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COLLEGE OF BASIC AND APPLIED SCIENCES

GENOTYPIC CHARACTERIZATION OF HIV-1 FROM PATIENTS IN TWO REGIONS OF GHANA

BY

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(10513087)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES DEGREE

DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

JULY, 2016
DECLARATION

I declare that this thesis is a result of my own research work carried out at the Virology Department of the Noguchi Memorial Institute for Medical Research, under the supervision of Dr. Osbourne Quaye of the Department of Biochemistry, Cell and Molecular Biology and Dr. Evelyn Yayra Bonney of the Noguchi Memorial Institute for Medical Research. I further declare that, any intellectual property used in this thesis that is not mine has been duly referenced and that this work has not been submitted to any other institution for the purposes of obtaining a degree.

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DEDICATION

To the memory of my late brother and father, Gameli Derek and Ben Ellis Deletsu. I wish you were here to celebrate this milestone with me.
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All said and done, I say, thank God for His grace and mercies!
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<tr>
<td>µl</td>
<td>Microlitre</td>
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<tr>
<td>3TC</td>
<td>Lamivudine</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
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<tr>
<td>ABC</td>
<td>Abacavir</td>
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<td>ABI</td>
<td>Applied Biosystems Inc.</td>
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<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td>ART</td>
<td>Antiretroviral therapy</td>
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<td>ARV</td>
<td>Antiretroviral</td>
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<td>AZT</td>
<td>Azidothymidine</td>
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<td>bp</td>
<td>Base pair</td>
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<td>Cluster of differentiation 4</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8</td>
</tr>
<tr>
<td>CDC</td>
<td>Centres for Disease Control</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<td>Full Form</td>
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<td>CRFs</td>
<td>Circulating recombinant forms</td>
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<td>Stavudine</td>
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<td>Didanosine</td>
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<td>ddNTPs</td>
<td>Dideoxynucleotide triphosphates</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide triphosphates</td>
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<tr>
<td>DRM</td>
<td>Drug Resistance Mutation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<tr>
<td>Efavirenz</td>
<td>Efavirenz</td>
</tr>
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<td>Env</td>
<td>Envelope</td>
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<td>FDA</td>
<td>Food and Drugs Administration</td>
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<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>Gag</td>
<td>Group-specific antigen gene</td>
</tr>
<tr>
<td>Glycoprotein</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type 1</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>------------------------------------------------</td>
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<tr>
<td>HIV-2</td>
<td>Human immunodeficiency virus type 2</td>
</tr>
<tr>
<td>HIVDB</td>
<td>HIV database</td>
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<tr>
<td>HSS</td>
<td>HIV sentinel survey</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
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<tr>
<td>IN</td>
<td>Integrase</td>
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<tr>
<td>LPV/r</td>
<td>Lopinavir/Ritonavir</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix</td>
</tr>
<tr>
<td>MEGA</td>
<td>Molecular evolutionary genetics analysis</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NACP</td>
<td>National HIV/AIDS/STI Control Program</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative regulatory factor</td>
</tr>
<tr>
<td>NFV</td>
<td>Nelfinavir</td>
</tr>
<tr>
<td>NMIMR</td>
<td>Noguchi Memorial Institute for Medical Research</td>
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<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NVP</td>
<td>Nevirapine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-integration complex</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulatory factor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>ss</td>
<td>Single-stranded</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>TAMs</td>
<td>Thymidine analogue mutations</td>
</tr>
<tr>
<td>Tat</td>
<td>Transactivator</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>TDF</td>
<td>Tenofovir</td>
</tr>
<tr>
<td>Tris</td>
<td>Trisaminomethane</td>
</tr>
<tr>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>United Nations joint programme on HIV/AIDS</td>
</tr>
<tr>
<td>URFs</td>
<td>Unique recombinant forms</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>v</td>
<td>Version</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>VL</td>
<td>Viral load</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein r</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein u</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

The HIV/AIDS pandemic is still a global health issue despite the many concerted efforts put in by various stakeholders to curtail it. It has become clear however that understanding the epidemiology of the highly variant virus is key in eliminating it. It has been shown that the subtype of infecting HIV-1 plays a crucial role in disease progression among other things. It is therefore important to continually monitor the circulating HIV-1 subtypes in the population.

Equally important, is the need to monitor the occurrence of drug resistance mutations in the population. With the continuous increase in the prevalence of drug resistance HIV-1 strains, it is key to be aware of the prevalence of drug resistance in the population in order to combat it.

This study aims to determine the common circulating HIV-1 subtype and to identify the drug resistance mutations present, if any, in the sampled population.

Viral RNA was extracted from sixty-three (63) archival plasma samples collected from four groups of HIV-1 patients in a previous study. The HIV protease and Reverse Transcriptase genes were amplified by polymerase chain reaction and positive amplification products sequenced and analysed to determine the subtype and to identify drug resistance mutations.

The protease and reverse transcriptase genes were successfully amplified from 46% and 33% of samples respectively. From these, 27.6% and 69.1% were successfully sequenced for the protease and reverse transcriptase genes respectively.

All (100%) of the protease subtypes were CRF02_AG. Of the reverse transcriptase subtypes, 77% were CRF02_AG, 13% G and 10% were CRF01_AE subtype.

Drug resistance mutations were identified in 37.5% of protease sequences and 53.9% of reverse transcriptase sequences. The most prevalent NRTI mutation was the M184I which translated
to a high frequency of lamivudine resistant samples. About 85.7% of reverse transcriptase sequences were resistant to both NNRTIs (Nevirapine and Efavirenz) in use in Ghana.

This study provides evidence that the \textit{CRF02\_AG} is the common circulating subtype in the sampled population and indicate the presence of high frequency of resistance to Nevirapine and Efavirenz.
CHAPTER ONE

1 INTRODUCTION

The scourge of the Human Immunodeficiency Virus (HIV) and the Acquired Immune Deficiency Syndrome (AIDS) is still a very pressing health issue despite all the research and attention that has been devoted to it. AIDS was discovered in 1981 when it was realised that homosexual men were falling victim to opportunistic infections due to a compromised immune system (Gottlieb et al., 1981; Siegal et al., 1981). Later on, in 1983, the causative HIV was isolated (Barré-Sinoussi et al., 1983). Since the beginning of the AIDS epidemic, an estimated 75 million people worldwide had died by 2012 (UNAIDS, 2013). Globally, in 2014, 36.9 million people were living with HIV and 1.2 million had died as a result of AIDS (UNAIDS, 2015; World Health Organization, 2015).

In Ghana, national figures quoted by the National HIV/AIDS/STI Control Program (NACP) in 2013 include a prevalence of 1.3% with 224,488 persons estimated to be living with HIV and 10,074 deaths due to AIDS (National AIDS/STI Control Program, 2014). At the forefront of issues in HIV research is vaccine development. However, issues relating to the genetic diversity of HIV-1 and also the development of resistance to antiretroviral therapy (ART) leading to treatment failure are fast becoming major problems.

