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DRUG RESISTANCE MUTATIONS IN HIV PATIENTS ON ANTIRETROVIRAL THERAPY IN GHANA

By

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This thesis is submitted to the University of Ghana, Legon in partial fulfillment of the requirements for the award of **PhD Biochemistry degree**

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DECLARATION

I hereby declare that this thesis is a result of my own research work carried out under the supervision of Professors Sammy Tawiah Sackey, William Kwabena Ampofo and Alexander Kwadwo Nyarko and that references made to other people's work have been duly acknowledged. I further declare that this work has not been submitted in part or whole to any other Institution for the purpose of acquiring a degree.

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DEDICATION

For all that she was to me; I dedicate this thesis to the memory of my late mother

Letitia Afua Bertha Kwame

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LIST OF ABBREVIATIONS AND ACRONYMS

µg	Microgram
µl	Microlitre
3TC	Lamivudine
A	Adenine
ABC	Abacavir
ABI	Applied Biosystems Inc
AIDS	Acquired immune deficiency syndrome
APOBEC	Apolipoprotein B editing complex
APV	Amprenavir
ART	Antiretroviral therapy
ARVs	Antiretrovirals
ATP	Adenosine triphosphate
ATV	Atazanavir
AZT	Azidothymidine
BLAST	Basic local alignment search tool
Bp	Base pair
C	Cytosine
CA	Capsid
CCR5	Chemokine coreceptor 5
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CDC	Centers for Disease Control
cDNA	Complementary DNA
CRFs	Circulating recombinant forms
CT	Cut-off threshold
CXC4	Chemokine coreceptor 4
D4T	Stavudine
dA	Deoxyadenosine
DC	Dendritic cells
DDI	Didanosine
ddNTPs	Dideoxynucleotide triphosphates
dG	Deoxyguanosine
DLV	Delavirdine
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DR	Drug resistance
DRM	Drug resistance mutations
Ds	Double-stranded
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
EIA	Enzyme immune assay
ELISA	Enzyme linked immunosorbent assay
Env	Envelope
EtBr	Ethidium bromide
ETR	Etravirine

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FDA	Food and Drugs Administration
FI	Fusion inhibitor
FOS-APV	Fosamprenavir
FRET	Fluorescent resonance energy transfer
FTC	Emtricitabine
G	Guanine
Gag	Group-specific antigen gene
GALT	Gut-associated lymphoid tissue
Gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HIVDB	HIV database
HIVDR	HIV drug resistance
HLA	Human leukocyte antigen
HR 1	Hydrophobic region 1
HR 2	Hydrophobic region 2
HSS	HIV sentinel survey
IC	Inhibitory concentration
IDs	Identification numbers
IDV	Indinavir
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IN	Integrase
LPV/r	Lopinavir/Ritonavir
LTR	Long terminal repeat
M	Molar
MA	Matrix
MEGA	Molecular evolutionary genetics analysis
$MgCl_2$	Magnesium chloride
Ml	Millilitre
mRNA	Messenger ribonucleic acid
NACP	National AIDS/STI Control Programme
NBT	Blue tetrazolium
NC	Nucleoprotein
Nef	Negative regulatory factor
NFV	Nelfinavir
NK	Natural killer
NMIMR	Noguchi Memorial Institute for Medical Research
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
nRTIs	Nucleoside and nucleotide reverse transcriptase inhibitors
NVP	Nevirapine
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

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PHI	Primary HIV-1 infection
PI	Protease inhibitor
PIC	Pre-integration complex
Pol	Polymerase
PPi	Pyrophosphate
PR	Protease
pVL	Plasma viral load
qPCR	Quantitative polymerase chain reaction
rDNA	Recombinant DNA
Rev	Regulatory factor
RLS	Resource-limited setting
RNA	Ribonucleic acid
RPV	Rilpivirine
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
RTV	Rotonavir
SIV	Simian immunodeficiency virus
SP1	Spacer peptide 1
SQV	Saquinavir
Ss	Single-stranded
ssDNA	Single-stranded deoxyribonucleic acid
SU	Structural unit
T	Thymine
T-20	Enfuvirtide
TAE	Tris acetate EDTA
TAMs	Thymidine analogue mutations
Tat	Transactivator
Tev	Tat env rev
TBE	Tris borate EDTA
TDF	Tenofovir
TE	Tris-EDTA
TLR	Toll-like receptor
TM	Transmembrane
TPV	Tipranavir
TRIM	Tripartite motif
Tris	Trisaminomethane
U	Uracil
U.S	United States
UNAIDS	United Nations joint programme on HIV/AIDS
URFs	Unique recombinant forms
USA	United States of America
UV	Ultra violet
V	Voltage
Vif	Viral infectivity factor
VL	Viral load
Vpr	Viral protein r
Vpu	Viral protein u
WHO	World Health Organization

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ABSTRACT

Highly active antiretroviral therapy (HAART) is known to improve treatment in Human Immunodeficiency Virus (HIV)-infected patients but the emergence of drug resistance is an obstacle in the effective management of HIV infection and Acquired Immune Deficiency Syndrome (AIDS). Plasma viral load monitoring is the gold standard used in high-income countries for monitoring treatment but it is not available in many resource-limited settings. Although limited viral load testing is now available in Ghana, viral load is not routinely used for monitoring majority of patients on HAART. Physicians in Ghana therefore depend mainly on CD4 counts and clinical symptoms to monitor treatment. Thus, a significant proportion of patients may suffer virologic failure while continuing to take first-line antiretroviral therapy (ART). This may encourage the development and accumulation of drug resistance mutations and compromise future treatment efforts.

The aim of this study was to investigate the presence of HIV drug resistance mutations in patients on ART in Ghana, relate these mutations to treatment regimens in order to inform policy on ART monitoring and patient management in the country.

Venous blood was obtained from 338 patients on ART from the Korle-Bu Teaching, St. Martin de Porres, Atua Government and Kumasi South hospitals in Ghana. Personal information and ART history of the patients were also collected using a sample collection form. The CD4 counts and viral loads of patients were determined. HIV ribonucleic acid (RNA) and proviral deoxyribonucleic acid (DNA) were extracted from the plasma and peripheral blood mononuclear cells (PBMC), respectively. The HIV protease and reverse transcriptase genes were amplified from the RNA and DNA by polymerase chain reaction.

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The positive amplification products were sequenced and analyzed for drug resistant mutations using the Stanford HIV Drug Resistance Database.

The mean age of patients was 42 years and 72% of patients were female. The mean CD4 counts increased from 161cells/µl at start of therapy to 454cells/µl at time of sampling. Only 7% of patients had detectable viral loads at time of sampling. Most of the patients (87%) were on first-line regimen. Physicians rating showed that 86% of patients were doing well and the rest were either not doing so well (11%) or were failing (3%). The reverse transcriptase gene was successfully sequenced from 65 (19%) and 99 (29%) of plasma and PBMC respectively while protease gene was successfully sequenced from 54 (16%) of plasma and 76 (23%) of PBMC. Out of these, 46 % and 49% of the plasma sequences had nucleoside reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance mutations respectively. From PBMC, 25% and 26%, respectively had NRTI and NNRTI mutations. Protease inhibitor (PI) resistance mutations were found in 28% of plasma and 9% of PBMC sequences. The most common NRTI mutation found was M184V and that of NNRTI was K103N. Thymidine analogue mutations (TAMs) including M41L, D67N, T215Y/F, L210W and K219E/Q were mostly found in patients on second-line regimen. Protease inhibitor mutations were mainly found in patients on second-line regimen and they included M46I, V82I and N88S. Similar resistance profiles were observed in paired sequences from plasma and PBMC of the same patients.

The results showed that even patients who were doing well, based on their CD4 counts, viral load and physicians assessment, were harbouring drug resistance mutations including TAMs that could render the NRTIs ineffective. The results suggest that CD4 count is an insufficient marker for monitoring treatment since it continues to increase even in the presence of drug

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resistance mutations. Patients on ART in Ghana may therefore not be deriving optimal benefits from treatment because of the current monitoring system. Therefore drug resistance testing will be useful before switching regimens in order to decide drugs in the new regimen. This will reduce the accumulation of multi-nucleoside and thymidine analog mutations in patients and preserve future drug options.

The study has provided vital drug resistance data to guide policy on ART monitoring in Ghana, improved protocols for HIV genotyping at the Virology Department of the NMIMR and built capacity for drug resistance analyses of patients that fail ART in Ghana.

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CHAPTER ONE

1.0 INTRODUCTION

The human immunodeficiency virus (HIV) is the causative agent of the acquired immune deficiency syndrome (AIDS). Since 1981 when the first cases of AIDS were reported, the pandemic has claimed the lives of many adults and children throughout the world. The biggest disease burden is in sub-Saharan Africa where majority (about 68%) of HIV-infected people live. AIDS is known to have killed 30 million people since 1981while about 34 million people are currently living with HIV in the world (WHO, 2012; amfAR 2012).

Since its approval for use in humans, about three decades ago, antiretroviral drugs have significantly improved the life of HIV-infected persons by slowing disease progression and reducing morbidity and mortality. Five distinct classes of antiretroviral drugs are currently used to treat HIV infection. These agents act at different stages in the replication cycle of the virus (Clavel and Hance, 2004). The entry or fusion inhibitors block the penetration of HIV into the target cells. The nucleos(t)ide reverse transcriptase inhibitors (NRTIs) act as DNA-chain terminators and inhibit reverse transcription of the viral RNA genome into DNA. The non-nucleoside reverse-transcriptase inhibitors (NNRTIs) bind in a hydrophobic pocket close to the active site of reverse transcriptase, and restrict the conformational change needed for the catalytic activity of the RT. The integrase inhibitors inhibit the action of integrase, the enzyme responsible for incorporating HIV DNA into the host cell DNA for transcription of the viral mRNA. The protease inhibitors (PIs) target and inhibit the viral protease, the enzyme required for the cleavage of precursor proteins (*gag and gag–pol*) into functional proteins, allowing the final assembly of the inner core of viral particle (Clavel and Hance, 2004).

Unfortunately, the effectiveness of antiretroviral therapy can be markedly reduced by the emergence of drug resistance. Clinical experience with all the drugs has shown that the virus is able to easily evade the antiviral effects of the drugs, when administered as monotherapy, through the rapid accumulation of amino acid mutations in the target proteins (Hartman and Buckheit, 2012). These mutations arise due to the high rate of replication of the virus coupled with the error- prone nature of the reverse transcriptase enzyme (Shafer *et al,* 2000). These lead to the generation of quasispecies of the virus in an infected person. The drug-resistant variants are thought to exist prior to the use of the drugs and are selected for due to the drug pressure. Some HIV variants are known to exhibit intrinsic or “primary” resistance to some antiretroviral agents, but most drug resistance develops as a result of exposure to the drugs (Shafer *et al,* 2000; Arts and Hazuda, 2012). Even in the presence of virologic suppression, during therapy of HIV infection, drug resistance can still occur due to residual virus replication. HIV may replicate in body sites lacking adequate exposure to the antiretrovirals (ARVs) resulting in selection of the drug-resistant mutants (Martinez-Picado *et al.* 2000).

Combinations of three drugs from at least two classes, called highly active antiretroviral therapy (HAART), are now used for the treatment of HIV infection. HAART regimens generally comprise three antiretroviral drugs usually two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI) [Yeni *et al,* 2002]. The use of drugs from different classes with different mechanisms of action helps to control the development of drug resistance. However, drug resistance can develop during HAART and against more than one drug within a class. In such cases, the emergence of drug-resistant variants complicates treatment by rendering current ARVs ineffective and potentially limiting future remedies (Kantor *et al*, 2004; Sigaloff *et al,* 2011). As treatment efforts intensify, drug resistance has become common as

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both a cause and a result of virologic treatment failure and incomplete virus suppression (Kantor *et al,* 2005). HIV drug resistance testing is therefore recommended for persons on treatment based on the mounting evidence that it improves the pharmacotherapy of HIV infection (Cohen *et al,* 2002).

Most of the current knowledge of HIV drug susceptibility and resistance, and interpretations of genotypic changes in HIV reverse transcriptase (RT) and protease (PR), are based on data obtained from HIV-1 subtype B viruses prevalent in North America, Western Europe, and Australia. Worldwide, however, the majority of people with HIV are infected with non-B subtypes, which differ from subtype B by as much as 30% in *envelope* gene and 15% in *polymerase* gene (Osmanov *et al,* 2000). Thus, there is increasing need to generate data on sustained viral suppression and the evolution of drug resistance with antiretroviral drugs for the non-B subtypes prevalent in sub-Saharan Africa which bears the greatest disease burden.

Antiretroviral therapy (ART) was introduced in Ghana in 2003 on a pilot basis. The programme has been scaled up from just 2 sites in 2003 to 160 sites at the end of 2011 (NACP, 2011). Ideally, all patients receiving antiretroviral therapy (ART) should undergo regular viral load testing in order to assess treatment failure or success. This is however not the case in Ghana since viral load testing is expensive and requires dedicated laboratory with highly trained staff. Immune status determined via CD4 counts are easier to measure and has been used in combination with clinical symptoms to inform decisions on switching ART regimen. The World Health Organization (WHO) guidelines recommend a CD4 count decline of 50% from the previous peak, or a one-third decline in the previous six months, as a trigger for changing treatment in resource limited settings where viral load testing is not routinely done (WHO, 2006). The use of mainly CD4 counts and clinical symptoms to monitor

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treatment outcomes is a limitation to Ghana's ART program since these markers do not provide enough information on drug resistance mutations and other causes of treatment failure. Despite using the WHO recommendations (WHO, 2006), it is likely that persons who have not yet attained the 50% drop in CD4 counts may still harbor drug resistance mutations selected for by the ARVs used over the period. Maintaining such persons on the same ARVs in the presence of the drug resistance mutations eventually renders the treatment ineffective. Furthermore, future therapy with drug combinations containing some of the drugs that the virus is resistant to are compromised and this limits the choice of alternate regimens (Sigaloff *et al*, 2011). Also, limited ARVs from the reverse transcription and protease inhibitor drug classes are procured and used in Ghana and their use can be optimized if HIV drug resistance data is available. In Ghana, the current practice for HIV-infected patients who have failed first-line therapy (consisting two NRTI and one NNRTI) is to switch them to a second-line therapy, consisting two NNRTI and one PI (NACP/MoH/GHS, 2010). This decision to switch the drug regimen is mostly based on clinical symptoms and significant drops in CD4 count. The NRTIs applied in the second-line regimen are usually different from those used in the first-line regimen. However, some of the NRTI drug resistance mutations acquired during first-line therapy may have cross-resistance to some of the drugs in the second-line regimen (Shafer et al, 2000).There is therefore a need to analyze the RT and PR genes in persons on treatment in order to characterize mutations associated with resistance to antiretroviral drugs.

HIV drug resistant testing protocols usually depend on plasma for the amplification of viral genes (Sarmati *et al,* 2002; Kabamba- Mukadi *et al,* 2010). However, this approach provides information mainly on circulating major viral population at the time of analysis. Analyzing proviral DNA from the peripheral blood mononuclear cells (PBMC) is an alternative approach (Kabamba-Mukadi *et al,* 2010). This could provide additional information on other

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HIV variants that the persons might have haboured over time and which are likely to resurface in immunological failure or drug interruption (Noe *et al,* 2009 and Rangel *et al,* 2009).

This study therefore looked at drug resistance mutations from both plasma and peripheral blood mononuclear cells of persons on ART. It sought to provide data on the drug-resistance mutations at the time of sampling and over the treatment period. This information serves to provide a means to review progress of the patients on ART, redesign treatment regimens and enable appraisal of the policy on ARV use in Ghana.

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1.1 AIM

The main aim of this study was to investigate the occurrence and type of HIV drug resistance mutations in patients on ART in Ghana and relate these mutations to treatment regimens in order to inform policy on ART monitoring and patient management in the country.

The Specific Objectives were:

- To identify HIV-infected persons on ART for a minimum of 6 months and document their drug histories
- To determine the HIV serotype, and measure the immunologic and virologic markers
- To amplify and sequence the protease and reverse transcriptase genes to find drug resistance mutations
- To relate the mutation patterns observed to the drug histories of the patients by comparing data for patients on first-line to those on second-line regimens
- To compare drug resistance mutations in plasma to those in peripheral blood mononuclear cells

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Human Immunodeficiency Virus (HIV)

2.1.1 General Introduction

The human immunodeficiency virus (HIV) is a lentivirus and belongs to the family Retroviridae. HIV is an enveloped virus and has two positive sense RNA strands as its genetic material (Santos and Soares, 2010). Retroviruses are characterized by the possession of the enzyme reverse transcriptase, which allows viral RNA to be transcribed into DNA and incorporated into the host cell genome by the help of another enzyme, integrase. Glycoproteins on the surface of HIV bind to the target cell and help the virus to enter the cell. The virus encodes multiple regulatory proteins, which control the life cycle and viral expression (Frankel and Young, 1998).

2.1.2 Life Cycle

As shown in Fig. 1, when the virus attaches itself to the host cell membrane, there is fusion of the viral envelope with the cell membrane resulting in the virus entering the cell. Once inside the cell, there is uncoating and the viral core undergoes a slow dissolution process (Arts and Hazuda, 2012). The process ensures the protection of the viral RNA but permits access to deoxyribonucleotide triphosphates (dNTPs) necessary for reverse transcription and proviral DNA synthesis. The single-stranded RNA genome is converted into double-stranded DNA by the help of reverse transcriptase (RT). Reverse transcriptase is a multifunctional enzyme with RNA-dependent DNA polymerase, RNase-H, and DNA-dependent DNA polymerase activities (Hughes and Hu, 2011). These enzyme activities are all required to convert the single-stranded HIV RNA into a double-stranded DNA (Hughes and Hu, 2011).

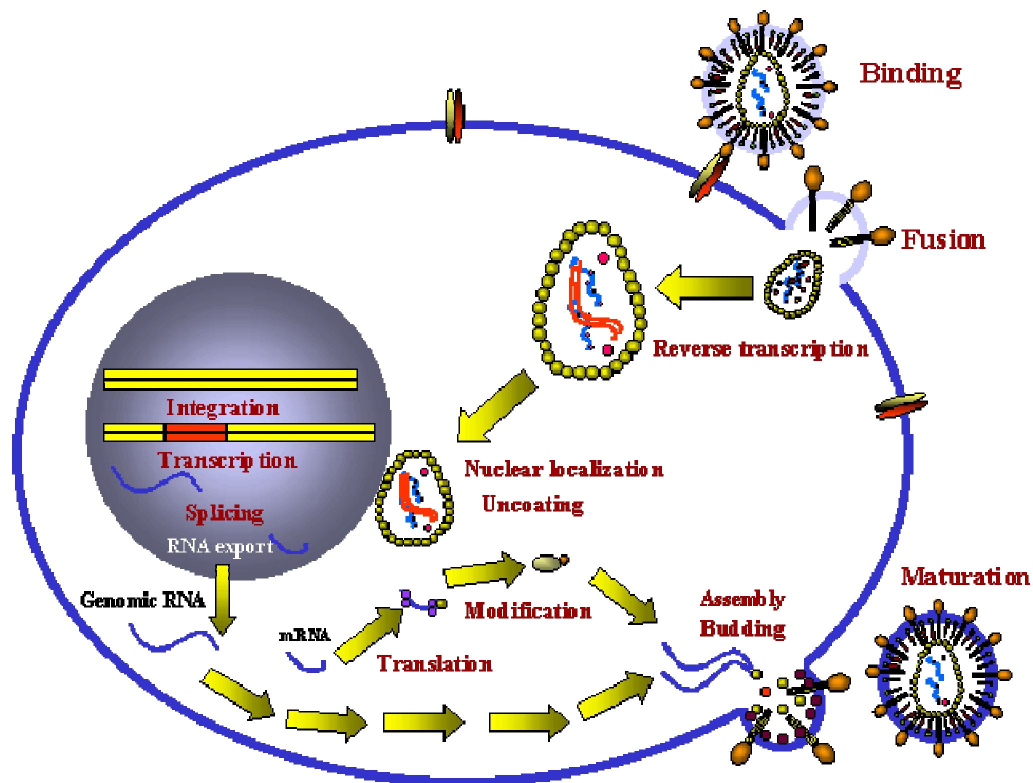

Figure 1: A schematic diagram of the life cycle of HIV showing the various steps involved in the replication of the virus in a human host
(Source: http://pathomicro.med.sc.edu/lecture/hivstage.gif)

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However, reverse transcriptase does not have a proofreading activity and so makes errors when copying the viral RNA into DNA. At the end of reverse transcription, a viral pre-integration complex (PIC) is formed. The PIC, which is made of viral as well as cellular components, is transported to the nucleus where the second essential HIV enzyme, integrase, catalyzes the integration of the viral DNA into the host DNA (Craigie and Bushman, 2011). Integration of the HIV DNA is required to maintain the viral DNA in the infected cell and is essential for expression of HIV mRNA and viral RNA (Arts and Hazuda, 2012) to ensure the replication of the virus within the host. Thus, the virus hijacks the cellular machinery for its own replication. In the final step of the replication cycle, the viral polypeptide is cleaved by the viral protease during proteolysis to produce the viral proteins needed to make an infectious viral particle (Sundquist and Kra¨usslich 2011). The viral RNA and proteins are assembled and packaged into a new virion that buds off the host cell membrane to infect a new cell and continue the process of replication.

2.1.3 Genome Organization

The HIV genome (Fig. 2), which is approximately 9.7Kbp, has several major genes that are common to all retroviruses and some accessory genes that are unique to HIV (Mushahwar, 2007). The *gag* (group-specific antigen) gene encodes the nucleocapsid, the *pol* (polymerase) gene encodes the viral enzymes and the *env* (envelope) gene encodes the envelop proteins. The functions of these genes are further explained below:

i. *gag*: codes for the gag polyprotein, which is processed during maturation to MA (matrix protein, p17); CA (capsid protein, p24); SP1 (spacer peptide 1, p2); NC (nucleocapsid protein, p7); SP2 (spacer peptide 2, p1) and p6.

ii. *pol*: codes for viral enzymes, reverse transcriptase, integrase, and HIV protease.

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iii. *env*: codes for gp160, the precursor to gp120 and gp41, proteins found as spikes on the viral envelope, which enables the virus to attach to and fuse with target cells.

iv. *tat* (transactivator), *rev* (regulatory factor) and *vpr* (viral protein r) are transactivators of gene expression.

v. *vif* (viral infectivity factor), *nef* (negative regulatory factor) and *vpu* (viral protein u) are regulatory proteins.

vi. *tev*: has been described in a few HIV isolates. It is a fusion of parts of the *tat, env*, and *rev* genes, and codes for a protein with some of the properties of *tat*, but little or none of the properties of *rev*.

All these genes may be altered by mutation and with exception of *tev,* they all exist in all known variants of HIV.

The *pol* gene is of particular interest to this study since it encodes the viral enzymes; reverse transcriptase, integrase and protease. These enzymes are the targets of most of the current antiretroviral agents (Arts and Hazuda, 2012). The *pol* gene is therefore analyzed to determine HIV- related drug resistance mutations.

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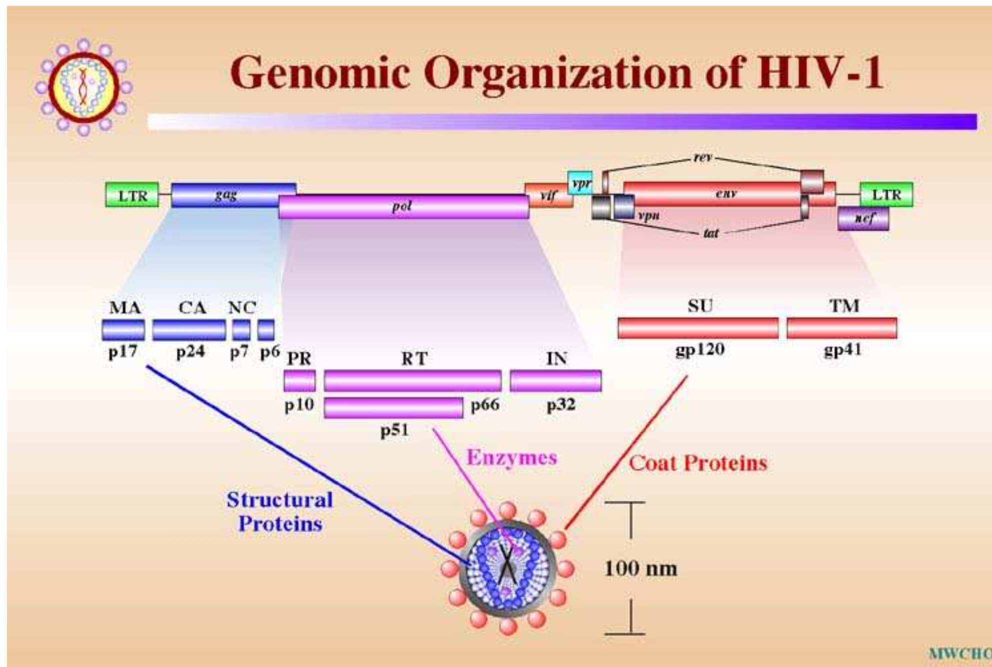

Figure 2: A genome map of HIV-1 showing the various genes that make up the virus and their corresponding proteins. MA-matrix; CA- capsid; NC-nucleocapsid; SU-structural unit; TM-transmembrane; PR-protease; RT-reverse transcriptase; IN-integrase; LTR-long terminal repeat
(Source: http://www.stanford.edu/group/virus/retro/2005gongishmail/HIV-1b.jpg).

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2.1.4 Genetic variability

HIV is known to have a very high genetic variability (Kantor *et al,* 2005; Buonaguro *et al,* 2007). This diversity is due to its extremely high replication rate and the error-prone nature of its reverse transcriptase. These two factors result in the generation of many variants of HIV even within a single infected person per day. In addition, different strains of HIV are able to infect one cell and this may lead to recombination between strains and the generation of recombinant forms.

Two main types of HIV have been characterized and known to cause AIDS. Human immunodeficiency virus type I (HIV-1) was first recorded in the United States and subsequently in Europe and the eastern and central parts of Africa (CDC, 1981). Human immunodeficiency virus type 2 (HIV-2) was isolated in 1986 from two HIV-1 seronegative individuals from West Africa with AIDS (Clavel *et al*., 1986). Whereas HIV-1 infection is worldwide, that of HIV-2 appears to be restricted to West Africa (Temin, 1993; Ho *et al*., 1995). Both HIV-1 and HIV-2 cause AIDS in West Africa but HIV-2 has been shown to be sexually and perinatally less transmissible than HIV-1. Also, the rate of disease progression of HIV-2 is much slower than HIV-1 (Pepin *et al*., 1991; Whittle *et al*., 1994; Marlink *et al*., 1994). The basis of these differences in the natural history of both infections remains unclear and may result from the host alone or in association with viral factors (Barin *et al*., 1985; Kanki *et al*., 1986; Clavel *et al*., 1986; Schim van der Loeff *et al.,* 1999).

Both HIV types are distinct; HIV-1 is known to be related to simian immunodeficiency virus (SIV) from chimpanzees (Gao *et al,* 1999) while HIV-2 is closely related to SIV from sooty mangabeys (Gao *et al,* 1992).

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HIV-1 has been characterized into four groups (Gao *et al,* 1999; Van Heuverswyn *et al*, 2006). These are groups M, O, N and P. Group M is the major HIV-1 group and is further divided into subtypes A, B, C, D, F, G, H, J, K and circulating recombinant forms (CRFs). These subtypes are different from each other by 10-12% in nucleotides and 5-6% in their amino acids (Kantor *et al,* 2005). Group N (new, non-M, non-O) was identified in a few individuals in Cameroon (Simon *et al,* 1998; Ayouba *et al,* 2000). Group O (outlier) contain diverse viruses but it is not commonly found. Viruses belonging to this group are thought to be transmitted to humans by gorillas (Van Heuverswyn *et al*, 2006). The virus classified as belonging to HIV-1 group P was isolated from a Cameroonian woman and it is closely related to SIV gor (Plantier *et al,* 2009. The group M viruses are responsible for most of the HIV-1 epidemic in the world (Buonaguro *et al,* 2007; Santos and Soares, 2010).

HIV-2 has been characterized into 7 main clades now called groups (www.hiv.lanl.gov, 2013). These groups (A, B, C, D, E, F and G) used to be known as subtypes but are now called groups because it is thought that each of these types resulted from separate introductions of SIV into the human populations. HIV-2 Groups A and B are known to be responsible for most of the HIV-2 infections (Lemey *et al*, 2002; Trevin *et al,* 2011). Recombination is rare in HIV-2; the first CRF of HIV-2 (CRF01_AB) was described in 2010 (Ibe *et al*, 2010).

The differences among HIV types and subtypes could have implications for clinical management. Most of our current knowledge about antiretroviral drugs, their development and mechanisms of action and the mechanisms of viral resistance to these drugs are gained from studies using HIV-1 subtype B virus.

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This virus subtype occurs in North America, Western Europe and Australia and is responsible for only 10% of the global infections (Kantor *et al.*, 2005). Majority of HIV infections are known to occur in sub Saharan Africa where other HIV-1 subtypes are predominant. Some previous studies have looked at drug resistance in HIV subtypes besides subtype B (Kantor *et al*, 2005; Soares et al, 2007; Martinez-Cajas *et al,* 2008; Martinez-Cajas *et al,* 2009). Studies in Ghana are however limited and involved mainly patients naïve to ART (Kinomoto *et al,* 2005; Sagoe *et al,* 2007; Delgado *et al*, 2008), thus there is a need to study viruses occurring in Ghana and in ART-experienced patients.

