DRUG RESISTANCE MUTATIONS IN HUMAN IMMUNODEFICIENCY VIRUS TYPE 2 STRAINS FROM PATIENTS IN TWO TREATMENT CENTERS IN GHANA

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DECLARATION

I hereby declare that this is the product of my own research undertaken under the supervision of Professor William Kwabena Ampofo and Dr. Kwamena William Coleman Sagoe and that references made to other people's work have been duly acknowledged. I also declare that this work has neither been presented in whole nor in part for another degree elsewhere.

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DEDICATION

To my dear parents Sampabilla and Talata Abana, for their wonderful encouragement and support for crossing another academic hurdle. To my uncle Justice Anafo and my sisters Yenpoka, Yenpokbill and Yenpokbilla.

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3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired Immunodeficiency Syndrome
AZT	Zidovudine
Bp	Base pair
CCR5	Chemokine Receptor Type 5
CD4	Cluster of Differentiation 4
CDC	Centres for Disease Control and Prevention
CXCR4	Chemokine Receptor Type 4
d4T	Stavudine
DDI	Didanosine
ddNTPs	Dideoxynucleotide triphosphates
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide triphosphate
DRV/r	Darunavir boosted with ritonavir
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
FTC	Emtricitabine
Gp	Glycoprotein
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LPV/r	Lopinavir boosted with ritonavir
MHC	Major Histocompatibility Complex
NACP	National AIDS/STIs Control Programme
Nef	Negative factor

NMIMR	Noguchi Memorial Institute for Medical Research
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NVP	Nevirapine
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PR	Protease
Rev	Regulatory factor
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SQV/r	Saquinavir boosted with ritonavir
TAE	Tris Acetate EDTA
Tat	Transactivator
TBE	Tris Borate EDTA
TDF	Tenofovir
UNAIDS	Joint United Nations Programme on HIV/AIDS
Vif	Viral Infectivity Factor
Vpr	Viral protein r
Vpx	Viral protein x
WHO	World Health Organization

ABSTRACT

Background

Antiretroviral therapy (ART) and drug resistance studies have focused almost exclusively on human immunodeficiency virus type 1 (HIV-1). Thus there is limited information on patients infected with HIV type 2 (HIV-2).

In Ghana, the HIV epidemic is characterized by the domination of HIV-1 with the co-circulation of HIV-1 and HIV-2. HIV-2 is known to be naturally resistant to some antiretroviral drugs including non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors, due to natural polymorphisms in the viral genes. However, these drugs are used to treat patients irrespective of their HIV type resulting in situations where patients infected with HIV-2 will develop resistance to the ART regimen. Thus, the monitoring of drug resistance against HIV in patients with HIV-2 only or dual HIV-1/2 infections is necessary for the effective clinical management of such patients.

Aim

The aim of this study was to describe drug resistance mutations in HIV-2 strains in patients in Ghana.

Method

In this cross sectional study, venous blood was obtained from treatment naive and treatment-experienced HIV-2- and dual HIV-1/2 infected persons from two treatment centers in Southern Ghana. Ribonucleic acid (RNA) and DNA was extracted from plasma and peripheral blood mononuclear cells (PBMC) and used in polymerase chain reaction (PCR) assays to amplify the reverse transcriptase (RT) and the protease (PR)-coding domains of the *pol* gene. The amplicons were sequenced, and analyzed for drug resistance mutations and subtype information.

Results

In this study, 18 patients, 11 infected with HIV-2 only and 7 dually HIV-1/2 infected, were enrolled. Sequences were successfully obtained from 4 treatment- naïve and one treatment-experienced patient. There were no drug resistance mutations in the patients that were ART- naïve. The ART-experienced patient however had M184V, K65R and Y115F, which confer resistance against the NRTI drug class. All three mutations were observed in both plasma and PBMC of the patient.

Conclusion

The results clearly show the presence of drug resistance mutations in HIV-2 strains circulating in Ghana and illustrate the need for regular monitoring of drug resistance to improve clinical management of patients.

CHAPTER ONE

1.1 Introduction

The 2014 Joint United Nations Programme on HIV AIDS (UNAIDS) estimate reveals that approximately 34 million people worldwide are living with the human immunodeficiency virus (HIV); with sub-Saharan African bearing 68% of the HIV burden (UNAIDS, 2014).

Two types of HIV, namely HIV type 1 (HIV-1) and HIV type 2 (HIV-2), have been described. HIV-1 is responsible for over 90% of the global AIDS pandemic while HIV-2 is restricted to West Africa and a few European and Asian countries. Co-infections of HIV- 1 and HIV- 2 are relatively common in West Africa representing 0.3–1% of all HIV-infections (De Silva *et al*., 2010; Nsagha *et al*., 2012). In 2013, the estimated adult national HIV prevalence for Ghana was 1.3% with 97.1% HIV-1, 0.8% HIV-2 and 2.1% dual HIV- 1/2 infections. (Ghana Health Service/National AIDS/STI, 2014). Two million people are known to be infected with HIV-2, most of which are found in West Africa. (Gottlieb *et al*., 2008; Menéndez-Arias and Alvarez, 2014). HIV-2 infection has also spread to nineteen different countries in Europe, Asia and North America (Gurjar *et al*, 2009; Valadas *et al*, 2009; Torian *et al*., 2010; Menéndez-Arias and Alvarez., 2014) with a high prevalence of 5.4 % HIV-2 infection reported in Portugal (5.4%) (Barin *et al*., 2007; Valadas *et al*., 2009; Menéndez-Arias and Alvarez, 2014).

HIV-1 and HIV-2 infections have similar routes of transmission, however, patients infected with HIV-2 have lower viral loads than those infected with HIV-1(Popper *et al.*, 2000) resulting in reduced transmission and longer disease progression rate among the HIV- 2-infected (Campbell-Yesufu *et al*., 2011; Styer *et al*., 2013;).

HIV- 2 was first isolated in 1986 (Clavel *et al*., 1986) and has been classified into groups A to H (Damond *et al*., 2004; Santiago *et al*., 2005; Menéndez-Arias and Alvarez, 2014). Among the eight groups, A and B are known to cause epidemics. Group A is mostly prevalent in Guinea Bissau while

Group B is frequently found in Ivory Coast and Ghana (Rowland-Jones, 2006). The HIV epidemic in Ghana is characterized by the co-circulation of HIV-1 and HIV-2 (Bonney *et al.* 2008; Sagoe *et al.* 2008).

Antiretroviral therapy (ART) was introduced in Ghana in 2003 on a pilot basis at two HIV care centers in the Manya Krobo District of the Eastern Region. The number of treatment sites in Ghana has increased to 175 by the end of 2013. As at December 2013, the cumulative number of people with HIV infection receiving ART was 75,762. This has contributed significantly to the reduction of HIV-related morbidity and mortality (Ghana Health Service/ National AIDS/STI Control Programme, 2014). Due to the co-circulation of HIV-1 and HIV-2 types in Ghana, the national guidelines have made provision for persons infected with HIV-2 strains. The current Ghana ART guidelines recommend the use of a Protease Inhibitor (PI) –based regimen in place of NNRTI for HIV-2 and dual HIV-1/2 infected patients. (Guidelines for Antiretroviral therapy in Ghana, 2014).

Despite the gains made with ART in Ghana, the emergence of drug resistant strains during treatment is a major obstacle to the success of any ART program. Both HIV-1 and HIV-2 are known to develop resistance against currently approved antiretroviral drugs. The development of drug resistance in HIV-1 infections has been examined in several studies. However, information is scarce on HIV-2 drug resistance (Ntemgwa *et al*., 2009). While transmission of drug-resistant HIV-1 strains is a well-documented phenomenon, occurring in approximately 10% of subjects in Western countries (Little *et al*., 2002; de Mendoza *et al*., 2005) there is limited global data on transmission of resistant HIV-2 (Jallow *et al.*, 2009; Silva *et al.*, 2010).

With increasing access to antiretroviral therapy in West Africa and other resource-limited settings, it is likely that HIV-2 drug resistant strains will emerge as has been observed for HIV-1. Hence a study of the HIV- 2 response to highly active antiretroviral therapy (HAART) and the development of resistance is crucial.

1.2 Problem Statement

There is limited experience in the treatment of HIV-2 infection in Ghana. This could be attributed mainly to the lower prevalence of HIV-2 compared to HIV-1 and the restriction of the HIV-2 mainly to West Africa (Jallow *et al*., 2009). The antiretroviral drugs that are currently approved: nucleoside reverse transcriptase inhibitors (NRTI); non-nucleoside reverse transcriptase inhibitors (NNRTI); integrase inhibitors; protease inhibitors and fusion/entry inhibitors, were all designed against HIV-1. These drugs could be used for HIV-2 due to the conserved protease (PR) and reverse transcriptase (RT) genes of both types (Poveda *et al*., 2004; Nkengasong *et al*., 2004).

However, HIV- 2 has been found to be naturally resistant to NNRTIs and the entry inhibitor T-20, and it may have reduced susceptibility to some protease inhibitors (Hightower *et al*., 2003; Rhodes *et al*., 2006). Therefore, drugs used for the treatment of HIV-2 should be carefully selected to allow optimal and durable viral suppression.

In Ghana, there is currently no optimized HIV-2 genotyping protocol to guide HIV-2 drug resistance studies. It is therefore imperative to optimize an HIV-2 genotyping protocol and investigate drug resistance mutations in HIV-2 strains circulating in the country. This will generate useful data to inform treatment options targeted at HIV-2 infected patients.

1.3 Rationale

Resistance-associated mutations are reported to develop in HIV-2 patients on therapy (Damond *et al*., 2004). Genotypic algorithms used to predict drug resistance in HIV-1 may not be applicable to HIV-2, because pathways and mutational patterns leading to resistance may differ (Ruelle *et al.*, 2008). The T cell CD4 cell recovery on therapy may be poor (Drylewicz *et al.*, 2008), suggesting that more reliable methods for monitoring disease progression and treatment efficacy in HIV-2 infection are needed.

In order to assess patient failure or success, all patients receiving antiretroviral therapy (ART) are expected to undergo regular viral load testing. Monitoring of virologic response in HIV-2 patients

is problematic because of the lack of commercially available HIV-2 viral load assays. Clinical symptoms and CD4 count improvements can be used to assess treatment response. Genotypic testing is recommended as the preferred monitoring tool to guide therapy in antiretroviral (ARV)-naive patients (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2009). Previous studies in Ghana have documented drug resistance mutations in treatment naïve or experienced HIV-1 patients (Kinomoto *et al*., 2005; Sagoe *et al*., 2007; Delgado *et al*., 2008; Brandful *et al*., 2012; Bonney *et al*; 2013a; Bonney *et al*; 2013b)

However there is limited drug resistance data on HIV-2 infected patients in Ghana. It is therefore important to establish an HIV-2 genotyping protocol to determine the patterns of HIV-2 drug resistance in Ghana.

The data generated will help to review progress of HIV-2 patients on ART and inform their clinical management during ART. The study results will provide a local information source for reviews of the national policy on ART for HIV infected patients in Ghana.

1.4 Aims and Objectives

Aim

The aim of this study is to determine the drug resistance mutations in HIV-2 strains in patients in Ghana.

Specific Objectives

1. To identify HIV-2 and dual HIV-1/HIV-2 seropositive patients at selected hospitals in Ghana.
2. To determine the mutations in the reverse transcriptase (RT) and (PR) of the polymerase gene and analyze the sequence data for drug resistance mutations.
3. To compare drug resistance mutations in the plasma and peripheral blood mononuclear cells (PBMC).

CHAPTER TWO

2.0 Literature Review

2.1 Structure of HIV

In 1981, acquired immune deficiency syndrome (AIDS) was first recognized as a new disease in young homosexual men who presented with unusual opportunistic infections and rare malignancies (Friedman-Kien *et al*., 1981; Greene *et al*., 2007). A retrovirus, termed human immunodeficiency virus type 1(HIV-1), was subsequently identified as the causative agent (Barre-Sinoussi*et al*., 1983; Gallo *et al*., 1984; Popovic *et al*., 1984).

HIV belongs to the viral family Retroviridae, subfamily Lentivirinae, and genus Lentivirus (Groenink *et al.*, 1993). The structure of HIV is made up of 2 strands, positive-sense ribonucleic acid (RNA) genome of about 9.7 kilobases (Figure 1). Each of these HIV RNA strand has a copy of the virus's nine genes. The viral envelope is composed of a lipid bilayer membrane, formed from the cellular membrane of the host cell during budding of the newly formed virus particle. Host-cell proteins, such as the major histocompatibility complex (MHC) antigens and actin, remain embedded within the viral envelope, along with the viral envelope protein. Embedded within the viral envelope are glycoproteins which are used to bind to cell surface proteins. (Oelrichs *et al*., 2004).