The first cases of HIV in Ghana were reportedly diagnosed in 1986. From then to 2000, the reported and estimated number of infected people were 43,587 and 185,000 respectively. Within the same period, 150,000 Ghanaians had already died from HIV/AIDS (National HIV/AIDS/STI Control Program, 2001). Key statistics from the HIV Sentinel Survey 2013 (National AIDS/STI Control Program, 2014) include,
- Highest prevalence (3.7%) – Eastern Region
- Lowest prevalence (0.8%) – Northern and Upper West Regions
- HIV-1 prevalence – 97.1% of cases
- HIV-2 prevalence – 0.8% of cases
- Mixed infections – 2.1% of cases

HIV-1 is classified into three major phylogenetic groups; M (Main), O (Outlier) and N (Non M/O) with group M causing the majority of HIV-1 infections worldwide (Buonaguro et al., 2007). HIV-1 group M is further divided into subtypes A-D, F-H, J, K and Circulating Recombinant Forms (CRFs) with subtype B being the most dominant worldwide (Hemelaar et al., 2006; Requejo, 2006). For epidemiological reasons, it is very necessary to monitor the circulating strains of HIV-1. Although all subtypes and Unique Recombinant Forms (URFs) have been found all over Africa, studies have shown that the CFR02_AG and A are the most common circulating forms in West Africa, subtype B in Northern Africa, subtype C predominant in Southern and East Africa and D limited to Central and East Africa (Buonaguro et al., 2007; Esbjörnsson et al., 2011). In Ghana, the predominant circulating subtype is CRF02_AG (Fischetti et al., 2004; Nii-Trebi et al., 2013). Surveillance of the circulating subtypes is important so as to monitor recombination which occurs frequently in the case of HIV. Subtype has also been linked in various studies to diagnosis (Mlisana et al., 2013), disease progression (Amornkul et al., 2013; Baeten et al., 2007; Kiwanuka et al., 2008; Touloumi et al., 2013) as well as mode of transmission (Chalmet et al., 2010; Yang et al., 2003) giving even more weight to the continual surveillance of the circulating subtype of HIV.

Globally, 15 million people leaving with HIV were accessing Antiretroviral Therapy (ART) by March 2015 (UNAIDS, 2015). An estimated 125,396 Ghanaians needed ART
in 2013 (National AIDS/STI Control Program, 2014) and 83,712 of them had access to ART by 2014 (UNAIDS, 2014). With the scale-up of ART there has been a recorded emergence and increase in the development of drug resistance (Aghokeng et al., 2011; Gupta et al., 2012; Hamers et al., 2011a, 2012, 2013; Rhee et al., 2015; Ssemwanga et al., 2015). This illustrates the need to monitor the occurrence and trend of resistance development to better inform therapy policies.

There has been substantial amount of work done on antiretroviral drug resistance all over in Africa (Aghokeng et al., 2011; Hamers et al., 2011a, 2012, 2013; Kamoto et al., 2008; Kantor et al., 2014; Ndembí et al., 2011; Price et al., 2010; Ssemwanga et al., 2015) and also in Ghana (Bonney et al., 2013; Delgado et al., 2008; Kinomoto et al., 2005; Nii-Trebi et al., 2013; Sagoe et al., 2007) which all report a low but increasing prevalence of antiretroviral drug resistance. It is therefore imperative that the trend be continually monitored.

This study therefore aims to add on to work already done in documenting the circulating subtypes of HIV-1 and also monitor emergence of drug resistance in patients at two hospitals in Ghana.

1.1 Justification

The necessity of this study is hinged on the fact that work done on genotyping HIV-1 subtypes in circulation, as well as on drug resistance, in Ghana is limited and needs to be updated. After establishing a drug resistance threshold, it is now necessary to follow up with further studies so as to establish a trend. Furthermore, studies, including one carried out by (Rhee et al., 2015), shows that certain drug resistance mutations are more prevalent in certain subtypes of HIV-1. This implies that it is necessary to correlate drug resistance with virus subtype in drug resistance studies.
1.2 Aim and Objectives

The aim of my study is to, through genotypic analysis, determine the subtypes of HIV-1 in circulation and also, detect the presence of any drug resistance mutations in plasma samples from HIV positive patients visiting two hospitals in Ghana.

Objectives include:

- To sequence the protease (PR) and reverse transcriptase (RT) genes of HIV-1 from patient plasma
- To characterize viruses into subtypes
- To identify drug resistance mutations present in analysed samples
CHAPTER TWO

2 LITERATURE REVIEW

2.1 Introduction

The Human Immunodeficiency Virus (HIV) is a retrovirus of animal origin (Telenti, 2005) belonging to the lentivirus genus and transmitted through blood, genital fluids and breastmilk (O’Cofaigh and Lewthwaite, 2013). It is an enveloped virus and has as its genomic material made of two identical copies of positive sense, single stranded ribonucleic acid (RNA). The hallmark of HIV, as with all retroviruses, is the possession of the reverse transcriptase enzyme which is used to transcribe the genomic ribonucleic acid (RNA) of HIV into deoxyribonucleic acid (DNA) (Coffin et al., 1997). Two types of the virus have been characterized, HIV-1 and HIV-2. Both types cause Acquired Immune Deficiency Syndrome (AIDS), however, HIV-1 is responsible for the global pandemic (Santos and Soares, 2010).

2.2 HIV and AIDS History and Pandemic

AIDS was discovered in 1981 in four homosexual men who had opportunistic infections including, Pneumocystis carinii, Candida albicans and cytomegalous viral infections, as well as having recurrent perianal ulcers and herpes simplex infections (Gottlieb et al., 1981; Siegal et al., 1981). They were said to show evidence of decreased cell mediated immunity. Later on, in 1983, a T-lymphotropic, type C RNA virus was isolated from men showing similar symptoms as those preceding AIDS and this was confirmed as the causative agent of the disease (Barré-Sinoussi et al., 1983). The first recorded case of the infection in Ghana was diagnosed in 1986 (National HIV/AIDS/STI Control Program, 2001).
From thence, HIV/AIDS has grown to pandemic proportions with an estimated 75 million people worldwide dying from the pandemic by 2012 (UNAIDS, 2013). As at the end of 2014, 36.9 million people globally were living with HIV and 1.2 million had died as a result of AIDS (UNAIDS, 2015; World Health Organization, 2015).

In Ghana, and from 1986 to the year 2000, 43,587 people have been reported as having gotten infected with HIV. Estimates however predict that about 185,000 have been infected and 150,000 Ghanaians had already died from HIV/AIDS (National HIV/AIDS/STI Control Program, 2001).

As at 2013, a prevalence of 1.3% was reported in Ghana with 224,488 persons estimated to be living with HIV and 10,074 deaths due to AIDS (National AIDS/STI Control Program, 2014).