2.1.5 Pathogenesis

Human immunodeficiency virus (HIV) infection is generally characterized by an acute phase of intense viral replication and dissemination to lymphoid tissues; a chronic, often asymptomatic phase of sustained immune activation, viral replication and establishment of stable tissue reservoirs; and an advanced phase of marked depletion of CD4+ T helper cells that leads to acquired immune deficiency syndrome (AIDS). Figure 3 shows the different phases of HIV infection, the cell types involved and the timelines associated with the various events.

Major insight into HIV transmission and each phase of infection have been gained from studies on blood and tissue specimens obtained from HIV-infected individuals, as well as from studies using the simian immunodeficiency virus (SIV)-infected rhesus monkey model of AIDS (Moir *et al,* 2011). Immediately after infection, there is an early burst of viremia and rapid dissemination of the virus to lymphoid organs, particularly the gut-associated lymphoid tissue (Moir *et al,* 2011). Although there are vigorous cellular and humoral immune responses during primary HIV infection, the virus succeeds in escaping immune-mediated clearance.

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Therefore, once infection once established, is never eliminated completely from the body (Fauci and Lane, 2005). The course of disease and its deleterious effect on the human host are influenced by the ability of the virus to rapidly replicate in CD4+ cells, maintain HIV reservoirs in tissues and cells and the action of restriction host factors.

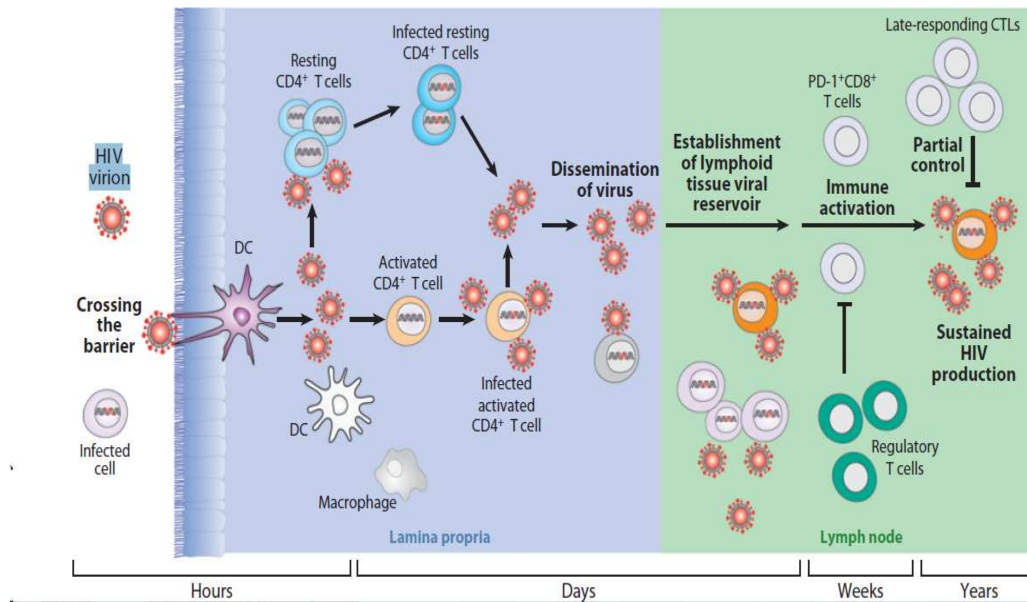

Figure 3: Phases of infection following exposure to human immunodeficiency virus (HIV). Infection begins with transmission across a mucosal barrier. Early propagation occurs in partially activated CD4+ T cells, followed by massive propagation in activated CD4+ T cells of the gut-associated lymphoid tissue lamina propria. The virus then spreads to other secondary lymphoid tissues where stable tissue viral reservoirs are established. Immune response is usually delayed and provides only partial control of viral replication. (Adapted from Moir *et al,* 2011)

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2.1.5.1 Rapid Replication of HIV in CD4+ cells

It is difficult to identify infected individuals soon after exposure therefore the early events leading to HIV infection in the rectal mucosa or genital tract are not well understood (Moir et al, 2011). *In vivo* models of simian immunodeficiency virus (SIV), epidemiological studies and *ex vivo* models have provided some clues into the transmission of HIV (Zhang, 1999; Haase 2005; Hladik and McElrath, 2008). Partially activated CD4+ T cells of the genital mucosa are the first targets of productive viral replication within a one week after exposure (Fauci, 2007). This is followed by the local propagation of SIV in the less abundant but more susceptible activated CD4+ T cells. As shown in Figure 3, the virus then migrates to the gut-associated lymphoid tissue (GALT) and causes massive depletion of memory CD4+ T cells in the intestinal lamina propria (Guadalape *et al.*, 2003; Brenchley *et al.*, 2004; Mehandru *et al*, 2004).

2.1.5.2 Establishment and maintenance of HIV reservoirs

Human immunodeficiency virus tends to 'hide' and persist in certain tissues and cells of an infected person and remain there until there is a trigger for replication (Moir et al, 2011). Whilst in hiding, the virus continues to replicate, although at a slower pace, and mutates during the process. Therefore the viruses that are produced during reactivation of the reservoirs are mostly the drug-resistant viruses (Richman *et al,* 2009). The rapid establishment and persistence of various HIV reservoirs remain two of the most important impediments to achieving complete eradication of the virus in infected individuals, even in an era of clinically effective ART (Richman *et al*, 2009).

Latent reservoirs can be divided into two main categories (Moir *et al*, 2011): (*a*) lymphoid tissues that provide HIV with an abundance of target cells, close contacts for efficient cell-to-

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cell propagation, and reduced drug penetration; and (*b*) cellular reservoirs that consist mainly of CD4+ T cells that are highly susceptible to HIV replication when activated but can also carry latent virus.

2.1.5.3 Host restriction factors

Replication of HIV and other retroviruses may be controlled at the intracellular level by intrinsic host factors that function in a virus-specific and, in many cases, species-specific manner (Strebel *et al* 2009). Such restriction factors include members of the apolipoprotein B editing complex (APOBEC) cellular deaminase family such as APOBEC3G (A3G) and APOBEC3F (A3F). These proteins modify cytidine by removing the amine group thus converting it to uridine in the transient (-) ssDNA replication intermediate. This change is later reflected as G-to-A changes in the (+) strand (Mangeat *et al,* 2003; Zhang *et al,* 2003; Amoedo *et al*., 2011).

Apolipoprotein B editing complex group of proteins are packaged within viral particles and, upon subsequent entry into a new target cell, induces dG-to-dA hypermutations in the nascent proviral DNA. They (A3G and A3F) target dinucleotide motifs within DNA and causing GG to AG and GA to AA substitutions in proviral DNA respectively. These proteins (A3G and A3F) exhibit potent anti-HIV-1 activity (Sheehy *et al.*, 2002; Liddament *et al.,* 2004; Bishop *et al.,* 2006) and are expressed in lymphocytes, the major target cells for HIV-1 infection (Liddament *et al*., 2004; Wiegand *et al.,* 2004). The HIV accessory protein *vif* is known to counter the crippling effect of APOBEC3G/3F on HIV DNA (Chiu *et al.,* 2005).

A second intrinsic cellular restriction factor is Trim-5α, a member of the tripartite protein family. Trim-5α targets the retrovirus capsid protein for degradation (Strebel *et al.,* 2009).

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The activity of Trim-5α is highly dependent on species-specific compatibility. HIV *Gag* escapes the effects of human Trim-5α and SIV *Gag* escapes the effects of most simian Trim-5α (Moir *et al,* 2011. The inability of HIV to infect non human primates is in part due to the inhibitory effects of simian Trim-5α on HIV (Moir *et al.,* 2011).

The two types of restriction factors (APOBEC and Trim-5α) are considered to be part of the innate immune response. Their effect could be harnessed to reduce the early deleterious effects of HIV infection and promote a more effective adaptive immune response against HIV. Receptors and HLA molecules associated with natural killer cell function and members of the toll-like receptor family associated with the various antiviral IFN-α-based pathways have also been associated with slower disease progression (Moir *et al.,* 2011).

2.1.6 The Course of HIV Infection and Disease

Majority of HIV-infected individuals experience an acute HIV syndrome approximately two to four weeks following the transmission of the virus (Fig. 4). This is defined as flu-like clinical manifestations associated with high plasma viremia and often fever and lymphadenopathy. Other symptoms have been reported but their severity varies from person to person (Gurunathan *et al.,* 2009). During this early phase, HIV often replicates extremely aggressively due to the lack of an immune response and viral loads reach levels as high as 10 million copies per milliliter (Piatak *et al*., 1993; Little *et al*., 1999). Without ART, plasma viremia typically peaks at three to four weeks post exposure (Little *et al.,* 1999; Fiebig *et al*., 2003), then declines spontaneously for several months before reaching a steady state or viral set point. The level of the viral set point is an important determinant of the rate of disease progression in HIV-infected individuals who are not treated with ART (Mellors *et al.,* 1996).

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Approximately 12 weeks after transmission, neutralizing antibodies begin to rise and evolve. However, this result of immune response is inadequate, too late and too narrow. The neutralization-sensitive virus is quickly replaced in succession by the neutralization-resistant variants (Wei *et al*., 2003; Richman *et al.,* 2003). The envelope of the escaping population of HIV tend to be more highly glycosylated, preventing the binding of neutralizing antibodies and promoting viral persistence (Wei *et al.,* 2003).

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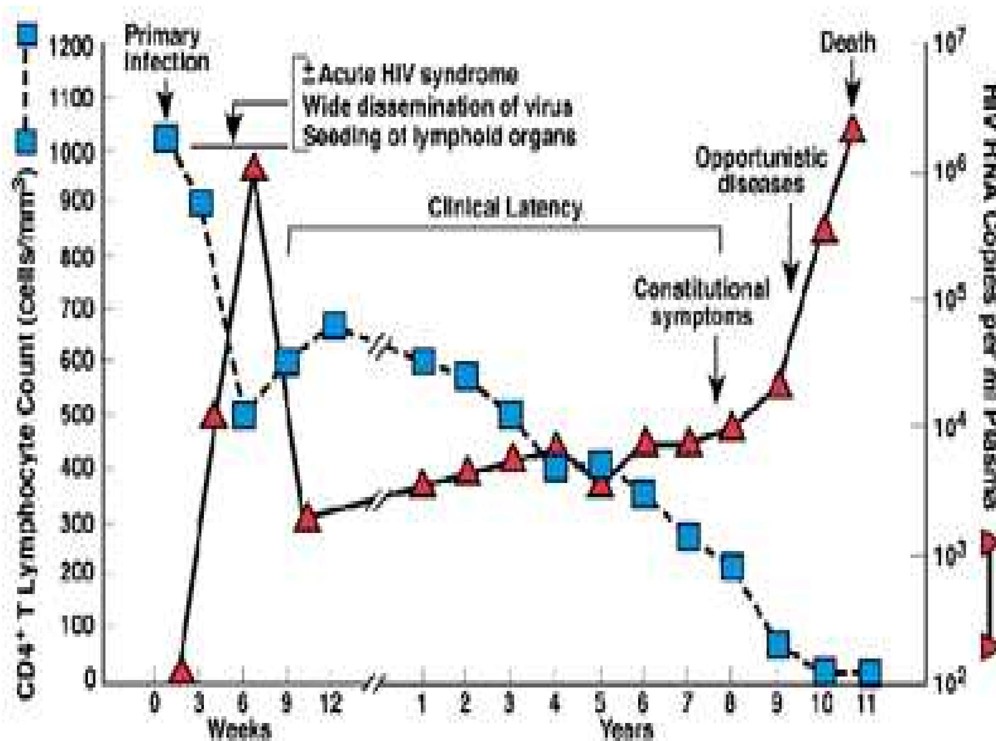

Figure 4: The natural course of HIV infection in the absence of antiretroviral therapy. (Source: Modified from Fauci *et al.,* 1996).

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Early CD8+ T cell response also contributes to the decline in HIV plasma viremia during the acute phase of infection (McMichael *et al.,* 2010). However, there is rapid evolution characterized by mutations in epitopes recognized by CD8+ T cells leading to escape from CD8+ T cell epitopes similar to that of neutralizing antibodies (Goonetilleke *et al.,* 2009). This occurs earlier than the appearance of neutralizing antibodies, continues throughout the course of disease and contributes to viral persistence.

HIV targets and destroys CD4+ T cells, a major constituent of the immune system. It also induces immunologic dysfunction of CD8+ T cells, B cells, natural killer (NK) cells, and non-lymphoid cells through mechanisms that include increased cell turnover, activation, differentiation, and homeostatic responses (Moir *et al,* 2011). All these factors lead to qualitative changes within each immune cell population and ultimately affect the strength of the immune system. A progressive depletion of CD4+ T cells occurs in the majority of HIV-infected individuals who remain untreated and can also occur in individuals receiving ART. In most patients on ART, there is dramatic increase in CD4+ T cell counts. However, some individuals have low CD4+ T cell counts throughout treatment even on virologically-suppressive ART (Moir *et al*, 2011).

2.1.7 Diagnosis of HIV Infection

Laboratory tests to detect the presence of HIV in infected persons use blood, serum, plasma or mucosal swabs. The tests may detect viral antibodies, antigens, viral nucleic acids by PCR or grow the virus in culture (Fearon, 2005). The tests could be further grouped into serological and molecular methods.

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2.1.7.1 Antibody tests

The enzyme-linked immunosorbent assay (ELISA), or enzyme immunoassay (EIA) are the most commonly used assays because they have high sensitivity and can easily be automated and used for bulk testing (Fearon, 2005). Enzyme immunoassays have evolved from first-generation EIAs (e.g. Vironostika HIV-1 Microelisa System) and second-generation EIAs (e.g. Genetic Systems rLAV EIA; Bio-Rad Laboratories) that detect IgG antibodies against HIV-1 to third-generation EIAs. The third-generation EIAs use "antigen sandwich" techniques and detect both IgG and IgM antibodies against HIV-1. There are kits that detect antibodies against HIV-1 and HIV-2 e.g. HIVAB HIV-1/HIV-2 (rDNA) EIA (Abbott Laboratories) and others that detect HIV-1/HIV- 2 Plus O antibodies. Fourth-generation EIA, identify HIV infection even earlier because they detect both HIV antibody and p24 antigen (Branson, 2007).

In an ELISA test, a person's serum is diluted and applied to a plate with wells pre-coated with HIV antigens. If antibodies to HIV are present in the serum, they may bind to these HIV antigens. The plate is then washed to remove all other components of the serum. A specially prepared "secondary antibody" — an antibody that binds to human antibodies - is then applied to the plate, followed by another wash. This secondary antibody is chemically linked in advance to an enzyme. Thus the plate contains enzyme in proportion to the amount of secondary antibody bound to the plate. A substrate_for the enzyme is applied, and catalysis by the enzyme leads to a change in color or fluorescence. ELISA results are reported as absorbance of the colour produced at a specific wavelength.

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2.1.7.1.1 Rapid HIV tests

Rapid HIV tests are single-use EIAs that contain all necessary reagents and yield results in less than 30 minutes. They usually contain antigens made from intact viruses and therefore detect antibodies to most types of HIV (Bulterys *et al*, 2004). These tests are useful to screen people since results are quickly available to enhance treatment decisions. Rapid tests can be used to determine the HIV status of pregnant women in labour to decide on antiretroviral therapy initiation to prevent mother-to-child transmission (Bulterys *et al,* 2004; Jamieson *et al*, 2007). An index patient or sample can also be tested after an occupational exposure, to allow prompt initiation of antiretroviral prophylaxis to the exposed health care worker or researcher (Bulterys *et al*, 2004; Panlilio *et al*, 2005). In high-volume, high-prevalence settings, such as emergency departments, rapid tests can make testing more feasible and generate results quickly enough to influence clinical management (Lyss *et al,* 2007). All samples tested positive for HIV antibody on EIA must be repeated on another EIA and confirmed on a Western blot (Dodd and Feng 1990; Fearon, 2005).

2.1.7.1.2 Western Blot

Western blot assays detect antibodies to HIV but unlike the ELISA, the antibodies are detected to individual proteins (core and envelope) of the virus that have been purified and fixed onto a membrane (Nuwayhid, 1995; Jackson *et al,* 1997). Some commercially prepared Western blot test kits contain the HIV proteins already on a cellulose acetate strip. The Inno-lia HIV1/II Score (Innogenetics, Belgium), for example, detects antibodies to specific recombinant proteins and synthetic peptides from HIV-1 and HIV-2 and a synthetic peptide from HIV group O coated as discrete lines on a nylon strip with plastic backing. Five HIV-1 antigens: *sgp*120 and *gp* 41, which detect specific antibodies to HIV-1 and p31, p24 and p17,

which may cross react with antibodies to HIV-2 and HIV-1 group O peptides were included. HIV-2 specific antigens *gp* 36 and *sgp* 105 are also applied to the strip to detect HIV-2.

During western blot assays, the diluted serum is applied to the membrane and antibodies in the serum bind to the HIV proteins. Coloured bands are developed on the strip when an alkaline phosphatase labeled antihuman IgG is added followed by a colour developing solution. The bands, representing the antigens present in the person's blood are visualized and judged for positivity based on the manufacturer's protocol (Jackson *et al*, 1997; Fearon, 2005).

The interpretation of western blot results varies from one manufacturer to the other but it is generally determined by the number and type of viral bands that are present on the strip after running the assay (Dodd and Fang, 1990; Nuwayhid, 1995; Fearon, 2005). If no viral bands are detected, the result is negative. If at least one viral band for each of the *gag, pol*, and *env* gene-product groups is present, the result is positive. Tests in which less than the required number or type of viral bands is detected are reported as indeterminate (Nuwayhid,, 1995).

2.1.7.2 Antigen tests

The viral capsid or core antigen (p24 antigen) test detects the presence of p24, the capsid protein of HIV. The assay uses p24 antibody to capture the p24 antigen in the person's blood sample (Darr *et al,* 1991). The test involves adding p24-specific monoclonal antibodies to the blood sample to be tested. If p24 protein is found in the blood, it will bind to the monoclonal antibody. An enzyme-linked antibody will then bind to the antigen-antibody complex and cause a color change. The p24 antigen test is not recommended for general diagnostics due to low sensitivity and its ability to work only during the short period after infection before antibodies are produced by the body (Allain *et al,* 1986).

2.1.7.3 Qualitative PCR

Polymerase chain reaction (PCR) assays amplify viral nucleic acid from a person's blood. These assays are sensitive and specific and can pick very small numbers of viral particles (Jackson *et al*, 1993). The assay uses specific primers that target specific HIV genes such as *gag*, *env* or *pol*. Polymerase chain reaction is particularly useful in diagnosing babies born to HIV-infected mothers in whom maternal antibodies can persist till 15 months (European Collaborative Study Group, 1988; De Rossi *et al,* 1992; Fearon, 2005). It may also be used in clarifying indeterminate western blot results. The primers used in PCR may be HIV type and subtype specific therefore negative PCR results could be due to subtype variations in specimens and must be carefully interpreted (Jackson *et al,* 1993; Jackson *et al,* 1997; Fearon, 2005). Polymerase chain reaction can also be used to amplify HIV genes from plasma, peripheral blood cells and whole blood for genotyping and identification of drug resistance mutations (Sarmati *et al,* 2002; Shafer 2002; Steegena *et al*, 2006; Kabamba- Mukadi *et al,* 2010). In such cases, the HIV gene of interest (protease, reverse transcriptase, integrase or envelope) is amplified by PCR and sequenced to identify the genotype and drug resistance mutations if present in the patient's sample (Steegena *et al,* 2006).

2.1.8 Diagnosis of HIV infection in Ghana

Rapid antibody assays such as First Response (Premier Medical Corporation Ltd, India) and OraQuick (OraSure Technologies Inc., USA) are used to screen individuals for their HIV status. The algorithm requires that results are declared when the outcome of two rapid assays are consistent. Inconsistent results are clarified on a line immunoblot assay such as Inno-lia HIV I/II Score (Innogenetics, Belgium), Inno-lia is also used to determine the type of HIV infection: HIV-1 or HIV-2. For large scale screening, during surveillance and after a blood donation exercise, ELISAs are used. Fourth generation ELISAs are used for detection of both

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antigen and antibody (Branson 2007). When there are occupational exposures to infectious agents, qualitative PCR is used to determine HIV infection status within 2 weeks of exposure. Qualitative PCR is also used to clarify the HIV status of babies born to HIV infected mothers.

2.2 Antiretroviral drugs and therapy

The development of inhibitors of the reverse transcriptase and protease, two of three essential enzymes of HIV-1 in the mid-1990s revolutionized the treatment of HIV-1 infection (Arts and Hazuda, 2012). The introduction of drug regimens that combined these agents further enhanced the overall efficacy and durability of antiretroviral therapy (Collier *et al,* 1996; D'Aquila *et al*, 1996; Staszewski *et al*, 1996).

Drugs from five distinct classes are available to treat HIV infection (Arts and Hazuda, 2012). The drug classes are:

1. Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs)
2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
3. Protease inhibitors (PIs)
4. Entry inhibitors or fusion blockers
5. Integrase inhibitors

Reverse transcriptase (RT) was the first HIV enzyme to be exploited for antiretroviral drug discovery (Young 1988). This enzyme is the target for two distinct classes of antiretroviral agents; the nucleoside reverse transcriptase inhibitors (NRTIs) and the non-nucleoside reverse transcriptase inhibitors (NNRTIs). Nucleoside reverse transcriptase inhibitors were the first class of drugs to be approved by the FDA (Young, 1988). Nucleoside reverse transcriptase inhibitors act as DNA-chain terminators and inhibit reverse transcription of the viral RNA

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genome into DNA, a crucial event occurring at an early stage of the viral life cycle. They are administered as pro-drugs and require host cell entry and phosphorylation by cellular kinases into their functional forms before enacting an antiviral effect (Mitsuya *et al,* 1985; Furman *et al,* 1986; Mitsuya and Broder, 1986; St Clair *et al*, 1987; Hart *et al,* 1992). Non-nucleoside reverse transcriptase inhibitors bind near the active site of RT, causing conformational change in the enzyme's active site and inhibiting reverse transcription of the viral RNA (Kohlstaedt *et al*, 1992; Tantillo *et al*, 1994; Spence *et al*, 1995).

Protease inhibitors target the viral protease, an enzyme required for the cleavage of precursor proteins (*gag* and *gag–pol*), and inhibit the final assembly of the inner core of viral particles (Hartman and Buckheit, 2012). The integrase inhibitors prevent the incorporation of the viral DNA into the host genome by the enzyme integrase while the entry inhibitors block the fusion and penetration of HIV virions into their target cells (Arts and Hazuda, 2012).

Twenty-three additional therapeutic agents have been approved for use in humans since the approval of zidovudine or azidothymidine (AZT) for the treatment of HIV-1 infection (US Food and Drug Administration, 2011; Arts and Hazuda, 2012). These drugs are listed below and the chemical structures of some of them are shown in figure 5.

1. NRTIs - abacavir (ABC, Ziagen), didanosine (DDI, Videx), emtricitabine (FTC, Emtriva), lamivudine (3TC, Epivir), stavudine (d4T, Zerit), zidovudine (AZT, Retrovir), and Tenofovir disoprovil fumarate (TDF, Viread), a nucleotide RT inhibitor;
2. NNRTIs - Rilpivirine (Edurant), Etravirine (Intelence), Delavirdine (DLV, Rescriptor), Efavirenz (EFV, Sustiva), Nevirapine (NVP, Viramune);

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3. Protease Inhibitors - Amprenavir (APV, Agenerase), Tipranavir (TPV, Aptivus), Indinavir (IDV, Crixivan), Saquinavir mesylate (SQV, Invirase), Lopinavir/Ritonavir (LPV/r, Kaletra), Fosamprevir calcium (FOS-APV, Lexiva), Ritonavir (RTV, Novir), Darunavir (Prezista), Atazanavir sulfate (ATV, Reyataz) and Nelfinavir mesylate (NFV, Viracept);
4. Fusion inhibitor: Enfuvirtide (T-20, Fuseon); entry inhibitors: Maraviroc (Selzentry) and
5. HIV integrase strand transfer inhibitors - Raltegravir (Isentress).

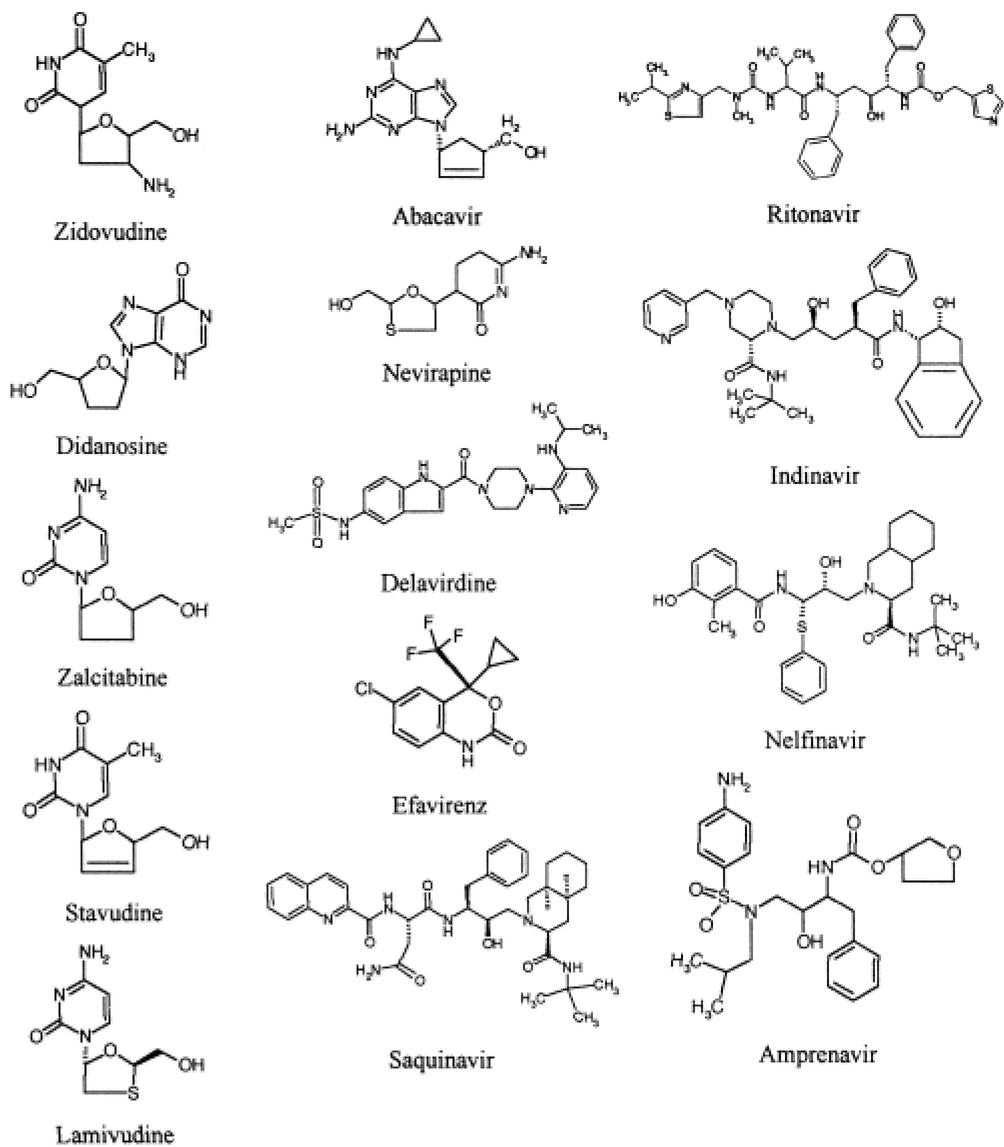

Figure 5: Chemical structures of some antiretroviral drugs (Source: Izzedine *et al.*, 2001)

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Clinical experience with all HIV agents has shown that HIV is able to clearly evade the antiviral effect of any drug used as mono-therapy through the rapid accumulation of amino acid changes in the targeted proteins - reverse transcriptase, protease, envelope, and integrase (Larder, 1995). This is due to the error-prone nature of the reverse transcriptase enzyme that catalyses the synthesis of a DNA copy of the viral RNA genome resulting in many quasispecies of the virus within an infected person. Some of the mutant are escape variant and are therefore become resistant to the antiviral in use as a monotherapy.

Highly active antiretroviral therapy (HAART), which involves the combination of antiretroviral drugs from different drug classes, has improved the outcome of treatment of HIV infection. HAART regimens generally comprise three antiretroviral drugs; usually two nucleoside analogues and either a protease inhibitor or a non-nucleoside reverse-transcriptase inhibitor (Yeni *et al.,* 2002). The use of agents from different classes is instrumental in controlling the development of resistance.

2.3 Mechanism of resistance to antiretroviral drugs

Various mechanisms of resistance have been identified that differ for different drug classes and in some cases for drugs in a given class (Clavel and Hance, 2004). Drug resistance is a result of amino acid changes that occur in the genes of the HIV proteins that the drugs target (Larder et al, 1995). These mutations are presented as a letter (the name of amino acid in the drug sensitive HIV), followed by a number (the amino acid codon at which the change has occurred) and another letter (the amino acid in the drug-resistant HIV). For example the mutation K103N in the RT gene represents the substitution of Lysine (K) with Asparagine (N) at codon 103 in the reverse transcriptase gene. Appendix VI has the one-letter codes and acronyms of the amino acids.

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2.3.1. Resistance to nucleoside and nucleotide reverse-transcriptase inhibitors

Nucleoside and nucleotide reverse transcriptase inhibitors (NRTI) arrest the synthesis of viral DNA by reverse transcriptase. These drugs resemble the natural nucleoside used for DNA polymerization except they lack a 3' OH group. They are incorporated into the growing viral DNA chain instead of the normal nucleoside and because they lack a 3' hydroxyl group needed to form the phosphodiester bond, there is chain termination, and the synthesis of viral DNA is aborted. Chain termination can occur during RNA-dependent DNA or DNA-dependent DNA synthesis, inhibiting the production of either the minus or plus strands of the HIV-1 proviral DNA (Cheng *et al*., 1987; Balzarini *et al*., 1989; Richman, 2001). Two distinct mechanisms are involved in the resistance by HIV to NRTIs (Zdanowicz, 2006: (1) impairment of the incorporation of the analogue into DNA and (2) removal of the analogue from the prematurely terminated DNA chain. These two mechanisms are illustrated in Figure 6.

