1C.UG.99, A1CD.KE.00, A1D.KE.00, A1D.KE.00 A1D.TZ.01,

A1D.TZ.96, A1D.TZ.96, A1D.UG.99, A1G.BE.94, A1G.KE.99 A1U.CM.01,

A1U.SE.93, A2C.ZA.98, A2D.KE.99, A2G.CD.97, AC.KE.97.M AC.ZA.04.0,

ACD.TZ.01, AD.US.99.9, AF2.CM.02, BC.CN.96.Y, BC.MM.99.m BC.MM.99.m,

BF.AR.00.A, BF.AR.99.A, BF.AR.99.A, BF.AR.99.A, BF1.BR.99. BF1.BR.99,

CD.KE.00.M, CD.TZ.01.A, CD.TZ.97.9, CD.UG.99.9, O.FR.92.VA  O.US.97.97, CPZ.CD.90,  CPZ.CM.01,  CPZ.GA.88,  Sample 5,  Sample 81  Sample 122

## APPENDIX 8: VIRAL LOAD RESULTS (COPIES/ML)

<400, <400, <400, 49700, 49300, <400, <400, <400, 2680, <400, <400, <400, <400, 1180, <400, <400, <400, <400, <400, <400, 71100, <400, <400, <400, <400, 5360, 183000, <400, <400, <400, 255000, 154000, <400, <400, 55300, <400, <400, <400, <400, <400, 88800, 83600, <400, <400, <400, <400, <400, <400, 104000, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, 42600, <400, <400, <400, <400, <400, <400, <400, <400, 25400, <400, <400, <400, 3730, <400, <400, <400, <400, <400, 542000, <400, 156000, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, 47500, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, 768, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, 17600, <400, <400, <400, <400, <400, <400, <400, <400, 364000, <400, 1010, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, <400, 275000, 49200, <400, >1000000, <400, <400, <400, <400, <400