2.3 The Virus

2.3.1 The Viral Structure

Mature HIV virions have spherical morphology of 100–120 nm in diameter and consist of a lipid bilayer membrane that surrounds a dense truncated cone-shaped nucleocapsid (Sierra et al., 2005). This lipid bilayer forms the envelope of the HIV and is derived from the plasma membrane of the infected host cell (Engelman and Cherepanov, 2012). On the envelope are anchored viral glycol proteins, gp120 (Engelman and Cherepanov, 2012), as well as host cell surface membrane proteins like the major histocompatibility complex antigens (Malim and Emerman, 2008).

Within the envelope, from outside inwards are the; matrix (MA) proteins which serve to anchor and give structural stability to the virion; capsid (CA) protein which forms the conical core of the viral particle (Hope and Trono, 2000) within which the nucleocapsid...
encapsulated genomic RNA, RT and integrase (IN) enzymes are located (Engelman and Cherepanov, 2012). Also in the virion are various other accessory and regulatory proteins.
Figure 1: Structure of the HIV showing the viral genome, reverse transcriptase (RT) and integrase (IN) enzymes and Tat within the viral capsid (CA) which is then surrounded by the matrix (MA) within which the protease enzyme is found. The matrix protein is surrounded by a lipid membrane onto which the envelope-glycoprotein complex is anchored.

2.3.2 Viral Genome

The viral genome is approximately 9.8 kilobases in length. It has three open reading frames and codes for three main types of proteins, i.e. structural proteins, regulatory proteins and accessory proteins (Hope and Trono, 2000).

http://ugspace.ug.edu.gh/
**Figure 2:** Structure of the HIV-1 Genome showing the position of the various genes and the gene products

*Source: Hoffmann and Rockstroth, 2015*
As described by Los Alamos National Laboratory (2014) and Hoffmann and Rockstroth (2015), the Gag genomic region is the first gene on the first reading frame. It encodes the precursor of the capsid proteins. This precursor is then processed into the MA (p17), CA (p24), Nucleocapsid (NC, p7) and the p6 proteins prior to viral assembly and maturation.

The Pol gene encodes the viral enzymes essential for viral replication and production of mature viral proteins. These proteins, the protease, reverse transcriptase, RNase and integrase, are always expressed as the Gag-Pol precursor polyprotein due to a ribosomal frameshift event at the end of the Gag gene.

Aside the gag and pol genes, the rest of the structural HIV proteins are expressed from the env gene. This is produced as a 160kD glycoprotein precursor which later forms the transmembrane gp41 and the external gp 120 envelope glycoprotein for HIV-1 and gp35 and gp105 for HIV-2.

Other accessory genes coded for by the viral genome include the tat, rev, vif, vpr, vpu and nef genes.

2.3.3 Life Cycle

The HIV life cycle starts with entry into the host cell, mainly T Helper cells, through fusion mediated by the viral envelope glycoproteins which associate with the CD4+ receptor and then subsequently the CCR5 or the CXCR4 chemokine coreceptor of host cells in the case of HIV-1 (Clapham and McKnight, 2002). After fusion, the viral core is released into the host cell cytoplasm (Sierra et al., 2005) where it undergoes a slow dissolution process which protects the genomic RNA but allows access to cellular deoxyribonucleoside triphosphates (dNTPs) needed for reverse transcription and proviral DNA synthesis (Arts and Hazuda, 2012). Reverse transcription is catalysed by the reverse
transcriptase enzyme which has RNA dependent DNA polymerase, DNA dependent DNA polymerase and RNase-H activity (Sarafianos et al., 2009). A preintegration complex (PIC) is then formed consisting mainly of the provirus and the integrase proteins and this complex is then integrated into the host cell DNA (Craigie and Bushman, 2014). Once integration occurs, proviral transcription and translation starts mediated mainly by host cell factors. The proteins and precursor proteins produced are then processed, assembled and packaged into new virions which bud out of the cell (Sierra et al., 2005).
Figure 3: HIV life cycle showing some of the processes and factors involved in binding, fusion, transcription, integration, translation release and maturation as well as the sites of action of different classes of antiretroviral drugs.

Source: Maartens et al., 2014
2.3.4 Pathogenesis and Disease Progression

The pathogenesis of HIV is mainly due to the progressive depletion of immune cells, especially the CD4+ T-Helper cells, either through direct killing of infected cells by cytotoxic T cells, immune activation or others (Maartens et al., 2014). In early disease, there is a dramatic loss of CD4+ T cells from the gastrointestinal tract resulting in impaired mucosal immunity, reduced peripheral CD4+ T-cell count, and increased systemic T-cell activation (Mehandru et al., 2004). In addition to this, there is a preferential loss of T-helper 17 which produces IL17A, IL22, IFNγ, and TNFα, cytokines important in mucosal immunity (Cosgrove et al., 2013). All these contribute to immune dysfunction which leads to disease.

Course of infection as described by (Coffin and Swanstrom, 2013), progresses as such; there is an initial eclipse phase where the virus is freely replicating but viremia is undetectable. From approximately 2 to 4 weeks after infection, the primary infection phase ensues, characterized by relatively high levels of viremia and most times, flu like symptoms. At this stage, towards the peak of viremia, immune response is initiated; initially in the form of neutralizing antibodies to viral proteins and then activated CD 8+ T-cell response against infected cells. By the end of this phase, the level of viremia decreases sharply due to immune control as well as exhaustion of activated target cells. There is also a decline in plasma CD4+ T cells. Following this, there is a clinical latency stage that can extend for years. This stage is marked by a fairly constant level of viremia and a gradual decline in CD4+ T cells. The latent phase graduates to the AIDS phase when CD4+ T cell count declines below 200 cells/µL mark. Opportunistic diseases begin to appear and there is a resurgent rise in viremia.
Figure 4: Time course of a typical HIV infection showing some landmark events and the levels of CD4+ T cells and HIV RNA with respect to time after infection

Source: Coffin and Swanstrom, 2013
Apart from immune factors, disease progression can also be influenced by infecting subtype of HIV-1. A disease progression study in a seroconverter cohort in Sub-Saharan Africa established that, patients with HIV-1 subtype C infection progressed faster to the selected CD4+, viral load and clinical AIDS endpoints than in subtype A infected patients. Also, as compared to subtype A, subtype D infections progressed faster to viral load endpoint and twice as fast to clinical AIDS than subtype A (Amornkul et al., 2013).

This was found to be the case in a Kenyan women cohort, though similar viral load levels were observed in both subtype A and D infections (Baeten et al., 2007), as well as in studies done in Uganda (Kiwanuka et al., 2008; Ssemwanga et al., 2013). In a group of South African women however, faster disease progression was found to be associated with subtype C infections (Mlisana et al., 2014). Other studies looking at the rate of CD4+ decline found faster rate of decline in infections with subtype D as compared to subtype A and recombinant subtypes (Kiwanuka et al., 2010) and a faster rate of decline in subtype B infections as compared to subtypes A, C and CRF02 (Touloumi et al., 2013).