2.3.1.1 Impairment of Analogue Incorporation

Several mutations or groups of mutations in reverse transcriptase can promote resistance by selectively impairing the ability of reverse transcriptase to incorporate an analogue into DNA. The substitution of methionine by valine at position 184 in the reverse transcriptase, which is described as M184V, is the main mutation that confers resistance to lamivudine. Methionine 184 is found at the heart of the catalytic site of reverse transcriptase. A valine substitution at this position interferes with the proper positioning of lamivudine triphosphate within the catalytic site because valine has a different side chain and this affects the folding of the protein in the active site (Sarafianos *et al*., 1999).

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Other mutations include the Q151M complex of mutations and the K65R mutation (Zdanowicz, 2006). The group of mutations referred to as the Q151M complex is most often selected in the course of the failure of regimens containing stavudine and didanosine (Iversen *et al*., 1996). This pathway always starts with the Q151M substitution, located in the immediate vicinity of the nucleotide binding site of reverse transcriptase. Once Q151M develops, other secondary mutations that enhance resistance follow and increase the activity of the enzyme (Kosalaraksa *et al.,* 1999). The Q151M complex is relatively rare (less than 5 percent of all HIV-1 strains with resistance to nucleoside analogues) but can confer high-level resistance to all NRTI except lamivudine and tenofovir (Iversen *et al*., 1996, Shafer *et al,* 2000). The Q151M mutation complex is more commonly found in HIV-2 drug resistance (Trevin *et al,* 2011). The K65R mutation is often associated with treatment failure after tenofovir or abacavir use. This mutation appears to confer resistance to most analogues, with the exception of zidovudine (Zdanowicz, 2006).

2.3.1.2 Removal of the Analogue from the Terminated DNA Chain

Removal of the nucleoside analogue from the terminated DNA chain is associated with a group of mutations commonly called “thymidine analogue mutations” (TAMs). Mutations from this group most frequently arise during treatment with drug combinations that include thymidine analogues, such as zidovudine and stavudine. These mutations can however promote resistance to almost all nucleoside and nucleotide analogues, including tenofovir (Larder and Kemp, 1989; Shafer *et al.,* 1996; Picard *et al.,* 2001). These mutations occur gradually, and their order of emergence can vary (Boucher *et al.,* 1992).

Thymidine analogue mutations (M41L, D67N, K70R, L210W, 215Y/F and K219Q/E) promote resistance by promoting adenosine triphosphate (ATP)- or pyrophosphate (PPi) -

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mediated removal of nucleoside analogues from the 3' end of the terminated DNA strand (Arion *et al.*, 1998; Meyer *et al.,* 1999). Adenosine triphosphate and pyrophosphate are abundant in normal lymphocytes but are usually not involved in the DNA-polymerization reaction. However, when reverse transcriptase expresses TAMs, its acquired structure allows entry of ATP and pyrophosphate into a site close to the incorporated analogue (Boyer *et al*, 2001; Chamberlain *et al.,* 2002). In this position, ATP or pyrophosphate can attack the phosphodiester bond that links the analogue to the growing DNA chain leading to the removal of the analogue. This promotes the building of the growing DNA chain and enhances viral replication.

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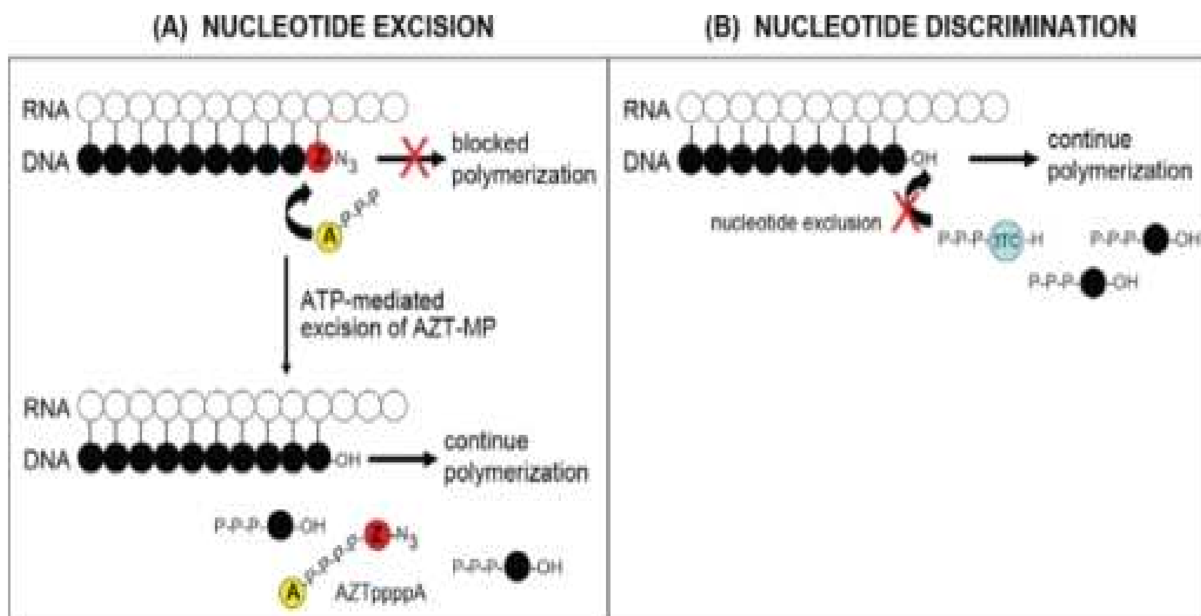

Figure 6: An illustration of two mechanisms of resistance to nucleoside reverse transcriptase inhibitors (NRTIs). (A) Mutations in the RT gene, such as thymidine analogue mutations, aid the ATP- or PPi- mediated removal of the incorporated NRTI such as zidovudine (AZT) from the growing DNA chain and allows polymerization to continue. (B) Mutations in the RT gene cause stearic hindrance in the active site of the enzyme and prevent certain NRTI such as lamivudine from binding and being incorporated during reverse transcription thus permits polymerization in the presence of the drug. RNA is shown with white circles and DNA with black circles. Red 'Z' stands for AZT and Black 'A' stands for ATP.

(Source: www.openi.nlm.nih.gov)

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2.3.2 Resistance to non-nucleoside reverse-transcriptase inhibitors

Non-nucleoside reverse-transcriptase inhibitors (NNRTIs) are small molecules that have a strong affinity for a hydrophobic pocket located close to the catalytic domain of the reverse transcriptase. The binding of the inhibitors prevents the conformation change needed at the active site of the enzyme, thereby inhibiting the enzyme's ability to synthesize DNA (Esnouf *et al.,* 1997). Figure 7 is an illustration of the mechanism of action and resistance to non-nucleoside reverse transcriptase inhibitors.

The mutations that are selected for after the failure of treatment with NNRTIs are all located in the pocket targeted by these compounds, and they reduce the affinity of the drug (Boyer *et al*., 1993; Richman *et al.,* 1994; Bacheler *et al.,* 2000; Ren *et al.,* 2001; Hsiou *et al.,* 2001). The mutations that emerge are however drug-dependent due to some differences in the interaction of the different NNRTIs with the hydrophobic pocket (Boyer *et al.,* 1993). For instance, nevirapine resistance is often associated with Y181C but other mutations, such as Y188C, K103N, G190A, and V106A also occur (Shafer *et al,* 2000). Initial resistance to efavirenz is generally characterized by the K103N mutation, but the Y188L mutation is also seen (Boyer *et al.,* 1993).

Cross resistance usually occurs with NNRTI because of the similarity in their mechanism of action. Therefore when resistance develops from the use of one drug, it is highly likely that other drugs in the same class may be affected (Zdanowicz, 2006). A crystal structure of HIV-1 reverse transcriptase showing positions of drug resistance mutations is presented in Figure 8.

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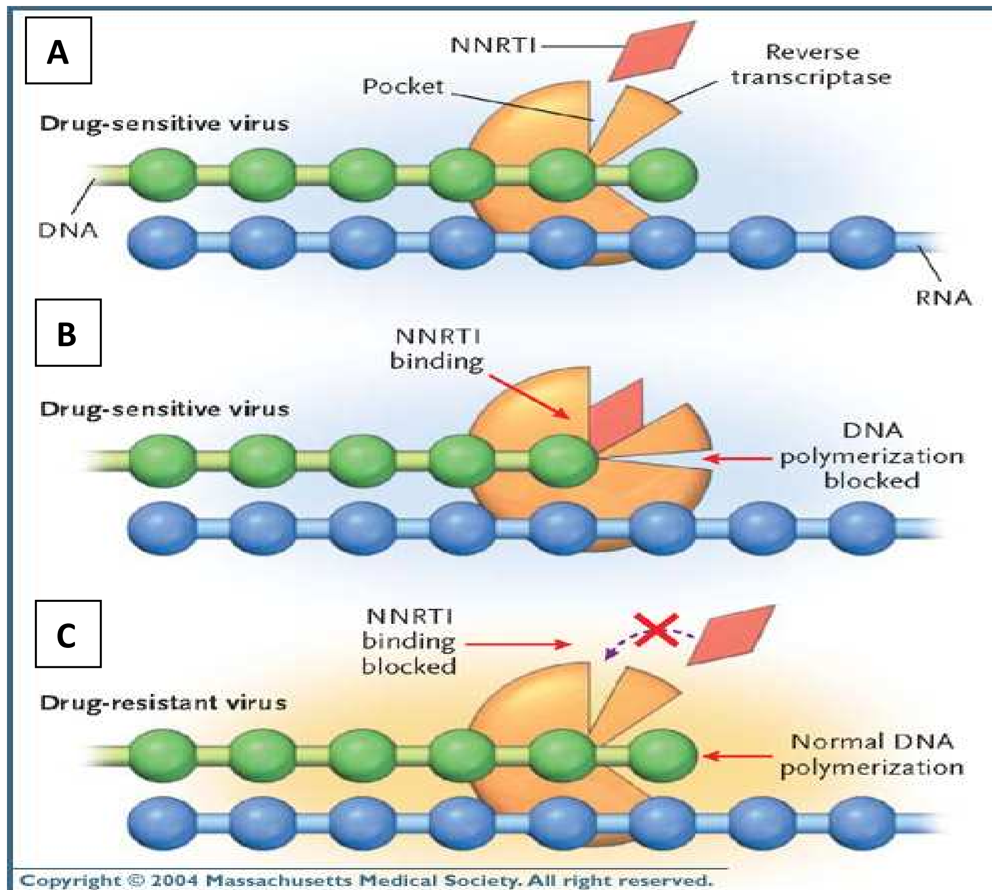

Figure 7: An illustration of the mechanism of action of and resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs). The NNRTIs bind in a hydrophobic pocket adjacent to the active site of the reverse transcriptase enzyme as shown in (A) and inhibit the binding of substrates at the active site for polymerization to occur. In a drug-sensitive HIV-1 strain (B) polymerization is blocked. In a drug-resistant HIV-1 strain (C), mutations in the hydrophobic pocket prevent NNRTI from binding therefore polymerization continues in the presence of the drug. (Source: www.depts.washington.edu)

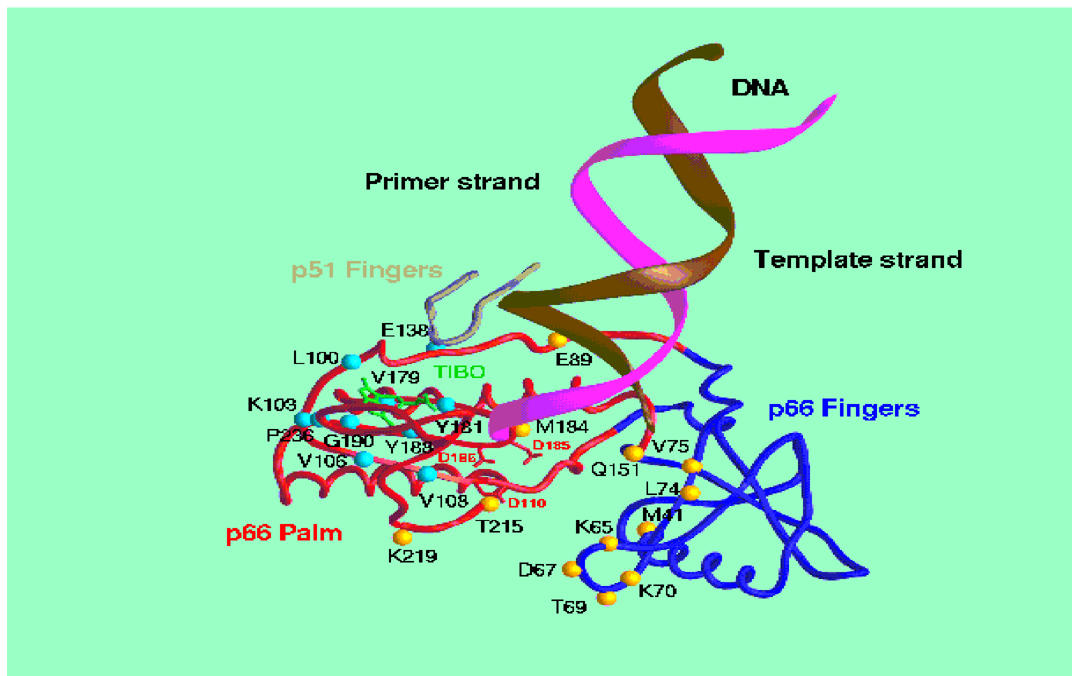

Figure 8: Structure of HIV-1 reverse transcriptase coupled with HIV RNA template (pink ribbon) and primer (Brown ribbon) showing positions of drug resistance mutations. Light blue balls indicate positions of NNRTI resistance mutations and yellow balls indicate positions of NRTI resistance mutations. The active is shown by a white arrow.

(Source: http://home.ncifcrf.gov)

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2.3.3 Resistance to protease inhibitors

Viral proteins are first synthesized as large polypeptides and then processed by the viral protease into functional proteins. The protease inhibitor (PI) group of drugs is designed to block this step in the replication of the virus (Roberts et al, 1990; Erickson and Kempf, 1994). The HIV protease cleaves large polyprotein precursors at specific sites, releasing the structural proteins and enzymes necessary for the assembly of infectious viral particles. When the protease action is inhibited, viral particles are produced, but they are immature and are not infectious (Clavel and Hance 2004).

The protease of HIV is composed of non-covalently associated structurally identical monomers, each 99 amino acids long (Shafer *et al,* 2000). It has a central, symmetric, substrate-binding cavity (Zdanowicz, 2006) covered by a mobile flap that moves to allow substrate polypeptides in and functional protein products out (Shafer *et al*, 2000). Protease inhibitors are similar in structure to the viral peptides that are normally recognized and cleaved by the protease and so compete with the normal substrates for the binding site of the enzyme (Roberts *et al.,* 1990; Erickson and Kempf, 1994). These compounds display a stronger affinity for the active site of the HIV protease and inhibit the catalytic activity of the enzyme in a highly selective manner.

Resistance to protease inhibitors is the consequence of amino acid substitutions that emerge either inside the substrate-binding domain of the enzyme or at distant sites and affects the folding of the protease (Kaplan *et al.,* 1994; Condra *et al.,* 1995; Molla *et al.,* 1996; Zhang *et al,* 1997). These amino acid changes affect the binding of the inhibitors to the active site of the protease, thereby reducing their affinity for the enzyme compared to the natural substrate (Chen *et al.,* 1995, Ridky *et al.,* 1998, Hong *et al.,* 2000; Prabu-Jeyabalan *et al.,* 2002).

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Protease inhibitor mutations can be classified into three: those occurring in the substrate binding site (protease substrate cleft mutations), those occurring in the flap region that covers the substrate binding cleft and those occurring at other sites close to the active site of the enzyme (Shafer *et al,* 2000). Mutations occurring at positions 23, 30, 32, 47, 48, 50, 82 and 84 are substrate binding cleft mutations (Shafer *et al,* 2000; Shafer and Schapiro, 2008). These mutations alter the size of the substrate binding domain and enhance the preferred binding of the natural viral protein substrate in the presence of the most inhibitors (Prabu-Jeyabalan *et al.,* 2002). The protease flap mutations occur at codon 46 and 54. This region is known to extend over the substrate binding cleft and must be flexible enough to allow for the polypeptide substrate to enter and the functional proteins to exit the substrate binding domain. Changes in amino acids in this region therefore inhibit the flexibility required for the protease activity. Mutations at codons 76, 88 and 90 are known to be close to the active site of the protease but their mechanism of resistance is not known (Shafer *et al*, 2000). Figure 9 is a crystal structure of HIV-1 protease indicating positions of drug resistance mutations.

Due to differences in the chemical structure of the inhibitors and their peculiar interaction with the substrate-binding domain of the enzyme, some mutations are selected for only specific protease inhibitors. However, there is considerable overlap between the combinations of mutations and this is responsible for the wide cross-resistance that is generally observed within this drug class (Schapiro *et al.,* 1999; Hertogs *et al.,* 2000).

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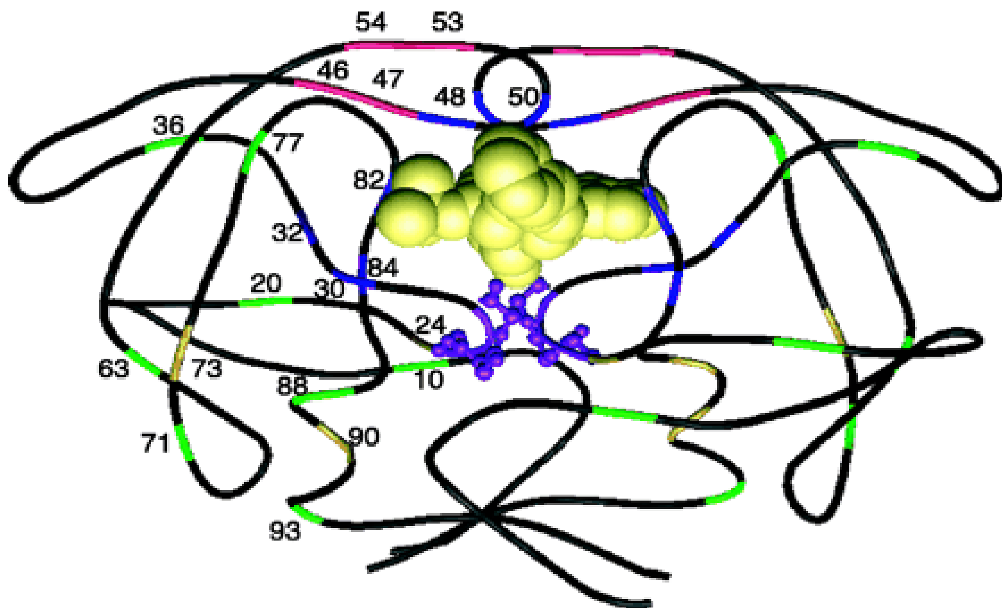

Figure 9: The structural model of HIV-1 protease homodimer showing positions of protease inhibitor resistance mutations in one subunit. The polypeptide backbone of both protease subunits (positions 1 to 99) is shown. The active site (codons 25 to 27 from both subunits), is displayed in ball and stick mode. The protease was co-crystallized with indinavir, which is displayed in space-fill mode. This model is based on a structure published by Chen et al, 1994

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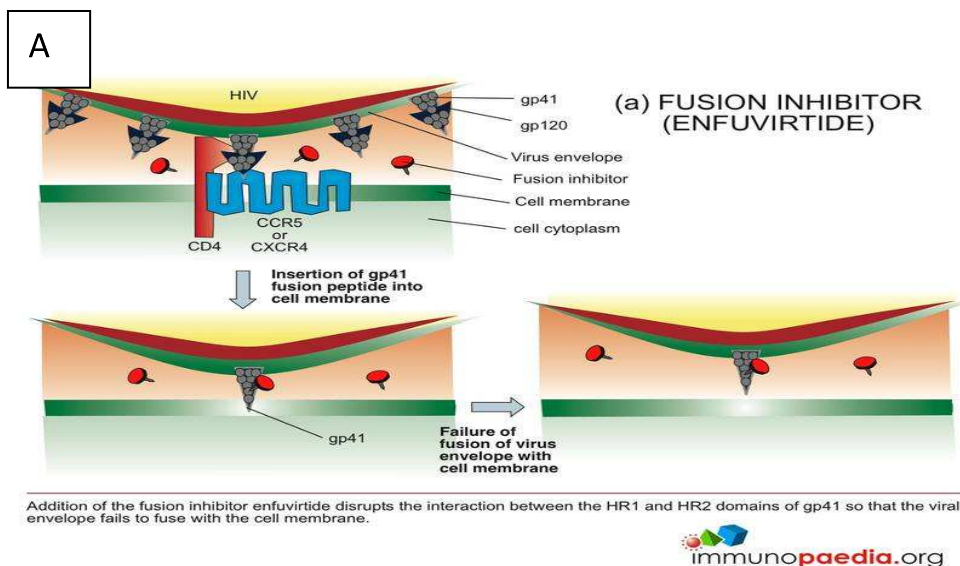
2.3.4. Resistance to fusion inhibitors

Human immunodeficiency virus enters target cells through an intricate sequence of interactions between the HIV envelope glycoprotein (gp) complex (gp120–gp41) and specific cell-surface receptors CD4, CCR5 and CXC4 (Kilby and Eron, 2003). First, gp41 interacts with the cell membrane and draws the virus closer to its target. The distal hydrophobic region of gp41, HR2, folds onto a more proximal hydrophobic region, HR1. This brings the membranes of the virus and target cell into close proximity and enhances the fusion of the membranes to allow for viral entry.

Enfuvirtide, a 36-aminoacid peptide derived from HR2, destabilizes this process by binding to HR1, preventing the folding of gp41 and blocking the fusion of the target cell and viral membranes and consequently viral entry (Fig. 10). Viral resistance to enfuvirtide usually results from mutations located in a stretch of 10 amino acids within HR1 (Rimsky *et al.,* 1998; Wei *et al.,* 2002). These mutations prevent enfuvirtide from binding to HR1 and thus allow the fusion of the virus to the cell membrane and enhance viral entry (Fig. 10). Changes in amino acids in gp41 outside HR1 — and even changes in gp120 — appear to be associated with significant differences in the susceptibility of the virus to enfuvirtide (Derdeyn *et al.,* 2001; Reeves *et al.,* 2002).



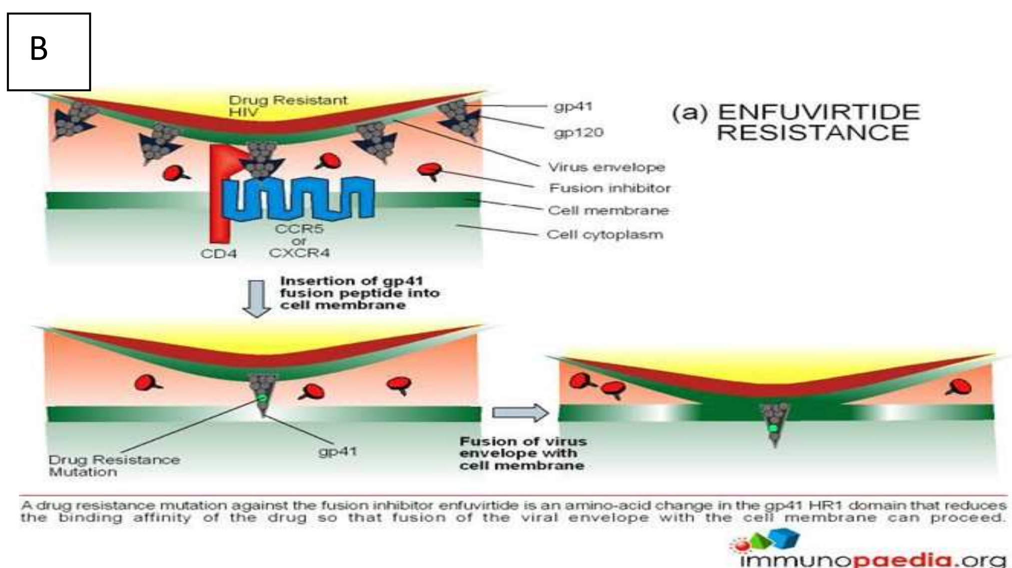


Figure 10: Illustration of mechanisms of action of and resistance to a fusion inhibitor Enfuvirtide (T-20). A) The inhibitor disrupts the interaction between the HR1 and HR2 domains of gp41 and prevents the fusion of the viral envelop with the host cell membrane. B) Drug resistance mutations in the envelop gene of HIV-1 (gp 41) reduce the ability of the inhibitor to bind therefore permits the fusion of the viral envelope to the host cell membrane. (Image Source: www.immunopaedia.org.za)

2.4 Development of HIV Drug Resistance

Many factors influence the rapid and widespread emergence of drug resistance that is seen in HIV. Key among them are the extremely high replication rate of HIV and the lack of proof reading activity by the viral RT (Clavel and Hance, 2004). In an untreated individual, there are 10^4–10^5 or more HIV-1 particles per ml of plasma, which turn over at a rate of ~10^{10} per day (Ho *et al*., 1995; Wei *et al*., 1995; Perelson *et al.,* 1996) due to the exceptionally high rate of replication. Owing to the error-prone reverse transcription process, it is estimated that one mutation is introduced for every 1000–10,000 nucleotides synthesized (Mansky and Temin, 1995; O'Neil *et al*., 2002; Abram *et al.,* 2010). With a genome size of ~10,000 nucleotides in length, 1 to10 mutations may be generated in each viral genome with every replication cycle. Majority of these mutations are base substitutions but insertions and duplications can also occur (Zdanowicz, 2006). This means that numerous HIV ''variants'' or quasi species are rapidly formed and propagated within an infected person. With this enormous potential for generating genetic diversity, HIV-1 variants with reduced susceptibility to any one or two drugs will often preexist in the viral quasi species before initiation of therapy (Coffin, 1995).

While some HIV variants may exhibit intrinsic or ''primary'' resistance to antiretroviral agents, most drug resistance develops as a result of exposure to the drugs. The presence of the drug pressure is known to select for the drug resistance variants. Even during successful therapy, when there is viral suppression, drug resistance can still occur due to residual viral replication (Martinez-Picado *et al,* 2000). Any mutations that confer a selective advantage to a particular viral variant will allow that particular viral variant to predominate. Thus, the very use of antiviral drugs exerts a ''selective pressure'' that favors propagation of resistant viruses. The emergence of resistant single mutants can occur in a matter of weeks (Clavel and Hance, 2004). However some single mutations can only induce low-level resistance to some

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drugs. In such cases, high levels of resistance or complete resistance requires the gradual accumulation of additional mutations.

Combination therapy can block this selection process for two reasons. First, multiple mechanisms are required for resistance to occur to all drugs in the regimen. Even if a small number of variants with the potential for resistance to individual agents exist before treatment, they may not be able to resist all the drugs in the regimen. Secondly, multiple drugs suppress viral replication more effectively than single agents (Gulick *et al.,* 1997; Hammer *et al.,* 1997). In patients who receive a triple combination of antiretrovirals from the onset of therapy, emergence of drug resistance results only if HIV continues to replicate in the presence of drugs. In these cases, drug levels are insufficient to block viral replication completely but sufficient to exert positive selective pressure on variants with decreased drug susceptibility. Under these conditions, viruses with resistance to all the components of the regimen will gradually emerge. Thus during HAART, drug resistance is most often the consequence of initial treatment failure. The resistance subsequently leads to increased treatment failure (Clavel and Hance, 2004).

Additional factors that may contribute to the development of HIV drug resistance include poor patient adherence, sub-therapeutic blood levels of antiretroviral agents, and inappropriate choice of antiretroviral agent(s) (Zdanowics, 2006). Poor oral absorption, alteration of drug metabolizing enzymes by other agents, and various drug-drug interactions could affect blood levels of the drugs (Zdanowicz, 2006). Highly active antiretroviral therapy becomes successful when drug combinations that decrease the probability of selecting virus variants with multiple mutations and conferring resistance to a three-antiretroviral-drug regimen are used.

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Cross resistance of HIV is also of great concern. This phenomenon involves development of HIV resistance to drugs within a particular class or with similar mechanism of action, but to which the patient has not been exposed (Zdanowicz, 2006). The emergence of cross-resistant viruses during treatment tends to limit future drug options.

2.5 Markers for monitoring patients on ART

As antiretroviral treatment (ART) for HIV infection became increasingly available in resource-limited settings, there was great concern regarding the development of drug resistance (Spacek *et al.,* 2006). While there was general agreement that potential drug resistance should not deter treatment efforts, it was necessary to initiate the monitoring of the development and prevalence of drug resistance (W.H.O. Draft Guidelines for Surveillance of HIV Drug Resistance, 2003). Individual drug resistance testing was recommended for treatment monitoring but is largely unavailable in resource-limited settings. CD4 cell count is available in many such settings and HIV viral load in some but genotyping tests are rarely performed (Badri *et al*, 2008; WHO Draft Guidelines for Surveillance of HIV Drug Resistance, 2003).

2.5.1 Using immunologic and virologic markers

In 2003, the vast majority of Africans treated with ART were not monitored with viral load testing. This was due to the cost and complexity of providing a reliable quantitative HIV RNA viral load service in resource-limited settings (Crowe *et al.,* 2003; Fiscus *et al.,* 2006). Early detection of virologic failure is however important for optimal management of HIV-infected patients receiving ART.