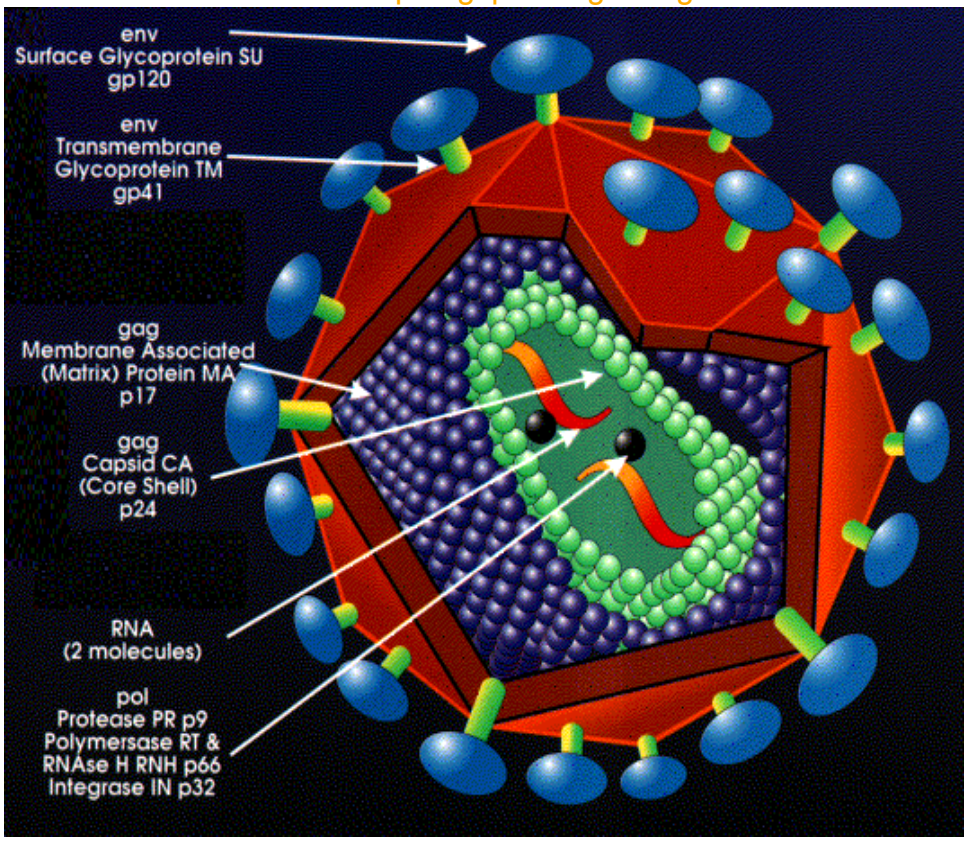

Figure 1: Structure of HIV

HIV is icosahedral in shape and like other viruses, HIV does not have a cell wall or a nucleus. The viral envelope, the outer coat of the virus, consists of two layers of lipids; different proteins are embedded in the viral envelope, forming "spikes" consisting of glycoproteins which are used for attachment to host cells. The HIV matrix proteins lie between the envelope and core. The viral core (or capsid) is usually bullet-shaped and houses three enzymes required for HIV replication called reverse transcriptase, integrase and protease. Also held within the core is HIV's genetic material, which consists of two identical strands of RNA (http://www.yale.edu/bio243/HIV/hivstructure.html on 8/05/2015

2.2 HIV type 2 Groups

After the discovery of HIV-1 as causative agent of AIDS (Barre-Sinoussi *et al*., 1983; Gallo *et al*., 1984; Popovic *et al*., 1984), a second virus, HIV-2, was isolated from West African patients in 1986. HIV-2 infection was associated with a longer asymptomatic phase and slower progression to AIDS than HIV-1 infection (MacNeil *et al*., 2007). The slower progression of HIV-2 was also observed in HIV-1 and HIV-2 co-infections where HIV-1 outgrew HIV-2. (Esbjörnsson *et al*., 2012; Raugi *et al*., 2013). HIV-2 virus is said to be restricted mainly to West African countries such as Ghana, Senegal, Guinea Bissau, and the Gambia where its prevalence ranges from 1 to 10% of the adult HIV population (Colson *et al*., 2004).

HIV-2 is distantly related to HIV-1 and the viruses originated from separate transfers of simian immunodeficiency virus (SIV) into the human population, HIV-1 from chimpanzees and HIV-2 from sooty mangabeys (Sharp *et al*., 2011).

Since the first isolation of HIV-2 in 1986, eight distinct groups of HIV-2 (group A through to H) have been identified (Damond *et al*., 2004; Santiago *et al*., 2005; Menéndez-Arias and Alvarez, 2014), each of which appears to represent an independent host transfer. Most HIV-2 infections worldwide are caused by isolates from group A although it is most prevalent in Guinea Bissau. In contrast, infections caused by HIV-2 group B are more frequent in Ivory Coast and Ghana (Rowland-Jones, 2006). All other HIV-2 groups have been identified only in single individuals suggesting that they represent incidental infection with very limited or no secondary spread (Sharp and Halm, 2011; Menéndez-Arias and Alvarez, 2014)

HIV-2 groups C to G infections have been in at least one or two individuals, however the infections in those cases did not lead to immune suppression (Gao *et al*., 1994). HIV-2 group H has also been isolated in Ivory Coast (Damond *et al*., 2004; Menéndez-Arias and Alvarez, 2014)

Recombinant HIV-2 strains have been previously identified in Ghana, Cameroon and Cote d'Ivoire (Gao *et al*., 1994; Takehisa *et al*., 1997; Yamaguchi *et al*., 2008) and more recently as HIV-2 CRF01_AB (Ibe *et al*., 2010; Menéndez-Arias and Alvarez, 2014). A novel HIV-2 variant (HIV-2-

017C-TNP03) has also been identified in Ivory Coast and was not found to be related to any of the previously identified HIV-2 groups (Ayouba *et al*., 2013).

2.3 HIV replication cycle and drug targets

The HIV life cycle, shown in figure 2 (http://www.thebody.com/content/art40989.html on 15/03/2015) presents many potential opportunities for therapeutic interventions.

1. **Viral entry and Fusion:** HIV begins its life cycle when it binds to a CD4 receptor and one of two co-receptors on the surface of a CD4+ T- lymphocyte. The virus then fuses with the host cell. After fusion, the virus releases RNA, its genetic material, into the host cell. Viral entry and fusion with the host cell membrane allow for uncoating of the viral core and initiate a slow dissolution process that maintains protection of the viral RNA genome while permitting access to deoxyribonucleoside triphosphates (dNTPs) necessary for reverse transcription and proviral DNA synthesis.

   Several classes of antiretroviral agents: attachment inhibitors, chemokine receptor antagonists and fusion inhibitors target the first step in the HIV replication cycle, which is the entry of the virus (Wilen *et al*., 2012; Arts and Hazuda, 2012)
2. **Reverse Transcription:** An HIV enzyme called reverse transcriptase (RT) converts the single-stranded HIV RNA to double-stranded HIV DNA. RT is a multifunctional enzyme with RNA-dependent DNA polymerase, RNase-H, and DNA-dependent DNA polymerase activities, all of which are required to convert the single-stranded HIV viral RNA into double-stranded DNA (Hughes and Hu, 2012). Two distinct classes of antiretroviral agents: the Nucleoside Reverse Transcriptase Inhibitors (NRTIs) which are analogs of native nucleoside substrates, and the Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), which bind to a non-catalytic allosteric pocket on the enzyme, target the reverse transcriptase enzyme. These two classes account for nearly half of all approved antiretroviral drugs.

Although the NRTIs and NNRTIs differ with respect to their site of interaction on the enzyme and molecular mechanism, both affect the DNA polymerization activity of the enzyme and block the generation of full-length viral DNA (Arts and Hazuda, 2012).

3. **Integration:** The newly formed HIV DNA enters the host cell's nucleus, where an HIV enzyme called integrase catalyzes the integration of the viral DNA with the host DNA. The integrated HIV DNA is called a provirus. 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. The cellular machinery can initiate transcription after integration. The class of approved ARVs, integrase inhibitors, specifically inhibit strand transfer and block integration of the HIV-1 DNA into the cellular DNA (Arts and Hazuda, 2012)
4. **Transcription:** The provirus uses a host enzyme called RNA polymerase to create copies of the HIV genomic material, as well as shorter strands of RNA called messenger RNA (mRNA). The mRNA is used as a blueprint to make long chains of HIV proteins. During the last 15 years a variety of candidate small-molecule inhibitors of HIV transcription have been identified but none of these compounds unfortunately were sufficiently potent to progress beyond phase 1 clinical trials (Hsu *et al*., 1991; Cupelli and Hsu 1995; Hamy *et al*., 1997; Hwang *et al*., 2003; Arts and Hazuda., 2012).
5. **Assembly:** An HIV enzyme called protease cuts the long chains of HIV proteins into smaller individual proteins. As the smaller HIV proteins come together with copies of HIV's RNA genetic material, a new virus particle is assembled.

   Protease inhibitors (PIs) block proteolysis of the viral polyprotein, a step required for the production of infectious viral particles (Sundquist and Kräusslich, 2011). PIs require a boosting agent such as ritonavir to enhance drug levels.
6. **Budding:** The newly assembled virus pushes out ("buds") from the host cell. During budding, the new virus takes part of the cell's outer envelope. This envelope, which acts as a

covering, is studded with protein/sugar combinations called HIV glycoproteins. These HIV glycoproteins are necessary for the virus to bind CD4 and co- receptors. The new copies of HIV can now move on to infect other cells. Although the assembly and maturation of HIV on the inner plasma membrane is also a target area for antiretrovirals, insufficient antiviral activity prohibited the development beyond early phase clinical trials (Smith *et al*., 2007).

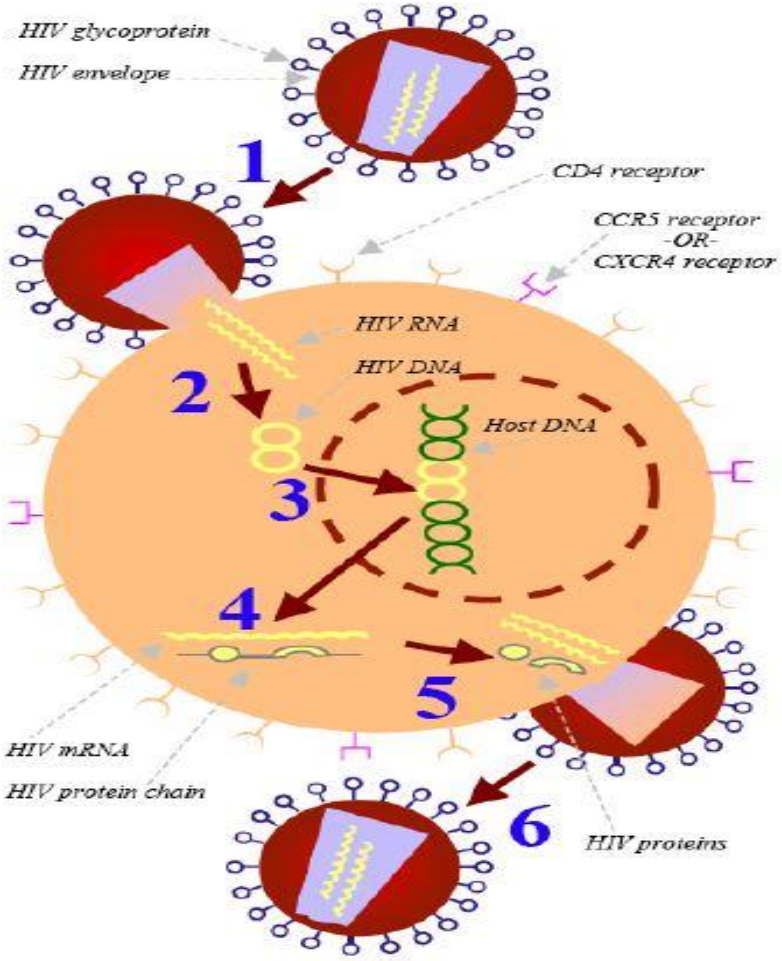

Figure 2: HIV replication cycle

The steps in the replication cycle are as follows: (1) Binding to the CD4 receptor and co-receptors; (2) Fusion with the host cell membrane; (3) Uncoating of the viral capsid; (4) Release of the HIV RNA and proteins into the cytoplasm; (5) Reverse transcription of HIV RNA to DNA and translocation into the nucleus. Once in the nucleus the viral DNA is integrated into the host DNA and subsequently transcribed and translated to form new viral RNA and viral proteins that translocate to the cell surface to assemble into new immature virus forms. The new viruses bud off and are released. Finally, during maturation, the protease enzyme cleaves the structural polyprotein to form mature gag proteins, resulting in the production of new infectious virions.

Source: http://www.thebody.com/content/art40989.html on 15/03/2015

2.4 HIV-2 genome organization

The genomic organization of HIV-2 is similar to that found in other lentiviruses, with three major genes, arranged in the order 5'-gag-pol-env-3', and a series of accessory and regulatory genes comprising *vif, vpx, vpr, tat, rev and nef*. (Figure 1). The major genes of the HIV-2 genome may be broadly classified into structural, catalytic, regulatory, and accessory groups (Oelrichs, 2004) as summarized in (Table 1).

Most current antiretroviral agents target the enzymes in the pol gene (protease, reverse transcriptase and integrase) (Arts and Hazuda, 2012). The current ART drugs used in Ghana are made up of reverse transcriptase and protease inhibitors. In this study, the reverse transcriptase and protease enzymes were analyzed for HIV-2 drug resistance mutations.

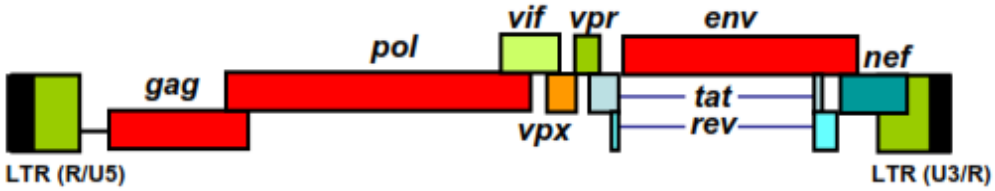

Figure 3: HIV-2 genome organization

Major (gag, pol, env) and accessory and regulatory genes (vif, vpx, vpr, tat, rev, nef) in the HIV-2 genome. Antiretroviral drugs targets the protease, the reverse transcriptase and the integrase genes which are encoded within the pol gene. Adapted from (Menéndez-Arias and Alvarez, 2014)

Table 1: The nine major genes of HIV-2 and their associated gene products (Oelrichs, 2004)

Protein Class	Gene	Gene Products	Functions
Structural	Gag	P17 (Matrix protein)	Binds cyclophilin A in the virion
		P24 (Capsid protein)	Forms nucleocapsid along with P6
		P6 (Nucleocapsid core protein)	Forms nucleocapsid along with P24
		P7 (Nucleic acid binding protein	Binds to viral RNA
Catalytic	Pol	Protease (P10)	Proteolytic cleavage of gag, pol and env precursor polypeptides
		Reverse transcriptase (P66/51)	Polymerase and ribonuclease H activity
		Integrase (P32)	Integration of viral DNA into host cell chromosome
Regulatory	Tat	Trans-activator of transcription (P14)	Major viral transactivator
	Rev	Regulator of expression of virion protein (P19)	Enhances expression of unspliced and singly spliced RNA molecules; regulates transport of messenger RNA; shuttles back and forth between nucleus and cytoplasm
Accessory	Vpx	Viral protein x (Vpx) – P25	Packaged into the virion
	Vif	Virus infectivity factor (Vif)- P23	Efficient cell free transmission; required for proper assembly of nucleoprotein core
	Vpr	Viral protein r (Vpr)- P15	Enhances viral replication in primary cells; G2/M phase arrest; nuclear localization in cell; virion-associated protein
	Nef	Negative factor (Nef)- P27	Inhibits or enhances viral replication, depending on strain and cell type; down-regulates CD4 and MHC class I receptor

2.5 Impact of HIV Drug Resistance

Highly active antiretroviral therapy (HAART) changed the face of the AIDS epidemic from a death sentence to a treatable, chronic infectious disease (Pomerantz & Horn., 2003). HAART has had a dramatic effect on mortality; slowing down disease progression and raising the quality of life of infected individuals (Gullick *et al*., 1997; Hammer *et al*., 1997; Palella *et al*., 1998). However, the high mutation rate of HIV and incomplete viral suppression due to suboptimal therapy inevitably result in the emergence of drug-resistant viruses. Suboptimal therapy is associated with low concentration of drugs in the blood due either to lack of adherence to toxic and complex regimens or to problems with drug absorption or metabolism (Little *et al*., 2002; Pomerantz *et al*., 2002).