2.3.5 Genetic Variability

HIV originated from multiple zoonotic transmissions of simian immunodeficiency virus (SIV) from non-human primates into humans in West and Central Africa (Hemelaar, 2012). There are two types of HIV, HIV-1 which is more closely related to the SIV from chimpanzees (Gao et al., 1999) and HIV-2 which is closely related to SIV from sooty mangabeys (Santos and Soares, 2010). The nature and prevalence of the infections caused by the different HIVs differ, with HIV-2 being restricted to West and Central Africa and causing less transmissible and slower progressing infections (Maartens et al., 2014; O’Cofaigh and Lewthwaite, 2013). HIV-1 infections are the cause of the HIV/AIDS pandemic (Santos and Soares, 2010). The HIV-1 can be classified into 4 main groups; M
(Main), N, O (Outliers) and P, with each group derived from a distinct SIV chimpanzee cross-species infection (Santos and Soares, 2010).

Studies have shown that three of the four groups of HIV-1; group M, N and O, arose due to individual cross species infection of humans by SIVs which are subspecies specific to the Pan troglodytes troglodytes chimpanzee (SIVcpzPtt) (Gao et al., 1999; Heuverswyn et al., 2007; Van Heuverswyn et al., 2006; Keele et al., 2006). The most recent addition to the HIV-1 groups is the group P, the sole member of which is a new HIV-1 virus which is most closely related to the gorilla SIV (SIVgor). It was isolated from a Cameroonian woman and is thought to have resulted from direct transmission rather than evolution (Plantier et al., 2009).

HIV-1 group M is responsible for the majority of HIV-1 infections and this group further classified into subtypes A, B, C, D, F, G, H, J, K and Circulating Recombinant Forms (CRFs) which arise through genome recombination of two or more subtypes (Hemelaar et al., 2006; Requejo, 2006). The CFR02_AG subtype for instance is derived from a recombination of subtypes A and G and was first identified and isolated in Nigeria (Howard and Rasheed, 1996).

In general, genetic variation is of 25–35% and 15–20% between and within subtypes, respectively (Santoro and Perno, 2013). This genetic variability is due to many factors including the rapid replication of HIV-1 in-vivo and the error prone nature of the HIV reverse transcriptase (Buonaguro et al., 2007).

Geographically, studies have shown that the CFR02_AG and A are the most common circulating forms in West Africa, subtype B in Northern Africa, subtype C predominant in Southern and East Africa and D limited to Central and East Africa (Buonaguro et al.,

http://ugspace.ug.edu.gh/
2007; Esbjörnsson et al., 2011), but subtype B is the most widely distributed worldwide (Peeters, 2013).
Figure 5: Geographical distribution of HIV types and subtypes

Source: Peeters, 2013

http://ugspace.ug.edu.gh/
Osmanov et al. (2002), estimated that the largest proportion of HIV-1 infections in the year 2000 was due to subtype C strains (47.2%). Subtype A/CRF02_AG was estimated to be the second leading cause of the pandemic (27%), followed by subtype B strains (12.3%). The same analysis confirmed an increasing role of HIV-1 CRFs in the pandemic. Seven years on, the distribution of the subtypes from 2004–2007 as determined by Hemelaar et al. (2011), is as follows. Subtype C accounted for nearly half (48%) of all global infections, followed by subtypes A (12%) and B (11%), CRF02_AG (8%), CRF01_AE (5%), subtype G (5%) and D (2%). Subtypes F, H, J and K together cause less than 1% of infections worldwide. Other CRFs and URFs are each responsible for 4% of global infections, bringing the combined total of worldwide CRFs to 16% and all recombinants (CRFs plus URFs) to 20%. It is therefore evident that recombinant forms may play an increasing role in the pandemic.

Phylogenetic classification or subtyping of HIV-1 is done mainly by sequencing and comparing the nucleotide sequences of the gag, pol or env gene or of the whole genome (Buonaguro et al., 2007).
Figure 6: A phylogenetic tree showing the evolutionary relatedness of Human Immunodeficiency and Simian Immunodeficiency Virus types

Source: https://upload.wikimedia.org/wikipedia/commons/2/2f/HIV-SIV-phylogenetic-tree.png
2.4 Treatment

There is still no cure for HIV/AIDS. Treatment comes in the form of antiretroviral therapy (ART) which consists of a combination of antiretroviral (ARV) drugs to maximally suppress the replication and stop the progression and transmission of the infection (World Health Organization, 2016).

2.4.1 Antiretroviral Drugs

Azidothymidine (AZT), a thymidine analogue which was later called zidovudine (ZDV), was the first drug approved, in 1987, for the treatment of HIV infections (Coffey, 2014; Stefano Vella, 2012). In 1985, (Mitsuya et al., 1985), showed the in vitro ability of 3’-Azido-3’-deoxythymidine to inhibit the infectivity and cytopathic effect of the human T-lymphotropic virus type III. Approval for the use of the drug by the Food and Drugs Administration (FDA) was later given in 1987 based on the evidence shown by (Fischl et al., 1987), in a double blind placebo trial of azidothymidine in 282 AIDS patients. AZT as well as other nucleoside analogues form the nucleoside reverse transcriptase inhibitor (NRTI) class of HIV drugs. Later on, in the mid 1990’s, the discovery of a new class of drugs which inhibited the reverse transcriptase and protease enzymes and the emergence of HIV strains resistant to monotherapy ushered in the multidrug therapy of antiretroviral therapy (Arts and Hazuda, 2012). Saquinavir (SQV), a protease inhibitor (PI), was approved in 1995 for use and then Nevirapine...
(NVP), the first non-nucleoside reverse transcriptase inhibitor (NNRTI) approved for use in 1996 (Stefano Vella, 2012).

Now, five different classes of antiretroviral drugs are available for the treatment of HIV infections (Arts and Hazuda, 2012; Santos and Soares, 2010). These are;

1. Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs): these are compounds that are structurally similar to deoxyribonucleotide triphosphate (dNTPs) but do not have the hydroxyl (-OH) group at the 3ʹ position and hence cannot form the phosphodiester linkage necessary for DNA chain elongation (Warnke et al., 2007). These drugs work by competitively inhibiting the incorporation of dNTPs into DNA strands that are being synthesized. In so doing, when these analogues bind to the active site of the polymerase, reverse transcription is terminated since no additional nucleotides can be added. A common example of this class is Lamivudine (3TC): the approval of this drug in 1995 for use in combination with ZDV was due to evidence provided by (Eron et al., 1995), showing that combination therapy was associated with greater increases in CD4+ cell count as well as greater decreases in HIV-1 RNA plasma levels as compared with ZDV or 3TC alone (Coffey, 2014).