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In the absence of viral load monitoring however patients continue to take first-line ART for as long as their CD4 counts are increasing or stable. During this period some may experience undetected virologic failure which may lead to accumulation of drug resistance mutations. Moreover, accumulation of multiple antiretroviral drug resistance mutations may compromise the response to future drugs and fuel the spread of primary drug resistance within communities (Boucher *et al.,* 1993; Kantor *et al.,* 2004; Napravnik *et al.,* 2005) even in patients whose viral loads are being monitored. Martinez-Picado *et al* (2000) also reported that antiretroviral-resistant HIV can be selected from residual virus replication during HAART in the absence of sustained rebound of plasma HIV-1 RNA (Martinez-Picado, *et al.,* 2000).

The World Health Organization (WHO) has recommended the use of CD4 cell count measurements and clinical outcomes for monitoring ART in the absence of viral load (WHO, 2006). However, the clinical outcomes and CD4 cell count changes that are able to predict virologic failure have not been identified. It is not clear whether the variability in CD4 cell count measurements adequately reflects the variability in viral load (Badri *et al.,* 2008). Jevtovic *et al* (2005) reported that HAART may allow for the reconstitution of immune functions in most treated HIV patients but cause discrepant responses in some patients. These responses may include failure to achieve a significant increase in circulating CD4+ T cells despite undetectable plasma viral loads or a good immunological response while not reaching undetectable viremia (Jevtovic et al, 2005). Some other researchers (Florence *et al.,* 2004; Bisson *et al.,* 2006; Moore *et al.,* 2006) found that a gain in CD4 cell count was useful to detect viral suppression in patients on ART. However, Badri *et al*. (2008) have shown that although changes in CD4 cell count correlated significantly with viral load at a group level, they had very poor predictive value when being used to assess individual patients. Meya *et al.*

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(2009) also found no significant difference in CD4 lymphocyte count gain between those with and without viral failure and concluded that CD4 cell count gain from baseline was not associated with viral outcome. Thus, CD4 cell count measurements cannot be used to substitute viral loads for measuring virologic failure.

2.5.2 HIV Drug resistance testing

The use of drug resistance testing has become an integral part of HIV clinical care. The first clinical description of HIV resistance to antiretroviral drugs was published in 1989, in patients taking zidovudine monotherapy (Larder *et al.,* 1989). Accumulation of mutations within the reverse transcriptase gene resulted in a marked increase in drug resistance (Larder *et al.,* 1989). Human immunodeficiency virus variants resistant to every available antiretroviral agent have been identified in viral culture in the presence of drug and in treated HIV-infected patients (Shafer and Schapiro, 2008). The evolution of drug resistance has significant clinical implications for choosing effective antiretroviral regimens (Hirsch *et al*., 2008). Drug resistance testing is recommended by the International Society of AIDS, USA panel of experts for the management of HIV/AIDS patients. Two types of assays, phenotypic or genotypic, are used for resistance testing. These assays detect resistance in fundamentally different ways, although the results generally correlate with each other (Zdanowicz, 2006).

2.5.2.1 Genotypic Assays

Genotypic testing is the most commonly used method of detecting resistant HIV-1 strains and is one of the earliest applications of gene sequencing for clinical purposes (Shafer, 2002). Genotypic testing has the ability to detect mutations present as mixtures, even if the mutation is present at a level too low to affect drug susceptibility in a phenotypic assay. Genotypic assays also provide insight into the potential for resistance to emerge and are advantageous

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because they can detect transitional mutations that indicate the presence of selective drug pressure but do not cause drug resistance by themselves (Shafer, 2002). Genotypic assays detect drug-resistance mutations present in relevant viral genes. Most genotypic assays involve sequencing of the *pol* gene that encodes the reverse transcriptase (RT) and protease (PR) genes to detect mutations that are known to confer drug resistance (Shafer, 2002). Genotypic assays that assess mutations in the integrase and gp41 (envelope) genes are also commercially available. Genotypic assays can be performed rapidly with results available within 1–2 weeks of sample collection.

Plasma is the main source of virus used for tests of HIV-1 drug resistance in clinical settings. HIV-1 genotyping requires extraction, reverse transcription and amplification of viral gene. The sensitivities of most genotypic assays is generally reduced to 1000 RNA copies/ml (Shafer 2002). Direct sequencing of PCR products is usually done in clinical settings since it is faster and more affordable than clonal sequencing, which is mostly used in research settings. Direct sequencing is able to detect nucleotide mixtures when the least common nucleotide is present in at least 20% of the total virus population (Larder *et al,* 1993; D'Aquila *et al,* 2000).

Dideoxynucleotide sequencing is the most commonly used method for genotyping (Shafer 2002). Commercial HIV-1 reverse transcriptase and protease genotyping kits such as ViroSeq and TRUGENE are available but are more expensive than in-house methods. The commercially available kits may have stronger quality control and validation profiles but they have similar performances when compared to in-house assays based on the dideoxynucleotide sequencing method (Erali *et al,* 2001; Shafer 2002).

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Previous studies that compared results from different laboratories showed that the dideoxynucleotide sequencing method was intrinsically reliable and results were reproducible for HIV-1 genotyping of cultured isolates (Husumi *et al*, 1992). The reproducibility of the dideoxynucleotide method was further attested by results from two clinical laboratories that sequenced protease and reverse transcriptase genes using plasma aliquots of 46 heavily treated patients. Although these laboratories used different in-house protocols with reagents from Applied Biosystems (Foster City, California), there was 99% sequence concordance between them (Shafer 2002).

Quality control is required for genotyping assays and aims at preventing PCR contamination and sample mix-up. Physical separation of pre-amplification and post-amplification areas is usually recommended (Kwok and Higuchi, 1989). Inclusion of negative controls in each stage of the PCR process is also advised. Construction of phylogenetic trees with newly generated sequences and other sequences generated earlier from the same laboratory can also help to detect high levels of similarity between different sequences due to cross- contamination (Shafer 2002).

Interpretation of test results requires knowledge of the mutations that different antiretrovirals select for and of the potential for cross resistance to other drugs conferred by certain mutations (Garcia-Lerma and Heneine 2002). The International AIDS Society-USA (IAS-USA) maintains a list of updated significant resistance-associated mutations in the reverse transcriptase, protease, integrase, and envelope genes. The latest of these lists was published in 2011 (Johnson *et al,* 2011). The Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu) also provides helpful guidance for interpreting genotypic resistance test results (Liu and Shafer, 2006). Other tools such as RegaDB (Rega Instituut, KU Leuven,

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French 'Agence Nationale de Recherche sur le Sida'- ANRS program (France), Visible Genetics Inc. (Toronto) are also available to assist in interpreting genotypic test results (Torti *et al.,* 2005; Flandre and Costagliola, 2006; Vercauteren and Vandamme, 2006; Gianotti *et al.,* 2006). Clinical trials have demonstrated the benefit of consultation with specialists in HIV drug resistance to improve virologic outcomes (Tural *et al.,* 2002).

2.5.2.1.1 Genotypic DR testing using peripheral blood mononuclear cells

The majority of HIV-1 genotyping assays analyze viral RNA from plasma. Proviral DNA from peripheral blood mononuclear cells is an alternative marker for studying drug resistance (Chew *et al,* 2005). Proviral DNA is known to persist in infected cells, even after prolonged highly active antiretroviral therapy (HAART) has reduced plasma RNA viral load to undetectable levels (Chew *et al*, 2005).

Data regarding detection of HIV-1 drug resistance mutations in proviral DNA has increased. Some researchers found key mutations in proviral DNA that were not present in plasma viral RNA (Riva *et al.,* 2001; Chew *et al*., 2005; Bona *et al*., 2007; Wang *et al.,* 2007). Using direct sequencing, these researchers observed that key mutations conferring resistance to reverse transcriptase inhibitors were found more frequently in proviral DNA than in plasma viral RNA. Major mutations in the protease region were only found in PBMCs. Wang *et al*. (2007) observed concordance between HIV strains in plasma and PBMC of treatment-naïve patients. Early establishment of the viral reservoir in patients acquiring resistant strains at primary HIV-1 infection has been previously described (Ghosn *et al*., 2006). They also showed that resistance associated mutations with similar profiles in paired plasma RNA and PBMC DNA persisted in such patients. In a case study, Usuku *et al* (2006) followed the changes in drug resistance mutations in a patient receiving HAART. They found out that the mutations

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detected in the plasma were infrequently detected in the proviral DNA. While plasma contains population of actively replicating viruses, the peripheral blood mononuclear cells contain a mix of newly synthesized HIV RNA, integrated and un-integrated HIV DNA. Thus the turnover in plasma is much higher than that in the peripheral blood mononuclear cells (Perelson *et al.,* 1996; *Kaye et al.,* 1995). Patterns of drug resistance mutations showed that the viral populations in PBMCs are more heterogeneous; that PBMCs included archival species that reflected the treatment history of the patient, with all the changes accumulated over the treatment period as well as the wild type viruses (Shafer 2002; Quan *et al.,* 2008). The plasma viruses are related to the most recent treatment and represent a more homogeneous virus population during treatment. Using archival HIV DNA present in PBMCs will provide additional information on drug resistance mutations that could emerge after a failing therapy is changed based on genotypes detected in plasma. Also, sequencing HIV DNA from PBMCs may be useful in patients with undetectable viral loads (Sarmati *et al.* 2003).

2.5.2.2 Phenotypic Assays

Phenotypic assays measure the extent to which an antiretroviral drug inhibits virus replication *in vitro* (Garcia-Lerma and Heneine, 2002). Reverse transcriptase and protease genes sequences and, more recently, integrase and envelope sequences derived from patient plasma HIV RNA are inserted into the backbone of a laboratory clone of HIV or used to generate pseudotyped viruses that express the patient-derived HIV genes of interest. These viruses are cultured at different drug concentrations, monitored by expression of a reporter gene and are compared with the growth of a reference HIV strain. The drug concentration that inhibits viral replication by 50% (IC50) is calculated, and the ratio of the IC_{50} of test and reference viruses

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is reported as the fold increase in IC_{50} (i.e., fold resistance) [Garcia-Lerma and Heneine, 2002].

Automated commercial phenotypic assays are commercially available with results reported in 2–3 weeks (Garcia-Lerma and Heneine, 2002). These include Antivirogram (Tibotec-Virco, Belgium), PhenoSense HIV-1 Drug Resistance Assay (ViroLogic Inc., South San Francisco, USA) and PhenoScript (VIRalliance, Paris, France). However, phenotypic assays cost more to perform than genotypic assays. Although clinically significant fold cut-offs are now available for some drugs, the interpretation of phenotypic assay results is complicated by incomplete information on specific resistance level (i.e. fold increase in IC_{50}) that is associated with drug failure (Lanier *et al.,* 2004; Miller *et al*., 2004; Naeger and Struble, 2006, 2007; Flandre *et al.,* 2007).

2.6 The HIV/AIDS Epidemic

Human Immunodeficiency Virus (HIV) was identified as the aetiologic agent of Acquired Immune Deficiency Syndrome (AIDS) in the early 1980s (Barre-Sinoussi *et al.*, 1983, Popovic *et al*., 1984; Gallo *et al.,* 1984). Since then the disease has continued to spread throughout the world claiming the lives of many adults and children and in some cases families and households. According to the statistics of the global HIV and AIDS epidemic published by UNAIDS, WHO and UNICEF in November 2011, 30 million people have died of AIDS since the beginning of the epidemic and 34 million people were living with HIV/AIDS by the end of 2010. Of these, 22.9 million live in sub-Saharan Africa. With around 68 % of all people living with HIV residing in sub-Saharan Africa, the region carries the greatest burden of the epidemic. Epidemics in Asia have remained relatively stable and are still largely concentrated among high-risk groups. Conversely, the number of people living

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with HIV in Eastern Europe and Central Asia has more than tripled since 2000 (UNAIDS, 2011). HIV/AIDS has had a great impact on society, both as an illness and as a source of discrimination. The disease also has significant economic impacts.

2.6.1 HIV/AIDS in Ghana

Ghana is a West African country located on the coast with a land mass of 238,537 sq. km (www.ghana.gov.gh, 2013). It is an English-speaking country that shares boarders with three francophone countries: Togo to the East, La Cote D'Ivoire to the West and Burkina Faso to the North. The south of the country is bounded by the Gulf of Guinea. The map of West Africa showing the location of Ghana is presented in Fig. 10. Ghana's population was estimated at 25 million in 2010 (www.ghana.gov.gh, 2013). The country has 10 administrative regions namely Greater Accra, Western, Eastern, Volta, Central, Ashanti, Brong Ahafo, Northern, Upper West and Upper East regions. The country is further divided into 212 districts (ghana.gov.gh, 2013).

Figure 11: The map of West Africa showing Ghana (arrowed).

Source: http://maps.google.com

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The first case of HIV in Ghana was reported in 1986 (Neequaye et al, 1987; Hishida *et al,* 1994). In that year, 1100 persons were screened and 10% of them were seropositive. The positive cases were skewed towards the females in a ratio of 9:1. These earlier infections with HIV were due mainly to HIV-2 (Kawamura *et al,* 1989; Hishida *et al*, 1994, Takehisa *et al,* 1997). Later, Brandful *et al*. (1997) found increasing numbers of HIV-1 infections in Ghana in the mid 1990s (Brandful *et al,* 1997; Ampofo *et al,* 1999). Since then, the number of reported cases has steadily increased and HIV infections in the country have been predominantly due to HIV-1 (Brandful *et al,* 1997, Brandful *et al,* 1998), with HIV-2 and HIV-1/HIV-2 co-infections contributing to approximately 5% of the infections (Bonney *et al,* 2008). The HIV Sentinel Survey (HSS) by the National AIDS/STI Control Programme of the Ghana Health Service, which was started in 1992, has consistently provided epidemiological data on HIV trends in Ghana. An estimated 217,428 persons, representing 1.42% of the adult populations, were infected with the virus in 2011 (National AIDS Control Programme, Ghana, 2011). This prevalence is known to be on a decline from the estimated 1.57% in 2009 and 1.49% in 2010. HIV-1 contributed 98% of the infection while 0.7% was due to HIV-2. HIV-1/HIV-2 co-infections made up 1.3% (National AIDS Control Programme, Ghana, 2011) is The predominant HIV-1 subtype is CRF02_AG circulating in Ghana with unique recombinant forms (URFs) [Delgado *et al.,* 2008; Brandful *et al.,* 2012].

The number of adults receiving ART in Ghana has greatly increased from 1,804 in 2004 to 73,339 in 2012 (NACP, 2013). However, this figure represents 69% of adults needing ART in the country. Antiretroviral drugs available in Ghana include the NRTIs: stavudine, lamivudine, abacavir, didnosine, tenofovir, zidovudine, emtricitabineNNRTIs: efavirenz and nevirapine and PIs: lopinavir/ritonavir and nelfinavir (NACP/MoH/GHS, 2010). With the scale up in ART, there is a need to monitor the emergence of drug resistance which is an

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inevitable outcome of antiretroviral use. A survey for transmitted drug resistance, conducted by the National AIDS/STI Control Programme, at the two sites in the Eastern region where ART was first started in 2003. This survey showed that the level of transmitted drug resistance at the sites was low (<5%) according to the WHO guidelines for such surveys (HIVDR Threshold Survey, unpublished data). Previous studies have found low levels of drug resistance among drug-naïve persons in Ghana (Kinomoto *et al.,* 2005; Sagoe *et al.,* 2007; Delgado *et al.,* 2008; Brandful *et al.,* 2012). Thus transmitted drug resistance appears not to be a problem in Ghana. However, persons on treatment need to be monitored for drug resistance in order to guide their clinical management and inform the country's ART policy.

Ghana's National ART Guidelines are based on the World Health Organization's ART guidelines for resource-poor countries. The national guidelines recommend the use of two NRTI and one NNRTI in the first-line regimen and two NRTI and one PI in the second-line regimen (NACP/MoH/GHS, 2010). The national guidelines recommend the switch from first-line to second line when there is evidence of treatment failure confirmed by CD4 monitoring and viral load where available (NACP/MoH/GHS, 2010). However, due to unavailability of viral load to most patients, the change in regimen is determined by physicians based on CD4 counts and clinical symptoms. In the absence of routine viral load and drug resistance testing, drugs in the new regimen are decided without any information on the resistance profile of the virus circulating in the individual. The lack of virologic and genotypic tests leads to the accumulation of resistance mutations even in the presence of increased or sustained CD4 counts and may limit future treatment options (Kantor *et al.,* 2004; Gupta *et al.,* 2007). Patients on treatment may habour drug resistance mutations to NRTIs at the time of the switch to second- line. Some of these mutations may confer cross-resistance to some of the NRTIs in the second-line regimen and render them ineffective (Sigaloff *et al.,* 2012).

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2.7 Nucleic acid extraction/isolation methods

The extraction or isolation and purification of nucleic acid are key steps for most molecular biology protocols and all recombinant DNA techniques (Brown, 2003). The extraction of nucleic acids from biological material requires cell lysis, inactivation of cellular nucleases, and separation of the desired nucleic acid from cellular debris. Conventional methods usually employ a lysis procedure which is rigorous enough to fragment the complex starting material (e.g. blood or tissue) and inactivate nucleases; yet gentle enough to preserve the target nucleic acid (Roche Applied Science, 2012).

Traditional methods for purifying nucleic acids from cell extracts are often combinations of extraction, precipitation, chromatography, centrifugation, electrophoresis, and affinity separation. Solvent extraction is usually used to eliminate contaminants from nucleic acids. There are several methods for removing the proteins from the lysed cell suspension and these include shaking with phenol (Kirby, 1968), shaking with a mixture of phenol, chroroform and isoamyl alcohol (Marmur, 1963) and enzymatic degradation with Pronase or Proteinase K (Hotta and Bassel, 1965). The nucleic acid is selectively precipitated by the addition of absolute ethanol or isopropanol. Precipitation with alcohol concentrates the high molecular weight nucleic acids whilst eliminating the small nucleic acid fragments, detergent and organic solvents used in the removal of proteins (Rodriguez and Tait, 1983). A subsequent wash with 70% ethanol and a brief centrifugation removes salt and moisture (Brown, 2003). Unfortunately, most of these methods require extensive handling of toxic chemicals (*e.g.* phenol or ethidium bromide), need expensive equipment (*e.g.* ultracentrifuges), and are time consuming. To minimize these problems, mini columns and other products were develop for the purification of nucleic acids (Roche Applied Science, 2012).

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2.7.1 Column-based nucleic acid purification

Column-based nucleic acid purification is a solid phase extraction method to quickly purify nucleic acids. This method relies on the fact that the nucleic acid may bind (adsorption) to the solid phase (silica or other) depending on the pH and the salt content of the buffer, which may be a Tris-EDTA (TE) buffer or phosphate buffer (used in DNA microarray experiments due to the reactive amine groups in tris). The Quick Spin columns may also contain gel filtration matrices (either G-25 or G-50 Sephadex) which allow large molecules (*e.g.* DNA or RNA) to pass through quickly while retaining small molecules (*e.g.* nucleotides).

The Quick Spin format improves the molecular sieving concept by using centrifugation to separate DNA or RNA rapidly and cleanly from small contaminants (Roche Applied Science, 2012). Prior to the major techniques employed today it was known that DNA binds to silica, glass particles or to diatoms which shield their cell walls with silica in the presence of chaotropic agents, such as sodium iodide or sodium perchlorate. This property was used to purify nucleic acid using glass powder or silica beads under alkaline conditions (Marko *et al.,* 1982). This was later improved to guanidinium thiocyanate or guanidinium hydrochloride as the chaotropic agent (Boom *et al*., 1990). The use of beads was later changed to mini columns.

2.8 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was developed for the *in vitro* amplification of the DNA or RNA of an organism or gene defect. This technique is versatile, specific and sensitive and has been widely used in molecular biology, microbiology, genetics, diagnostics, clinical laboratories, forensic science, environmental science, food science, hereditary studies, paternity testing, and many other applications (Horizon Press, 2012). Polymerase chain reaction takes

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advantage of an enzyme 'polymerase' that uses defined segment in a strand of DNA as a template for assembling complementary strand.

PCR requires three-step cycling process 1) denaturing of a double-stranded DNA 2) annealing of primers and 3) strand extension. If an RNA sequence is to be amplified, a DNA copy of the RNA (cDNA) must first be synthesized using the reverse transcriptase enzyme before the PCR is begun. The PCR reaction contains a mixture of buffers, nucleotides, primers, enzyme and the nucleic acid from the specimen of interest. Denaturation is achieved by heating at 92^oC-100^oC and separates the complementary strands by breaking the hydrogen bonds that holds them together. In the annealing process, primers are attached to the denatured DNA strand when the temperature is reduced. Once annealing has occurred, the enzyme catalyzes the synthesis of a new strand of DNA at the extension step. The enzyme, DNA polymerase, adds on nucleotides complementary to those in the unpaired DNA strand onto the annealed primers. The reaction is achieved in a thermal cycler that is programmed to cycle the appropriate temperatures required for each step of the reaction process. The original molecules of DNA doubled in the first cycle of denaturation, annealing and extension. The cycle is then repeated a number of times in a chain reaction leading to the exponential amplification of the original DNA (Sambrook and Russel, 2001).

Polymerase chain reaction is simple, robust, speedy and flexible. An enormous number of variations to the method have been described and entire journals and books have been devoted to the technique (Sambook and Russel, 2001).

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2.9 Gel Electrophoresis

Gel electrophoresis is a method used in clinical chemistry to separate proteins by charge and or size. In biochemistry and molecular biology, it is used to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge (Kryndushkin *et al,* 2003).

Electrophoresis can be done using either agarose or polyacrylamide gels to separate, identify and purify DNA fragments. The technique is simple, rapid to perform and capable of resolving fragments of DNA that cannot be separated adequately by other procedure such as density gradient centrifugation. The most common buffers used for electrophoresis are Tris/Acetate/EDTA (TAE), Tris/Borate/EDTA (TBE) for nucleic acids. Borate can polymerize, and/or interact with cis diols such as those found in RNA. TAE has the lowest buffering capacity but provides the best resolution for larger DNA (Brody and Kern, 2004). After electrophoresis is complete, DNA may be visualized using ethidium bromide (EtBr) which, when intercalated into DNA, fluoresce under ultraviolet light.

Polyacrylamide gels are most effective for separating small fragments of DNA (5-500bp). Their resolving power is extremely high and DNA fragments that differ by 1bp in size or 0.1% in mass can be separated from one another (Sambrook and Russel, 2001).

2.9.1 Agarose gel electrophoresis

Agarose is a linear polymer composed of alternating residues of D- and L- galactose joined by α-(1-3) and β – (1-4) glycosidic linkages. The L-galactose residue has an anhydrous bridge between the three and six positions (Sambrook and Russel, 2001). Agarose is extracted from seaweed. Purified agarose is in powdered form, and is insoluble in water (or buffer) at room

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temperature but it dissolve on heating. When it starts to cool, it polymerises; the sugar polymers crosslink with each other, causing the solution to "gel" into a semi-solid matrix. DNA is a negatively charged molecule, and is moved by electric current through a matrix of agarose. Molecules of double-stranded DNA migrate through the gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs (Helling *et al*, 1974). Thus, larger molecules migrate more slowly because of greater frictional drag and because they move less efficiently through the pores of the gel than smaller molecules (Thorne, 1966, 1967). Agarose gels have lower resolving power compared to that of polyacrylamide gels but they have a greater range of separation from 50bp to several mega bases in length.

2.10 Real-Time (Quantitative) PCR

Traditionally, PCR is performed in a tube and when the reaction is complete the products of the reaction (the amplified DNA fragments) are analyzed and visualised by gel electrophoresis. However, real-time PCR permits the analysis of the products while the reaction is actually in progress. This is achieved by using various fluorescent dyes which react with the amplified product and can be measured by an instrument. This also facilitates the quantification of the DNA (Arya *et al*, 2005). Quantitative PCR (qPCR) is used to measure the quantity of a PCR product to extrapolate the starting amounts of DNA, cDNA or RNA. Real-time PCR is rapid since it is not necessary to perform electrophoresis or other procedure after the DNA amplification reaction.

The development of fluorescent methods for a closed tube polymerase chain reaction has greatly simplified the process of quantification. Current approaches use fluorescent probes that interact with the amplification products during the PCR to allow kinetic measurements of product accumulation (Horizon Press, 2012). There are also a number of strand-specific

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probes that use the phenomenon of fluorescent resonance energy transfer (Bernard and Wittwer, 2000). The development of instruments that allowed real-time monitoring of fluorescence within PCR reaction vessels was a very significant advance in PCR technology. The technology is very flexible and many alternative instruments and fluorescent probe systems are currently available. Identification of the amplification products by probe detection in real-time is highly accurate compared with size analysis on gels (Ririe *et al*, 1997). Analysis of the progress of the reaction allows accurate quantification of the target sequence over a very wide dynamic range, provided suitable standards are available. Sequence variants including single base mutations could be detected by further analysis (Horizon Press, 2012). Quantitative PCR is now established as the method of choice for the detection of nucleic acids (Arya *at al.,* 2005).

2.11 DNA Sequencing

DNA sequencing is the process of reading the nucleotide bases in a DNA molecule. It includes any method or technology that is used to determine the order of the four bases-adenine, guanine, cytosine, and thymine-in a strand of DNA. Knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields such as diagnostic, biotechnology, forensic biology, archaeology and anthropology. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA genomes of numerous types and species of life, including the human genome and other animal, plant, and microbial species. DNA sequencing is promoting new discoveries that are revolutionizing the conceptual foundations of many fields (Franca *et al,* 2002).

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2.11.1 Sanger Chain Termination Method

The DNA sequencing method, described by Sanger and his co-workers in 1977 revolutionized the field of genomics (Sanger *et al,* 1977). The method known as the chain termination method or the dideoxynucleotide method consisted of a catalyzed enzymatic reaction that polymerizes the DNA fragments complementary to the template DNA of interest (unknown DNA). A ^{32}P-labelled primer is annealed to a specific known region on the template DNA, which provides a starting point for DNA synthesis. In the presence of DNA polymerases, catalytic polymerization of deoxynucleoside triphosphates (dNTPs) onto the DNA occurs until the enzyme incorporates a modified nucleoside called a terminator or dideoxynucleoside triphosphate (ddNTP). The growing chain is then terminated because there is no free –OH group available for forming the phosphodiester bond with the next nucleoside triphosphate (Sanger *et al,* 1977).

This method was performed in four different tubes, each containing the appropriate amount of one of the four terminators. All the generated fragments had the same 5'-end, whereas the residue at the 3'-end was determined by the dideoxynucleotide used in the reaction. After all four reactions were completed; the mixture of different-sized DNA fragments was resolved by electrophoresis on a denaturing polyacrylamide gel, in four parallel lanes. The pattern of bands showed the distribution of the termination in the synthesized strand of DNA and the unknown sequence was read by autoradiography (Sanger *et al*. 1977).

Although other sequencing methods, such as the chemical method, were later described (Maxam and Gilbert, 1977), the chain-termination method developed by Frederick Sanger and coworkers became the method of choice due to its relative ease and reliability (Sanger *et al,* 1977; Sanger and Coulson, 1975). Various modifications have been made to the Sanger

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method to develop the current line of sequencing methods that are fully automated, require one-tube reactions and ready-to-use reaction mixtures. The overwhelming majority of current DNA sequencing assays are based on variations of the dideoxynucleotide method.

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 General Introduction

This section outlines all the materials and procedures used in the study. It includes the procedures used to collect and process blood samples into component plasma and peripheral blood mononuclear cells (PBMC), analyze plasma for HIV serotypes and extract nucleic acids from the plasma and PBMC. The section also describes procedures used to determine the plasma viral load for HIV-1, amplify protease and reverse transcriptase genes by polymerase chain reaction (PCR), sequence the genes and analyze for HIV-1 drug resistance mutations. The protocols used for PCR and sequencing were optimized for HIV-1 as the predominant type.

3.2 Materials

The details of reagents, equipment, consumables, software and their sources and/or manufacturers, used to collect and process the venous blood from HIV-infected persons on ART to obtain drug resistance mutation data are shown in appendix IV.

3.3 Methods

3.3.1 Study Population

HIV-infected persons who had been on ART for a minimum of 6 months, from St. Martin de Porres Hospital, Agomanya, Atua Government Hospital Atua, Korle-Bu Teaching Hospital, Accra and Kumasi South Hospital, Kumasi in the Eastern, Greater Accra and Ashanti Regions respectively were enrolled in the study. After informed consent was obtained, blood samples were collected from 338 persons (98 from Korle-Bu, 101 from St. Martin de Porres, 96 from Atua Government and 43 from Kumasi South).

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This study was approved by the Scientific and Technical Committee of Noguchi Memorial Institute for Medical Research (NMIMR). Ethical clearance for this study was also obtained from the Institutional Review Board of NMIMR in March 2010 (Certificate No. NMIMR-IRB CPN 036/09-10). The clearance was reviewed annually for each year of the study. Copies of the ethical clearance certificates can be found in Appendix V.

3.3.2 Blood sample collection from study participants

Seven millilitres of venous blood samples were collected into EDTA vacutainer tubes from eligible persons after obtaining their informed consent. A sample collection form (Appendix I), detailing date of collection and other personal data, CD4 counts, drug history, adherence information, herbal medicine use and Physician's comment, was completed for each participant. The samples were labeled with hospital identification numbers, packaged in cold boxes and transported by road to the Virology Department of the Noguchi Memorial Institute for Medical Research (NMIMR) for laboratory analyses. Samples were received at NMIMR within 48 hours of collection. Upon receipt, blood samples were checked with the sample collection and consent forms (Appendix II and III). The adequacy of the sample was ascertained and the hospital identity number was cross-checked with the information on the accompanying form. The samples were given laboratory identification and the accompanying information was entered into a Microsoft Excel sample collection database.