The development of drug resistance has posed a major obstacle to the effective treatment of HIV, limiting both the magnitude and the duration of the response to treatment as well as reducing the number of active antiretroviral (ARV) drugs available for HAART (Little *et al*., 2002). Fortunately, the availability of drug resistance testing has improved the ability of clinicians to deal knowledgeably with HIV drug resistance. Research on drug resistance testing has enabled investigators to more effectively develop and study both novel and older therapeutics to enable tailoring treatment for patients with varying resistance profiles.

Drug resistance mutations in HIV-2 are selected at the same positions as in HIV-1, although with different frequency. Polymorphisms in the RT and PR associated with drug resistance in HIV-1 as compensatory changes are common in untreated HIV-2 subjects (Treviño *et al*., 2011). These findings however, highlight the need for specific guidelines for interpreting genotypic resistance patterns in HIV-2 infection.

ARV drugs were and are still designed and optimized for the treatment of HIV-1. These drugs were expected to have similar efficacy for HIV-2 patients as well as HIV-1 patients, due to the high structural and enzymatic function similarities between the protease and RT genes and proteins of these two viruses (Nkengasong *et al*., 2004).

However, HIV-2 is naturally resistant to NNRTIs and the entry inhibitor T-20 and could thus have reduced susceptibility to some protease inhibitors (PIs) (Rodes *et al*., 2006). These resistance properties restrict the treatment of HIV-2-infected and dually infected patients with HAART by limiting the drugs available for the second and subsequent regimens. The use of potent and effective first line regimen for HIV-1 results in full viral suppression for at least 7 years (da Silva *et al.*, 2005).

There were no randomized trials to address the question of when to start ART or the choice of initial or second-line therapy for HIV-2 infection (Gottlieb *et al*., 2008) thus, the optimal treatment strategy was not defined. In vitro data suggested that HIV-2 was sensitive to nucleoside reverse transcriptase inhibitors (NRTIs), although with a lower barrier to resistance than HIV-1 (Smith *et al*., 2009). Variable sensitivity among protease inhibitors (PIs) was also been reported; lopinavir (LPV), saquinavir (SQV), and darunavir (DRV) were known to be more active against HIV-2 than other approved PIs (Brower *et al*., 2008). The integrase inhibitor, raltegravir (RAL*)* (Roquebert *et al*., 2008), and the CCR5 antagonist, maraviroc (MVC), appeared active against some HIV-2 isolates, although no approved assays to determine HIV-2 co-receptor tropism existed and HIV-2 was known to utilize multiple minor co-receptors in addition to CCR5 and CXCR4 (Owen *et al*., 1999). Several small-scale studies suggested poor responses among HIV-2 infected individuals treated with some ARV regimens, including dual-NRTI regimens, regimens containing two NRTIs plus NNRTI, and some unboosted PI-based regimens including nelfinavir (NFV) or indinavir (IDV) plus zidovudine (ZDV) and lamivudine (3TC) (Gottlieb *et al*., 2009). Clinical data on the utility of triple-NRTI regimens gave conflicting results (Matheron *et al*., 2006). In general, boosted PI-containing regimens resulted in more favorable virologic and immunologic responses (Ruelle *et al*., 2008).

Some groups have recommended specific preferred and alternative regimens for the initial therapy of HIV-2 infection *(*Gilleece *et al*., 2010) although there are no controlled trial data to reliably predict their success. It is however recommended that, until more definitive data are available in an ART-

naive patient with HIV-2 mono-infection or with HIV-1/HIV-2 dual infection who require treatment, clinicians should initiate a regimen containing two NRTIs and a boosted PI *(*Gilleece *et al*., 2010).

2.4 Mechanisms of HIV Drug Resistance

HIV makes about one to ten billion viral copies in a day (Ho *et al*., 1995; Wei *et al*., 1995; Perelson *et al*., 1996). Owing to the lack of an accurate proof reading mechanism of the 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). Therefore at every replication cycle, there are different strains of the virus: the wild type HIV strain and the resistant or mutant HIV strain. These strains differ from one another by random mutations in their genetic structures. Some of these mutations are minor (base substitutions or amino acid substitutions) whilst other mutations are more significant as they involve combinations of amino acid substitutions, deletions or insertions.

However with the start of a single antiretroviral agent (Fig 4a), it is likely that treatment will be initially effective in reducing the dominant, usually the wild type (drug sensitive) strain of HIV. The viral load declines as wild type (drug sensitive) strain of HIV disappears (Fig 4b). However, among the diverse population of virus, there will likely be at least the drug resistant strain harboring a particular mutation that confers a small survival advantage in the presence of the particular antiretroviral drug. If this variant strain is permitted to continue replicating, it will continue to diversify with some progeny virus accumulating additional mutations that may confer greater resistance to the antiretroviral agent being used. Eventually, a variant will likely emerge that harbor enough key mutations to fully resist the agent being used thereby rendering it the uncontested dominant strain. If a second drug is used to combat the virus that has become resistant to the first drug, the process repeats itself (Fig 4c) (Clavel, 2004).

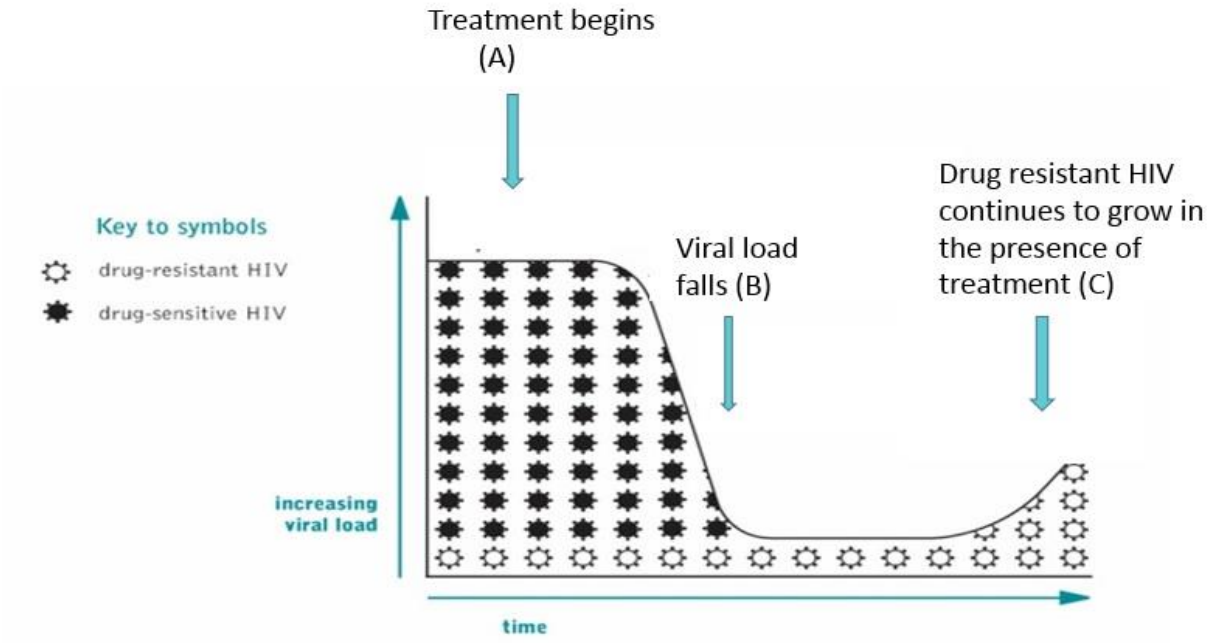

Figure 4: Mechanism of HIV drug resistance

This figure explains how resistance to anti-HIV drugs can develop. The line represents viral load. Before starting treatment, the viral load is high and is made up of both drug-sensitive and drug-resistant HIV. In figure 4A, when treatment begins, it is effective in reducing amounts of drug sensitive HIV and the viral load falls (figure 4B) leaving drug resistant HIV. As drug resistant HIV continues to grow and diversify, progeny virus emerge that harbor mutations that may confer greater resistance to the ART being used. Over time, the growth of these viruses will cause viral load to rise again (figure 4C). If a second drug is introduced to combat progeny virus that become resistant to the first drug, the process repeats itself (adapted from www.aidsmap.com on 21/05/2015)

2.4.1 Comparing drug resistance mutations in PBMC and plasma

Some drug resistance mutations studies have demonstrated more drug resistance mutations in plasma (RNA) compared to PBMC (DNA) (Smith *et al*., 1993; Chew *et al*., 2005; Saracino *et al*., 2008); some have also found specific drug resistance mutations in PBMC that were not detected in plasma (Ellis *et al*., 2004; Bon *et al*., 2007; Parisi *et al*., 2007) and some studies have observed no significant difference in drug resistance mutations from the two sources (Vicente *et al*., 2007; Bonney *et al*., 2013b). Most of these studies have suggested the use of PBMC (DNA) with plasma (RNA) sequencing to increase the sensitivity of drug resistance testing while other studies have suggested the use PBMC (DNA) as an alternative to plasma (Chew *et al*., 2005; Bonney *et al*., 2013b).

2.5 Treatment of HIV-2 infection

Treatment of HIV-2 infection poses challenges in comparison to HIV-1 infections. Firstly, the lack of commercial assays for HIV-2 viral load to monitor treatment response. Secondly, the absence of guidelines and ample research targeting HIV-2 therapy. It is important to start therapy as soon as possible, always before advanced immunodeficiency develops. A third problem is that antiretroviral drugs designed against HIV-1 are not effective inhibitors of HIV-2 propagation. Thus, HIV-2 is broadly resistant to various non-nucleoside reverse transcriptase inhibitors (NNRTIs) and fusion inhibitors. In addition, several protease inhibitors licensed to treat HIV-1 infection show weak or no inhibitory activity against HIV-2.

Against this background, preferred initial treatments for HIV-2, consist of combinations of two nucleoside reverse transcriptase inhibitors (NRTIs) (either tenofovir plus emtricitabine or lamivudine, or zidovudine plus lamivudine) and an appropriate boosted protease inhibitor (usually ritonavir-boosted lopinavir or darunavir) (Gilleece *et al*., 2010; Vandamme *et al*., 2011; Menéndez-Arias and Alvarez, 2014). Integrase inhibitors recently licensed for treating HIV-1 infection are also effective against HIV-2 but information available is limited (Menéndez-Arias and Alvarez, 2014)

2.5.1 HIV-2 RT inhibition by NRTIs and mechanisms of resistance

There are basically two mechanisms by which resistance to nucleoside reverse transcriptase inhibitors (NRTIs) can occur. The first which is the primary mechanism involves mutations e.g. (M184V, K65R, Q151M) that occur at or near the drug binding site of the reverse transcriptase gene resulting increased drug discrimination by this gene (Clavel *et al*., 2004). The second mechanism is by nucleotide excision. This involves key mutations that essentially work to undo the action of these drugs even if they do manage to bind correctly within the RT gene. As shown in figure 5A, an NRTI is incorporated into nascent DNA by RT, and the absence of a 3′ OH group results in termination of DNA synthesis. This effectively terminates chain extension and ultimately inhibits replication of the virus. This process can be reversed by a reverse transcriptase reaction that removes the chain terminating residue and reinstates an extendable primer (Clavel *et al*., 2004). This reverse reaction of DNA polymerization termed pyrophosphorolysis enables reverse transcription and DNA synthesis to resume. Pyrophosphorolysis can be enhanced by thymidine analogue mutations (TAMs) (i.e. M41L, D67N, K70R, L210W, T215F/Y and K219E/Q). These mutations appear to select and are hobbled by many of the same mutations in reverse transcriptase that confer drug resistance.

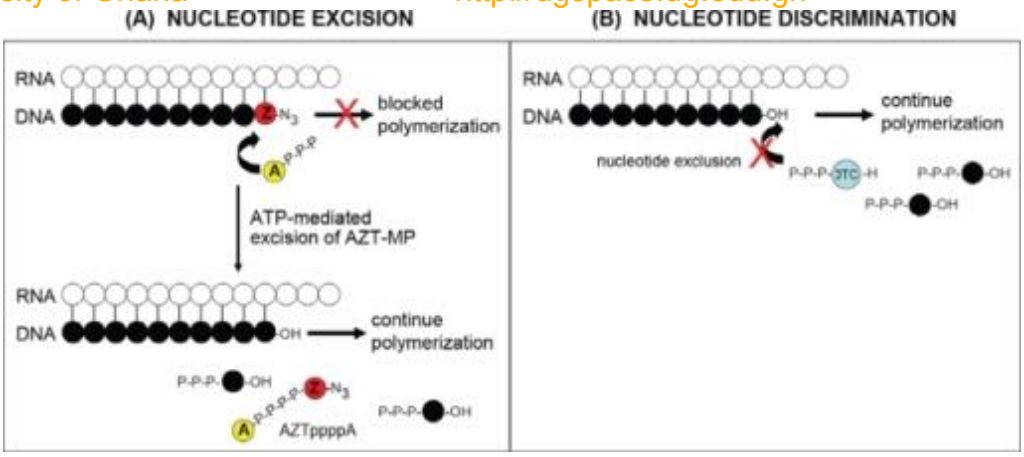

Figure 5: Mechanism of NRTI resistance

A: Nucleotide excision mechanism (Clavel et al., 2004). Mutations such as thymidine analogue mutations (TAMs), aid in the adenosine tryphosphate (ATP)-mediated removal of an incorporated (Zidovudine) AZT monophosphate (AZT-MP) yielding an AZTppppA excision byproduct.

B: Nucleotide discrimination (Clavel et al., 2004). Mutations at the active site excludes certain drugs for instance Lamivudine (3TC), from being incorporated during reverse transcription. Both examples yield a complex competent for polymerization. Yellow circle with the letter A and three phosphates, ATP; black circles with three phosphates, dNTPs; red circle with the letter Z and the N3 azido group, AZT-MP; blue circle with three phosphates, 3TC-triphosphate; P, phosphate group. RNA is depicted with white circles; DNA is depicted with black circles

Clinical studies have shown that the nucleotide excision mechanism is rarely used by HIV-2 RT to acquire resistance to NRTIs (van der Ende *et al*., 1996; Rodés *et al*., 2000; Brandin *et al*., 2003; Castro *et al*., 2012; Menéndez-Arias and Alvarez, 2014).