![Figure 8: Chemical structure of Lamivudine](http://ugspace.ug.edu.gh/)

2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs): these are reverse transcriptase inhibitors which work by binding to a hydrophobic pocket on the reverse transcriptase enzyme and causing a conformational change in its active
site resulting in a disruption in its catalytic activity. An example is Nevirapine (NVP). NVP was the first non-nucleoside reverse transcriptase inhibitor (NNRTI) approved for use by the FDA, in 1996 (Stefano Vella, 2012). This was based on a placebo-controlled trial, results of which showed that patients who were placed on NVP in addition of the previous regimen of ZDV and ddI had an 18% higher mean absolute CD4+ cell count, a 0.32\log_{10} lower mean infectious HIV-1 titre and a 0.25\log_{10} lower mean plasma HIV-1 RNA level after 48 weeks of therapy (D’Aquila et al., 1996).

3. Protease inhibitors (PIs) bind to the viral protease enzyme, either at the active site or elsewhere, and inhibit the processing of the precursor polyproteins hence preventing viral assembly and maturation.

Nelfinavir (NFV) is one such drug. It received approval from the U.S. Food and Drug Administration (FDA) in 1997 for the treatment of adults and children with HIV infection. Initial approval was based on studies showing increases in CD4+ T-lymphocyte counts and reductions in HIV viral load in individuals receiving nelfinavir either alone or in combination with 1 or 2 nucleoside analogues. These changes were superior to those seen with nucleoside analogues alone (Coffey, 2014).
Lopinavir/Ritonavir (LPV/r) is another commonly used PI. It is a combination of lopinavir and ritonavir in a 4:1 ratio. Studies comparing this formulation with nelfinavir showed that after 48 weeks, 75% of patients receiving lopinavir-ritonavir in combination with stavudine and lamivudine had fewer than 400 copies of HIV RNA per millilitre and 67%, fewer than 50 copies per millilitre as compared to 63% and 52% respectively for patients on nelfinavir combined with stavudine and lamivudine (Walmsley et al., 2002). The drug received FDA approval in 2000 for use in adults and children 6 months or older and in 2008 for use in infants (Coffey, 2014).

4. Integrase inhibitors prevent the incorporation of the viral DNA into the host cell genome. These include raltegravir (RAL) which was approved in 2009 and elvitegravir (EVG) which was approved in 2012 (Coffey, 2014).

5. Fusion/Entry inhibitors prevent the fusion and incorporation of the virion into the host cell. Enfuvirtide (ENF) was approved by the FDA in 2003 for use in adults, and in children ages 6 and older, with advanced HIV infection. The first agent to be approved in the class of fusion inhibitors, enfuvirtide functions by binding a region of the HIV envelope glycoprotein gp41 and preventing viral fusion with the target cell membrane (Coffey, 2014).

The Highly Active Antiretroviral Therapy (HAART) involves the combination of at least three antiretroviral drugs from different classes for the management of HIV infections. In their consolidated ARV guidelines, June 2013, the World Health Organization...
recommends the use of simplified, less toxic and more convenient regimens which have a non-thymidine NRTI backbone plus one NNRTI (World Health Organization, 2013). The regulations in Ghana recommend the following class combinations for use in adolescents and adults (National HIV/AIDS/STI Control Program, 2010);

- 2 Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and 1 Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI)
- 2 NRTIs and 1 Protease Inhibitor (PI)
- 2 NRTIs and 2 PIs. The 2 PIs are considered as one ARV, as the second PI, usually ritonavir, is in low dose and is used to boost the blood level of the first PI.

The specific drug combinations recommended are elucidated in the tables below.
Table 1: Recommended first line ARV drug combinations in Ghana detailing contraindications and alternatives

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Contraindications</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zidovudine + Lamivudine + Nevirapine</td>
<td>Zidovudine is contraindicated in severe anaemia</td>
<td>Replace with Stavudine</td>
</tr>
<tr>
<td></td>
<td>Nevirapine is contraindicated in liver dysfunction and hypersensitivity</td>
<td>Replace with Efavirenz</td>
</tr>
<tr>
<td>Zidovudine + Lamivudine + Efavirenz</td>
<td>Efavirenz is contraindicated in Pregnancy and CNS presentations</td>
<td>Replace with Nevirapine</td>
</tr>
</tbody>
</table>
**Table 2:** Recommended second line ARV drug combinations in Ghana detailing contraindication and alternatives

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Contraindications</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abacavir + Tenofovir + Nelfinavir</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First alternative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abacavir + Tenofovir + Lopinavir/r</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Didanosine + Abacavir + Nelfinavir</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second alternative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Didanosine + Abacavir + Lopinavir/r</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypersensitivity of Abacavir</td>
<td>Switch to</td>
<td></td>
</tr>
<tr>
<td>Abacavir</td>
<td>Tenofovir</td>
<td></td>
</tr>
</tbody>
</table>

**INTEGRIS PROCEDAMUS**
2.4.2 Antiretroviral Drug Resistance

Despite the HAART regimen, introduced to curtail the rapid development of resistance to monotherapy drugs, resistance is still a major issue in the management of HIV/AIDS.

Resistance to nucleoside and nucleotide analogues can occur in two ways. Certain mutations in the reverse transcriptase gene cause the enzyme to selectively prefer cellular nucleotides against the drug analogues resulting in resistance. Also, mutations known as thymidine analogue mutation (TAMs) enable the removal of nucleoside analogues from the 3’ end of the terminated DNA chain (Clavel and Hance, 2004).

NNRTI resistance occurs due to mutations which change the structure of the hydrophobic pocket to which these drugs bind to, reducing their affinity to the enzyme and hence reducing their therapeutic effect (Boyer et al., 1993).

Protease inhibitor resistance mutations also reduces the affinity of the inhibitors to the enzyme by modifying the binding site of the inhibitor on the enzyme (Miller, 2001).

The two main ways of determining antiretroviral drug resistance is through genotypic assay, thus analysing the nucleotide sequence of the target genes to identify resistance conferring mutations, and phenotypically, by determining the IC₅₀ of the drug in vitro (Vella and Palmisano, 2005). The list of drug resistance conferring mutations recommended for use in genotypic assays is frequently updated to include newly determined mutations (Bennett et al., 2009; Wensing et al., 2014).