3.3.3 Separation of blood into plasma and peripheral blood mononuclear cells

Blood samples were processed into plasma and peripheral blood mononuclear cells as follows: the blood was mixed and layered onto 3 ml of lymphocyte separation medium (Histopaque®-1077) in a 15ml centrifuge tube. This was centrifuged (Centrifuge H-900 (Kokusan, Japan) at 2500 rpm for 15 minutes to separate the various components into four

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layers. The upper layer (plasma) was carefully taken into new sterile and labeled cryovials and stored frozen in 1ml aliquots at minus 35°C until use. The next layer (buffy coat) containing the peripheral blood mononuclear cells (PBMCs) was carefully removed into another sterile 15ml centrifuge tube and the rest (lymphocyte separation medium and red blood cells) discarded. Five milliliters of PBS was added to the PBMCs, mixed and centrifuged at 2500 rpm for 5 minutes. The supernatant was discarded and the pellet re-suspended in 5ml of PBS. This washing step was repeated. The final pellet was re-suspended in 1.0ml of freezing medium, consisting of 1% DMSO in FBS, and stored in two aliquots of 0.5 ml each at minus 35°C.

3.3.4 Determination of the HIV type

A line immuno-assay [Inno-lia HIV-1/II Score (Innogenetics, Belgium)] was used to confirm the presence of HIV antibody and also determine the specific type as HIV-1 or HIV-2. The test was performed according to the manufacturer's protocol in test troughs provided with the kit (User Manual, Inno-lia^{TM} HIV I/II Score, 2011). The presence and intensity of bands, representing gp120, gp41, P24, P17, gp105 and gp36 (Fig. 12) were used to interpret the results and based on the manufacturer's instructions, a sample was declared as HIV-1 positive, HIV-2 positive or HIV-1/HIV-2 dual positive.

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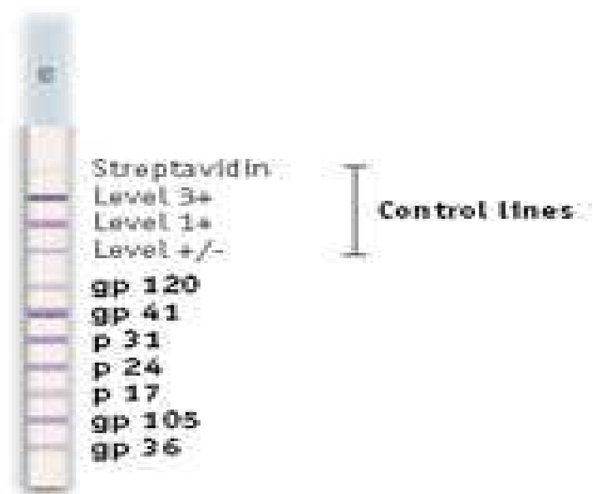

Figure 12: A photo of Inno-lia strip (taken after the test) showing the various antigen bands coated onto it. The control band (3+, 1+, +/-) were used to grade the intensity of bands that developed during the test and this is used in result interpretation

(Source: www.innogenetics.com).

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3.3.5 Extraction and purification of nucleic acids from plasma and PBMC

QIAamp Viral RNA extraction kit (QIAGEN, USA) or QIAamp Blood DNA kit (QIAGEN, USA) or Nucleic Acid Purification kit (Roche Diagnostics, Germany) was used to extract and purify nucleic acids from the plasma and peripheral blood mononuclear cell samples.

3.3.5.1 Extraction and purification of viral RNA from plasma by QIAamp Viral RNA mini kit (QIAGEN, USA)

Ribonucleic acid (RNA) was extracted from thawed plasma samples using the QIAamp viral RNA mini kit according to the manufacturer's instructions (User Manual, QIAamp viral RNA mini kit, 2007) and with the following modifications. A starting volume of 200µl of plasma was used instead of the recommended 140µl. The elution volume was also changed to 50µl from the recommended 60-80µl. These modifications were made to synchronize sample working volumes of the QIAGEN and Roche kits. The eluted RNA was stored at -35°C until use.

3.3.5.2 Extraction and purification of viral RNA from plasma using Nucleic Acid Purification kit (Roche Diagnostics, Germany)

The manufacturer's instructions (User manual, Nucleic Acid Purification Kit, 2010) were followed to extract and purify RNA from plasma by the nucleic acid purification kit. In brief, lysis/binding buffer supplemented with poly (A) was added to 200µl of plasma and mixed. The mixture was carefully transferred to the labeled high pure filter tubes and centrifuged at 10,000rpm for 15 seconds. The collection tube with the flow through was discarded and the filter tube transferred into a new collection tube. To wash the membranes, inhibitor removal buffer (500 µl) was added to the filter tubes and spun at 10,000rpm for 1min. This was followed by the addition of wash buffers. The filter tubes were again spun at 10,000rpm for

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1min and 13,000rpm for 10sec with wash buffers 1 and 2 respectively and transferred into fresh RNase-free 1.5ml tube. Fifty microliters (50µl) of elution buffer was added to the filter tube and centrifuged at 10,000rpm for 1 minute to elute the purified RNA. The RNA was stored at -35° C until use.

3.3.5.3 Extraction and purification of proviral DNA from PBMC using QIAamp DNA blood mini kit (QIAGEN, USA)

A starting volume of 200µl of peripheral blood mononuclear cells was taken through the DNA extraction procedure as instructed by the manufacturer of the kit. The lysis, separation, washing and elution were all done according to the manufacturer's protocol (User manual, QIAamp DNA Blood mini kit, 2007). The DNA was eluted in 50µl of elution buffer.

3.3.5.3 Extraction and purification of proviral DNA from PBMC using Nucleic acid purification kit (Roche, Germany)

The manufacturer's protocol (User manual, Nucleic acid purification kit, 2010) was followed to extract and purify proviral DNA from 200 µl of peripheral blood mononuclear cells. The DNA was eluted in 50 µl of elution buffer.

3.3.6 Determination of HIV-1 viral load

A quantitative real-time PCR (qPCR) assay was used for plasma viral load determination. The purpose was to quantify the amount of virus in the patient's plasma. The LTR region of HIV-1 was amplified in a real time PCR assay using primers

F1: 5'GCCTCAATAAAGCTTGCCTTGA-3' (sense) and

R1: 5'GGCGCCACTGCTAGAGATTTT3' (anti sense).

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A probe (pILNA) 5'FAM - CAGTACATGCAGGGCCTATTCCACCAG–TAMRA 3' and Taqman® One-step RT-PCR reagents (ABI, USA) were used for the assay. The RT-PCR reaction was prepared according to the manufacturer's instructions (Kit insert, Taqman® One-step RT-PCR Master Mix Kit, 2001) Amplification plots were analyzed real time and cut-off threshold (CT) values were obtained and used in a formula below to estimate the viral load.

RNA copy number = 2^(plasmid Ct – Sample Ct) * (Plasmid copy number) * (volume of RNA extracted / volume of RNA used for PCR) * (1000 µL / volume of plasma used for extraction)

Plasmid concentration 5 µg/µl =1.14X104 copies/ml

3.3.7 Reverse-Transcription Polymerase Chain Reaction (RT-PCR) for protease and reverse transcriptase genes from RNA extracts

This procedure was used to amplify the protease and reverse transcriptase genes from the RNA that was previously purified from the plasma samples. The QIAGEN One-step® RT-PCR kit was used for the amplification according to the manufacturer's protocol (Handbook, QIAGEN One-Step RT-PCR, 2010). Briefly, a total of 25µl reaction mix was prepared. The reaction mix consisted of 0.4mM dNTPs, 15µl of RT-PCR mix, 1µl of enzyme mix by and 10µl of RNA. Primers, DRRT1L/DRRT4L for RT gene and DRPRO5/DRPRO2L for the PR gene were used as previously described (Fujisaki et al, 2007).The primer sequences can be found in Table 1. The thermal cycling conditions were as previously described (Villahermosa *et al,* 2000).

3.3.8 Polymerase Chain Reaction (PCR) for DNA extracts from PBMC

Peripheral blood mononuclear cells of an HIV-infected person contain the viral DNA that had been integrated into the human DNA (proviral DNA). This procedure was therefore to

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amplify the PR and RT genes from the proviral DNA. Proviral DNA samples from PBMC were amplified using AmpliTaq Gold® PCR Reagents (ABI, USA). Primers, DRRT1L/DRRT4L for RT gene and DRPRO5/DRPRO2L for the PR gene were used as previously described (Fujisaki et al, 2007). A total reaction of 25µl consisting 1X reaction buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.25µl AmpliTaq Gold® and 10µl of DNA extract was used. The sequences of the primers can be found in Table 1. The thermal cycling conditions used were modified from Villahermosa *et al,* 2000 as follows: 94°C for 2 minutes followed by 40 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 60 seconds plus 72°C extension for 5 minutes.

3.3.9 Amplification of RT-PCR and PCR products by nested PCR

AmpliTaq Gold® reagents (ABI) were used to re-amplify PCR products from the first round (RT-PCR and PCR) in a nested PCR reaction. A total reaction of 25µl consisting 1X reaction buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.25µl AmpliTaq Gold® and 5µl of RT-PCR or round one PCR products was used. Primers DRRT7L/DRRT6L and DRPRO1M/DRPRO6 were used for the RT and protease genes respectively (Fujisaki *et al,* 2007). The sequences of the primers are presented in Table 1. The thermal cycling conditions were as previously published (Villahermosa *et al,* 2000).

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Table 1: Details of the primers used for PCR and sequencing

Name	Position (HXB2)	Sequence (5'-3')	Purpose
DRPRO5	2074-2095	AGACAGGYTAATTTTTTAGGGA	Round 1 PCR
DRPRO2L	2716-2691	TATGGATTTTCAGGCCCAATTTTTGA	(PR GENE)
DRPRO1M	2148-2167	AGAGCCAACAGCCCCACCAG	Nested PCR
DRPRO6	2611-2592	ACTTTTGGGCCATCCATTCC	(PR gene)
DRRT1L	2388-2410	ATGATAGGGGGAATTGGAGGTTT	Round 1 PCR (RT gene)
DRRT4L	3425-3402	TACTTCTGTTAGTGCTTTGGTTCC	
DRRT7L	2485-2509	GACCTACACCTGTCAACATAATTGG	Nested PCR
DRRT6L	3372-3348	TAATCCCTGCATAAATCTGACTTGC	(RT gene)
PRTS	2157-2177	AGC CCC ACC AGA AGA GAG CTT	Sequencing (PR gene)
P3G	2198-2217	CAACTCCCTCTCAGAAGCAG	Sequencing (PR gene)
A2	2583-2601	TTAAAGCCAGGAATGGATG	Sequencing (RT gene)
RT-sec-1-S	2692-2716	CAA AAA TTG GGC CTG AAA ATC CAT A	Sequencing (RT gene)
PRSec2A	2811-2838	TGGGAAGTTCAATTAGGAATACCACATC	Sequencing (PR and RT gene)

The name, location on the HXB2 sequence, the primer sequence from 5'to 3' and the purpose of each primer is shown. HXB2 is a reference HIV-1 subtype B sequence. All the PCR primers and two sequencing primers (P3G and A2) were previously published by Fujisaki *et al* (2007). PRTS, RT-sec-1-s and PRSec2A were previously published by Villahermosa *et al* (2000).

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3.3.10 Agarose Gel Electrophoresis

Two gel concentrations (1.5% and 2.0%) were used to analyze the reverse transcriptase and the protease gene products respectively. This was to cater for the difference in expected product sizes of 463bp and 887bp respectively of the protease and RT genes. A 100bp ladder molecular weight marker (Invitrogen, USA) was used to estimate the size of the PCR products. The gel was viewed with a Gel Logic 100 Imaging system (Eastman Kodak Company, USA) and a photograph taken. A product of size 887bp and 463bp for the reverse transcriptase and protease genes respectively, indicated positive amplification of corresponding genes.

3.3.11 Purification of PCR products

The purpose of this procedure was to separate the amplification products from left- over reaction components such as dNTPs and enzymes. All PCR products with expected sizes were purified using QIAquick PCR purification kit (QIAGEN) according to the manufacturer's protocol (User manual, QIAquick PCR purification kit, 2010). The purified DNA was eluted in 50µl of elution buffer and at minus 20°C and used for sequencing.

3.3.12 Direct sequencing of purified PR and RT PCR products

The sequencing method used was a variant of the Sanger dideoxynucleotide method. Cycle sequencing was done using Big Dye Terminator cycle sequencing kit version 3.1 (ABI, USA). The reaction mixture consisted of 1µl of purified PCR products, 1.6µl of sequencing primer (2µM), 3.4µl of nuclease-free water, 2µl each of the Big Dye Terminator mix and 5X Sequencing buffer in a total reaction mixture of 10µl (Villahermosa *et al*, 2000). The thermal conditions were 94°C for 2 minutes followed by 25 cycles of 94°C for 30 seconds; 50°C for 15seconds; 60°C for 4 minutes. Three primers A2, PRSec2A and RTSec1s were used for

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sequencing RT gene while two primers PRTS and P3G were used for sequencing the PR gene (Villahermosa *et al*, 2000; Fujisaki *et al*, 2007). The sequences of the primers used are shown in Table 1.

3.3.13 Purification of cycle sequenced products

Cycle sequencing products were purified to remove excess dye terminators, primers and dNTPs using the AgenCourt CleanSeq Dye Terminator Removal kit according to manufacturer's protocol (User manual, AgenCourt CleanSeq Dye Terminator Removal kit, 2006). Briefly, magnetic particles and 85% ethanol were added to the cycle-sequenced products and mixed. The tube or plate containing the mixture was placed in a magnetic field to enable DNA bound to the magnetic particles to attach to the walls. The liquid portion was discarded and the particles were washed with 85% ethanol and dried. The purified sequenced DNA was eluted from the particles with nuclease-free water, transferred into the wells of a 96-well optical plate and loaded onto the Genetic Analyzer.

3.3.14 Sequence Analysis on the 3130 ABI Genetic Analyzer

This procedure is an automated polyacrylamide gel electrophoresis (PAGE). The sequenced samples were run using a polymer (POP-7) that was automatically injected into the capillary array. The 96-well optical plate, with purified samples, was properly assembled by first sealing with the 96-well septa, then placing it in the plate base with the correct orientation and covering it with the plate retainer. The monitor, computer and analyzer were switched on (in order of their appearance in this text). When the instrument status light was stable green, the Data Collection v3.0 software was launched. The manufacturer's instructions (Applied Biosystems 3130/3130xl Genetic Analyzers, Getting Started Guide, 2004) were followed to

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load and run the plate in the analyzer in order to generate and store sequence data appropriately.

At the end of the run, the stored sequence data was recalled by launching into the Sequence Analysis Software v5.2 and adding the samples. The electropherogram, details of run, base sequences and other parameters of the run were viewed in this software. The sequence data was saved and further analyzed by other software discussed in the next section.

3.3.15 Sequence data analysis

Sequences were edited using the Seqman software (DNASTAR, USA) and aligned in BioEdit (http://www.mbio.ncsu.edu/Bioedit/bioedit.html). The HIV BLAST programme (http://www.hiv.lanl.gov) was used for subtype reference alignment and MEGA 4.2 (http://www.megasoftware.net/) was used for generating phylogenetic trees. Mutation data and drug resistance interpretations were obtained by submitting the sequences to the Stanford University HIV Drug Resistance Database. One or more sequences in fasta format were pasted into a textbox in the database and analyzed; a maximum of 100 sequences could be analyzed at a time. Nucleotide sequences were aligned to the consensus subtype B HIV-1 *pol* amimo acid sequence using a nucleotide to amino acid sequence local alignment program (Huang and Zhang, 1996). Nucleotide triplets containing mixed bases were translated into each of the possible amino acids they encode. Mutations were defined as the differences from consensus B reference sequence and further characterized as RT mutations (NRTI or NNRTI), PI mutations (Major and minor) and other mutations. Other mutations were those associated with drug resistance but are primarily accessory and polymorphic. Patients' sequences were categorized as being susceptible or having potentially low-level, low-level, intermediate or high-level resistance according to the Stanford algorithm (Liu and Shafer, 2006).

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For subtype determination, each sequence was compared to a list of reference sequences for each HIV-1 group M subtypes A, B, C, D, F, G, H, J, K, CRF01_AE and CRF02_AG. The subtype of the closest reference is assigned to the submitted sequence. This method is generally accurate (Gifford *et al,* 2006) however, with exception to CRF01_AE and CRF02_AG, it does not accurately characterize circulating recombinant forms and unique recombinant forms. An HTML output was generated with the mutation data and subtype information.

3.3.16 Data Analyses

Descriptive statistics such as means and median were used to describe the study population. Tables and charts were used to summarize the demographic data and clinical history of participants. The Pearson's Chi Square was used to find the association between the presence of major drug resistance mutations, duration on treatment, viral load and the difference in CD4 count over the treatment period. The Pearson's Chi Square was also used to find the association between the presence of major drug resistance mutations, adherence to antiretrovirals and herbal medicine use.

3.4 Staff training and capacity building

The techniques and protocols used in the study were passed on to research assistants in the Virology Department of NMIMR through training and practical sessions.

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4.0 RESULTS

4.1 Study population

Blood samples were obtained from three hundred and thirty-eight (338) patients at four study sites. The distribution of these among the sites is shown in Table 2.

4.2 Patient Information

The mean age, gender composition and mean CD4 counts at time of sampling are shown in Table 2. The trend of mean CD4 counts over the treatment period is shown in Figure 13. Majority (93%) of the patients had undetectable viral loads. Twenty-four patients, representing 7%, had detectable viral loads with a mean of 1664 copies/ml (range 158 - 8751 copies/ml). The spread of viral loads is presented in Figure 14. HIV-1 infections were most prevalent (91.7%) while 0.9% was HIV-2 and 7.4% were HIV-1 and HIV-2 dually seropositive (Figure 15).

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Table 2: Summary of the characteristics of the study population

Hospital	**Number**	**Males**	**Females**	**Mean Age**	**^^Mean CD4 (cells/µl)**
Korle Bu Teaching	98	39	59	42	389
St. Martin de Porres	101	26	75	43	470
Atua Government	96	22	74	42	467
Kumasi South	43	8	35	41	534
Total	**338**	**95**	**243**	**42**	**454**

The summary demographic data of the study populations is shown for the four study sites. The respective numbers for patients enrolled, their gender distribution, mean age and mean CD4 count in cells/µl are presented.^^Mean CD4 represents the mean of CD4 counts taken at the time of sampling for all patients enrolled from the site. The values for the entire study population are shown under Total.

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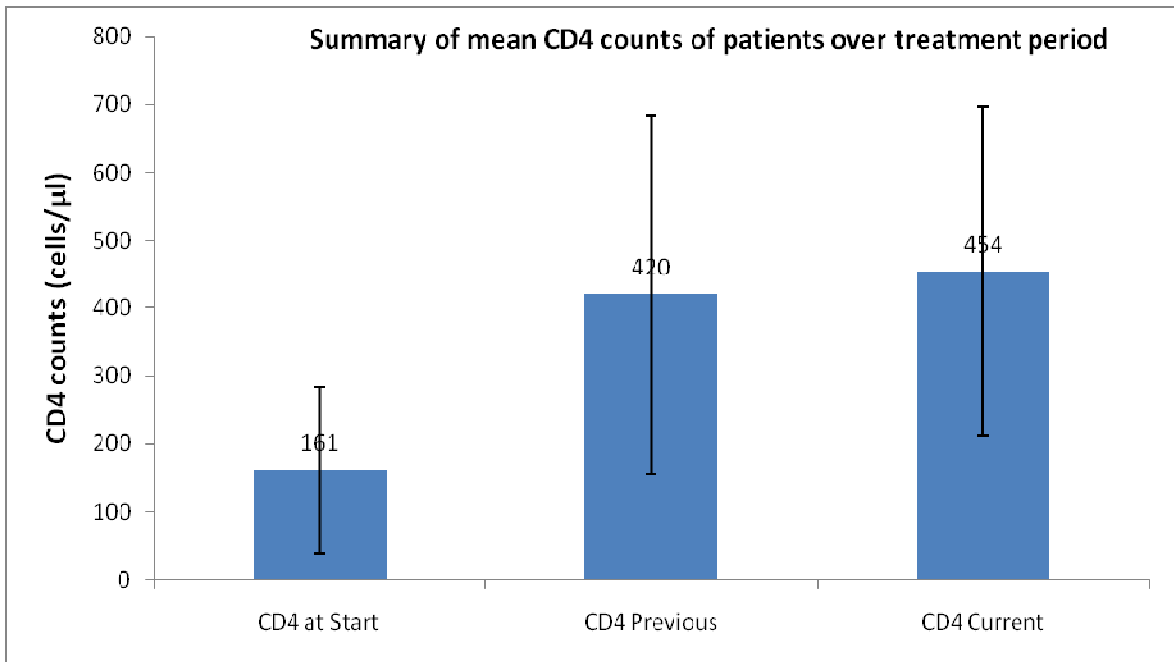

Figure 13: Mean CD4 counts (cells/µl) for all enrolled patients from start of therapy to time of sampling. Start CD4 represents the mean CD4 count of patients at start of therapy; Previous CD4 represents the mean CD4 count during the visit (6 months on average) prior to the sampling date and Current CD4 represents the mean CD4 count at the time of sampling. CD4 counts were measured using BD FACSCount Instrument and reagents (BD Biosciences, USA).The chat shows increasing trend from start to time of sampling.

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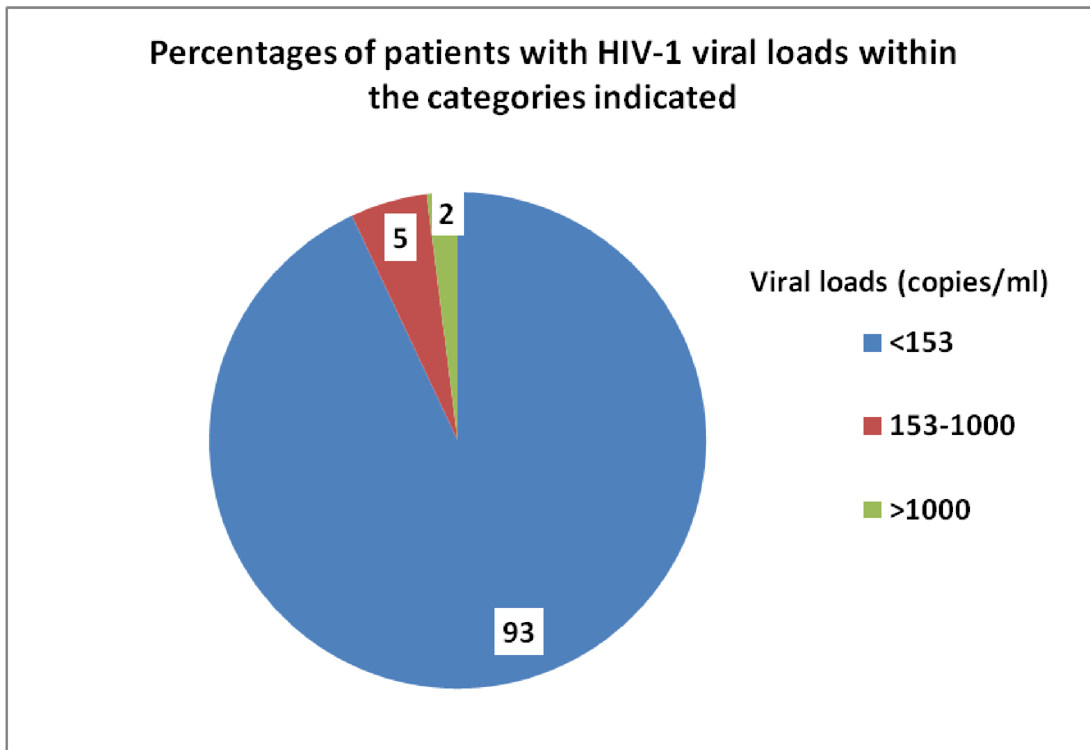

Figure 14: Summary of HIV-1 viral loads for all enrolled patients at time of sampling. The percentages of patients with viral loads within the categories indicated are shown. A quantitative real-time PCR assay with detection limit of 153copies/ml was used to determine the viral loads. Majority (93%) of the patients had viral loads below the detection limit. Patients with detectable viral loads were further divided into two categories: those with viral loads less than 1000 copies/ml (i.e. 153-999 copies/ml) were 5% and those with viral loads greater than or equal to1000copies/ml (i.e. 1000 - 8751copies/ml) were 2%.

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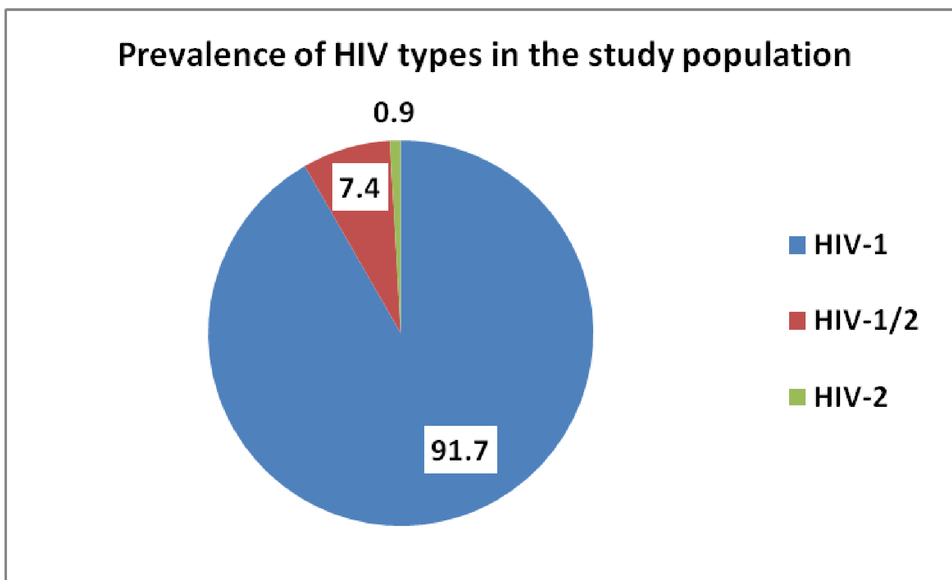

Figure 15: Summary results of the serological typing of samples by Inno-lia HIV I/II Score assay. The percentage of study population (N=338) that had antibodies to HIV-1 only; HIV-2 only and both HIV-1and HIV-2 are shown. Majority of the patients (92%) had HIV-1 antibodies, confirming HIV-1 as the predominant HIV type in Ghana.

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4.3 History of antiretroviral therapy in patients studied

All patients had been on antiretroviral therapy for at least 6 months in a range 6 to 30 months with the mean duration of treatment being 30 months. The majority (87%) of the participants were still on the first-line regimens. The first-line regimen comprised a combination of zidovudine (or stavudine), lamivudine and nevirapine (or efavirenz) for the majority (92%) of the patients. Combinations involving tenofovir, abacavir, didanosine, efavirenz nelfinavir and nevirapine were also used by the some patients (Table 3). Fourteen percent (N= 47) of patients were on second-line regimen and their regimen consisted primarily didanosine, abacavir and ritonavir-boosted lopinavir or nelfinavir. The details of the drug combinations taken by patients on second-line are presented in Table 3.

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Table 3: ART histories of patients showing drugs taken as first-line and second-line regimens

Regimen	NRTI	NNRTI	Patients N (%)
	Zidovudine, Lamivudine	Nevirapine	88 (26)
	Zidovudine, Lamivudine	Efavirenz	83 (24)
First-line N=338	Stavudine, Lamivudine	Nevirapine	68 (20)
	Stavudine, Lamivudine	Efavirenz	73 (22)
	Other combinations		26 (8)
	NRTI	**PI**	**No of Patients (%)**
	Didanosine, Abacavir	Lopinavir/r	30 (64)
	Abacavir, Didanosine	Nelfinavir	5 (11)
Second-line N=47	Lamivudine, Stavudine	Nelfinavir	2 (4)
	Lamivudine, Didanosine	Nelfinavir	1 (2)
	Zidovudine, Lamivudine	Nelfinavir	3 (6)
	Other combinations		6 (13)

Proportions of patients on the various first-line or second-line drug combinations are shown. NRTI - nucleoside reverse transcriptase inhibitors; NNRTI - non-nucleoside reverse transcriptase inhibitors; PI - protease inhibitors; Lopinavir/r –ritonavir-boosted Lopinavir. All patients on second-line regimens had previously been on one of the first-line combinations.

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4.4 Adherence to ART and herbal medicine use

In order to assess adherence to the drugs, patients were asked if they took their drugs daily as scheduled. Nine percent (9%) of patients said they had not taken their drugs as scheduled for at least one month. Herbal medicine use was also investigated and 2% admitted they had used some form of herbal medicine for at least one month while on the ARVs.

4.5 Physicians assessment of patients

Assessment of physicians' rating of patients based on CD4 counts and clinical status showed that most patients (86%) were doing well. However, 11% were not doing so well and 3% were failing (Figure 16).