Virological studies have shown that HIV-2 RT favors the Q151M discrimination pathway for resistance to zidovudine and other NRTIs (Ruelle *et al*., 2008; Gottlieb *et al*., 2009; Jallow *et al*., 2009; Ntemgwa *et al*., 2009; Menéndez-Arias and Alvarez, 2014)

In general, NRTI resistance in HIV-2 has a lower genetic barrier than in HIV-1. Apart from Q151M that confers resistance to all NRTIs except tenofovir, K65R and M184I or V are frequently found in viral isolates and confer class wide NRTI resistance (Smith *et al*., 2009; Menéndez-Arias and Alvarez, 2014)

The Q151M mutation alone contributes to high-level resistance to zidovudine (AZT). However, Q151M had a small impact on tenofovir resistance (Damond *et al*., 2005; Bennett *et al*., 2007). In HIV-1 RT, the Q151M mutational pathway involves additional changes around the dNTP binding site (i.e. A62V, V75I, F77L and F116Y) (Menéndez-Arias, 2008). In HIV-2 RT, the Q151M complex contains V75I (present in the wild-type enzyme) and very often V111I. Selection of V111I together with Q151M in HIV-2 isolates resulted in decreased viral susceptibility to all tested NRTIs (i.e. zidovudine, lamivudine, stavudine, didanosine, abacavir and tenofovir), although in the case of tenofovir, a smaller effect was observed (Damond *et al*., 2005).

Other mutations involved in class wide NRTI resistance such as K65R or M184V have been identified in HIV-2-infected patients exposed to tenofovir or abacavir (Rodés *et al*., 2008; Peterson *et al*., 2011; Menéndez-Arias and Alvarez, 2014) or in patients treated with lamivudine (van der Ende *et al*., 2003), respectively.

2.5.2 HIV-2 RT resistance to NNRTIs

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) exert their antiviral effect against HIV by binding to reverse transcriptase in a hydrophobic pocket located next to the active site of the enzyme (figure 6A), causing a conformational change in this enzyme that blocks the process of DNA polymerization (figure 6B). The region of binding predominantly involves amino acid codons 98-108 and 179-190 in the hydrophobic pocket (Bacheler *et al*., 2000). Resistance to NNRTIs occurs as a result of mutations that inhibit effective binding of the NNRTI, thus allowing DNA polymerization to proceed in an unrestricted manner (figure 6C). The specific mutations associated with resistance to NNRTIs correlate with amino acid changes in the pocket where the NNRTI drug preferentially binds.

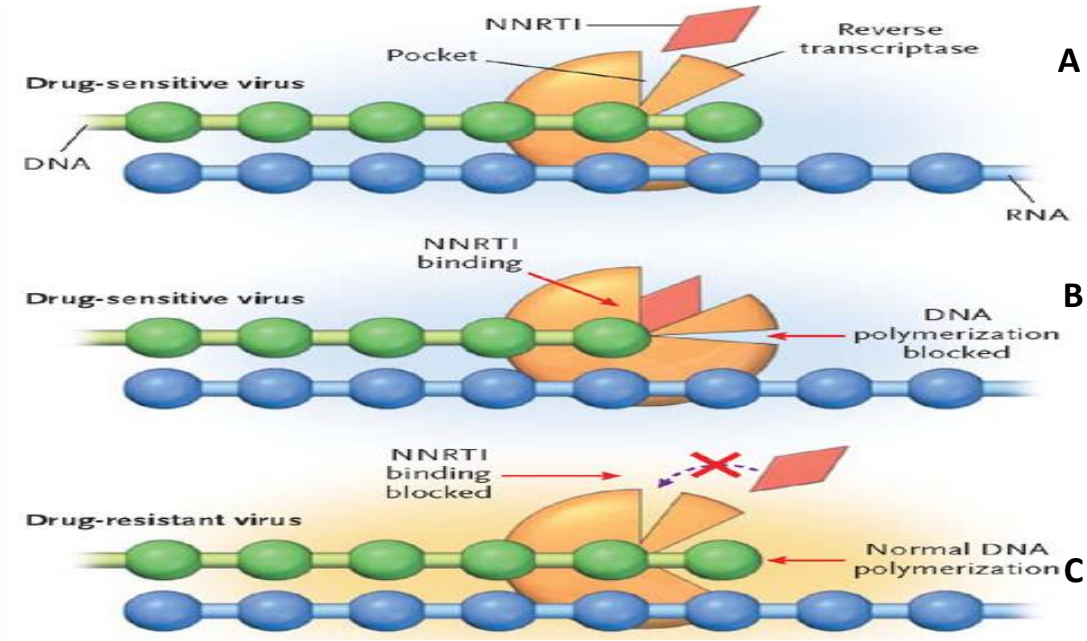

Figure 6: Mechanism of action and resistance of NNRTI

Mechanism of action and resistance of NNRTI. In A, NNRTI binds to the hydrophobic pocket near the reverse transcriptase and DNA polymerization is blocked (B). Resistance to NNRTIs occurs as a result of mutations that inhibit effective binding of the NNRTI, thus allowing DNA polymerization to proceed in an unrestricted manner (C). (http://hiv.uw.edu on 18/06/2015)

HIV-2 RT is intrinsically resistant to NNRTIs (Tuaillon *et al*., 2004), the *Y188L* polymorphism appears naturally in all HIV-2 isolates. Reversion to *Y188* restores the reverse transcriptase sensitivity to some NNRTIs, including efavirenz and delavirdine (Isaka *et al*., 2001)

In general, NNRTIs inhibit HIV-2 at effective concentrations that are at least 50-fold higher than those that inhibit HIV-1 (Witvrouw *et al*., 2004), making the use of these drugs for HIV-2 infection problematic.

2.5.3 Protease Inhibitors and Resistance

The HIV protease functions as a homodimer- a complex of two identical protein molecules both consisting of two identical chains made up of 99 amino acids- that processes gag (p55) and gag-pol (p160) polyprotein products into functional core proteins and viral enzymes (Clavel *et al*., 2004). During or immediately after budding, the polyproteins are cleaved by HIV protease at nine different cleavage sites to yield the structural proteins (p17, p24, p7 and p6), as well as the viral enzymes reverse transcriptase, integrase and protease. Protease inhibitors are based on amino acid sequences recognized and cleaved in HIV proteins. They all bind to active site amino acids within a pocket at the center of the homodimer (Clavel *et al*., 2004).

HIV-2 and HIV-1 proteases have similar substrate specificities (Tözsér, 2010). However, there are relevant differences between HIV-1 and HIV-2 that affect substrate binding sites and therefore to the mutational patterns conferring resistance to various protease inhibitors. Phenotypic susceptibility assays carried out with the HIV-2 reference isolate and virus obtained from infected patients showed that saquinavir, lopinavir and darunavir were the most potent protease inhibitors against HIV-2, and should be preferred as first-line options for HIV-2-infected patients (Desbois *et al*.,2008; Menéndez-Arias and Alvarez, 2014). However, HIV-2 expresses natural polymorphisms in the protease that may be implicated in emergent drug resistance and accelerate time to development of PI resistance. (Ntemgwa *et al*., 2007).

2.5.4 Integrase Inhibitors and Resistance

The double-stranded DNA resulting from reverse transcription is transported into the host cell nucleus where it is integrated into the cellular DNA. This process is catalyzed by the viral integrase and involves two reaction steps. First, the integrase cleaves a dinucleotide from each viral DNA terminus to produce reactive CpA 3-hydroxyl ends (30-end processing), and then the 300-end processed DNA is covalently joined to the host DNA during a strand transfer reaction (Delelis *et al*., 2008; Craigie and Bushman, 2012; Menéndez-Arias and Alvarez, 2014). Antiretroviral drugs that target the HIV-1 integrase (i.e. raltegravir, elvitegravir and dolutegravir) bind the catalytic site of the integrase and act as inhibitors of the strand transfer reaction.

Despite a 40% difference in amino acid sequence between HIV-1 and HIV-2 integrase inhibitors, phenotypic assays carried out with reference strains or clinical isolates have shown that, these drugs (i.e. raltegravir, elvitegravir and dolutegravir) are effective against HIV-2 (Roquebert *et al*., 2008; Charpentier *et al*., 2010; Koh *et al*., 2011; Smith *et al*., 2011). There is limited information on the mutational pathways that could lead to HIV-2 resistance to integrase inhibitors. However, available evidence indicates that mutations conferring resistance in HIV-1 and HIV-2 are essentially the same (Charpentier *et al*., 2011; Smith *et al*., 2012; Menéndez-Arias and Alvarez, 2014). In both viruses, raltegravir and elvitegravir have shown extensive cross resistance. Dolutegravir retains activity against raltegravir and elvitegravir-resistant HIV-1 strains (Geretti *et al*., 2012), but information on the efficiency against HIV-2 strains bearing integrase inhibitor resistance mutations are limited (Charpentier *et al*., 2010).

2.5.5 Fusion Inhibitors

Fusion inhibitors in the clinical treatment of HIV-1 infection are represented by enfuvirtide. This drug is a peptide of 36 amino acids that derives from the C-terminal region of the heptad repeat 2 (HR2) (i.e. residues 127–162), located within the N-terminal ectodomain of the HIV-1 trans membrane glycoprotein (TM,gp41). HR2-based peptides target the HR1 region and block the formation of a stable six-helix bundle during the fusion process (Menéndez-Arias and Alvarez, 2014)

HIV-2 strains show natural resistance to enfuvirtide. Thus, in phenotypic assays, reference HIV-2 ROD and HIV-2 strains showed 64 and 88-fold decreased susceptibility to the drug, respectively (Witvrouw *et al*., 2004). Most HIV-2 clinical isolates tested in vitro in another study involving patients naïve to therapy, showed increases in the IC50 (drug concentration) for enfuvirtide that ranged from 7- to >500-fold (Borrego *et al*., 2012).

2.5.6 Treatment of patients with HIV-2 and dual HIV-1/2 infections in Ghana

- Confirmation of HIV infection and type (HIV-1, HIV-2 or dual HIV-1/2)
- CD4 count less than 350 cells/µl
- If patient is HIV-2 or dual HIV-1/2 infected, use a protease inhibitor (PI) –based regimen lopinavir boosted with ritonavir (LPV/r) for treatment in place of Non-nucleoside reverse transcriptase inhibitor (NNRTI)

Source: (Guidelines for Antiretroviral therapy in Ghana, 2014)

2.6 Laboratory diagnosis of HIV

Laboratory diagnosis of HIV infection is essential for detecting and monitoring infection. HIV infection is identified either by the detection of HIV-specific antibodies in serum or plasma or by demonstrating the presence of the virus by nucleic acid detection using polymerase chain reaction (PCR) or p24 antigen testing. Antibody testing is however, the method most commonly used to diagnose HIV infection (Fearon, 2005). HIV assays can be divided into two categories: (i) screening

assays, designed to detect all infected individuals; and (ii) confirmatory assays designed to differentiate those persons who test falsely reactive by screening assays from those who are truly infected. Screening tests must have a high degree of sensitivity (proportion of true positives correctly identified by a test), whereas confirmatory assays must possess a higher specificity (proportion of true negatives correctly identified by a test). In most cases screening and confirmatory tests are performed in tandem to produce results that are highly accurate and reliable.

2.6.1 Screening assays for HIV infection

Most screening tests are based on the EIA (enzyme immune assay) principle. The EIA uses the basic concept of an antigen binding to its specific antibody and this allows detection of very small quantities of proteins, peptides, hormones, or antibody in a fluid sample. Four generations of EIAs have been developed (Gan and Patel, 2013). The first-generation EIA relied on the detection of antibodies directed against a coated well with whole-cell lysate of infected cells. The second-generation EIA substituted the whole-cell lysate for recombinant-produced HIV antigens. In the third generation, antibodies are detected through the "antigen sandwich" technique, in which the enzyme is linked to the antigen rather than to the antibody. This technique detects both immunoglobulin G (IgG) and immunoglobulin M (IgM), therefore allowing earlier antibody identification. In the fourth-generation EIA, the wells are coated with both p24 antibodies and HIV-1 antigens. Host-derived p24 antigens or antibodies directed at these molecules are detected using an enzyme-labeled antibody (Fearson, 2005).

In Ghana, rapid HIV tests such as the First Response HIV 1-2 Card Test (Premier Medical Corporation, India) and Oraquick (Orasure Technologies, Pennsylvania) tests are used as the screening assays for HIV infection. These rapid test detects antibodies of all classes specific to HIV-1 (including Group O) and HIV-2 in human serum, plasma or whole blood. The First Response HIV 1-2.O Card Test is based on the principle of immune-chromatography in which a nitrocellulose membrane is coated with recombinant HIV-1 capture antigens (gp41 including Group O and p24)

on test band "1" region and with recombinant HIV-2 capture antigen (gp36) on test band "2" region. Upon addition of blood containing HIV -1 and HIV-2 antibodies, a conjugated antigen-antibody complex moves through the nitrocellulose membrane and binds to the corresponding immobilized HIV 1 antigens and HIV 2 antigen (Test Lines). This leads to the formation of visible colored lines indicating HIV antibody reactive results.

2.6.2 Confirmatory assays

Most commonly used confirmatory assays for HIV infections are western blot (WB) for instance HIV Blot 2.2 Western Blot Assay (MP Diagnostics, Singapore) and line immune assays (LIA) such as Inno-lia HIV-1/2 score (Fujirebio Europe N. V, Belgium). These confirmatory assays are usually carried out on reactive samples from screening assays or to evaluate discordant results between two screening assays or rapid tests or to distinguish HIV-1 and HIV-2 infections. Such discriminatory immunoblot assays include antigens from both HIV-1 and HIV-2. The operational principle of the Inno-lia HIV-1/2 score (Fujirebio Europe N. V, Belgium) is that HIV denatured proteins are blotted on strips of a nitrocellulose membrane, which are then incubated with patient serum or plasma. If the serum or plasma contains antibodies against the various viral proteins, they will bind to the corresponding protein (Fearson, 2005). Recombinant proteins and synthetic peptides from HIV-1 and HIV-2, and a synthetic peptide from HIV-1 group O, are coated as discrete lines. Five HIV-1 antigens are applied: sgp120 and gp41, which detect specific antibodies to HIV-1, and p31, p24, and p17, which may also cross-react with antibodies to HIV-2. HIV-1 group O peptides are present in the HIV-1 sgp120 band. The antigens gp36 and sgp105 are fixed to detect antibodies to HIV-2. In addition to these HIV antigens, four control lines are coated on each strip: anti-streptavidine line, ± cut-off line (human IgG), 1+ positive control line (human IgG) and one strong 3+ positive control line which is also the sample addition control line (anti-human Ig). In the INNO-LIA™ HIV 1/2 Score, the test sample is incubated in a test trough together with the multiple antigen-coated test strip. HIV antibodies, if present in the sample, will bind to the individual HIV antigen lines on the

strip. Afterwards, a goat anti-human IgG labelled with alkaline phosphatase is added and will bind to any HIV antigen/antibody complex previously formed. Incubation with enzyme substrate (BCIP/NBT) produces a dark brown color in proportion to the amount of HIV antibody present in the sample. Color development is stopped with sulfuric acid.