Not all mutations are drug resistance mutations and not all drug resistance mutations are major drug resistance mutations. A mutation can lead to a high level of resistance or a moderate level of resistance or contribute to resistance together with other mutations.
2.4.2.1 Nucleoside Reverse Transcriptase Inhibitor Resistance Mutations

Thirty-four (34) mutations at 15 amino acid positions were listed for the surveillance of HIV-1 NRTI drug resistance in 2009 (Bennett et al., 2009). This was updated to 18 amino acid positions by Wensing et al. (2014). Insertion at position 69 and change from Glutamine (Q) to Methionine (M) at position 151 (Q151M) are classified as multidrug resistance mutations which reduce susceptibility to all NRTIs (Rhee et al., 2003; Shafer, 2006; Wensing et al., 2014). NRTI drug resistance mutations which result in high levels of resistance, as listed in (Rhee et al., 2003; Shafer, 2006), include:

- K65R: high resistance to ABC, ddI and TDF
- Insertions at position 69: high resistance to ABC, ddI, TDF, d4T and ZDV
- L74V: high resistance to ABC and ddI
- Q151M: high resistance to ABC, ddI, d4T and ZDV
- M184V: high resistance to 3TC
- T215F/Y: high resistance to d4T and ZDV

2.4.2.2 Non-Nucleoside Reverse Transcriptase Inhibitor Resistance Mutations

Surveillance drug resistance mutations (SDRMs) for monitoring resistance to NNRTIs include 19 mutations at 10 positions (Bennett et al., 2009), later updated to 16 positions (Wensing et al., 2014). Mutations which result in high levels of resistance to NNRTIs, as listed in (Rhee et al., 2003; Shafer, 2006), include:

- K101E/P, K103N/S, V106A/M, Y181C/I/V, Y188L/C/H, G190A/S/E and M230L which show high resistance to NVP
2.4.2.3 Protease Inhibitor Resistance Mutations

There are 40 SDRMs at 18 positions that give rise to protease inhibitor resistance (Bennett et al., 2009). The major mutations that cause high levels of resistance to nelfinavir and lopinavir/ritonavir are D30N, G48V/M, I54V/T/A/L/M, V82A/F/T/S, I84V, N88D/S, L90M and I47A, L76V, V82A/F/T/S respectively (Rhee et al., 2003; Shafer, 2006).

2.4.2.4 Prevalence Of Antiretroviral Drug Resistance In Africa

There has been a lot of work profiling the prevalence of antiretroviral resistance in Africa especially, after the scale up of antiretroviral therapy.

In a Sub Saharan Africa cross-sectional study in antiretroviral naïve individuals recruited from Kenya, Nigeria, South Africa, Uganda, Zambia and Zimbabwe, overall prevalence of drug resistance was found to be at 5.6% (Hamers et al., 2011b). Similar studies in Malawi (Kamoto et al., 2008), Kenya (Kantor et al., 2014) and Uganda (Ndembi et al., 2011) reported <5%, 7% and 8.6% prevalence respectively. In Cameroon (Aghokeng et al., 2011), the prevalence of drug resistance mutations increased from 0% between 1996-1999 to 12.3% in 2007. In an Eastern and Southern African study, the prevalence of transmitted drug resistance mutations ranged from 3% in Lusaka to 20% in Cape Town (Price et al., 2010). A study into the drug resistance patterns in patients who had failed first line antiretroviral therapy showed that 70% of them carried one or more drug resistance mutations and 49% had dual class resistance (Hamers et al., 2012). These findings and patterns have been reviewed severally (Hamers et al., 2013; Ssemwanga et al., 2015).

Previous research in Ghana has shown very low prevalence in drug resistance. A 2005 study showed that though there were no known major drug resistance mutations in the protease genes of subject, these proteases were differentially less susceptible to protease
inhibitor (Kinomoto et al., 2005). Other studies reported either no transmitted drug resistance mutations (Nii-Trebi et al., 2013) or only minor drug resistance mutations (Sagoe et al., 2007) or only two major drug resistance mutations (Bonney et al., 2013)
CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Materials

The details of all reagents, equipment, consumables, software used for this work and their sources and/or manufacturers is listed in Appendix I.

3.2. Methods

3.2.1. Study Population

The study population was made up of consenting adult HIV-1 patients who were visiting the Korle-Bu Teaching and the Koforidua Regional Hospitals at the time of sampling. These were made of ART naïve patients, patients who were on first line therapy, second line therapy and patients who were long term non-progressors.

This study was approved by the Noguchi Memorial Institute for Medical Research in March 2016, and ethical clearance (NMIMR-IRB CPN 089/15-16) given by the Institutional Review Board of the same.

3.2.2. Samples

The samples used in this study were 63 (25 from Korle Bu and 38 from Koforidua) archival plasma samples previously processed from blood samples collected (between 2014 and 2015) for a parent study and stored at -80°C until use.

3.2.3. Extraction of Viral RNA from Plasma

The isolation of viral RNA from the thawed plasma samples was done using the QIAamp Viral RNA mini kit (QIAGEN, USA), according to the manufacturer’s protocol (QIAamp Viral RNA Mini Kit Handbook, 2014). Extracted RNA was stored at -30°C until further use.
3.2.4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of Protease and Reverse Transcriptase Genes from RNA Extract

Reverse transcription of the target genes was done using the kit and protocol provided by the QIAGEN One Step RT-PCR Kit (QIAGEN OneStep RT-PCR Kit Handbook, 2012). The reaction mix, 25µl total volume, consisted of 5µl 5X QIAGEN One Step RT-PCR buffer, 1µl each of dNTP and enzyme mix, 0.75µl each of forward and reverse primers and 11.5µl of nuclease free water. The primer sets DRPRO05/DRPRO2L and DRRT1L/DRRT4L, previously described by (Fujisaki et al., 2007), were used to amplify the PR and RT genes respectively under cycling conditions which have been previously described (Villahermosa et al., 2000).

3.2.5. Amplification of RT-PCR Products by Nested PCR

The RT-PCR products were further amplified using more specific primer sets DRPRO06/DRPRO1M for the protease gene and DRRT6L/DRRT7L for the reverse transcriptase gene (Fujisaki et al., 2007). The process was carried out using the OneTaq PCR Master Mix according to the manufacturers protocol (OneTaq® 2X Master Mix with Standard Buffer, 2016).

3.2.6. Agarose Gel Electrophoresis

The PCR products were analysed on 1.5% ethidium bromide stained agarose gels in 1X TAE. 100bp and 1000bp ladders, TrackIt DNA Plus ladders (Invitrogen, USA) were used to estimate the size of PCR products. The gels were viewed using the GelDoc-It Imaging Systems (UVP, USA) with the expected band sizes being 463bp and 887bp for the PR and RT genes respectively.
3.2.7. Purification of PCR Products

The amplified gene products were purified to remove the enzymes and other components that may interfere with downstream processes. The QIAGEN PCR Purification Kit and protocol was used (QIAquick Spin Handbook, 2008).

3.2.8. Cycle Sequencing of PR and RT Genes

To determine the nucleotide sequence of the target genes, cycle sequencing was done. This is a modification of the Sanger sequencing method in which dye labelled dideoxynucleotide triphosphates (ddNTPs) were used as chain terminators. The Big Dye Terminator cycle sequencing kit version 3.1 (ABI, USA) was used with modified conditions as described by (Villahermosa et al., 2000). Thermal cycling conditions used were 94°C for 2 minutes followed by 25 cycles of 94°C for 30 seconds; 50°C for 15 seconds; 60°C for 4 minutes. Three sets of primers were used to sequence the RT gene, A2, PRSec2A and RTSec1S, whilst PRTS and P3G were used for the PR gene (Fujisaki et al., 2007; Villahermosa et al., 2000).