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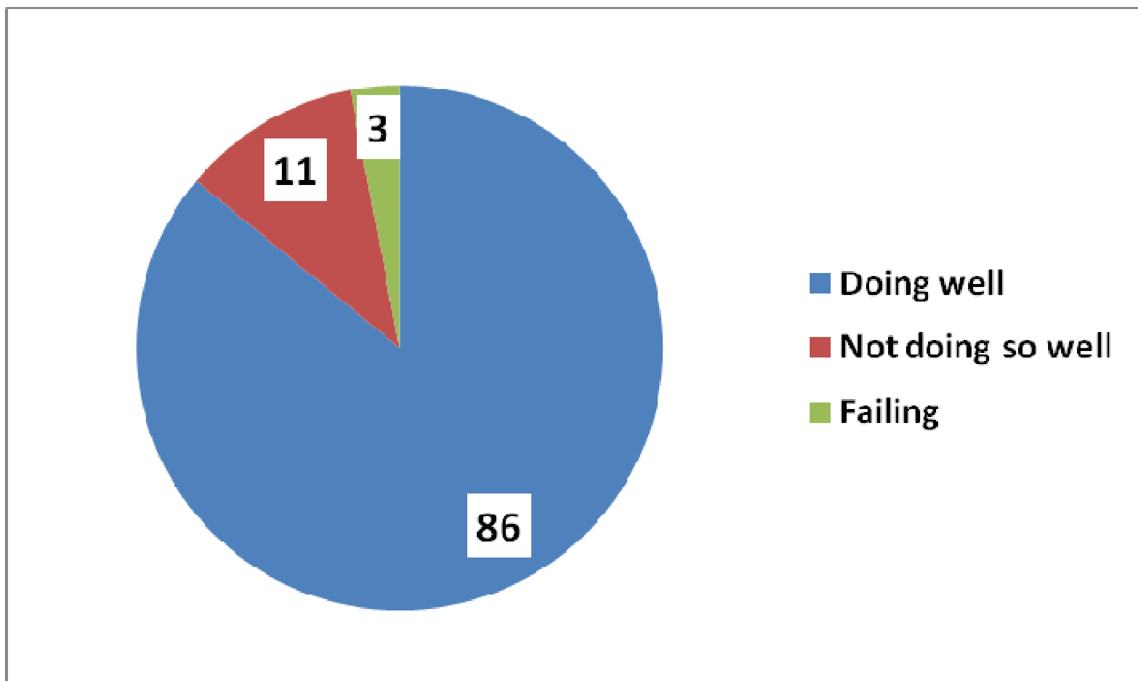

Figure 16: Classification of all study patients (N=338) based on the physicians' assesement of their clinical status. The figures shown are percentages of the study population that were either 'Doing well', 'Not doing so well' or 'failing' according to the physicians. This classification was guided by the CD4 counts and clinical symptoms of patients as stipulated in the guidelines for antiretroviral therapy in Ghana.

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4.6 Nested PCR

Out of the 338 RNA samples analyzed for protease and reverse transcriptase genes, 152 and 96 respectively were successfully amplified. Of the 338 DNA samples analyzed, 182 PR genes and178RT genes were successfully amplified. The details of these results are found in Table 4. Representative gel photographs are shown in Figure 17 and 18.

Table 4: Summary of PCR results showing numbers of samples with amplified genes of interest by study sites and type of sample

Study Sites	**PR Gene**		**RT Gene**	
	Plasma	**PBMC**	**Plasma**	**PBMC**
Korle-Bu Teaching (N=98)	34	73	37	67
St. Martin de Porres (N=101)	52	42	21	55
Atua Government (N=96)	54	44	23	33
Kumasi South (N=43)	12	23	15	23
Total (N=338)	152	182	96	178
Percent (%)	**49.9**	**53.8**	**28.4**	**52.6**

Nested PCR was used to attempt amplification of the protease (PR) and reverse transcriptase (RT) genes from plasma and peripheral blood mononuclear cells (PBMC) of all the samples collected from each study site. The numbers of positive amplicons obtained for each gene are shown for each site. The overall numbers and percentages of positive amplicons are shown under Total.

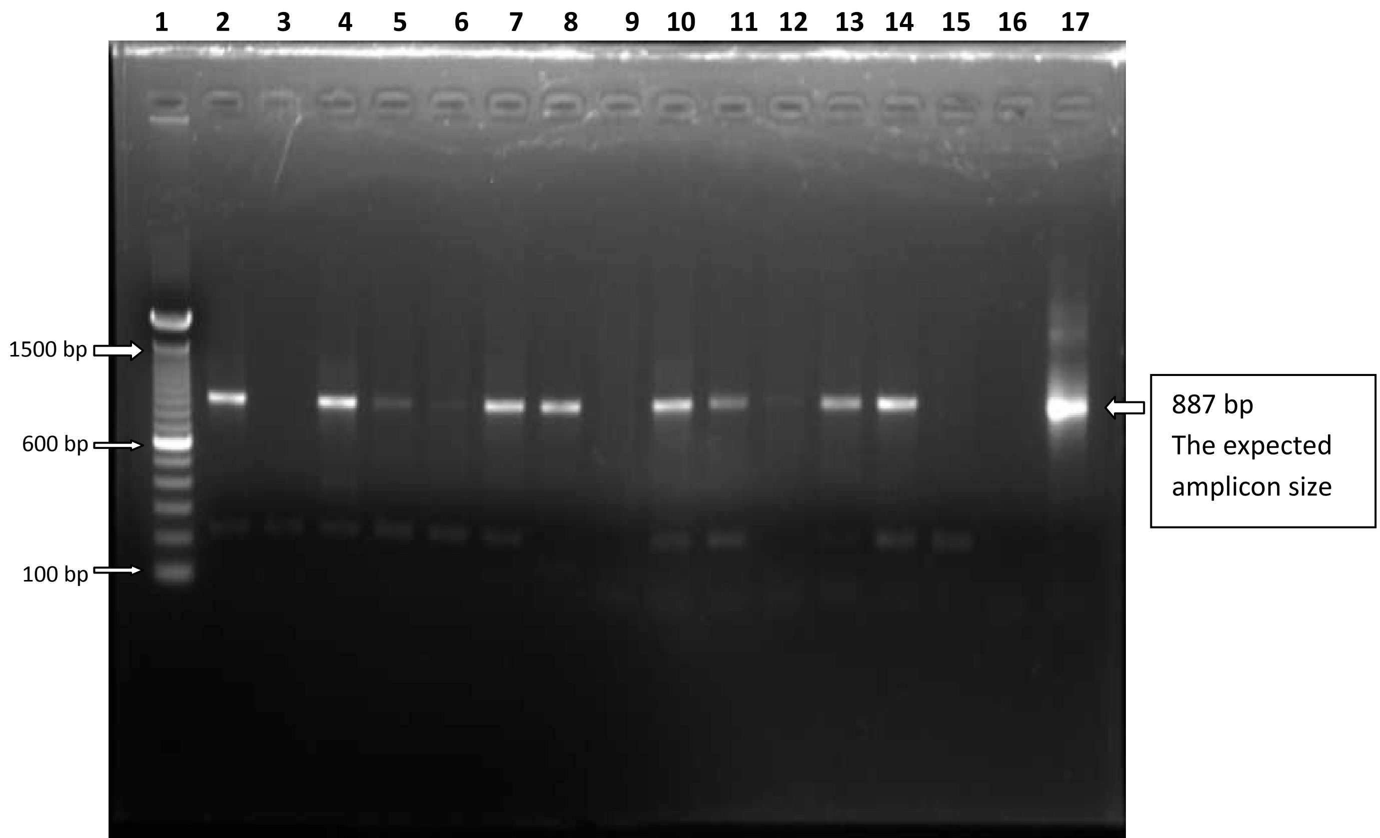

Figure 17: Representative gel photograph showing results of amplification of the RT gene. Agarose gel (1.5%) was run using 1X TAE buffer. Lane 1 contained Trackit™ 100bp DNA ladder (Invitrogen, USA). The band sizes of the molecular weight marker increased from 100bp, at the top of the gel, towards the sample wells in steps of 100. Lanes 2 to 15 contained samples from participants, Lane 16 contained a negative control and Lane 17 contained a positive control. The expected size of the positive product was 887bp. Samples in lanes 3 and 9 were considered as failed amplification while samples in the other lanes (2, 4-8 and 10-14) were successful amplifications.

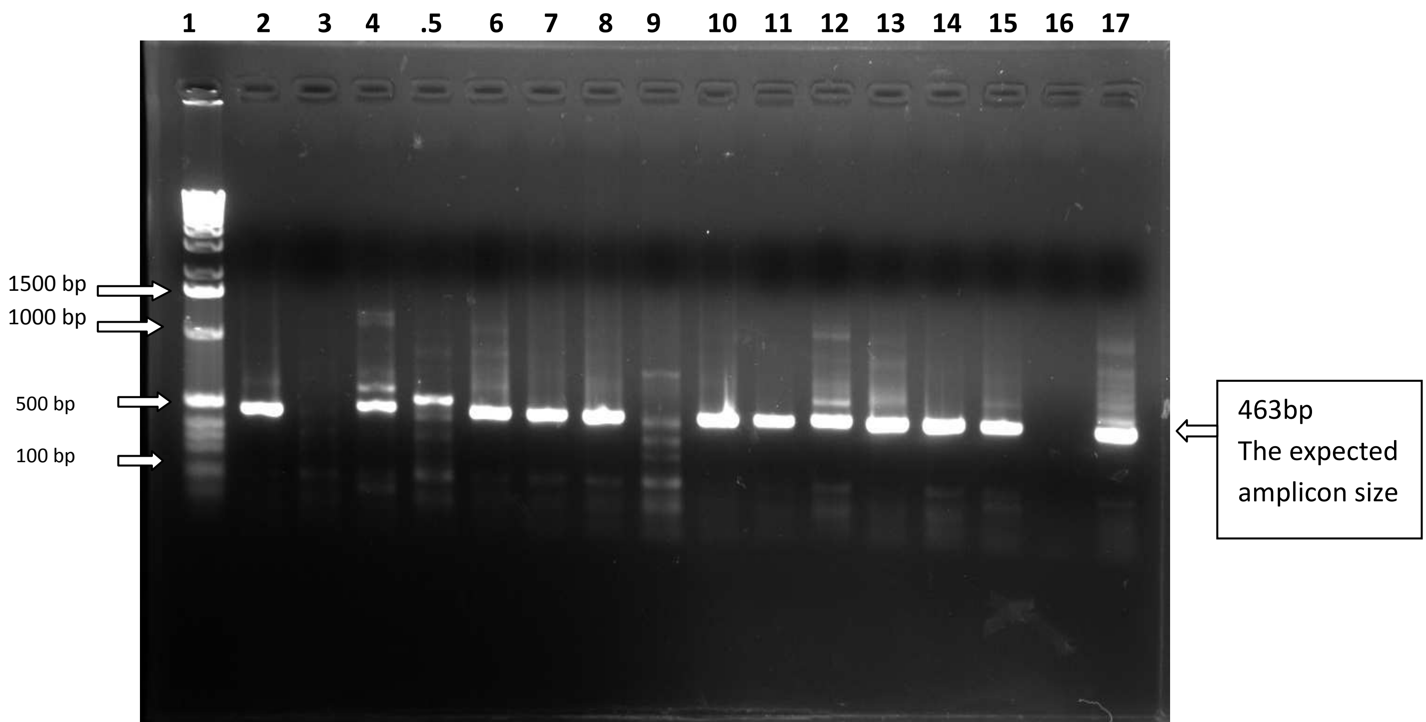

Figure 18: Representative gel photograph showing the result of amplification of the protease gene. Agarose gel (2%) was run in 1X TAE buffer. Lane 1 contained Trackit™ 100bp DNA ladder (Invitrogen, USA). The band sizes of the molecular weight marker increased from 100bp, at the top of the gel, towards the sample wells in steps of 100. Lanes 2 to 15 contained samples from participants, lane 16 contained a negative control and lane 17 contained a positive control. The expected size of the positive product was 463bp. Samples in lanes 3, 5 and 9 were considered as 'failed amplification' whiles the other lanes (2, 4, 6-8 and 10-15) were successful amplifications.

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4.7 Sequencing

Out of the samples successfully amplified, 67% (n=65) and 55% (n=99) were successfully sequenced for the RT gene from plasma and PBMC respectively compared to 35% (n=54) and 42% (n=76) for the PR gene from plasma and PBMC respectively (Table 5). Although more samples were sequenced from PBMC than from plasma, not all samples that were successfully sequenced from plasma were successfully sequenced from PBMC. These sequences were all edited and submitted online to the Stanford HIV Drug Resistance Database (HIVDB) to generate drug resistance mutation information. A summary of the number of sequences with drug resistance mutations for the three drug classes is presented in Table 5.

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Table 5: Summary data showing the number of samples successfully sequenced and those with drug resistance mutations

Sample type	**Plasma**		**PBMC**	
Gene	**PR**	**RT**	**PR**	**RT**
Sequences obtained (N)	54	65	76	99
Number (%) with *DRM	15 (28)	30 (46)-NRTI 32 (49)- NNRTI	7 (9)	25 (25)- NRTI 26 (26)- NNRTI

All positive amplicons were taken through direct sequencing. The total number of patients from whom protease (PR) and reverse transcriptase (RT) sequences were successfully obtained from either plasma or peripheral blood mononuclear cells is shown. The proportions (number and percentage) of these sequences that had drug resistance mutations (DRM) are also shown. RT sequences were analyzed for resistance to both nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) while PR sequences were analyzed for resistance to protease inhibitors.

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4.8 Prevalence of HIV drug resistance mutations

Drug resistance mutations were mostly observed in the RT gene. Forty-six percent (46%) of the RT sequences obtained from plasma had major drug resistance mutations to the nucleoside reverse transcriptase inhibitors (NRTI). Forty-nine percent had major drug resistance mutations to the non-nucleoside reverse transcriptase inhibitors (NNRTI). Twenty-six percent (25%) of the RT sequences obtained from PBMC had drug resistance mutations to NRTI and 26% had NNRTI resistance. The major drug resistance mutations observed in the PR genes were from 28% of the plasma and 9% of the PBMC samples. The RT gene sequences obtained from patients on second-line regimen (N= 19) had more NRTI and NNRTI mutations (79% and 68% respectively) compared to 33% for NRTI and 41% for NNRTI in RT sequences obtained from patients on first-line regimen (Figure 19).

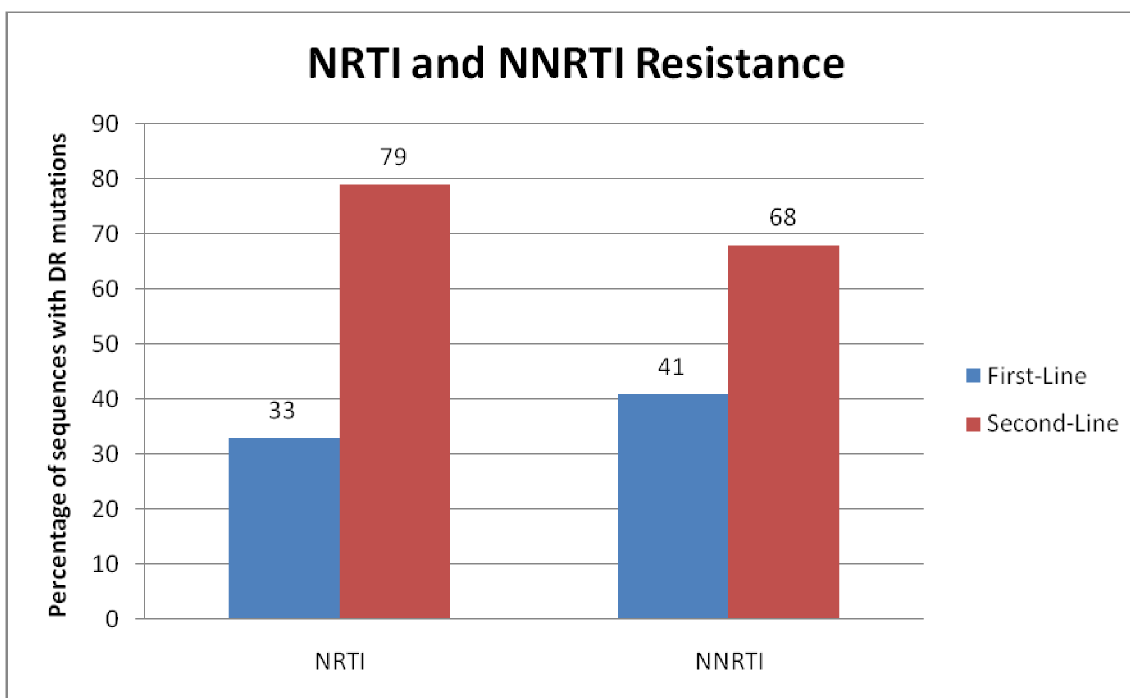

Figure 19: The proportions of RT sequences obtained from patients on first-line regimen that had nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) resistance mutations (blue bars) compared to the proportions of RT sequences obtained from patients on second-line regimen with NRTI and NNRTI resistance mutations (wine bars). Higher proportions of 79% and 68% were observed for in second-line sequences for NRTI and NNRTI respectively compared to 33% and 41% of first-line sequences for NRTI and NNRTI mutations.

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4.8.1 Types of drug resistance mutations found among persons on first-line regimen

The most frequently observed NRTI mutations among persons on first-line were M184V/I (78.6%), T215Y (28.6%) and K219E/Q/R (28.6%). Other less frequently observed drug resistance mutations were M41L, K65R, D67N and K70R. Three samples had T69D or T69S or T69N in RT (Table 6). Nine out of the thirteen patients whose RT sequences were analyzed had two or more NRTI mutations. Six patients had thymidine analogue mutations (TAMs) in addition to M184V (Table 6). Except for patient PDR 295 and PDR 71 who were on therapy for 14 and 21 months respectively before the study, all other patients with TAMs were on therapy for more than 50 months. The Q151M mutation was not found in the sequences analyzed.

The NNRTI mutation commonly observed in persons on first-line were K103N (53%) and V90I (33%). V179E, V106A/I, Y181C, Y188I/L and K238T were also observed less frequently. Other mutations; A98G, E138A, M230L, F227L and H221Y were each found in one person (6%). The details of the combinations of these mutations observed in the patients are shown in Table 7. Except for sample PDR 223 that had N88S and L10V in the PR gene, all other sequences obtained from patients on the first-line regimen had no major drug resistance mutations in the PR gene.

Table 6: Types of NRTI resistance mutations found in patients on first-line regimen and their clinical implications

No.	Patient ID	Months on ART	NRTIs taken	NRTI mutations	*NRTI resistance
1	PDR 71	21	AZT, 3TC	M41LM, M184V, T215Y	3TC, ABC, AZT, D4T, DDI, FTC
2	PDR 88	21	D4T, 3TC	M184MV	3TC, FTC
3	PDR 91	55	AZT, 3TC	M41L, T69D, M184V, T215Y	3TC, ABC, AZT, D4T, DDI, FTC,TDF
4	PDR 102	18	D4T, 3TC, AZT	T69AT, K70EK	DDI
5	PDR 117	12	AZT, 3TC	M184V	3TC, FTC
6	PDR 185	55	D4T, 3TC	K65R, T69d, K219R	3TC, ABC, AZT, D4T, DDI, FTC, TDF
7	PDR 223	58	AZT, 3TC	D67N, K70R, M184V, T215Y, K219Q	3TC, ABC, AZT, D4T, DDI, FTC
8	PDR270	36	D4T, 3TC	M184V, K219EK	3TC, FTC
9	PDR 281	72	AZT, 3TC,	M184V	3TC, FTC
10	PR 285	11	D4T, 3TC	T69S, M184I	3TC, FTC
11	PDR 295	14	AZT, 3TC	D67N, K70R, M184GV	3TC, FTC, AZT
12	PDR 304	48	AZT, 3TC	M184V	3TC, FTC
13	PDR 310	60	AZT, 3TC	D67N, K70R, M184V, T215Y, K219Q	3TC, ABC, D4T, AZT, FTC
14	PDR 351	44	AZT, 3TC	M184V	3TC, FTC

Patients on first-line regimen with RT gene sequences that had resistance mutations to nucleoside reverse transcriptase inhibitors (NRTI). The duration on treatment, the NRTIs they had taken, the list of mutations found and the drug resistance implications of the mutations are shown for each patient. *NRTI resistance shows the names (acronyms) of drugs that the patient is resistant to as a result of the mutations found. Lamivudine-3TC; Stavudine- D4T; Zidovudine- AZT; Abacavir-ABC; Didanosine-DDI; Emtricitabine-FTC; Tenofovir-TDF. Red font shows high level resistance and blue font depicts intermediate level resistance. Drugs with low or potentially low resistance are not shown.

Table 7: Types of NNRTI resistance mutations found in patients on first-line regimen and their clinical implications

No	Patient ID	Months on ART	NNRTIs taken	NNRTI mutations	*NNRTI resistance
1	PDR 71	21	EFV	V90I, A98G, K103N, E138A, V179EV	EFV, NVP
2	PDR 91	21	EFV	K103N, M230L	EFV, ETR NVP, RPV
3	PDR 117	12	NVP	V106A, F227L	EFV, NVP
4	PDR 121	28	NVP	V90I, Y181C	NVP
5	PDR 185	55	NVP	K103N, Y181C, H221Y	EFV, ETR, NVP, RPV
6	PDR 233	30	NVP	K103KN	NVP, EFV
7	PDR 281	72	NVP	K103N, K238T	EFV, NVP
8	PDR 285	11	NVP	V179E, Y188I	EFV, NVP
9	PDR 295	14	NVP	Y188L	EFV, NVP RPV
10	PDR 328	23	EFV	V90I, K103N	NVP, EFV
11	PDR 345	6	EFV	V90I, K103N	NVP, EFV
12	PDR 351	44	NVP	K103N, K238T	EFV, NVP

Patients on first-line regimen who had RT gene sequences with resistance mutations to non-nucleoside reverse transcriptase inhibitors (NNRTI). The duration on treatment, the NNRTIs they had taken, the list of mutations found and the drug resistance implications of the mutations are shown for each patient. *NNRTI resistance shows the names (acronyms) of drugs that the patient is resistant to as a result of the mutations found. Efavirenz-EFV; Nevirapine-NVP; Etravirine- ETR; Rilpivirine-RPV. Red font shows high level resistance and blue font depicts intermediate level resistance. Drugs with potentially low or low level resistance are not shown.

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4.8.2 Types of drug resistance mutations found among persons on second -line regimen

The patients on second-line are those who had failed the first line drugs, based on their CD4 counts and clinical symptoms, and were switched by their physicians to a new combination of drugs including protease inhibitors. The most frequently observed NRTI mutations were M184V (100%), T215Y/F/I (85%), M41L (54%), D67G/N (31%) and L210W (31%). Other mutations were observed at codon 215 in RT and these included T215ADSY and T215NSTY. One patient had two amino acid insertions at codon 69 in RT. E44D, T69D, K70R, L74V/I, V118I and K219Q were also observed in lesser proportions. The details of the combinations of resistance mutations observed in patients on second-line are shown in Table 8. The Q151M mutation was not found in any of the sequences analyzed.

Various NNRTI mutations were found among patients on second-line regimen; the most frequent being K103N/S which occurred in 52% of the samples with resistance mutations, K101P/E/Q and A98G each of which occurred in 31% of samples with resistance mutations. Other NNRTI mutations that occurred less frequently were E138A, V179L, P225H, K238T, V90I, G190A, V108I, Y181C, V106M, H221Y, F227L and M230L. Table 9 shows the details of these mutations found in the patients.

Resistance to protease inhibitors was observed in patients on second-line regimen (Table 10). The most frequently detected mutation was M46I, which occurred in 50% of sequences with PI resistance. Other DR mutations detected less frequently were N88S, I54V, I82F, L90M, E35G, A74V, L89V, 184V, V11I, L23I, L33I, G58E and L10I/V (Table 10).

Table 8: Types of NRTI resistance mutations found in patients on second-line therapy, their clinical implication and the current NRTI taken by patients

No.	Patient ID	NRTI mutations	*NRTI resistance	NRTI taken
1	PDR 1	M41L, E44D, L74V, V118I, M184V, L210W, T215Y	3TC, ABC, DDI, FTC, AZT, D4T, TDF	DDI, ABC
2	PDR 2	V75M, M184V, T215Y	3TC, ABC, DDI, FTC, AZT, D4T	TDF,3TC
3	PDR 4	L74V, M184V, L210W, T215Y	3TC, ABC, DDI, FTC, AZT, D4T, TDF	DDI, ABC
4	PDR 5	T69i, M184V, L210W, T215NSTY	3TC, ABC, DDI, FTC, AZT, D4T	DDI, ABC
5	PDR 6	M41L, D67G, K70R, V75M, M184I, T215F, K219Q	3TC, ABC, DDI, FTC, AZT, D4T, TDF	DDI, ABC
6	PDR 9	M41L, L74I, M184V, T215F, K219W	3TC, ABC, DDI, FTC, AZT, D4T, TDF	DDI, ABC
7	PDR 10	E44D, M184V, L210W, T215Y	3TC, FTC, ABC, AZT, D4T, DDI	DDI, ABC
8	PDR 11	D67G, K70R, M184MV, T215IT	3TC, AZT, FTC, ABC, D4T	DDI, ABC
9	PDR 19	M41L, E44D, D67N, M184V, T215F	3TC, ABC, DDI, FTC, AZT, D4T	DDI, ABC
10	PDR 20	M184V, T215ADSY	3TC, FTC, ABC	DDI, ABC
11	PDR 41	M41L, T69D, M184V, T215Y	3TC, ABC, DDI, FTC, AZT, D4T, TDF	DDI, ABC
12	PDR 90	M41L, M184V, T215Y	3TC, ABC, DDI, FTC, AZT, D4T	TDF, D4T
13	PDR 106	M41L, D67N, T69D, M184V, T215Y	3TC, ABC, DDI, FTC, AZT, D4T, TDF	DDI, ABC

Patients on second-line regimen who had RT sequences with resistance mutations to nucleoside reverse transcriptase inhibitors (NRTI). The list of mutations found, the drug resistance implications of the mutations and the NRTI being taken at time of sampling are shown for each patient. *NRTI resistance shows the names (acronyms) of drugs that the patient is resistant to as a result of the mutations found. Lamivudine-3TC; Stavudine- D4T; Zidovudine- AZT; Abacavir-ABC; Didanosine-DDI; Emtricitabine-FTC; Tenofovir-TDF. Red font shows high level resistance and blue font depicts intermediate level resistance. Drugs with low or potentially low resistance are not shown.

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4.8.3 Occurrence of thymidine analog mutations (TAMs)

Thymidine analog mutations were detected less in patients on first-line compared to those on second line. In those patients on first-line regimen, 6 out of the 15 (40%) of sequences with NRTI resistance mutations had at least 2 TAMs compared with 11 of 13 i.e. 85% of sequences from the second-line participants. Mutations occurring at position 215 and M41L were most frequently found in both groups of patients. Mutations such as L210W, D67N and K70R were detected mostly in patients on second-line regimen while K219Q was mainly found in those on first-line drugs. One patient on second-line had a double amino acid insertion at codon 69. Two patients on the first-line regimen had four TAMs each (D67N, K70R, T215Y and K219Q). Figure 20 shows a comparison of the prevalence of each TAMs between the two groups of patients.

4.8.4 Drug resistance mutations found in plasma and PBMC pairs

The drug resistance mutations found in plasma were generally similar to those found in the PBMC of same patients (Table 11). In a few cases, mutations were detected in plasma but not in the PBMC and *vice versa.* A phylogenetic analysis of paired plasma and RT sequences (Fig. 22) showed that the pairs were closely related to each other than to sequences from other patients.

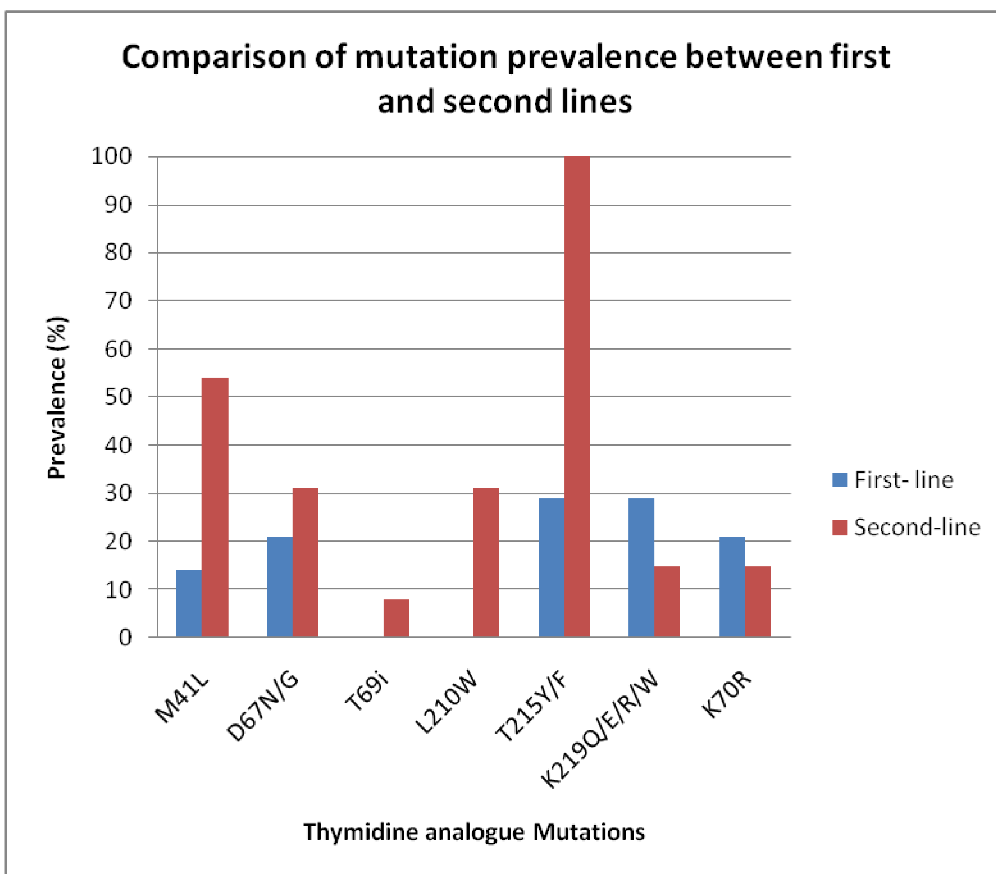

Figure 20: Occurence of thymidine analogue mutations (TAMs) in patients on first-line regimen and those on second-line regimen.The types and frequency of detection of TAMs in patients on first-line regimen and those in patients on second-line regimens are shown. TAMs, known to cause cross-resistance among NRTIs, were detected more frquently in patients on second-line (wine bars) compared to patients on first-line (blue bars). Two of the TAMs (T69i and L210W) were only found in patients on second-line and not in patients on first-line.