If the sample contains no HIV-specific antibodies, the labelled antihuman antibody will not be bound to antigen/antibody complex so that only a low standard background color develops.

2.7 Qualitative PCR for HIV detection

This technique uses a thermostable DNA polymerase to extend oligonucleotide primers complimentary to the viral DNA genome target (Saiki *et al*., 1988). Consecutive cycles of denaturation, annealing and extension result in an exponential accumulation of target DNA. In this approach, a second round PCR on the first round amplicon will increase the overall sensitivity of detection. The second round uses a different set of PCR primers internal to the first set and can therefore act as a confirmation that the correct amplicon was produced by the first round reaction.

2.8 Agarose gel electrophoresis

Agarose gel electrophoresis is routinely used method in molecular biology laboratories for separating proteins, DNA or RNA (Kryndushkin *et al*., 2003). The separation medium is a gel made from agarose, which is a polysaccharide derivative of agar. The agarose gel consists of microscopic pores that act as a molecular sieve, which separates molecules based upon charge, size and shape. These characteristics, together with buffer conditions, gel concentrations and voltage, affect the mobility of molecules in gels. The sieving properties of the agarose gel influence the rate at which a molecule migrates. The two most common buffers for nucleic acids are Tris/Acetate/EDTA (TAE) and Tris/Borate/EDTA (TBE). DNA fragments migrate at different rates in these two buffers due to differences in ionic strength. TBE has disadvantages as it polymerizes and/or interacts with cis-diols found in RNA. TAE on the other hand has the lowest buffering capacity but provides the best

resolution for larger DNA, which implies the need for lower voltage and more time for a better product (Yilmaz *et al.*, 2012).

This powerful separation method is frequently used to analyze DNA fragments generated by restriction enzymes, and it is a convenient analytical method for determining the size of DNA molecules in the range of 500 to 30,000 base pairs (Yilmaz *et al*., 2012).

2.9 DNA sequencing

DNA sequencing refers to methods for determining the order of the nucleotides bases adenine, guanine, cytosine and thymine in a molecule of DNA (Shedure and Ji, 2008). DNA sequencing play an important role in many different fields, such as research, diagnostics, biotechnology and forensic biology.

The first DNA sequences were obtained by academic researchers using methods based on 2-dimensional chromatography in the early 1970s (Sanger *et al*., 1977). With the development of dye based sequencing method with automated analysis, DNA sequencing has become easier and faster. The knowledge of DNA sequences of genes and other parts of the genome of organisms has become indispensable for basic research studies on biological processes, as well as in applied fields such as diagnostic or forensic research.

2.9.1 Chain-termination method by Sanger

The key principle of the Sanger method is the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators (Sanger *et al*., 1977). The chain termination method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, radioactively or fluorescently labelled nucleotides, and modified nucleotides that terminate DNA strand elongation. The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP, dTTP) and the DNA polymerase. To each reaction is added one of the four dideoxynucleotide (ddATP, ddGTP, ddCTP, ddTTP) which are the chain terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length (Sanger *et al*., 1977).

The newly synthesized and labelled DNA fragments are heat denatured, and separated by size by gel electrophoresis on a denaturing polyacrylamide-urea gel with each of the four reactions run in one of the four individual lanes (lanes A, T, G, C), the DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image. A dark band in a lane indicates a DNA fragment that is result of chain termination after incorporation of a dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP). The relative position of the different bands among the four lanes are then used to read (from bottom to top) the DNA sequence (Sanger *et al*, 1977)

2.9.2 Dye-terminator sequencing

Dye-terminator sequencing utilizes labelling of the chain terminator dideoxynucleotide (ddNTPs), which permits sequencing in a single reaction, rather than four reactions as in the labelled- primer method.

In dye-terminator sequencing, each of the four chain terminators is labelled with fluorescent dyes, each of which with different wavelengths of fluorescence and emission (Rosenblum *et al*., 1997).

Owing to its greater convenience and speed, dye-terminator sequencing is now the mainstay in automated sequencing.

The target sequence is determined by high-resolution electrophoretic separation of the single-stranded, end-labeled extension products in a capillary-based polymer gel. Laser excitation of fluorescent labels as fragments of discreet lengths exit the capillary, coupled to four-color detection of emission spectra, provides the readout that is represented in a Sanger sequencing 'trace (Rosenblum *et al*., 1997).

CHAPTER THREE

3.0 Materials and Methods

3.1 Study Site

The patients were recruited from the HIV/AIDS care centers at the Koforidua Regional Hospital and the Korle-Bu Teaching Hospital in the Eastern and Greater-Accra regions of Ghana. The Korle-Bu Teaching Hospital is the premier health care facility in Ghana with 2,000 beds and 17 clinical and diagnostic Departments/Units. It has an average daily attendance of 1,500 patients and about 250 patient admissions. The Koforidua Hospital is the regional hospital and main HIV/AIDS clinic in the Eastern region of Ghana. This hospital is also responsible for HIV prevention and intervention programs in the region and provides free ART and clinical care to HIV-infected patients. The Eastern Region has the highest regional HIV prevalence of 3.7% in Ghana according to the 2013 HIV Sentinel Survey (National AIDS/STIs Control Programme, 2014).

3.2 Study design and study population

This was a cross-sectional study comprising of two groups of HIV-2 as well as dual HIV-1/2 infected persons; an ART-naïve group and an ART-experienced group.

The ART naive group provided baseline sequence data to profile the type of mutations present in the absence of antiretroviral therapy. The ART experienced group provided sequence data in the presence of antiretroviral drugs. A comparison of data from both groups thus gave insight on drug resistance mutations acquired on treatment and their impact on therapy.

A formula by Sullivan *et al.*, 2009 for sample size determination was adopted with the national prevalence of HIV-2 (0.8%) in Ghana for 2013, *t* value of 1.96 at 95% confidence level and 0.05 confidence interval.

The formula for sample size determination by Sullivan *et al.*, 2009 is as follows:

$$\mathbf{n} = \frac{\mathbf{t^2 \times p(1-p)}}{\mathbf{m^2}}$$

Description:

n = required sample size

t = confidence level at 95% (standard value of 1.96)

p = estimated prevalence of HIV-2 in Ghana (0.8%)

m = margin of error at 5% (standard value of 0.05)

Application of this formula gave a minimum sample size of 12 patients but 18 patients were recruited to ensure the target was attained.

3.2.1 Recruitment of study subjects

The purposive sampling method was used by the attending physician or health care givers to recruit study subjects and any patient diagnosed as seropositive for HIV-2 or dual HIV-1/2 who gave informed consent was recruited. Both males and females above aged 18 years and above were recruited. The patients were thereafter grouped into ART-naïve and ART- experienced based on whether they had initiated antiretroviral therapy or not. Clinical histories were obtained for all recruited patients by data retrieval from their hospital folders. This data included age, gender, date of HIV diagnosis, symptoms, ART status, ART regimen, start date of ART for the experienced and CD4 counts.

3.3. Blood collection from patient and transportation to the NMIMR

Six milliliters (6ml) of venous blood samples were collected into EDTA vacutainer tubes (Becton Dickinson Company, UK) from eligible patients after obtaining their informed consent. A sample collection (Appendix A) form accompanied each sample. 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. The samples were received at NMIMR within 24 hours of collection. Upon receipt, the blood samples were cross checked with the sample collection and consent forms (Appendix B and C). The adequacy of the blood sample volume was checked and the hospital identification number was cross-checked with the information on the accompanying form. The samples were given laboratory identification numbers and the information on the sample collection form was entered into a Microsoft Excel sample collection database.

3.4 Peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells were separated from the whole blood samples by measuring 3mls of lymphocyte separating medium (Histopaue-1077) into labeled 15ml centrifuge tubes. Six milliliters of whole blood was then carefully layered with a 10ml disposable pipette onto the Histopaque so that the blood formed a separate layer. The mixture was spun at 2500rpm for 15 minutes. The top layer (plasma) was then carefully removed with a sterile Pasteur pipette into 2ml vials leaving the buffy coat intact. The buffy coat containing the PBMC was then carefully put into 15ml centrifuge tube containing 5ml PBS, mixed by gentle vortexing and spun at 1500rpm for 10 minutes to wash the cells. The supernatant was discarded and the cell pellet was re-suspended in 5ml PBS. The washing step was repeated and the PBMC pellet was gently resuspended in one milliliter of freezing medium (1% Dimethyl sulphoxide in Fetal Bovine Serum) and distributed in two aliquots of 0.5ml each into 2ml cryovials. The PBMC and plasma were stored in a –80°C freezer until further analyses.

3.5. Serological Tests for HIV Antibodies

All the blood samples collected were tested by First Response HIV 1-2 (Premier Medical Corporation Ltd, India) followed by Inno-lia HIV-1/2 Score (Fujirebio Europe N.V, Belgium) to determine the type of HIV infection that the patient had.

3.5.1 Screening Test: First Response HIV-1/2 Card Test

First Response HIV 1-2.O Card Test is a rapid test used for detection of HIV antibodies. The test was performed according to the manufacturer's instructions (User Manual, First Response HIV 1-2 Card Test). Briefly, one drop of plasma was added to the sample well followed by a drop of the assay diluent. Results were read visually after 15 minutes by inspecting the sample and control windows for bands. Depending on bands present, the result was interpreted according to the manufacturer's directions (User Manual, First Response HIV 1-2).

3.5.2 Confirmatory Test: Inno-lia HIV I/II Score

The Inno-lia HIV I/ II Score (Fujirebio Europe N.V, Belgium), was used as a confirmatory assay to corroborate the presence of antibodies against HIV-1 and HIV-2 and also differentiate between HIV-1 and HIV-2 infections. The test was performed according to the manufacturer's instructions (User Manual, Inno-lia™ HIV I/II Score, 2015) using the 16 hours sample incubation option. Testing was done in troughs provided in the kit. The troughs were labeled with sample identification numbers and 10µl of plasma, sample diluent and the nylon test strip were transferred to the trough and incubated over night for 16 hours at room temperature. At the end of incubation, each test strip was washed three times with 1ml wash solution. One milliliter (1ml) of conjugate solution was added to each test trough and incubated for 30 minutes. Each test strip was washed three times with wash solution following incubation. One milliliter of substrate solution was added to each test trough and incubated for 15 minutes at room temperature. After incubation, the liquid was aspirated and 1ml of stop solution was added. The stop solution was aspirated after 10 minutes and test strips were allowed to dry prior to interpretation of results.

The presence and intensity of bands representing sgp120, gp41, p31, p24, p17, sgp105 and gp36 were used to interpret the results and based on the manufacturer's instructions, a sample was declared HIV-2 positive, HIV-1 positive or HIV-1 and HIV-2 positive.

3.6 Ribonucleic acid (RNA) extraction

Ribonucleic acid (RNA) was extracted from 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 kits. The eluted RNA was stored at minus 35 °C.

3.7 Deoxyribonucleic (DNA) acid extraction

DNA was extracted from PBMCs by following the manufacturer's instruction using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The cells were first lysed using Buffer AL and Proteinase K. Buffering conditions were adjusted to provide DNA-binding conditions and the lysate was loaded onto the DNeasy Mini spin column. There was centrifugation twice using wash buffers AW1 and AW2 to ensure complete removal of any residual contaminants that might affect the DNA. During centrifugation, DNA was selectively bound to the DNeasy membrane as contaminants passed through. DNA was eluted in 60 µl Buffer AE. The eluted DNA was stored in minus 80°C freezer until further analyses.

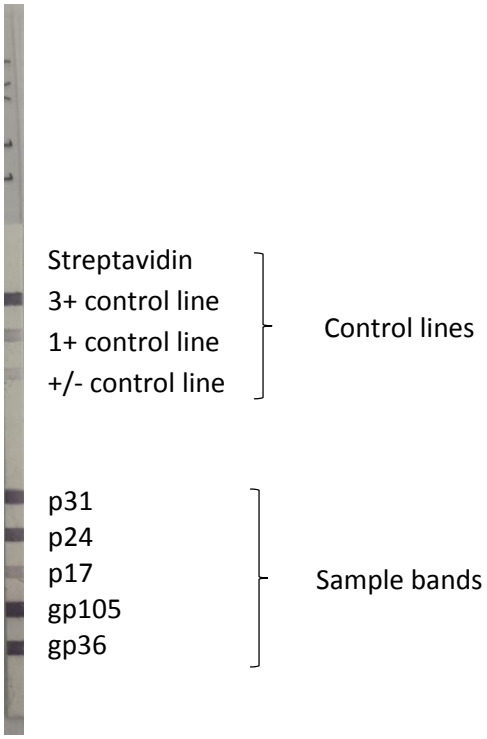

Figure 7: An Inno-lia strip showing bands after the test for presence of HIV antibodies.

A picture of Inno-lia strip (taken after the experiment) showing the antigen bands for HIV-2 coated onto it. The control band (3+, 1+, +/-) were used to grade the intensity of bands that developed during the test and this was used in result interpretation

3.8 Polymerase chain reaction (PCR) amplification

The protease (PR) and reverse transcriptase (RT) genes were amplified separately according to two protocols (Rodes *et al*., 2006). Polymerase chain reactions (PCR) for the PBMC samples were carried out in 25µl total volume containing 5µl of DNA, 12.5µl of Supermix (Life technologies, Invitrogen™, USA) and 25µM of each primer. The PCR for the plasma samples were carried in two steps: the first step PCR was carried using the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) following the manufacturer's protocol. The second step was carried out using the Amplitaq Gold PCR master mix kit (Applied Biosystems, USA). Primers were synthesized as previously described by Rodes *et al*., 2000. Outer RTC and RT2 and inner RT3 and RT4 primers were used during the first and second round PCRs. Nested PCR was carried out using 5µl of the first round PCR product. A genomic region of 1050bp encoding the RT was amplified by nested PCR.