3.2.9. Purification of Cycle Sequencing Products

After cycle sequencing, the removal of excess primers, dyes, ddNTPs, dNTPs and other unneeded components was done using the magnetic bead based purification method supplied by Agencourt Bioscience (Agencourt CleanSeq Dye-Terminator Removal protocol, 2006). The protocol briefly involves mixing sequencing products with 8µl magnetic bead solution and suspending the mixture in 85% ethanol. The mixture was then placed on a magnetic field thereby causing the magnetic beads, with DNA bound, to adhere to the wall of the tube. The solution was then carefully suctioned out taking care not to dislodge the beads from the wall. The beads were then
washed again with 85% ethanol and left to dry and then nuclease free water was added to unbind the now clean DNA from the beads. The solution, containing the DNA was then aliquoted into a 96-well plate for downstream analysis.
Table 3: A table showing the name, position on reference HXB2 genome, sequence and purpose of all primers used

Adapted from: Fujisaki et al., 2007; Villahermosa et al., 2000

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Position (HXB2)</th>
<th>Sequence (5’-3’)</th>
<th>Purpose</th>
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<tr>
<td>DRPRO5</td>
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<td>RT-PCR</td>
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<td>DRPRO2L</td>
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<td>TATGGATTTTCAGGCCCAATTTTGA</td>
<td>(PR Gene)</td>
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<td>DRPRO1M</td>
<td>2148-2167</td>
<td>AGAGCCAACAGCCCCCACCAG</td>
<td>Nested PCR</td>
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<tr>
<td>DRPRO6</td>
<td>2611-2592</td>
<td>ACTTTTGGGCCATCCATTCC</td>
<td>(PR Gene)</td>
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<td>DRRT1L</td>
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<td>ATGATAGGGGGAATTGGAGGTTT</td>
<td>RT-PCR</td>
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<td>DRRT4L</td>
<td>3425-3402</td>
<td>TACTTCTGTAGTGCTTTGGTTCC</td>
<td>(RT Gene)</td>
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<td>DRRT7L</td>
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<td>A2</td>
<td>2583-2601</td>
<td>TTAAAGCCAGGAAATGGATG</td>
<td>Sequencing</td>
</tr>
<tr>
<td>RTSEC1S</td>
<td>2692-2716</td>
<td>CAAAAATTGGGCTGAAAATCCATG</td>
<td>(RT Gene)</td>
</tr>
<tr>
<td>PRSEC2A</td>
<td>2692-2716</td>
<td>TGGGAAGTTCATTTGGAATACCACAC</td>
<td></td>
</tr>
</tbody>
</table>

http://ugspace.ug.edu.gh/
3.2.10. Genetic Sequencing

To determine the sequence of bases and obtain a sequence readout, the ABI 3130xl Genetic Analyser was used (ABI, USA). This is an automated 16 capillary electrophoresis machine with a laser detection system connected to a computer. Data generated from the machine is collected and displayed by the data analysis software supplied with the instrument. For this work, the performance optimized polymer vs 7 (POP-7) was used. In a nutshell, according to the manufacturers guide (Applied Biosystems 3130/3130xl Genetic Analyzers Getting Started Guide, 2010), the instrument was inspected to ensure that all components were in ideal condition. The purified cycle sequencing products in a 96-well plate covered with the appropriate septa was then loaded in the right orientation into the analyser. A plate map was then created on instrument software and linked to the plate. After the correct parameters had been chosen, the run was started. After the run, the data generated was viewed and stored for further analysis (Applied Biosystems Sequencing Analysis Software Version 5.2, 2004).

3.2.11. Sequence Analysis

The analyses of the sequences generated included cleaning and editing the reads and generating contigs using SeqMan Pro v 13 (DNASTAR, USA); alignment of generated contigs using BioEdit Sequence Alignment Editor v 7.2.5 (Thomas, 2016). Identification of sequence subtype and resistance conferring mutations in the generated sequences was done by inputting the sequence text (fasta formatted) into the Stanford University online HIV drug resistance sequence analyses program (Liu and Shafer, 2006). This program translates the input sequence and compares it to the reference sequence of the various HIV subtypes and then gives the closest related subtype and the level of similarity. To determine drug resistance mutations, the user
sequence was compared to the reference HIV sequence to identify amino acid differences. These differences/mutations, if they correspond to known drug resistance mutations, are then classified as PI (major or minor), NRTI and NNRTI mutations. The program also gives information about the type of mutation it is and its clinical significance, thus, low level, intermediate level or high level resistance. MEGA 5 software was used to construct Maximum Likelihood phylogeny trees at default settings of 1000 bootstrap replications.

3.2.12. Data Analysis

Descriptive statistics and tables and charts were used to summarize patient data. Kruskal–Wallis tests were used to compare variables across three or more groups, and where significant differences were observed, Mann–Whitney U-tests were conducted for pairwise comparisons. Statistical significance for all analyses was determined using a critical α-value of 0.05.
CHAPTER FOUR

4. RESULTS

4.1. Patient Information

Patient demographic data and clinical data is summarized in the tables and figures below. This includes age, sex, duration of infection, viral load (VL) and CD4 counts.
Table 4: Summary of patient demographic and clinical information showing the age, duration of infection, viral load and CD4 count per patient category and p-value.

<table>
<thead>
<tr>
<th>Category</th>
<th>Samples (n)</th>
<th>Male (n)</th>
<th>Female (n)</th>
<th>Age</th>
<th>Duration of Infection</th>
<th>Viral Load</th>
<th>CD4+ T cell count</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Line</td>
<td>19</td>
<td>6</td>
<td>13</td>
<td>48 (42-51.5)</td>
<td>9 (8-10)</td>
<td>21.9 (0-937)</td>
<td>534 (445-823)</td>
</tr>
<tr>
<td>Second Line</td>
<td>13</td>
<td>4</td>
<td>9</td>
<td>41 (34-43)</td>
<td>9 (7-10)</td>
<td>167 (63-25800)</td>
<td>238 (206-329)</td>
</tr>
<tr>
<td>Fast Progressors</td>
<td>19</td>
<td>8</td>
<td>11</td>
<td>45 (37-58)</td>
<td>3 (2-3)</td>
<td>7680 (1520-17050)</td>
<td>484 (325-726)</td>
</tr>
<tr>
<td>Long Term Non-Progressors</td>
<td>12</td>
<td>6</td>
<td>13</td>
<td>50 (44-52)</td>
<td>11 (9-11.5)</td>
<td>9145 (2648-36050)</td>
<td>525.5 (426-669)</td>
</tr>
</tbody>
</table>

p-value

Data are presented as median (Inter-quartile range) unless otherwise indicated

aKruskal Wallis test
bNot significantly different from First Line group
cNot significantly different from Second Line group
dNot significantly different from Long Term Non-Progressor group
**Figure 12**: A clustered column graph showing the number of patient samples per viral load category.

It shows the number of patients samples with viral RNA Not Detected, <20 copies/ml, 20-10,000 copies/ml and >10,000 copies/ml, stratified according to patient groups.
Figure 13: A clustered column graph showing the number of patient samples per CD4 count category.