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Table 9: Types of NNRTI resistance mutations observed in patients on second-line therapy and their clinical implications

No.	Patient ID	NNRTI mutations	*NNRTI resistance
1	PDR 1	K101P, K103N	RPV, EFV, NVP, ETR
2	PDR 2	K101P, K103S, E138A	RPV, EFV, NVP, ETR
3	PDR 4	A98G, K103N, V179LV, P225H, K238T	EFV, NVP, ETR
4	PDR 5	K103N, V108I	EFV, NVP
5	PDR 6	V90I, K101E, E138A, G190A	EFV, NVP, RPV
6	PDR 9	K103N, V108I	EFV, NVP
7	PDR 10	V90I, A98AG,Y181C, H221Y	NVP, ETR, EFV, RPV
8	PDR 19	V106M, H221Y, F227Y	EFV, NVP
9	PDR 20	K101KQ, K103N, Y181C, H221HY, P225HP	RPV, EFV, NVP, ETR
10	PDR 41	K103N, M230L	RPV, EFV, NVP, ETR
11	PDR 90	K103N, M230L	EFV, NVP

Patients on second-line regimen who had RT gene sequences with resistance mutations to non-nucleoside reverse transcriptase inhibitors (NNRTI). The list of mutations found and the drug resistance implications of the mutations are shown for each patient. *NNRTI resistance shows the names (acronyms) of drugs that the patient is resistant to as a result of the mutations found. Efavirenz-EFV; Nevirapine-NVP; Etravirine-ETR; Rilpivirine-RPV. Red font shows high level resistance and blue font depicts intermediate level resistance. Drugs with low or potentially low resistance are not shown.

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Table 10: PI resistance mutations in patients on second-line therapy, their clinical implications and the current PI taken by the patients

No.	Patient ID	PI mutations	*PI resistance	PI taken
1	PDR 1	M46I, N88S, L10V	ATV/r, NFV	NFV
2	PDR 2	M46I, I54V, I82F, L90M, L10V	ATV/r, IDV/r, NFV, SQV/r, FPV/r	LPV/r
3	PDR 11	E35G, M46I, A71V, I84V, L89V	NFV, ATV/r, FPV/r, IDV/r, SQV/r	LPV/r
4	PDR 19	M46I, L74V, I84V	FPV/r, IDV/r, NFV, ATV/r, LPV/r,SQV/r	LPV/r
5	PDR 106	L90M, L10V, A71T, L89V	NFV, ATV/r, FPV/r, SQV/r	NFV
6	PDR 199	L90M, L23I, L33I, G58E	NFV, SQV/r	NFV

Patients on second-line regimen who had protease gene sequences with resistance mutations to protease inhibitors. The list of mutations found and the drug resistance implications of the mutations are shown for each patient. *PI resistance shows the names (acronyms) of drugs that the patient is resistant to as a result of the mutations found. Nelfinavir-NFV; Fosamprenavir-FPV; Saquinavir-SQV; Lopinavir-LPV; Atazanavir-ATV; Indinavir-IDV; Ritonavir-r. Except for Nelfinavir, all other protease inhibitors were boosted with ritonavir denoted by /r. Red font shows high level resistance and blue font depicts intermediate level resistance. Drugs with low or potentially low level resistance are not shown.

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Table 11: Comparison of NRTI and NNRTI drug resistance mutations observed in sequences from plasma and PBMC pairs.

Patient ID	**NRTI Mutations**		**NNRTI mutations**	
	Plasma	**PBMC**	**Plasma**	**PBMC**
PDR 41	**M41L, T69D, M184V, T215Y**	**M41L, T69D, M184V, T215Y**	**K103N, M230L**	**K103N, M230L**
PDR 48	NONE	M184MV	*NONE	NONE
PDR 71	**M41L, M184V, T215Y**	**M41L, M184V, T215Y**	**V90I**, A98G, **K103N, E138A, V179EV**	**V90I, K103N, E138A, V179E**
PDR 90	M41L, M184V, T215Y	V75IV	**K103N**, V106I,V108I	K101P, **K103N**, E138A
PDR 106	**M41L, D67N, T69D, M184V, T215Y**	**M41L, D67DN, T69AT, M184V, T215Y**	**A98G**	**A98AG**
PDR 223	**D67N, K70R, M184V, T215Y, K219Q**	**D67N, K70R, M184V, T215Y, K219Q**	NONE	V90I
PDR 304	M184V	NONE	NONE	V90I
PDR 328	NONE	M184IM	**V90I, K103N**	**V90I, K103KN**, V108IV, M230ML

Patients who had protease (PR) and reverse transcriptase (RT) sequences from both plasma and peripheral blood mononuclear cells (PBMC) are shown. The mutations found in the PR and RT genes from plasma and PBMC are compared for each patient. *None implies absence of drug resistance mutations detected towards the particular drug class in the plasma or PBMC of that patient.

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4.9 Relationship between change in CD4 counts, viral load, duration on first line ART and the presence of drug resistance mutations

The 65 sequences obtained were categorized based on the change in CD4 counts, viral load at sampling and duration on first-line therapy (Table12). The Pearson's Chi Square test was used to test the relationship between these variables and the presence of drug resistance mutations. There was no statistically significant association between the variables tested and the presence of drug resistance mutations (Table 12).

4.10 Relationship between adherence to ART, herbal medicine use and the presence of drug resistance

Self-reported data on adherence to therapy and herbal medicine use during ART was available for 53 of the 65 patients whose RT genes were successfully sequenced from plasma. Out of the 11 patients who admitted herbal medicine use during ART, 3 had NRTI mutations while 5 had NNRTI mutations. Seventeen of the 42 patients who did not use any herbal medicine during ART each had both NRTI and NNRTI mutations (Table 13). For adherence to ART, the 2 patients that admitted non-adherence both had NNRTI mutations while only 1 had NRTI mutations. Among the 51 patients who adhered, 19 and 20 had NRTI and NNRTI mutations respectively. Analysis of the relationship between herbal medicine use, adherence to ART versus presence of drug resistance mutations by the Pearson's Chi Square test however revealed no statistically significant association between them (Table 13).

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4.11 Polymorphisms at drug resistance –associated positions in PR sequences

Majority (72%) of the sequences analyzed did not have major drug resistance mutations in the PR gene. However, polymorphisms at drug-resistance associated positions were frequently detected in the PR sequences (Fig. 23). M36I was found in 97% of sequences while 93% had K20I and 22% had L10IV. Two sequences (PDR 10 and PDR 251) each had one amino acid insertion; Isoleucine at codon 36 and Asparagine at codon 37 respectively.

4.12 Prevalence of HIV-1 subtypes

Out of the 65 RT sequences analyzed for HIV-1 subtypes, 61 (92.3%) were CRF02.AG recombinant, three (4.6%) were subtype G and one (1.5%) each was subtype B and K. These results were obtained from the Stanford HIV Drug Resistance Database and were confirmed by phylogenetic analysis (Figure 21)

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Table 12: Relationship between the change in CD4 counts, viral load at sampling, the duration on first-line antiretroviral therapy and the presence of drug resistance mutations

		Patients with mutations (%)	
Variable (*N=65*)		**NRTI**	**NNRTI**
difference in CD4 count	Neg (*n=25*)	48	56
	Pos (*n=40*)	45	45
	p Value	***0.813***	***0.388***
Viral load	Undetectable (n=56)	***46***	***48***
	Detectable (n=9)	***56***	***56***
	p Value	***0.542***	***0.683***
Duration on first-line ART	< 2yrs (n=24)	33	46
	2-5 yrs (n=32)	59	59
	>5yrs (n=9)	33	22
	p Value	***0.109***	***0.132***

Associations between parameters were analyzed using the Pearson's Chi Square test. The test of statistical significance was 2-sided and differences were considered significant at $P< 0.05$. Difference in CD4 count was 'Negative' if previous CD4 count was greater than current CD4 and 'Positive' if the opposite was true. Based on the *p*-values obtained, there was no statistically significant association between the development of drug resistance mutations and difference in CD4 count or viral load or duration on ART.

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Table 13: Relationship between adherence to ART, herbal medicine use and the presence of drug resistance mutations

Variable (N=53)		Mutations (n)	
		NRTI	**NNRTI**
Herbal Use	Yes (n=11)	3	5
	No (n=42)	17	17
	p Value	***0.421***	***0.765***
Adherence to ART	No (n=2)	1	2
	Yes (n=51)	19	20
	p Value	***0.715***	***0.087***

Associations between parameters were analyzed using the Pearson's Chi Square test. The test of statistical significance was 2-sided and differences were considered significant at $P < 0.05$. Adherence was defined as not skipping any day without taking antiretrovirals or skipping less than one month of taking drugs cumulatively since the start of therapy. These were self-reported by patients. Based on the *p*-values obtained, there was no statistically significant association between the development of drug resistance mutations and self-reported adherence to antiretroviral therapy or herbal medicine use during antiretroviral therapy.

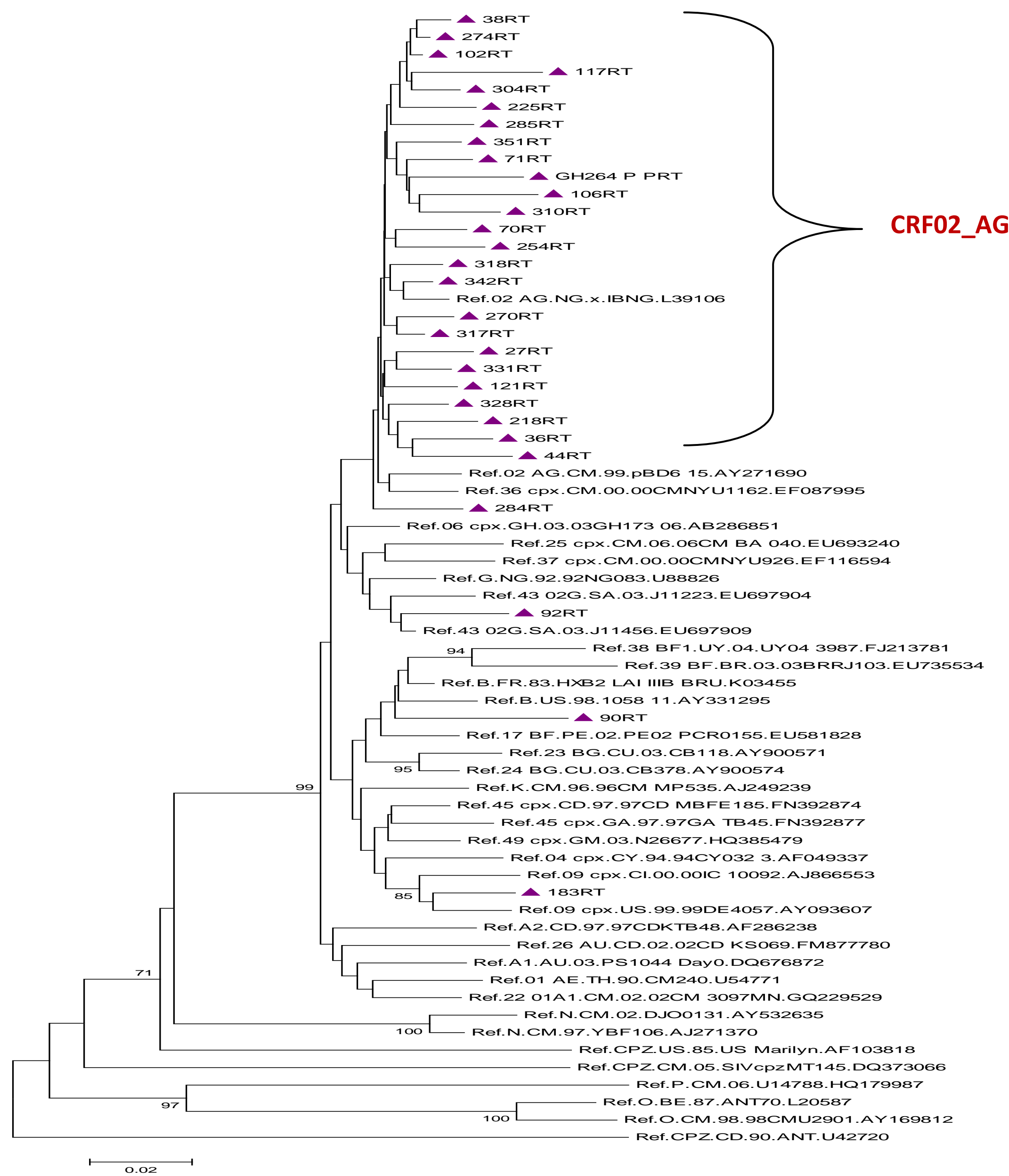

Figure 21: Phylogram (neighbour joining tree) of RT sequences (708 nucleotides) and selected HIV-1 subtype references. Phylogenetic analyses were conducted in MEGA 4 (Tamura *et al,* 2007).Bootstrap values 70% or greater are shown. Purple triangles represented study sequences. Majority of study sequences clustered with the circulating recombinant form (CRF02_AG). This subtype emerged due to the recombination of subtype A and G and it is the predominant HIV-1 subtype in West Africa. The study sequences clustered with reference CRF02_AG sequences *Ref. 02 AG NG .x. IBNG.L39106* and *Ref. 02 AG CM 99.pBDS 15 ay271690* from Nigeria and Cameroon respectively.

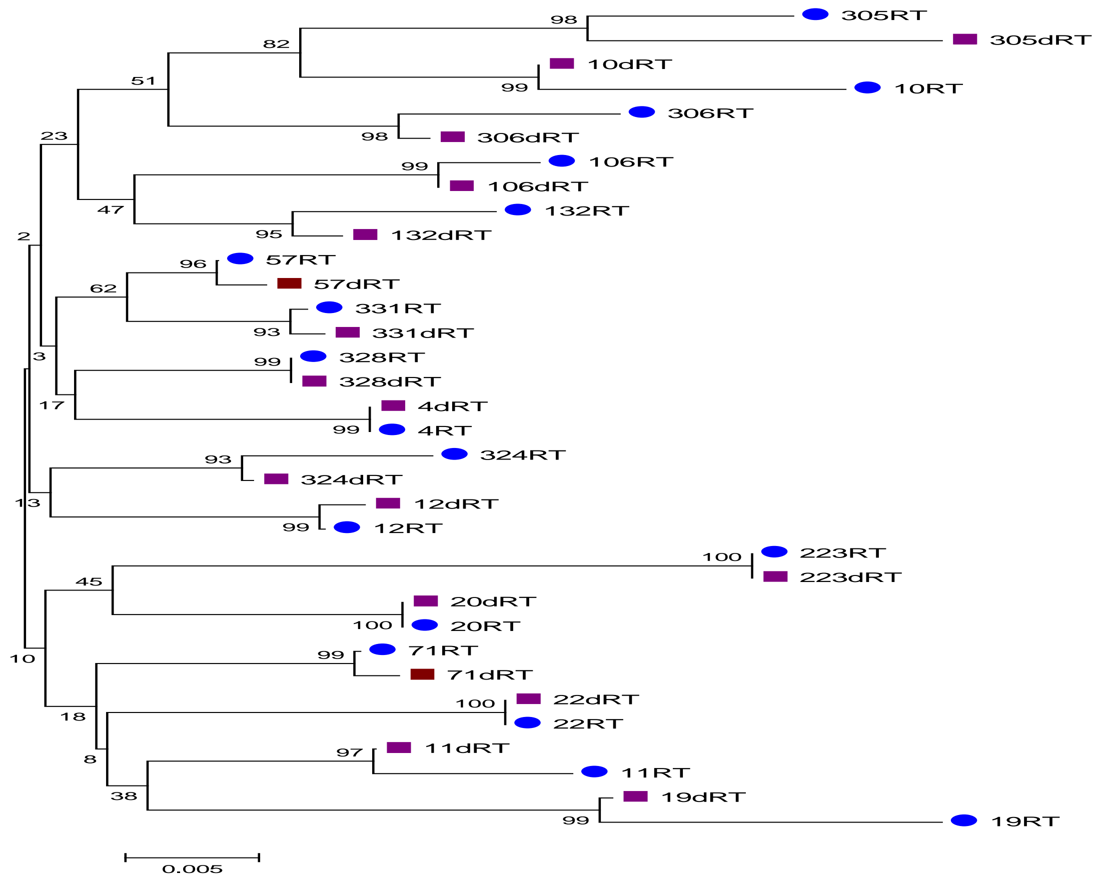

Figure 22: Phylogenetic tree of paired RT sequences (708 nucleotides) obtained from plasma and PBMC of same patient showing the relationship between the sequences. Phylogenetic analyses were conducted in MEGA 4 (Tamura *et al,* 2007).Blue circles represent plasma samples and purple squares represent PBMC sequences. Sequences obtained from paired plasma and PBMC from the same patients clustered together.

Figure 23: Dot plot of protease sequences from study aligned with the protease sequence of HIV-1 reference HXB2. All 99 amino acids (one-letter names) are shown for the reference sequence. The dots represent positions with similar amino acids in study sequences as the reference sequence. Alphabets are amino acid names and show positions where the amino acids in the study sequences are different from that at the same position of the reference sequence. The reference sequence is HIV-1 subtype B and majority of the study sequences are HIV-1 subtype CRF02_AG

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4.13 Capacity building at Virology Department, Noguchi Memorial Institute for Medical Research

As part of this study, an optimized protocol for nested PCR and sequencing of protease and reverse transcriptase genes of HIV-1 was developed. This protocol was modified from two different protocols (Villahermosa et al, 2000; Fujisaki et al, 2007). The protocol was used to establish standard operating procedures in 2011 for HIV-1 genotyping in the Virology Department, Noguchi Memorial Institute for Medical Research. Five research assistants were trained on this protocol to perform HIV-1 drug resistance testing for patients. Three research assistants were also trained on DNA sequencing techniques which are now applied in studies on Influenza and Polio viruses.

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CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

5.1.1 General Introduction

This study sought to investigate drug resistance mutations present in HIV-infected patients on antiretroviral therapy in Ghana and relate the mutations to their drug histories, virologic and immunological profiles. Patients who had been on antiretroviral therapy for a mean duration of 30 months (range 6-86months) were studied.

5.1.2 HIV type, immunologic and virologic markers

The majority of patients had HIV-1 infections (Figure 15) confirming findings of previous studies that HIV-1 is predominant in Ghana (Brandful *et al,* 1997; Ampofo *et al*, 1999; Fischetti *et al,* 2004; Brandful *et al,* 2012). However, the proportion of HIV-2 and HIV-1/2 dual infections (0.9% and 7.4% respectively) observed in this study (between November 2009 and July 2010) was higher than the recent national prevalence of 0.7% and 1.3%, respectively in 2011 (NACP, 2011). Access to highly active antiretroviral therapy (HAART) has expanded in resource-limited settings (RLS) such as Ghana. The laboratory infrastructure to monitor and identify patients failing treatment and requiring a switch in treatment regimen is however inadequate (Badri *et al,* 2008). Countries in such settings do not usually have routine plasma viral load monitoring, the gold standard used in developed countries for diagnosing virologic failure. Countries in sub-Saharan Africa such as Ghana therefore utilize the World Health Organization recommendations of CD4 cell count measurements and clinical symptoms to monitor ART in the absence of viral load (WHO, 2006). Investigations are therefore required into the usefulness of these markers as good indicators for ART success.

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In this study in Ghana, an increasing trend was observed in the mean values of CD4 counts recorded over the treatment period (Figure13). This trend suggested that the drugs used over the period had been effective. Also, since CD4 counts were used as a tool for monitoring treatment and switching therapy, this trend of increasing CD4 counts over the treatment period showed that the participants were generally doing well and there was no need to change their drugs. This finding was confirmed by the physician's assessment of patients' clinical status (Figure 16), which showed that 86% of the participants were doing well. Some individuals (data not shown) had decreased CD4 counts accounting for the 14% of the patients (Figure 16) rated by the physicians as not doing so well or failing on the drugs. However, analysis of the difference in CD4 counts at the time of sampling did not show significant association with the presence of drug resistance mutations in the RT gene. Although those with negative CD4 counts difference had relatively higher frequency of resistance mutations of 48% for NRTI and 56% for NNRTI compared to 46% for NRTI and 45% for NNRTI among those with positive CD4 count difference, these differences were however not statistically significant (Table 12) as also confirmed by the error bars (Figure 13). Thus the difference in CD4 count over a period could not indicate the presence or otherwise of drug resistance mutations. This observation was in agreement with an earlier report by Badri *et al* (2008) that measurement of CD4 count was an inadequate alternative to viral load measurement for the detection of virologic failure which is both a cause and consequence of the development of drug resistance mutations (Badri *et al,* 2008).

Viral load was measured in all patients using real time quantitative RT-PCR assay. Only 7% (n=24) had detectable viral load. This suggested that only 7% of the study population may be experiencing virologic failure and may have drug resistance mutations. Fourteen percent (14%) of the study population were classified as failing or not doing so well on the drugs and

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this is double the number that had detectable viral loads. Thus, some of the patients classified as failing by the physicians had viral loads below the detection limit. The RT gene of nine of the patients with detectable viral load, representing 38%, was successfully sequenced and analyzed. Out of these, five had major drug resistance mutations. These five represent only 16% of all sequences with drug resistance mutations in the RT gene. The majority (84%) of sequences with drug resistance mutations in the RT gene were derived from patients with viral loads below detection limit. When the differences were statistically tested using Pearson's Chi Square, no significant association was found between the presence of drug resistance mutations and detectable viral load (Table 12). This finding emphasized the observation that although several studies seek drug resistance mutations in patients experiencing virologic failure (Samati *et al*, 2003; Saravanan *et al*, 2012; Sigaloff *et al*, 2012), it is worthwhile to also investigate drug resistance mutations in patients on ART with persistently low or undetectable viral loads (Mackie *et al,* 2004; Martinez-Picado *et al,* 2007; Metzner *et al,* 2007).

5.1.3 PCR amplification of reverse transcriptase and protease genes

Nested PCR was used to separately amplify an 887bp fragment of the reverse transcriptase coding genes and 463bp fragment of the protease coding genes of the *pol* region of HIV-1. The amplification success of the RT gene from plasma was the least followed by that of RT gene from PBMC then PR gene from plasma and finally the PR gene from PBMC (Table 4). The low success in amplification, a major limitation of this study, could be attributed to the mostly undetectable and low viral loads observed in these patients. However, some samples with undetectable viral loads were amplified while others with detectable viral loads were not amplified (data not shown) making it difficult to attribute the failure of amplification solely to viral load differences. The lengths of the fragments (887bp for RT and 463bp for PR) could

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have contributed to the higher success observed in the PR as compared with the RT genes. Proviral DNA from PBMC is known to be more easily amplified by PCR compared to RNA from plasma particularly in patients with low or undetectable viral loads (Kabamba-Mukadi *et al,* 2009) and so the differences observed in amplifying from PBMC compared to plasma were as expected. Template volumes were increased but that did not improve rate of successful amplifications. Other parameters of the protocol need further modification to achieve better amplification rates.

5.1.4 Sequencing of reverse transcriptase and protease genes

Sequencing of the successfully amplified genes was done using reagents from Applied Biosystems on a Genetic Analyzer ABI 3130. The sequences were edited, assembled and aligned. For some patients, the RT or PR genes amplified were not successfully sequenced. This scenario was mostly observed for the protease gene. In some of these cases, some sequence data was obtained but the peaks in the electrophorogram looked so compressed like mixtures of bases at each position and therefore could not be edited and analyzed. Repeated sequencing using smaller amount of template DNA could not resolve these mixtures. Clonal sequencing could have resolved the mixtures involved but this was not within the scope of the current study.

5.1.5 Prevalence and characteristics of drug-resistance mutations

Drug resistance mutations were observed both in patients on first-line regimen and those on second-line. The types of mutations and their implication for therapy are discussed in the following sections.

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5.1.5.1 Drug resistance mutations among patients on first-line regimens

Among patients on first-line therapy, M184V was the most commonly detected mutation. This mutation was detected in 10 out of 15 participants, in addition to two other mutations at the same codon M184I and M184GV. Thus 80% of the sequences obtained from patients on first-line ART had a drug resistance mutation at codon 184 in the RT gene. This finding confirmed that of previous studies that M184V is the dominant NRTI mutation found among persons on first-line therapy (Shafer *et al*, 2000; Wallis *et al*, 2010; Shafer and Schapiro, 2008; Martinez-Cajas *et al*, 2009; Sigaloff *et al*, 2012). The mutations M184V and M184I cause high-level resistance to lamivudine and emtricitabine and low-level resistance to didanosine and abacavir. Thus the high frequency of M184IV observed in this study is not unusual since all these patients had been on lamivudine as part of their regimen. Previous findings by Ji and Loeb (1994) and Keulen *et al* (1999) showed that the M184I usually emerges before M184V due to the reverse transcriptase's preferential mutation of G to A over A to G resulting in ATG to ATA (Methionine to Isoleucine) more quickly than ATG to GTA (Methionine to Valine) . The enzymatic efficiency of M184I is however lower than that of M184V therefore all viruses with mutations at this codon eventually develop the M184V (Frost *et al,* 2000).

The observation of high prevalence of mutations at codon 184 in the RT gene is also in agreement with earlier findings that patients who develop virologic failure on their first treatment regimen are usually found to have HIV strains with resistance to only one drug in the regimen (Shafer, 2002; Gallego *et al,* 2001; Descamps *et al,* 2000; Murphy *et al,* 1999). According to these reports, the drug to which resistance most commonly developed is lamivudine or an NNRTI. Patients with M184V could however continue treatment with lamivudine or emtricitabine because these mutations increase susceptibility to zidovudine,

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tenofovir and stavudine and are associated with clinically significant decrease in HIV-1 replication (Miller *et al,* 2002; Diallo *et al,* 2003; Shafer and Schapiro, 2008). Results from this study also confirm that M184IV is the first NRTI mutation to emerge in patients treated with lamivudine-containing regimen (Descamps *et al*, 2000; Shafer, 2002) since it was the only mutation in patients that had a single drug resistance mutation (Table 6).

Other mutations commonly detected were T215Y and K219E/Q/R. These mutations occurred with M184IV, M41L and K70R in some cases and with K65R and D69D/N/S in others (Table 6). The M41L, D67N, K70R, T215Y/F and K219Q/E are thymidine analogue mutations (TAMs), which usually develop during treatment with thymidine analogues (zidovudine and stavudine). TAMs are known to enhance the entry of pyrophosphates (PPi) and adenosine triphosphate (ATP) into a site adjacent to the incorporated analogue. The PPi or ATP then attacks the phosphodiester bond between the analogue and the growing chain resulting in the removal of the analogue and allowing polymerization to continue (Shafer *et al,* 2000; Shafer 2002; Clavel and Hance, 2004 and Schapiro, 2008). Due to this mechanism, the TAMs induce resistance to all the NRTIs to some extent. The mutation, T215Y, develops during treatment with zidovudine and stavudine and reduces susceptibility to abacavir, didanosine, and tenofovir particularly in combination with M41L and L210W. These, T215Y and T215F mutations result from two base-pair changes. Other mutations at codon 215 are transitions between the wild type and the mutant. These mutations (T215ADSY, T215NSTY and T215I) found in patients on this study do not reduce susceptibility of the drugs but are indicators of the presence of drug pressure (Shafer, 2002). The K219Q/E mutations decrease zidovudine and probably stavudine susceptibility when present with K70R or T215Y/F, but have little if any effect on the remaining NRTIs. K219R occurs commonly in heavily NRTI-treated patients.

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The mutations; K65R and D69N were also observed. Position 64-72 in RT forms a loop between the beta 2 and beta 3 strands in the finger region. This loop makes contact with the incoming dNTP during polymerization (Huang *et al,* 1998; Sarafianos *et al,* 1999). Mutations in this area affect the contact between the loop and the incoming dNTPs thus inhibiting polymerization. Two sequences had T69D and T69S. The mutations at codon 69 are known to cause resistance to each of the NRTIs when they occur with the TAMs (Winters and Merigan, 2001). Abacavir therapy selects for K65R and this mutation reduces susceptibility to abacavir, didanosine and tenofovir (Miller *et al* 2004). According to Hawkins *et al* (2009), K65R is also selected during treatment with stavudine-containing regimens in patients infected with HIV-1 subtypes other than subtype B (Hawkins *et al* 2009; Wallis *et al*, 2010). This could explain the occurrence of K65R in a patient without Abacavir experience in this study. An amino acid deletion at codon 69 in RT was also found. Amino acid deletions (d) between codon 66 and 71 are rare and usually only occur in combination with either multiple thymidine analogue mutations (TAMs) or the Q151M complex and, in these contexts, they are often associated with high-level multi-NRTI resistance (Rhee *et al*, 2006). In this particular patient (PDR 185), the amino acid deletion occurred with K65R and K219R.

Generally, multi-nRTI resistance mutations were less frequently observed in patients on first-line regimen. The Q151M mutation was not observed in this study. This mutation, which is known to confer multi- NRTI resistance, is rare and occurs in only 5% of patients treated with DDI and AZT or d4T (Van Vaerenbergh *et al,* 2000; Shafer *et al,* 1995). It is therefore not strange that this mutation was not found in this study. In almost all of the patients on first-line regimen, lamivudine, stavudine and zidovudine were the NRTIs taken and the mutations observed were all indicative. Thymidine analogue mutations (M41L, T215Y, D67N, K70R

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and K219E/Q) were found among patients on first-line therapy and this is of great concern since these mutations could indicate cross resistance to the NRTIs (Shafer, 2002).