Cycling conditions were as follows: The first round of PCR had an initial denaturation of 94°C for 2mins. It was followed by 40 amplification cycles (94°C for 30s, 55°C for 1min and 72°C for 1min 30s) and then by an elongation step at 72°C for 7min. Nested PCR conditions were: denaturation at 94°C for 2min, followed by 40 amplification cycles (94°C for 30s, 56°C for 1min, and 72°C for 1min). The last step was an extension at 72°C for 5min.

The whole protease coding region (297bp) was amplified by nested PCR. The primers used were as follows. PR1 and PR2 were the outer primers. DP27 and PR3 were the inner primers. The cycling conditions for the first round and nested PCRs were the same as that for the RT gene. Nested PCR was carried out using 5µl of the first round PCR product. Details of the primers used are presented in Table 2 on page 43.

3.9 Gel electrophoresis

Gel electrophoresis was used to evaluate the PCR products. Five microlitres (5µl) each of the PCR products were subjected to gel electrophoreses on 1.5% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer (Life technologies, Ambion®, USA) with ethidium bromide (Life technologies, Invitrogen™, USA) staining. The PCR products were then visualized under ultraviolet trans-illumination using the KODAK Gel Logic 100 Imaging system (Eastman Kodak Company, USA)

Table 2: Details of oligonucleotide primers used to amplify HIV-2 sub-genomic fragments.

Name	Position HIV-2 ROD	Nucleotide sequence (5'-3')	Purpose/ Genomic region
RTC	2309-2339	ATGACAGGGGATCCCCCAATCAATATTTTTG	Round 1 PCR targeting RT gene
RT2	3593-3526	GAAGTCCCAGTCTGGGATCCATGTCACTTGCCA	
RT3	2474-2504	GAGGCATTAAAAGAGATCTGTGAAAAATAGG	Round 2 PCR targeting RT gene
RT4	3500-3529	TCCCCAAATGACTAGTGCTTCTTTTTCCTAT	
PR1	2419-2437	GGG AAAGAAGCCCCGCAACTTC	Round 1 PCR targeting PR gene
PR2	3109-3088	GGGTATTATAAGGATTAGTTGG	
PR3	2632-2652	GCTGCACCTCAATTCTCTCTT	Round 2 PCR targeting PR gene
DP27	2360-2380	TAGATTTAATGACATGCCTAA	
RT3	2474-2504	GAGGCATTAAAAGAGATCTGTGAAAAAATGG	Sequencing the RT gene
RT5-HIV2	3586-3506	GGATGATATCTTAATAGCTAG	
RT4	3500-3529	TCCCCAAATGACTAGTGCTTCTTTTTCCTAT	
RT6-HIV2	3505-3689	GATGTCATTGACTGTCC	
PR2	3109-3088	GGGTATTATAAGGATTAGTTGG	Sequencing the PR gene
PR3	2632-2652	GCTGCACCTCAATTCTCTCTT	

The name, location on HIV-2 ROD and the purpose of each primer has been shown. RTC/RT2 and RT3/RT4 were previously published by Gao et al., 2004; DP27 was reported by Pieniazek et al., 1999 and PR1, PR2, PR3 as well as the sequencing primers were described by Rodes et al., 2000

3.11 Purification of nested PCR products

3.11.1 Application of the QIAquick PCR purification system (Qiagen, Hilden, Germany)

For nested PCR products with the expected band, purification was carried out using QIAquick PCR purification system (Qiagen, Hilden, Germany). Procedures were performed according to manufacturer's recommendations (QIAGEN GmbH 2006). Briefly, 100 µl of binding buffer was added to 20 µl of PCR product and mixed by vortexing. The mixture was then transferred to a spin column and centrifuged for 1 minute at 13200rpm in (Eppendorf microcentrifuge 5415D, USA). The columns were washed by adding 0.75 ml washing buffer and centrifuging for 1minute at 13200rpm. DNA was eluted with 30µl elution buffer by centrifugation at 13200rpm for 1 minute.

3.11.2 Use of the QIAquick Gel Extraction kit

The QIAquick Gel Extraction kit was used for samples that had other bands aside the expected band. The remaining volume of PCR products (approximately 20µl) were run by agarose gel electrophoresis and the gel extraction method was used to purify the products. Expected DNA fragment was excised from the agarose gel with a clean, sharp blade and gel extraction was performed following the manufacturer's instructions. Briefly, the sliced gels were weighed in a tube and 3 volumes of solubilization buffer was added. The tubes were incubated at 50 ℃ for 10 minutes until the gel melted completely. One gel volume of isopropanol was added to increase the yield of DNA fragments. The QIAquick spin column was placed in a 2ml collection tube and the sample was applied to the spin column and centrifuged at 13200rpm in an Eppendorf microcentrifuge 5415 D for one minute to bind DNA. The flow through was discarded and 500 µlof solubilization buffer was added to the QIAquick column and spun for another 1minute at 13200 rpm. To wash, 0.75ml of wash buffer was added to the QIAquick column and centrifuged for 1minute at 13200rpm. The flow through was discarded and centrifugation was repeated to remove any remnants wash buffer. DNA was then eluted by adding 50 µl of elution buffer to the center of the QIAquick membrane and centrifuged for 1minute at 13200rpm. The eluted DNA was stored at -20℃ prior to usage.

3.12 Cycle sequencing of PCR products

Cycle sequencing was performed using the Big Dye terminator v3.1 Cycle Sequencing kit (Applied Biosystems, California, USA). The DNA fragments were sequenced on both strands with sense primers RT3 and RT5-HIV2 and antisense primers RT4 and RT6-HIV2 for the RT region and primers PR3 and PR2 for the protease region. A 10µl reaction mixture, consisting of 2µl each of 5X Big Dye Sequencing Buffer, Big Dye Terminator Mix, 2µM primer, nuclease-free water and purified PCR product, was used for cycle sequencing. The cycling conditions (Bonney *et al*., 2013b) were 94^{O}C for 2 minutes, 25 cycles of 94^{O}C for 30 seconds, 50^{O}C for 15 seconds and 60^{O}C for 4 minutes.

3.13 Purification of sequenced products

Purification of sequenced product was performed with the Agencourt CleanSEQ sequencing reaction clean-up system (Agencourt Bioscience, USA). Procedures were performed according to manufacturer's recommendations (Agencourt Bioscience Corporation: Protocol 000600v031 n.d.). Briefly, 5µl of magnetic particles (Agencourt CleanSEQ) were added to the cycle-sequenced products (10 µl) and 42µl of 85% ethanol was added to the mixture. The mixture was placed in on a magnetic plate (Agencourt SPRI Plate 96R ring magnetic plate) at room temperature for 3 minutes. After that, the liquid portion of the mixture was carefully taken out and discarded leaving the DNA bound to the beads on the wall of the tube. With the tube still on the magnetic plate, 100 µl of 85% ethanol was added to the tube and kept for 3 minutes. Again the liquid was carefully taken off leaving the DNA bound to the beads on the wall. The tube was left on the magnetic plate for 10 minutes for the ethanol to completely dry off. Forty microliters of nuclease-free water (Life technologies, Ambion, USA) (40µl) was then added and the tube taken off the magnetic plate for 3 minutes to ensure the complete re-suspension of beads. The tube was placed back on the magnetic plate and purified DNA transferred into a 96 well optical plate (Applied Biosystems, USA) to be loaded onto the 3130xl Genetic Analyzer (Applied Biosystems, USA) to generate raw sequence data. At the end of the run, the raw sequence data was retrieved and further analyzed.

3.14 Sequence data analyses

Sequences were edited using the Sequencher software v4.0 (Gene Codes Corporation, 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 6.0 (http://www.megasoftware.net/) was used for generating phylogenetic trees. Mutation data and drug resistance interpretations were obtained by submitting the sequences to the HIV.2EU online tool (Charpentier *et al*., 2013) for the interpretation of HIV-2 drug resistance data. This is an online tool set up by top notch European scientists used for HIV-2 drug resistance analyses. The fasta-formatted sequences were copied and analyzed. Nucleotide sequences were aligned to the consensus subtype A and B HIV-2 ROD pol. Mutations were defined as the differences from consensus A or B reference sequence and further characterized as RT mutations (NRTI or NNRTI) and PI mutations. Patients' sequences were finally categorized as being susceptible, intermediate resistance or resistance. The HIV-2 EU online tool also determined the subtype of each sequence that was uploaded.

3.15 Data Analyses

Tables and charts were used to summarize the demographic data and clinical history of patients and the HIV-2EU online tool was used to analyze drug resistance mutations.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic characteristics

A total of eighteen (18) patients that were seropositive for HIV-2 were enrolled from December 2014 to May 2015 from the Korle-Bu Teaching hospital, Accra (GA/R) and Koforidua Regional hospital, Koforidua (E/R). The patients were assigned to two groups based on their antiretroviral (ART) history as ART-naïve or ART experienced. Eleven (11) of the patients were ART-naïve and seven (7) were ART experienced. Of the 18 patients, 11 were females and 7 were males. The mean age of the patients was 50 years ranging from 24 to 65 years summarized in Table 3 on page 48.

4.2 ART Naïve patients

Of the eighteen (18) patients enrolled, 11 were ART naïve. Seven (7) were HIV-2 only and 4 were dual HIV-1/2 infected. The median CD4 cell count for these ART naive patients was 701 cells/ µl [Interquartile range (Xu – Xl): 880] as shown in Table 3 on page 48

4.3 Patients on ART

Seven out of the 18 HIV-2 seropostive patients enrolled had been on antiretroviral therapy for a median duration of 12 months [(Interquartile range (Xu – Xl): 18] The CD4 cell counts at start of ART and at enrollment into the study are presented in figure 8 on page 50. Of the 7 patients on therapy, 4 were HIV-2 only and 3 were dual HIV-1/2 infected. The 4 HIV-2 patients were on Tenofovir (TDF), Lamivudine (3TC) and Nevirapine (NVP), Efavirenz (EFV) or Lopinavir/r. However, treatment options differed in the two ART care centers for the HIV-1/2 dual infected patients. In Korle-Bu Teaching Hospital, the HIV-1/2 patients were treated with Zidovudine (AZT), Lamivudine (3TC) and Efavirenz (EFZ) whilst in Koforidua they were treated with Tenofovir (TDF), Lamivudine (3TC) and Nevirapine (NVP). The details of the composition of individual regimens are shown in Table 4 on page 49.

Table 3: Demographic characteristics and clinical characteristics of study patients

No.	Facility	Treatment Category	Pat. ID	Age	Sex	Current CD4	HIV Status
1	Korle - Bu	ART Naïve	KBAN-14-01	24	M	11	HIV-2
2			KBAN-14-02	60	F	701	HIV-2
3		ART Experienced	KBAE-14-01	53	F	111	HIV-2
4			KBAE-15-05	49	F	856	HIV-1/2
5			KBAE-15-06	42	F	482	HIV-1/2
6			KBAE-15-07	63	M	282	HIV-2
7	Koforidua	ART Naïve	KFAN-15-01	42	F	916	HIV-1/2
8			KFAN-15-02	44	F	36	HIV-2
9			KFAN-15-06	58	M	881	HIV-2
10			KFAN-15-07	38	M	145	HIV-2
11			KFAN-15-08	39	F	255	HIV-1/2
12			KFAN-15-09	65	F	836	HIV-2
13			KFAN-15-11	47	F	10	HIV-1/2
14			KFAN-15-12	60	F	1200	HIV-2
15			KFAN-15-13	60	M	930	HIV-1/2
16		ART Experienced	KFAE-15-01	49	M	76	HIV-2
17			KFAE-15-04	60	M	162	HIV-2
18			KFAE-15-05	52	F	195	HIV-1/2

Summary of demographic and clinical data of the 18 enrolled patients from two study sites. The CD4 cell count, ART status, sex and age distribution are presented. Eleven patients were ART naïve and 7 patients were on therapy

Table 4: Immunological status, HIV type and ART history of study patients

Part. ID	Start CD4	Current CD4	HIV Status	Current ARV	Duration (months)
KBAE-14-01	45	111	HIV-2	TDF, 3TC, LPV/r	6
KBAE-15-05	591	856	HIV-1/2	AZT, 3TC, **EFV**	10
KBAE-15-06	271	482	HIV-1/2	AZT, 3TC, **EFV**	96
KBAE-15-07	71	282	HIV-2	3TC, TDF, LPV/r	12
KFAE-15-01	27	76	HIV-2	TDF, 3TC, **NVP**	20
KFAE-15-04	131	162	HIV-2	TDF, 3TC, **EFV**	1
KFAE-15-05	112	195	HIV-1/2	TDF, 3TC, **NVP**	24

NRTI: Zidovudine (AZT); Lamivudine=3TC; Tenofovir= TDF; PI: Lopinavir/r= LPV/r
NNRTI: Efavirenz = EFV; Nevirapine (NVP)

A summary of immunological status, HIV type and ART history of study patients. The start CD4 count represent the CD4 count in cells/µl taken at the start of ART. The current CD4 represent the CD4 cell count in cells/µl taken at the time of enrollment. The current ARV regimen represents the drug regimen at the time of sampling. The duration represents the time interval from the start of ART till enrollment into the study.

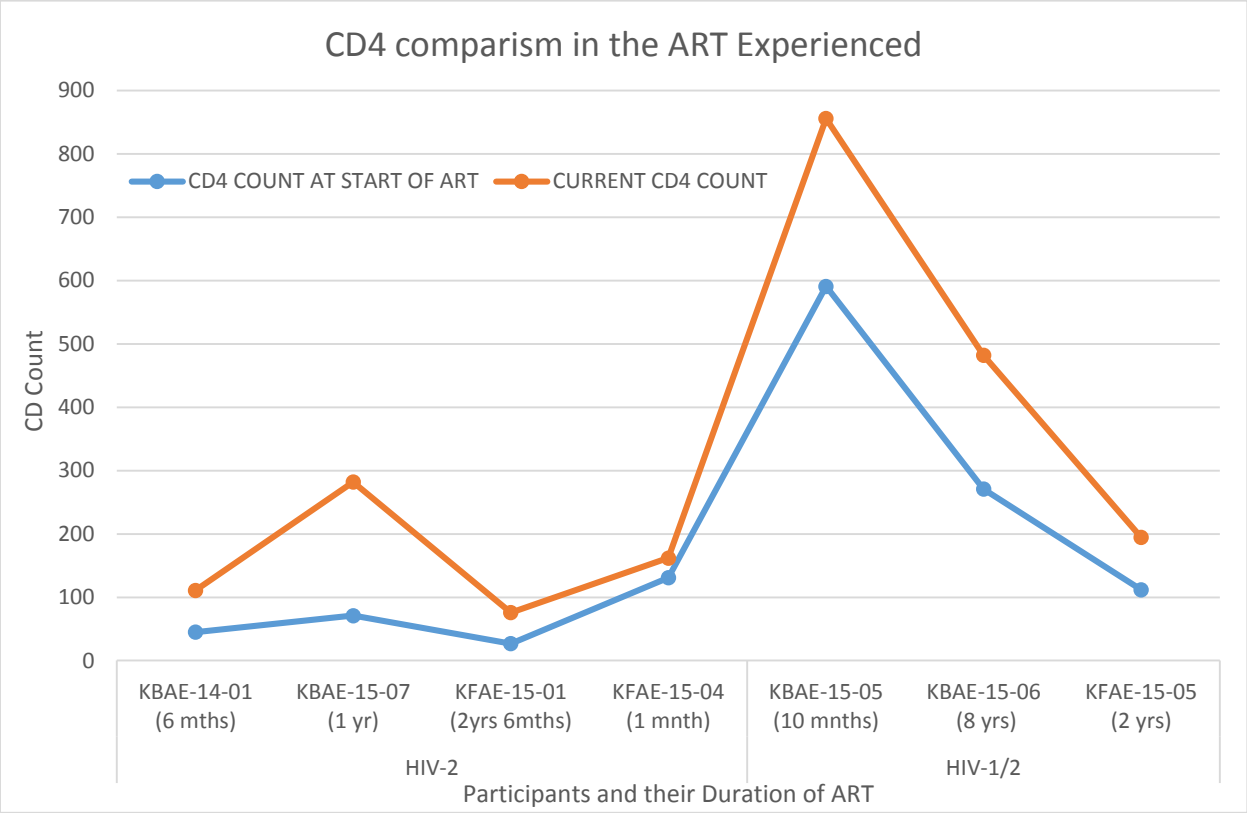

Figure 8: Comparison of CD4 counts in the ART experienced patients

The start CD4 represented by a blue line is the CD4 cell count in µl/ ml measured at start of ART.

The current CD4 represented by an orange line is the CD4 cell count in µl/ ml measured at enrollment.

4.4 PCR and sequencing from plasma and PBMC

There was successful amplification of 5 samples for the reverse transcriptase gene. Four samples were from PBMC and one was from plasma. Sequences were successfully obtained for all the 5 samples amplified. In the protease gene, 5 samples were also successfully amplified of which 4 were from PBMC and one was from plasma. Sequencing was however only successful for 2 samples out of the 4 from PBMC.

Out of the 11 ART- naïve patients studied, RT sequences and a PR sequence were successfully generated from the PBMCs of four patients and one patient respectively. One patient had both the RT and the PR genes successfully sequenced from PBMC. No sequence was generated from the plasma of the ART-naive patients.

From the 7 ART-experienced patients, RT sequences were generated from the paired plasma and PBMC of one patient. No PR sequences were however generated. The summary of the PCR and sequencing results obtained in this study can be found in Table 5 on page 52.

Table 5: Summary of PCR and sequencing from plasma and PBMC of seven patients

Gene	Sample type	Number of samples amplified (Out of 18)	Number of sequences obtained
Reverse Transcriptase	Plasma	1	1
	PBMC	4	4
Protease	Plasma	1	0
	PBMC	4	2

Summary of PCR and sequencing from plasma and PBMC from the reverse transcriptase and protease genes. The number of samples amplified and sequences obtained from the reverse transcriptase and protease genes are represented in the table.

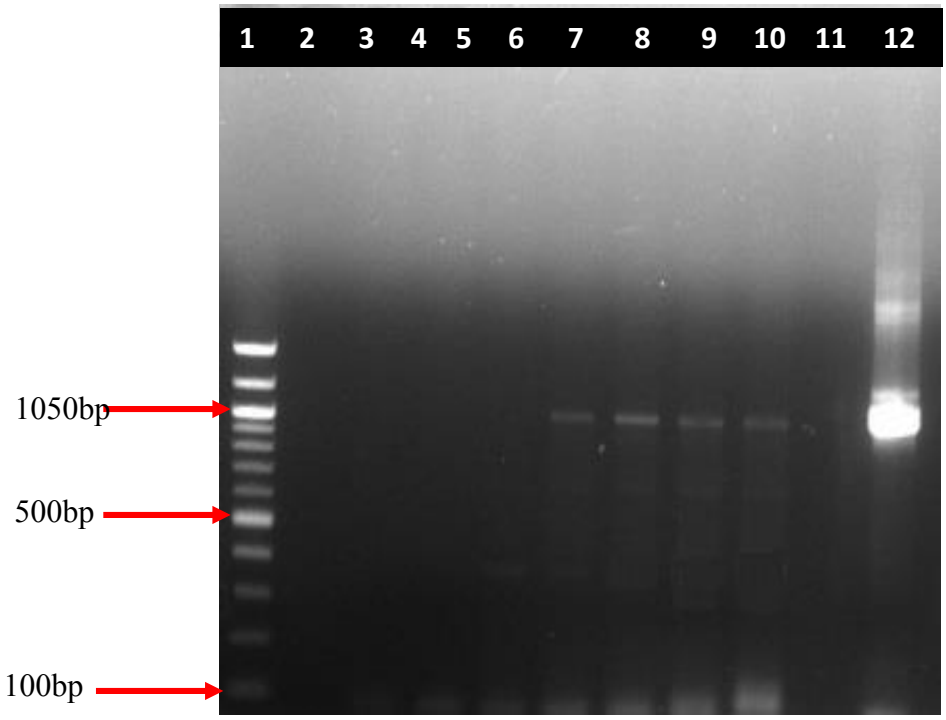

Figure 9: A gel picture of the RT gene from PBMC

Agarose gel (1.5%) was run using 1X TAE buffer. Lane 1 contained the 100bp DNA ladder (Biolabs, New England). Lanes 2 to 10 contained samples from participants; Lanes 11 has a negative control while lane 12 contained a positive control. The expected size of the positive product was 1050bp. Samples in lanes 2 to 6 were considered as failed amplification while samples in the other lanes (7 to 10) were successful amplifications

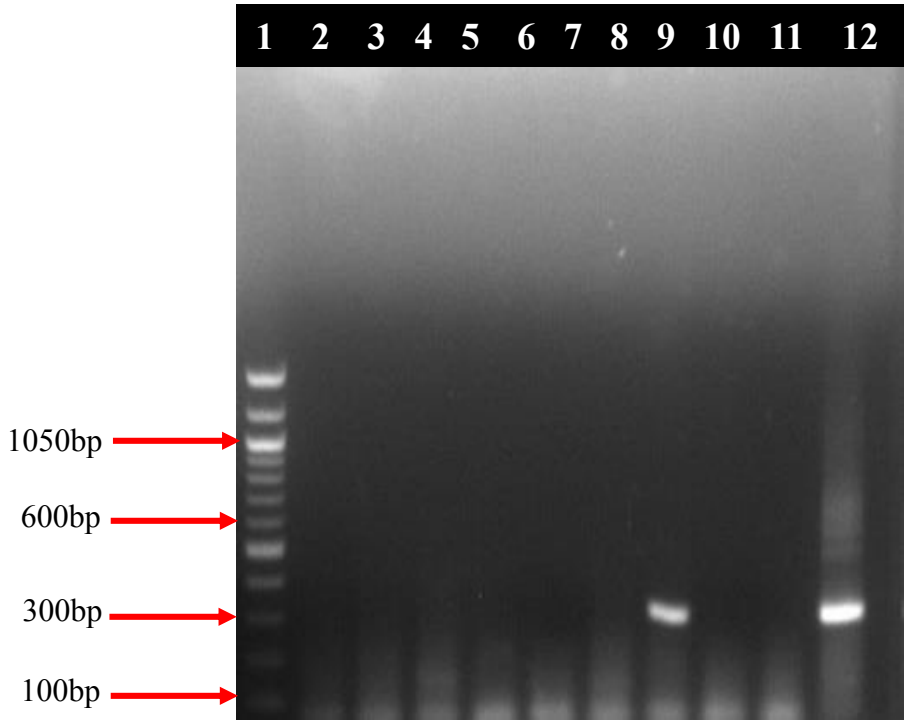

Figure 10: A gel picture of the PR gene from plasma

Agarose gel (1.5%) was run using 1X TAE buffer. Lane 1 contained the 100bp DNA ladder (Biolabs, New England). Lanes 2 to 10 contained samples from participants, Lanes 11 and 12 contained a negative control and a positive control respectively. The expected size of the positive product was 303bp. Samples in lanes 2 to 8 and 10 to 11 were considered as failed amplification while samples in the other lane 9 was successful amplification.

4.5 Drug resistance mutations and subtype information from study patients

4.5.1 Sequence analysis of ART naïve patients

There were no drug resistance-conferring mutations in any of the sequences derived from ART-naïve patients. Two sequences from the RT region were determined to belong to HIV-2 Group B and one was HIV-2 Group A. However, one sequence was HIV-2 Group A and B from the PR gene and RT gene respectively. The percentage similarity of the sequences to the closest HIV-2 reference isolate ranged from 83% to 97%. The details can be found in Table 6 page 57.

4.6 Sequence analysis of an ART experience patient

Three major drug resistance mutations were found in both the PBMC and plasma sequences obtained in a treatment-experienced patient. The resistance mutations were M184V, K65R and Y115F to the NRTI class of drugs. All three mutations were found both in the plasma and PBMC. This RT sequence was characterized as belonging to HIV-2 Group B by the HIV2EU online tool. Other details obtained on the sequences are shown in Table 7 page 57.

Table 6: Sequence analysis of ART naïve patients

Sample ID	Gene sequenced	Mutations	Drug Class	HIV-2 Group and % similarity to closest reference isolate
KFAN-15-01	RT	None	NRTI	B (88%)
KFAN-15-13		None		B (86%)
KBAN-14-01		**None**		**B (83%)**
KBAN-14-01	PR	**None**	PI	**A (93%)**
KFAN-15-06		None		A (97%)

Result of analyses of sequences from ART- naïve patients using the HIV-2 EU online tool. The sequences from the RT and PR genes are susceptible to the class of NRTI and PI drugs. The HIV-2 group and percentage similarity to closest reference isolate have been presented. Two sequences (in bold), were generated from the PR and RT gene of one patient. These sequences were HIV-2 group A from the PR gene and HIV-2 Group B from the RT gene.

Table 7: Analyses of sequences generated from Plasma and PBMC from an ART experienced patient

Sample ID	Treatment History	Source	NRTI mutations	NRTI resistance	HIV-2 Group and % similarity to closest reference isolate
KFAE-15-01	3TC, TDF, NVP	Plasma	M184V, K65R, Y115F	3TC, ABC, AZT, D4T, DDI, FTC, TDF	B (91%)
KFAE-15-01		PBMC	M184V, K65R, Y115F	3TC, ABC, AZT, D4T, DDI, FTC, TDF	B (92%)

Zidovudine=AZT; Lamivudine=3TC; Efavirenz=EFV; Tenofovir=TDF; D4T=Stavudine; DDI= Didanosine; FTC=Emtricitabine.

The table shows the sequence analyses of an ART experienced patient illustrating the mutations conferring resistance to NRTI.in plasma and PBMC components as well as the HIV-2 group and percentage similarity to the closest reference isolate.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1.0 Discussion

5.1.1 Identification of drug resistance mutations in ART experienced and ART naïve patients

The study sought to primarily determine drug resistance mutations in HIV-2 strains in patients infected with HIV-2 from two treatment centers in Ghana. This was done to generate data that was hitherto unavailable for the review of the progress of such patients to inform targeted management during ART. Previous studies in Ghana have clearly shown the presence of drug resistance mutations in HIV-1 strains in ART-naïve and ART-experienced individuals (Delgado *et al*., 2008; Brandful *et al*., 2012; Bonney *et al*., 2013a, Bonney *et al*., 2013b). This study thus presents the first report on drug resistance in HIV-2 infected patients in Ghana.

The study has revealed the presence of HIV-2 drug resistance mutations in an ART-experienced patient. The M184V, K65R, Y115F mutations which confer resistance to NRTIs were detected. These key mutations had been reported elsewhere from other genotypic studies (van der Ende *et al*., 2000; Damond *et al*., 2004; Descamps *et al*., 2004; Gottlieb *et al*., 2009). The K65R and Q151M substitutions are known to occur more commonly in HIV-2 than in HIV-1 infections, and often appeared together with M184V in patients that receive NRTI, as in the case of the ART-experienced patients in this study. The presence of K65R is indicative of class-wide nucleoside analogue resistance. The occurrence of Y115F, which is an abacavir-induced mutation, together with M184V, which reduces the susceptibility of lamivudine, emtricitabine and abacavir, confers resistance to all currently approved drugs in the NRTIs class.

While M184V is documented as the first mutation to emerge in patients on lamivudine-containing regimen (Descamps *et al*., 2000; Arnedo-Valero *et al*., 2005; Castagna *et al*., 2006) other studies have reported a high frequency of K65R in NRTI-treated patients infected with HIV-2 (Damond *et al*., 2004; Descamps *et al*., 2004). The patient in whose blood sample these mutations were detected, was on a regimen containing Lamivudine (3TC), Tenofovir (TDF) and Nevirapine (NVP). Thus it can be assumed that these mutations were induced by these drugs. The patient's regimen also contained Nevirapine, an NNRTI, which may not be of much therapeutic benefit, because HIV-2 strains are known to be naturally resistant to NNRTIs. HIV typing was not properly done to ascertain the exact type of HIV before initiating therapy. In effect, this patient was in reality, receiving dual therapy of Lamivudine and Tenofovir. This would encourage the development of more NRTI resistance mutations in the virus population and eventually lead to treatment failure. The CD4 count (76 cells/ul) for this patient at the time of sampling showed immunological failure but this was not used for a therapeutic change in regimen probably because routine CD4 counts were unavailable due to a nationwide shortage of reagents – the CD4 counts were measured due to reagents provided specifically by the research study. The trend in this patient's CD4 count (Table 4 on page 49); a paltry increase of just 50 cells/ul (27 at start and 76 at study) over 20 months of therapy is also a sign of poor prognosis. The absence of a commercial assay for viral load measurements specifically for HIV-2, is a major limitation for the monitoring of HIV-2 infected patients on therapy in Ghana. If viral load data had been available for this patient, it probably would have informed a change in regimen and curbed the emergence of more resistance mutations.