The duration on therapy seemed to have contributed to the number and types of drug resistance mutations found in the patients on first-line regimen. Majority of the patients on first-line therapy had been on the same drugs for a median of 36 months and had accumulated these mutations over the treatment period. Four out of the six patients who had RT sequences with >2 NRTI mutations with TAMs (Table 6) had been on therapy for more than 50 months. Thus the longer patients are maintained on same drugs; the more likely they are to develop more mutations including multi-drug resistance ones (Kantor *et al,* 2004). Although there was no statistically significant association found between duration on therapy and the presence of the mutations (Table 12), it was clear that these mutations accumulated with time in the presence of drug pressure. According to Kantor *et al,* 2004, the maintenance of patients on the same drugs in the presence of one or two drug resistance mutations leads to the development of more mutations particularly cross-resistant mutations that will render future drugs ineffective. It is important to determine at what time point in therapy multi-NRTI resistance mutations usually develop but the cross-sectional nature of this study could not provide this information. Even in patients that had more than two mutations at the time of analysis, it was difficult to tell, using a one-time sequence analysis, which of the mutations was first to develop, except for M184V/I, which is usually the first mutation to appear in patient who had taken lamivudine (Descamps *et al*, 2000; Shafer, 2002). A longitudinal study would provide data that will better explain the evolution of resistance mutations.

The mutations that are selected for after the failure of treatment with NNRTIs are all located in the pocket targeted by these compounds, and they reduce the affinity of the drug (Boyer *et*

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al., 1993; Richman *et al.,* 1994; Bacheler *et al.,* 2000; Ren *et al.,* 2001; Hsiou *et al.,* 2001). The mutations develop in a drug-specific manner due to the specific interaction of the drugs with the hydrophobic pocket but cause cross resistance to other drugs in the class. For example, K103N is an EFV- induced mutation but causes high resistance to both EFV and NVP. The Y181C mutation emerges during NVP use but induces high level resistance to EFV. The most commonly detected NNRTI resistance mutation among patients on first-line was K103N. This was expected since all the patients studied had been on either EFV or NVP as part of their regimen. The sequential use of NVP and EFV (in either order) is not recommended because of cross-resistance between these drugs (Antinori *et al,* 2002). However, this was done in some of the patients in this study perhaps due to drug availability. Although more patients used NVP compared to EFV (Table 3), the Y181C mutation was less frequently detected compared to K103N. The presence of the K103N mutation meant that there was high resistance to both NVP and EFV. Other NNRTI mutations detected are shown in Table 7. The V90I mutation, a substitution of valine by isoleucine, is a common polymorphism that is selected for by etravirine and rilpivirine and is associated with reduced etravirine susceptibility in combination with other NNRTI-resistance mutations (Liu and Shafer, 2006). The occurrence of this mutation in the study population at a level as high as 40% is contrary to expectation since neither etravirine nor rilpirvirine was used by any patient. This could mean that nevirapine and or efavirenz use may also select for this mutation but this needs further research. It has been reported that A98G reduces NVP and EFV susceptibility by about 5-fold and 3-fold, respectively. The V106A mutation causes high-level resistance to NVP and low-level resistance to EFV. Whilst V179D/E alone can reduce NVP and EFV susceptibility approximately2-fold, the combination of K103R and V179D reduces susceptibility to NVP and EFV approximately10-fold. The Nevirapine related mutation (Y181C) causes high-level resistance to NVP, and decrease susceptibility to EFV, ETR and

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RPV by approximately 2-fold and 5-fold respectively (Liu and Shafer, 2006). Other mutations found were Y188L/H/C and F227L that Y188L causes high-level resistance to NVP, EFV, and RPV. The F227L mutation usually occurs in combination with V106A and is associated with high level resistance to nevirapine and intermediate resistance to efavirenz while M230L causes intermediate/high-level resistance to each of the NNRTIs.

The mutations K238T and K238N are NNRTI-selected mutations that usually occur in combination with K103N. In combination with K103N, they cause high-level resistance to Nevirapine and efavirenz. While E138A may contribute to reduced etravirine and rilpilvirine susceptibility in combination with other NNRTI-resistance mutations, H221Y is seen in patients receiving NNRTI and contributes to decreased NNRTI susceptibility in combination with other NNRTI-resistance mutations. Therefore, all the NNRTI mutations found among patients in this study had contributed in one way or the other to decreased susceptibility of NVP and EFV in particular and may lead to decreased susceptibility to etravirine and rilpivirine that were not taken by patients in this study. All the patients on first-line regimen were still receiving either NVP or EFV. Generally, these patients were described as doing well by the Physicians but the resistance mutations found in them could reduce the effectiveness of the NNRTI in their regimen and contribute to poor clinical outcomes.

Protease inhibitor (PI) mutations were found in only one participant on first-line regimen, which is not surprising, since the first-line regimen does not usually contain PIs. Although there was no clinical history of PI use, this patient (PDR 223) had N88S and L10V mutations. The N88S mutation causes high-level resistance to NFV and ATV/r and low-level resistance to IDV/r; it increases susceptibility to FPV/r (Liu and Shafer, 2006). L10I/V/F/R/Y are associated with resistance to most PIs when present with other mutations (Liu and Shafer,

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2006). L10I/V also occurs in 5-10% of untreated persons. It is therefore likely that the L10V mutation was present before the patient begun treatment. The source of N88S could not be traced to any drugs used since the participant had no history of PI use. However, the presence of these mutations could affect future administration of second-line regimen which contains PIs.

The pattern of resistance observed in patients on first-line regimen indicates that drug resistance testing would be most useful in the choice of appropriate drugs for the second-line regimen to achieve optimum treatment outcomes.

5.1.5.2 Drug resistance mutations among patients on second-line regimen

In this study, various NRTI resistance mutations were found among patients on second-line regimen. The M184V was most frequently detected. This mutation was found in all the sequences that had drug resistance mutations and was closely followed by T215Y/F/I and M41L. The mutations D67GN, L210W, E44D, T69D, K70R, L74V/I and K219Q were also found in this study (Table 8). The presence of M184V in all the samples confirms previous use of lamivudine in the first-line therapy. Thymidine analogue mutations (M41L, D69N, K70R, L210W T215F/Y, and K219Q) were more frequently detected in these samples compared to samples from patients on first-line regimens. Thus, HIV strains in these patients were more resistant to many of the NRTIs. When compared with mutations detected among patients on first-line, the mutations observed among the first-line group were drug-specific while those observed in the second-line were class-specific. Thymidine analogue mutations were frequently observed in the second-line group in addition to the 69 insertion complex of mutations (Table 8).

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Some of the drug resistance mutations found affected the very drugs that the patients were taking at the time of study. Didanosine and abacavir were the most commonly used combination of NRTI for the second-line group and all of the viral sequences obtained had indications of either high level or intermediate level resistance to either or both (Table 8). This may render the NRTIs in the second-line regimen ineffective and further limit future options of drug choices. The accumulation of TAMs and other complex mutations, such as 69i causes decreased activity of NRTIs, with consequences for the effectiveness of the currently available second-line regimens in Ghana. Previous findings have shown that once cross-resistance has developed, standard second-line regimens will primarily offer the benefits of the boosted PI, with limited or no additional effect of the NRTI backbone (Sigaloff *et al* 2012). Patients will thus practically receive PI mono-therapy, which lowers the barrier for selection of PI resistance.

In the patients on second-line regimen, NNRTIs were not part of the regimen but NNRTI mutations similar to those detected among patients on first-line regimen were observed (Table 9). This observation was concordant with a previous study that majority (73%) of patients in whom NNRTIs had been discontinued still harboured NNRTI mutations (Saravanan *et al,* 2012). Persistence of NNRTI mutations long after discontinuation of the NNRTI may be related to the low overall impact on viral fitness of mutations such as K103N, Y181C and G190 (Iglesias-Ussel *et al,* 2002; Joly *et al,* 2004). However, the presence of these mutations needs to be evaluated in case the need arises to re-introduce NNRTIs in future regimens.

The protease inhibitor administered to patients on the study was NFV or LPV/r. The most frequently detected PI resistance mutations were M46I and L90M occurring in 50% and 37.5% of the sequences. The I84V mutation, which is an ATV/r-related mutation, was also

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observed in 20% of the patients although there was no ATV/r use. Other mutations observed less frequently and in various combinations included N88S, I54V, I82F, L74V, A71T, L89V, L23I, L33I, G58E and E35G (Table 10). The presence of PI mutations in these patients was expected since they had been on PIs as part of their second- line regimen. The levels of PI resistance mutations observed were lower than those of NRTI and NNRTI which could mean that the PIs were still effective. This difference could also be attributed to the longer duration of patients on NRTI and NNRTI compared to PI. The resistance profiles of patients on second-line mirrors the effect of switching drugs without drug resistance data. These patients have accumulated various NRTI resistance mutations in addition to PI mutations, rendering their regimen sub-optimal. Shafer (2002) alludes to the fact that patients whose physicians have access to drug resistance data respond better to salvage therapy than those whose physicians depend on other markers than drug resistance data and make 'blind' switches (Shafer 2002). It is therefore strongly recommended that patients failing second-line regimen in Ghana should undergo genotypic resistance testing to guide the composition of their subsequent regimens.

5.1.5.3 Drug resistance mutations in paired plasma and PBMC

Generally, the NRTI drug resistance mutations observed in plasma and their corresponding PBMC were similar (Table 11). One mutation M184MV or M184IM each was detected in PBMC and not in plasma in two patients (PDR 48 and PDR 328). In these cases, it was possible that these mutations were selected for earlier and archived while the patients were on lamivudine but disappeared from plasma after the drug was discontinued. These patients also showed the simultaneous presence of the mutant with the wild type indicating a gradual reversal to the wild type. Another patient (PDR 304) also had M184V in plasma and no mutation in PBMC. In this case, M184V, being the first mutation to emerge in patients using

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lamivudine-containing regimen (Shafer, 2002) may be recent and not yet archived in PBMC. In one patient (PDR 90), the three mutations (M41L, M184V and T215Y) found in plasma were different from that found in PBMC (V75IV). This was the only case of a completely different mutation profile in plasma compared to PBMC for NRTI. The PBMC sequence however showed hypermutation due to the presence of APOBEC 3G/F and this could explain why the resistance mutations were not observed in the PBMC. Apolipoprotein B editing complex (APOBEC) group of proteins are host restriction factors that inhibit HIV replication by inducing dG-to-dA hypermutations in the proviral DNA (Sheehy *et al.*, 2002; Liddament *et al.,* 2004; Bishop *et al.,* 2006). These proteins are expressed in lymphocytes, the major target cells for HIV-1 infection (Liddament *et al.*, 2004; Wiegand *et al.,* 2004). They reduce the rate of HIV replication and by extension the development of mutations.

The NNRTI mutations were also similar in plasma and PBMC (Table 11). Where there were more mutations detected in either plasma or PBMC, the cumulative implications of these mutations for drug resistance were similar for both compartments. Patient PDR 328 for example, had 2 more mutations (M230L and V108I) in PBMC in addition to V90I and K103N observed in both compartments but these mutations cumulatively had reduced NVP and EFV susceptibility so the clinical implications were the same.

PI mutations were observed mainly in plasma component of the patients on second-line therapy. The three patients that had mutations in both plasma and PBMC were PDR 4, PDR 11 and PDR 19. One of them (PDR 4) had the same mutation L10I in both plasma and PBMC. The patient PDR 11 had M46LM and A71AV in PBMC whereas its plasma that had I84V, E35G and L89V in addition. The additional mutations in plasma might have emerged recently and were not yet archived in PBMC since those mutations (M46LM and A71AV)

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found in the PBMC were co-existing with the wild type. Thus in general, the profile of mutations observed in plasma were similar to those observed in PBMC.

5.1.6 Effect of change in CD4 counts, viral load at sampling and duration on first-line ART on drug resistance mutations

Duration on ART, change in CD4 counts and viral loads are factors known to affect the presence of drug resistance mutations in HIV patients. In this study however, the differences observed were not statistically significant. This may be due to the small number of sequences (65) analyzed.

5.1.7 Effect of adherence to antiretrovirals or herbal medicine use on the presence of drug resistance mutations

Data on adherence to ART and herbal medicine taken during ART showed some differences between patients that took herbal medicine and those who did not and between those who adhered and those who did not. However, these differences were not statistically significant (Table 13). These differences are worth further investigations particularly in a longitudinal study.

5.1.8 Polymorphisms at DR sites in the PR sequences

Polymorphisms at resistance-associated positions were observed in the PR genes analyzed in this study. The mutations K20I and M36I were found in 96% and 97% of the sequences respectively. The L10I or L10V mutations were also observed in 22% of the sequences confirming the fact that polymorphisms associated with PI resistance in subtype B do occur naturally in non-B subtypes (Turner *et al,* 2004; Kantor *et al,* 2006). Majority of the sequences in this study were CRF02_AG and known to be associated with K20I, M36I and

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L10IV which have implications for nelfinavir and tipranavir susceptibility (Turner *et al,* 2004; Delgado *et al,* 2008). Kinomoto *et al* (2004) also reported that HIV-1 protease sequences obtained from ART- naive patients from Ghana were differentially less susceptible to protease inhibitors but did not directly link their findings to any of the mutations observed. Amino acid insertions at codons 35 and 37 in the protease gene, similar to those reported by Delgado *et al* (2008) were also detected in patients in this study. The implication of these mutations on PI susceptibility however needs further investigation.

5.1.9 HIV-1 Subtype information

The majority of the reverse transcriptase sequences were subtyped as CRF02_AG by the Stanford HIV Database. This finding agrees with those of previous studies that CRF02_AG is the predominant HIV-1 subtype in Ghana (Delgado *et al*, 2008; Brandful *et al*, 2012). The other subtypes found in this study were B, G and K. Subtype B is reported as the predominant HIV-1 subtype in Europe and North America (Osmanov *et al,* 2000; Kantor *et al,* 2005) and finding it in Ghana could be due to importation. Subtypes G and K have been previously reported in Ghana and other West African countries (Brandful *et al*, 1998; Kantor *et al*, 2005). Although unique recombinant forms are also known to occur in Ghana (Delgado *et al*, 2008), none were described in this study probably because detailed analysis was not done to identify recombinants.

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5.2 Conclusions

This study has clearly showed that HIV-1 patients on ART in Ghana may not be deriving optimal benefit because of the limited markers being used to monitor treatment and effect changes in regimens. The CD4 counts and viral loads of the patients studied confirmed the physicians' assertion that majority of the patients were doing well. However, 46% and 49% of these patients had major drug resistance mutations to NRTIs and NNRTIs respectively. Also, TAMs, which render NRTIs ineffective, were found in both patients on first-line (33%) and those on second-line (79%) regimens. There were PI resistance mutations present in 28% of the PR sequences obtained from patients on second-line regimen.

Similar patterns of drug resistance mutations were observed across study sites and were comparable to patterns observed elsewhere with similar drug regimens. Thus, the types of antiretroviral drugs taken by patients were the main driving force for the development of resistance mutations.

The profiles of drug resistance mutations found in patients on first-line regimens suggested that the duration of a patient on same drug combination has an influence on the type and number of drug resistance mutations developed. There was however no statistically significant association between duration on therapy and the presence of drug resistance mutations probably because this was a cross-sectional study. There was also no statistically significant association between adherence to therapy or herbal medicine use while on therapy and the presence of drug resistance mutations.

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The HIV-1 sequences obtained were mostly classified as CRF02_AG recombinants and this confirmed the dominance of this HIV-1 subtype in Ghana as shown by previous studies.

This study also found similar resistance profiles in plasma compared to peripheral blood mononuclear cells of paired sequences in both the RT and PR genes. Thus PBMC could be used as an alternative to plasma for drug resistance testing.

The drug resistance mutation data generated in this study indicates the clear need for the use of genotypic resistance data in patient management particularly to inform the choice of drugs to change regimens. This will reduce the accumulation of multi- nucleoside and thymidine analog mutations in patients and enhance treatment success. For patients who will fail the current second-line regimen, the data showed that there will be very little benefit with available drugs if switched without individual drug resistance profiling

The study has provided vital drug resistance data to guide policy on ART monitoring in Ghana. It has also contributed to improved protocols for HIV-1 genotyping at the Virology Department of the Noguchi Memorial Institute for Medical Research and helped to build capacity for drug resistance testing of patients who fail ART in Ghana.

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5.3 Recommendations

1. Regular viral loads should be used to monitor patients on ART in Ghana and drug resistance testing should be done before switching to a new regimen.

2. A longitudinal study is recommended to establish a cohort of patients on ART in Ghana to better understand the evolution of drug resistance mutations during treatment.

3. A phenotypic study is recommended to better understand the drug resistance implications of the polymorphisms observed in the protease gene of HIV-1 subtype CRF02_AG from Ghana.

4. Continuous surveillance for HIV drug resistance in the ART-naive population to alert the national program on the levels of transmitted HIV drug resistance,

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APPENDICES

Appendix I

Questionnaire for Sample Collection

CHARACTERIZATION OF HIV DRUG-RESISTANT STRAINS IN HIV-INFECTED PERSONS ON ANTIRETROVIRALS (ARV) IN SELECTED HIV CARE CENTRES IN GHANA

Virology Department, Noguchi Memorial Institute for Medical Research,
University of Ghana, Legon

1. Study Site____

2. Sample ID___________________ Age____________ Sex_______________

3. Date of Collection________________ Current CD4 Count____________________

4. Previous CD4 count______________ Date analyzed________________________

5. Date started ART________________ CD4 count at start of ART_________________

6. ARV currently on_______________________ Duration__________________

7. Treatment history:

   ARV_______________________________ Dates (from- to) ______________________

   ARV_______________________________ Dates (from- to) _______________________

Please make a cross (X) near the appropriate answer for questions 8-10

8. Have you missed any drug days since you started? Yes_______ No________

   8b. If Yes, for how long____

9. Have you taken any herbal medicine while on ART? Yes_______ No________

   9b. If Yes, for how long____

10. Physician's comment on performance of patient:

    Doing well___________Not doing so well__________ Failing_____________

    Other (please specify____

Thank you for the sample and your time!!!

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Appendix II

PATIENT CONSENT FORM

Research Title

Characterization of HIV Drug-Resistant Strains in HIV-Infected persons on antiretrovirals (ARV) in selected HIV Care Centres in Ghana

Principal Investigator

Evelyn Yayra Bonney (Mrs.)

Address

Noguchi Memorial Institute for Medical Research, University of Ghana, Legon Accra, Ghana

Introduction

This Consent Form contains information about the research named above. In order to be sure that you are informed about being in this research, we are asking you to read (or have read to you) this Consent Form. You will also be asked to sign it (or make your mark in front of a witness). We will give you a copy of this form. This consent form might contain some words that are unfamiliar to you. Please ask us to explain anything you may not understand

Reason for the Research

You are being asked to take part in research to find out whether the medicine that you are given at the clinic (ARV) can control the growth of the HIV you have in your blood now.

Research Purpose/General Information about Research

This is a research to find out whether the HIV in persons on ARV is being successfully managed with the drugs that are currently in use in Ghana. When the ARV can longer control the growth of the HIV a person is carrying, we say the virus is a drug-resistant strain. The development of drug-resistant strains makes ARV no longer useful to the person using it and sometimes affects the effectiveness of the other drugs that can be used by the patient if they belong to the same class as the current ARV. Some people seem to be doing well clinically but still have drug-resistant strains and these strains will eventually make all the treatment efforts fruitless. CD4 counts cannot tell whether a person has drug-resistant strain or not. This research will use a more advanced method to find out the types of strains people on ARV have so that the success of the current ARV program in the country can be evaluated and modified if necessary.

Your Part in the Research

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If you agree to be in the research, you will be asked to give 7ml of your blood sample, taken from your veins, only once, although the project is expected to last over a three year period. About 380 adults (both males and females) from 4 HIV care centres in Ghana, who have taken the drugs for at least 6 months, will take part in this study.

Possible Risks and Discomforts

Participants may have some discomfort and pain when blood sample is being taken.

Possible Benefits

The data generated from this research would be useful in monitoring therapy and redesigning treatment regimens. This will directly benefit participants by informing change in treatment regimens if necessary. The project will also benefit all persons living with HIV/AIDS by providing information for the better management of the infection.

Alternatives to Participation

You will continue to benefit from the ART programme even if you decline to participate in this research project

Confidentiality

We will protect information about you and your part in this research to the best of our ability. No report generated from this research will name, or be linked to, the persons involved. Where necessary however, the researcher and your doctor may need to discuss your specific results so as to enable a better management of your condition.

Compensation

You will not be paid for participating in this research, since you do not have to take part in this research.

If You Have a Problem or Have Other Questions

Please call Evelyn Yayra Bonney (0244 785677) if you have further questions about the research.

Your rights as a participant

This research project has been reviewed and approved by the IRB of the Noguchi Memorial Institute for Medical Research. If you have any questions about your rights as a research participant you may contact Rev. Dr. Ayete-Nyampong, Chairperson, NMIMR-IRB, mobile 0208152360

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Appendix III

PARTICIPANT'S AGREEMENT

The above document describing the benefits, risks and procedures for the research title **"Characterization of HIV Drug Resistant Strains in HIV-Infected persons on antiretrovirals in selected HIV Care Centres in Ghana"** has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

_________________________ ____

Date Signature or mark of volunteer

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

_________________________ ____

Date Signature of Witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

___________________________ ____

Date Signature of Person Who Obtained Consent

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Appendix IV
MATERIALS

A. Reagents for laboratory analyses

Lymphocyte separation medium- (Histopaque®-1077) [SIGMA, USA]

Phosphate buffered saline (PBS) [SIGMA, USA]

Foetal bovine serum (FBS) [SIGMA, USA]

Dimethyl sulphoxide (DMSO) [SIGMA, USA]

INNO-LIA HIV-1/2 Confirmatory Assay (Innogenetics, Belgium)

Absolute ethanol (molecular biology grade) [SIGMA, USA]

Nuclease-free water (Ambion, USA)

Nucleic acid purification kit (Roche Diagnostics, Germany)

QIAamp DNA Blood kit (QIAGEN, USA)

QIAamp viral RNA kit (QIAGEN, USA)

Taqman One-Step RT-PCR Reagents (ABI, USA)

One Step RT-PCR Kit (QIAGEN, USA)

AmpliTaq Gold Master Mix Reagents (ABI, USA)

Agarose (SIGMA, USA)

Ethidium bromide (SIGMA, USA)

Tris-Acetate-EDTA (TAE) [Ambion, USA)

DNA molecular weight 100bp ladder (Invitrogen, USA)

QIAquick PCR purification kit (QIAGEN, USA)

Big Dye Terminator Cycle Sequencing Kit vs. 3.1 (ABI, USA)

AgenCourt CleanSeq Dye Terminator Removal kit (Beckman Coulter, USA)

Sequencing Buffer with EDTA 5X (ABI, USA)

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Performance Optimized Polymer-POP 7 (ABI, USA)

Primers (Eurogentec, Belgium

B. Laboratory equipment

Biosafety Cabinet Class IIA (AirTech Services, India)

Biological Safety Cabinet Class II (LABGARD, USA)

Centrifuge (H-900) [Kokusan, Japan]

Platform rocker, STR6 (Bibby, UK)

Autoclave SS-325 (Tomy, Japan)

Vortex Genie-2 (Scientific Industries, USA)

Microcentrifuge 5415D (Eppendorf, USA)

Heat block (Haep labor Consult, Germany)

Pipetman Classic: p1000, p200, p20, p10 (Gilson S.A.S, France)

Aerosol-resistant pipette tips: 1000µl, 200µl, 20µl, 10µl (Gilson S.A.S, France)

MBP ART® self-sealing barrier tips: 1000µl, 200µl, 20µl, 10µl (SIGMA, USA)

AirClean 600 PCR Workstation (AirClean Systems, USA)

Real Time PCR System 7300 (ABI, USA)

GeneAmp PCR System 2700 and 2720 (ABI, USA)

Microwave oven (LG Electronics Inc., Ghana)

Electrophoresis system (Mupid-2 Plus) [Japan]

Gel logic 100 Imaging System (Eastman Kodak Company, USA)

High Performance Ultraviolet Transilluminator (UVP, UK)

Genetic Analyzer 3130 (ABI, USA)

C. Consumables for laboratory analyses

RNase-free 15ml centrifuge tubes (Ambion, USA)

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Nalgene 1.8ml cryovials (Nalge Nunc, USA)

Nalgene cryoboxes (Nalge Nunc, USA)

Latex examination gloves GN32; powder-free, Fine Touch (Hand Safe, UK)

RNase-free 1.5ml microfuge tubes (Ambion, USA)

Sterile RNase-free 0.2ml thin walled PCR tubes (Ambion, USA)

KimTech Science Precision wipes (Kimberly-Clark® Professional, USA)

D. Software for sequence analysis

Seqman (DNAStar, Madison, WI)

Bioedit (http://www.mbio.ncsu.edu/Bioedit/bioedit.html)

MEGA 4.1 (http://www.megasoftware.net/)

Stanford University HIVdb Program (http://hivdb6.stanford.edu)

Los Alamos HIV Sequence Database

(http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html)

BLAST (http://www.ncbi.nlm.nih.gov/blast/blast overview.shtml)

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Appendix V

ETHICAL CLEARANCE CERTIFICATES

The ethical clearance certificates received from the Institutional Review Board of Noguchi Memorial Institute for Medical research are found on Pages 179-182. The study first received ethical clearance in March 2010 (Page 182) and the clearance was renewed annually for its 4-year duration.

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NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH

Established 1979

A Constituent of the College of Health Sciences
University of Ghana

INSTITUTIONAL REVIEW BOARD

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Telex No: 2556 UGL GH

Post Office Box LG 581
Legon, Accra
Ghana

My Ref. No: DF.22
Your Ref. No:

7th March, 2013

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 036/09-10 ***revd. 2013*** **IORG 0000908**

On 7th March, 2013, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting conducted continuing review and approved your protocol titled:

TITLE OF PROTOCOL : **Characterization of HIV Drug-Resistant Strains in HIV-infected Persons on Antiretrovirals (ARV) in Selected HIV Care Centres in Ghana.**

PRINCIPAL INVESTIGATOR : **Evelyn Y. Bonney, PhD Cand.**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 6th March, 2014. You are to submit annual reports for continuing review.

Signature of Chairman: ....
Rev. Dr. Samuel Ayete-Nyampong
(NMIMR – IRB, Chairman)

cc: Professor Kwadwo Koram
Director, Noguchi Memorial Institute
for Medical Research, University of Ghana, Legon

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Established 1979

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My Ref. No: DF.22
Your Ref. No:

7th March, 2012

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 036/09-10 ***revd. 2012*** **IORG 0000908**

On 7th March, 2012, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting conducted continuing review of your protocol titled:

TITLE OF PROTOCOL : **Characterization of HIV Drug-Resistant Strains in HIV-Infected Persons on Anti retrovirals (ARV) in Selected HIV Care Centres in Ghana**

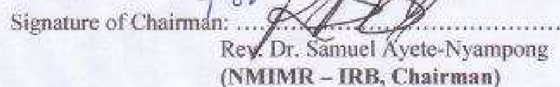
PRINCIPAL INVESTIGATOR : **Evelyn Yayra Bonney (PhD Candidate)**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 6th March, 2013. You are to submit annual reports for continuing review.

Signature of Chairman: for 
Rev. Dr. Samuel Ayete-Nyampong
(NMIMR – IRB, Chairman)

cc: Professor Alexander K. Nyarko
Director, Noguchi Memorial Institute
for Medical Research, University of Ghana, Legon

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NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
INSTITUTIONAL REVIEW BOARD

(UNIVERSITY OF GHANA)

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Telex No: 2556 UGL GH

P.O. Box LG581
Legon
Ghana

My Ref. No: DF.22

2nd March, 2011

Your Ref. No:

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 036/09-10 *revd. 2011* **IORG 0000908**

On 2nd March, 2011, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB), at a full board meeting conducted continuing reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **Characterization of HIV Drug-Resistant Strains in HIV-Infected Person on Anti retovirals (ARV) in Selected HIV Care Centres in Ghana**

PRINCIPAL INVESTIGATOR : **Evelyn Yayra Bonney (PhD Candidtate)**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 1st March, 2012. You are to submit annual reports for continuing review.

Signature of Chairman: ....
Rev. Dr. Samuel Ayete-Nyampong
(NMIMR – IRB, Chairman)

cc: Professor Alexander K. Nyarko
Director, Noguchi Memorial Institute
for Medical Research, University of Ghana, Legon

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NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
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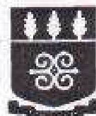

P.O. Box LG581
Legon
Ghana

My Ref. No: DF.22

3rd March, 2010

Your Ref. No:

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 0001276**

NMIMR-IRB CPN 036/09-10 **IORG 0000908**

On 3rd March, 2010, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB), at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **Characterization of HIV Drug-Resistant Strains in HIV-Infected Persons on Antiretrovirals (ARV) in Selected HIV Care Centres in Ghana**

PRINCIPAL INVESTIGATOR : **Evelyn Yayra Bonney (PhD Student)**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 2nd March, 2011. You are to submit annual reports for continuing review.

Signature of Chairman:
Rev. Dr. Samuel Ayete-Nyampong
(NMIMR – IRB, Chairman)

cc: Professor Alexander K. Nyarko
Director, Noguchi Memorial Institute
for Medical Research, University of Ghana, Legon

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Appendix VI

AMINO ACID NAMES, ONE-LETTER CODE AND ACRONYMS

Name	One letter code	Acronym
Alanine	A	Ala
Cysteine	C	Cys
Aspartic acid	D	Asp
Glutamic acid	E	Glu
Phenylalanine	F	Phe
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Lysine	K	Lys
Leucine	L	Leu
Methionine	M	Met
Asparagine	N	Asn
Proline	P	Pro
Glutamine	Q	Gln
Arginine	R	Arg
Serine	S	Ser
Threonine	T	Thr
Valine	V	Val
Tryptophan	W	Trp
Tyrosine	Y	Tyr