The study did not record any major drug resistance mutations to the NRTIs and PIs in HIV-2 strains found in ART naïve patients. However, on the contrary, (Ruelle *et al*. 2007) and Jallow *et al*. (2009) reported the occurrence of possible transmission of drug-resistant HIV-2 strains in some patients in Burkina Faso and Guinea Bissau respectively. Transmitted drug-resistant HIV-2 strains are probably

rare, possibly due to the small number of HIV-2 cases and the lower transmissibility of the virus compared to HIV-1.

It was also observed from the study that the CD4 cell counts of the ART- naïve patients ranged from 10 to 1200 cells /µl (Table 4 on page). Ideally patients with CD4 cell count lower than 350cells/µl must initiate therapy as per national treatment guidelines but this aberrance is surely due to the unavailability of reagents for routine CD4 measurements. Even though recently viral load testing for HIV patients on ART has been introduced, these assays are specifically for HIV-1 infections. The study also observed an increasing trend of CD4 cell count in the ART experienced patients (figure 8 on page 52) indicating ART regimen was successful in these patients. One patient's CD4 cell count at start of therapy was 591cells/µl. An unusually high CD4 cell count of 591cells/µl at initiation of therapy was noted for a pregnant patient, however, ART was possibly begun to protect the unborn baby.

5.1.2 HIV-2 subtypes

HIV-2 strains in this study were found to belong to groups A and B, confirming the epidemic potentials of only members of HIV-2 Groups A and B (Kanki *et al*., 1994; Plantier *et al*., 2004). Group B was found to be predominant which agrees with previous studies by Kanki *et al*., 1994; Plantier *et al*., 2004. This study also identified one intergroup (A/B) recombinant HIV-2 strain. This strain was classified as Group A in the PR gene and Group B in the RT gene. Similar recombinant HIV-2 strains had been documented previously in other West African countries. Gao *et al* (1994) identified an HIV-2 AB recombinant strain in Cote d'Ivoire, referred to as AB-CI-90-7312A and Yamaguchie *et al*., 2008 found a similar strain in Cameroun.

5.1.3 Comparison of drug resistance mutations in the plasma and PBMC

In this study, the profile of NRTIs resistance mutations observed in plasma was similar to that in paired PBMC from the same patient which is in consonance with earlier studies that documented matching drug resistance mutations in paired PBMC and plasma for HIV-1 (Vicenti *et al*., 2007; Bonney *et al*., 2013b). Although the previous studies were in HIV-1 infected patients, the findings from the current study for HIV-2 patients supports the recommendations for the use of PBMC as an alternative to plasma for resistance testing; particularly in patients with low plasma viral loads (Chew *et al*., 2005; Bonney *et al*., 2013b).

5.13 Identification of HIV-2 and dual HIV-1/ HIV-2 infected patients at the hospitals.

This study found patients with HIV-2 only and dual HIV-1/2 infections by the use of the INNO-LIA HIV-1/2 assay thus confirming the continuous co-existence of both HIV types in Ghana. This is in agreement with previous serological studies in Ghana in which Ampofo *et al*., 1999; Sagoe *et al*., 2008; Bonney *et al*., 2008 used INNO-LIA HIV-1/2 assay for the detection of HIV-2 and dual HIV-1/2 infections in Ghana.

This study successfully amplified and sequenced HIV-2 DNA from HIV-1/2 dual seropositive patients, as previously documented (Bonney *et al*, 2008; Sagoe *et al*, 2008), confirming the presence of HIV-2 virus in these patients.

5.2 Limitations

- The limitation of this study was the absence of a viral load system to determine the amount of HIV-2 RNA copies in the patients. Such an assay would indicate success or otherwise of therapy in these patients to inform changes in regimen if necessary.
- The inability to amplify genes of interest from all the 18 patients sampled was also a drawback since this would have provided more data to better understand the trends observed. The low amplification rates makes it rather difficult to generalize the results although the overall prevalence of HIV-2 infections are low.

5. 2 Conclusions

The study identified patients with HIV-2 only or dual HIV-1/2 infections confirming the co-circulation of both types of HIV in Ghana.

The presence of major HIV-2 drug resistance mutations to NRTIs in the ART experienced group were noted. These mutations; M184V, K65R and Y115F although occur also in HIV-1 strains, this work has now confirmed their association with HIV-2 strains as well.

The profile of drug resistance mutations was similar in a paired PBMC and plasma albeit for HIV-2. This finding supports the use of PBMC as an alternative to plasma for drug resistance testing.

This study, the first in Ghana to provide drug resistance data on HIV-2 strains, will inform targeted management of HIV-2 only and dual HIV-1/2-infected patients.

In addition, this investigation has for the first time in Ghana attempted to optimize an HIV-2 genotyping protocol that can be applied for HIV-2 drug resistance testing in Ghana at the Virology Department of the Noguchi Memorial Institute for Medical Research which is the national genotyping center for HIV drug resistance.

5.3 Recommendations

- Future studies should seek to establish an in-house HIV-2 viral load system to support the monitoring of HIV-2-infected patients in line with the use of viral load monitoring for patients on ART.
- The National AIDS/STIs Control Programme should ensure that clinicians in HIV care centers accurately type HIV patients before therapy and also follow the treatment guidelines for HIV-2 patients
- The Noguchi Memorial Institute for Medical Research should consider a collaboration with the National AIDS/STIs Control Programme /Ghana Health Service to establish an HIV-2 bio-bank to support future studies.
- A longitudinal study is recommended to better understand the patterns of HIV-2 drug resistance mutations in Ghana.

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APPENDICES

Appendix A: Questionnaire for ART Experienced Patients

Drug Resistance Mutations In Patients Infected With HIV Type 2 In Ghana.

Medical Microbiology Department, School of Biomedical and Allied Health Sciences

University of Ghana

1. Study Site_____
2. Outpatient Number (ART#) _____
3. Sample ID_____ Age_____ Sex_____
4. Date of Collection_____Current CD4 Count_____
5. Previous CD4 count_____ Date analyzed_____
6. Date started ART_____ CD4 count at start of ART_____
7. ARV currently on_____ Duration *of use*_____
8. Treatment history:

ARV_____ Dates (from- to) _____

ARV_____ Dates (from- to) _____

Appendix B: Questionnaire for ART Naïve patients

Drug Resistance Mutations In Patients Infected With HIV Type 2 In Ghana.

Medical Microbiology Department, School of Biomedical and Allied Health Sciences, University of Ghana

Study Site_____

Outpatient Number (ART#) _____

Sample ID_____ Age_____ Sex_____

Date of Collection_____Current CD4 Count_____

Does the patient currently have any of the following infections? (check all that apply)

TB ☐
PCP ☐
Candida ☐
Herpes Zoster ☐
Chronic Diarrhe ☐
None ☐

How was HIV-2 transmitted to patient? (check all that apply)

Heterosexual ☐
MSM ☐
IDU ☐
Sex worker ☐
MTCT (Mother to child transmission) ☐
Unknown ☐

Appendix C: Patient Consent Form

CONSENT FORM

Title: Drug Resistance mutations in patients infected with HIV type 2 in Ghana

Principal Investigator: Christopher Zaab-Yen Abana

Address: Department of Medical Microbiology

School of Medicine and Dentistry

University of Ghana

Phone: 0244144576

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 it read to you. You will also be asked to sign it (or make your mark in front of a witness). A copy of this form will be given to you. 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

There are two types of HIV, HIV type 1 and HIV type 2. You are being asked to take part in this research to find out whether the medicine (ARV) that you are given at the clinic can control the growth of the HIV type 2 you have in your blood now.

General Information about Research

This is a research to find out whether the HIV type 2 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 in a person, 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 unsuccessful. CD4 counts cannot tell whether a person has drug-resistant strain or not. This research will therefore use a more advanced

method to find out the types of strains HIV type 2 people on ARV have so that the success of the current ARV program in the country can be evaluated and modified if necessary.

Study Procedures

If you agree to be in this study, this is what will happen. You will participate in a survey with a staff member. The survey has questions about your background, health, and history of ART treatment. It will take up to 20 minutes to complete. Any information you provide will be kept confidential.

Why have I been asked to participate?

You have been chosen because you have tested HIV-2 or HIV-1/2 dual positive and have started drugs (antiretroviral therapy) or yet to start taking drugs (antiretroviral therapy)

Do I have to take part?

You are free to choose to participate or not to participate. If you decide not to participate this is will not affect the care you will receive in anyway. If you agree to participate you are free to end your participation at any time.

Will I be paid any money?

This is purely voluntary participation that is required of you and no monetary compensation is

available

What will happen to me if I take part?

If you take part, a trained staff member will collect information from your medical chart. You will be asked to provide a few teaspoons of blood for research purposes. The blood for this study will not have your name on it so there will be no way to provide the test results back to you. Any later test results cannot be linked back to you because I will not be writing your name on any of the blood test tubes. You may take part in the study without having your blood stored for later testing.

Will my residual sample be stored?

Yes. With your permission, I would like to store your residual blood sample for a later research work on other viral pathogens that is not mentioned in this study

Do you want your residual sample to be stored?

Yes ☐ No ☐

What is the risk of being in this research study?

The risk to you if you take part in the study is small. All the things that i use to take the blood are clean and safe. They have never been used before and will be thrown away after each use. Some people may feel dizzy when blood is drawn and there may be some pain or discomfort from the needle stick. You may develop a bruise or swelling where the needle goes into your arm. If you have any pain, bleeding, or swelling from taking blood, please contact the study staff

What will happen to the results of this research study?

The results will be written into my thesis which will not have any individual names. This thesis will be used by the Ministry of Health/ Ghana Health Service and other donors to improve HIV services in Ghana.

Do I receive the results?

No. The results will be given to your Clinician and it will be used to develop future programs and interventions to address any effects HIV-2 patients on ART have on public health in Ghana

Confidentiality

All information and blood samples will be private. Names are not written on the forms or the blood tubes. All information will be stored in a locked cabinet that can only be opened by an assigned health staff of the Koforidua Regional Hospital and the Korle-bu Teaching Hospital. Your samples collected for research purposes will be labelled with a code number and will be taken to the NMIMR for processing. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

Time Duration

If you agree to take part in this study, your involvement will last for less than 30 minutes. You will be asked to return to the clinic for a repeat sample if such circumstance arises.

Discomforts and Risks

The risk to you if you take part in the study is small. All the things that i use to take the blood are clean and safe. They have never been used before and will be thrown away after each use. Some people may feel dizzy when blood is drawn and there may be some pain or discomfort from the needle stick. You may develop a bruise or swelling where the needle goes into your arm. If you have any pain, bleeding, or swelling from taking blood, please contact the study staff

Benefits

You may not benefit directly from taking part in this research study. However the results of this research may guide the future treatment of HIV type 2 infected people. Health policy makers may also gain further understanding of HIV type 2 infection that might lead to formulation of policies and programs to benefit HIV infected individuals

Contacts for Additional Information

If you have any questions about this study you may contact Christopher Zaab-Yen Abana on 0244144576, if you are still not satisfied and need further explanation you can contact Prof. William Ampofo on+233 (0) 244 371 207 or Dr. Kwamena William Coleman Sagoe on +233(0)277408528.

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.mimcom.org

Appendix D: Volunteer Agreement Form

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title ***(Drug Resistance Mutations in Patients Infected with HIV type 2 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 Name and 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 Name and 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 Name Signature of Person Who Obtained Consent

Appendix E: Ethical Clearance Certificate from NMIMR

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979
A Constituent of the College of Health Sciences
University of Ghana

INSTITUTIONAL REVIEW BOARD

Phone: +233-302-916438 (Direct)
+233-289-522574
Fax: +233-302-502182/513202
E-mail: nirb@noguchi.mimcom.org
Telex No: 2556 UGL GH

Post Office Box LG 581
Legon, Accra
Ghana

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

4th March, 2015

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 063/14-15 **IORG 0000908**

On 4th March 2015, 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 : **Drug resistance mutations in patients infected with HIV type 2 in Ghana**

PRINCIPAL INVESTIGATOR : **Christopher Zaab-Yen Abana, Mphil 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 3rd March, 2016. You are to submit annual reports for continuing review.

Signature of Chair: .....
Mrs. Chris Dadzie
(NMIMR – IRB, Chair)

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

Appendix F: Ethical Clearance Certificate from University of Ghana School of Medicine and Dentistry

UNIVERSITY OF GHANA
SCHOOL OF MEDICINE AND DENTISTRY

Ref. No.:.....

18th February, 2015

Dr. Christopher Zaab-Yen Abena
Department of Medical Microbiology
SBAHS
Korle-Bu

ETHICAL CLEARANCE

Protocol Identification Number: **MS-Et/M.4 – P 4.3/2014-2015**

The Ethical and Protocol Review Committee of the School of Medicine and Dentistry on 11th February 2015 unanimously approved your research proposal.

TITLE OF PROTOCOL: **"Drug Resistance Mutations in Patients Infected with HIV Type 2 in Ghana"**

PRINCIPAL INVESTIGATOR: **Dr. Christopher Zaab-Yen Abena**

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till November, 2015.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed:.....
PROFESSOR ANDREW A. ADJEI
(FOR: CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE)

cc: Dean, SMD
Head of Department
Research Office

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Appendix G: Materials

A: Reagent for laboratory work

First Response HIV-1/2 Card Test (Premier Medical Corporation, India)

INNO-LIA HIV-1/2 Confirmatory Assay (Fujirebio Europe N. V, Blegium)

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

Phosphate buffered saline (PBS) [SIGMA, USA]

Dimethyl sulphoxide (DMSO) [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 (Biolabs, New England)

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)

Performance Optimized Polymer-POP 7 (ABI, USA)

B. Laboratory equipment

Biological Safety Cabinet Class II (LABGARD, USA)

Biosafety Cabinet Class IIA (AirTech Services, India)

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)

AirClean 600 PCR Workstation (AirClean Systems, USA)

GeneAmp PCR System 2700 and 2720 (ABI, USA)

Microwave oven (LG Electronics Inc., Ghana)

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

Genetic Analyzer 3130 (ABI, USA)

C. Sequence analysis software

Sequencher software v4.0 (Gene Codes Corporation, USA)

EU HIV-2 Internet Tool (http://www.hiv-grade.de/HIV2EU/deployed/grade.pl?program=hivalg)

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

MEGA 6 (http://www.megasoftware.net/)

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

Los Alamos HIV Sequence Database

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