It shows the number of patients samples with CD4 count <250, 250-500 and >500 cells/µl, stratified according to patient groups.
4.2. RT-PCR and Nested PCR

After electrophoretic analysis of PCR products, the average PCR positive rate was found to be 39.7%. **Table 5** shows the summary of the PCR results. The representative agarose gels of PR and RT amplification are shown in **Figure 14** and **Figure 15** respectively.
Table 5: Summary of PCR results showing number of successfully amplified targets and the PCR positive rate

<table>
<thead>
<tr>
<th></th>
<th>PR Gene</th>
<th>RT Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Number of RNA extracts</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>Number Successfully Amplified</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>Positive Rate (%)</td>
<td>46.0</td>
<td>33.3</td>
</tr>
</tbody>
</table>

http://ugspace.ug.edu.gh/
Figure 14: Representative Agarose gel (1.5% in 1X TAE) photograph of PR gene amplification analysis showing band of interest. Last lane (labelled M) contains 100bp molecular marker with lanes labelled 1-10 containing patient samples. Presence of bands of expected size in lanes labelled 3,6, and 10 indicated successful amplification.

M – Molecular Marker; NC – Negative Control; PC – Positive Control
Figure 15: Representative Agarose gel (1.5% in 1X TAE) photograph of RT gene amplification analysis showing band of interest. Last lane (labelled M) contains 100bp molecular marker with lanes labelled 1-13 containing patient samples. Presence of bands of expected size in lanes labelled 4,6,7,12 and 13 indicated successful amplification.

M – Molecular Marker; NC – Negative Control; PC – Positive Control
4.3. Sequencing

Of the successfully amplified gene targets, 44.8% yielded positive sequencing results. **Table 6** shows a summary of the sequencing results. Sequencing output files were in ABI file format which can be viewed as electrophorograms as shown in **Figure 16. Figure 17** shows sequencing which were aligned in BioEdit interface.
**Table 6:** Summary of sequencing results

<table>
<thead>
<tr>
<th></th>
<th>PR Gene</th>
<th>RT Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Number of Target Amplicons</strong></td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td><strong>Number Successfully Sequenced (%)</strong></td>
<td>8 (27.6)</td>
<td>13 (61.9)</td>
</tr>
</tbody>
</table>
Figure 16: An electrophorogram of one of the successfully sequenced amplicons showing base calls, base position and call quality.
Figure 17: A figure showing aligned sequences in BioEdit interface showing aligned sequences.
4.4. Subtyping

Results from subtype analysis shows that most of the samples are of the CRF02_AG subtype. All of the PR subtypes were CRF02_AG but RT subtype had a few G subtypes and one CRF01_AE. Details are shown below in Table 7.
Table 7: Summary of subtyping results.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>PR Subtype</th>
<th>RT Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR-14-01</td>
<td>CRF02_AG</td>
<td>CRF02_AG</td>
</tr>
<tr>
<td>AR-14-03</td>
<td>CRF02_AG</td>
<td></td>
</tr>
<tr>
<td>AR-14-09</td>
<td>CRF02_AG</td>
<td></td>
</tr>
<tr>
<td>AR-14-13</td>
<td>CRF02_AG</td>
<td></td>
</tr>
<tr>
<td>AR-15-06</td>
<td>CRF02_AG</td>
<td>G</td>
</tr>
<tr>
<td>AR-15-09</td>
<td>CRF02_AG</td>
<td></td>
</tr>
<tr>
<td>EL-14-01</td>
<td></td>
<td>CRF01_AE</td>
</tr>
<tr>
<td>EL-14-05</td>
<td>CRF02_AG</td>
<td>CRF02_AG</td>
</tr>
<tr>
<td>EL-14-09</td>
<td></td>
<td>CRF02_AG</td>
</tr>
<tr>
<td>EL-14-12</td>
<td></td>
<td>CRF02_AG</td>
</tr>
<tr>
<td>FG-15-02</td>
<td></td>
<td>CRF02_AG</td>
</tr>
<tr>
<td>FG-15-04</td>
<td>CRF02_AG</td>
<td>G</td>
</tr>
<tr>
<td>FG-15-05</td>
<td></td>
<td>G</td>
</tr>
<tr>
<td>FG15-14</td>
<td></td>
<td>G</td>
</tr>
<tr>
<td>FK-15-15</td>
<td></td>
<td>CRF02_AG</td>
</tr>
<tr>
<td>FK-15-16</td>
<td></td>
<td>CRF02_AG</td>
</tr>
</tbody>
</table>

Shaded cells indicate that the specific genes target sequence was not available for the sample in question.
Figure 18: A pie-chart showing the proportion of the various subtypes present in the RT gene samples.
Figure 19: Maximum likelihood phylogenetic tree showing the evolutionary relationship between the PR gene of patient samples (▲), selected subtype references (▲) and the HIV-1 reference subtype (▲).
**Figure 20:** Maximum likelihood phylogenetic tree showing the evolutionary relationship between the RT gene of patient samples (▲), selected subtype references (▲) and the HIV-1 reference subtype (▲).
4.5. Drug Resistance Analysis

Analysis of all the sequences obtained from the direct sequencing procedure gave information about the resistance profile of patient samples. The information obtained included samples harbouring resistance conferring mutations, position and nature of the mutations, type of resistance conferring mutation i.e., PI, NRTI and/or NNRTI resistance mutations and clinical implication of mutation as per the ARV drugs the mutation gives resistance to and what level of resistance it confers. All these information are captured in the tables (Table 8, Table 9 and Table 10) and figures (Figure 21 and Figure 22) below.
Table 8: Summary of drug resistance analysis showing the number and proportion of samples with DRM for PR and RT genes.

<table>
<thead>
<tr>
<th></th>
<th>PR Gene</th>
<th>RT Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sequences obtained</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Number with DRM (%)</td>
<td>3 (37.5)</td>
<td>7 (53.9) 7 (53.9)</td>
</tr>
</tbody>
</table>
Table 9: PI resistance mutation found in patients and their clinical implications.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>PI Mutation</th>
<th>PI resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR-14-01</td>
<td>I47I/V</td>
<td>LPV/r, NFV</td>
</tr>
<tr>
<td>AR-14-03</td>
<td>K20I</td>
<td></td>
</tr>
<tr>
<td>AR-14-09</td>
<td>K20I</td>
<td></td>
</tr>
<tr>
<td>AR-14-13</td>
<td>K20I</td>
<td></td>
</tr>
<tr>
<td>AR-15-09</td>
<td>V32I</td>
<td>LPV/r, NFV</td>
</tr>
</tbody>
</table>

Green font indicates low level resistance and red font indicates high level resistance to PIs in coloured font.

*K20I though a drug resistance mutation in reference HXB2 genome, I at amino acid position 20 is the consensus for CRF02_AG subtype.
Table 10: NRTI and NNRTI resistance mutations found in patient samples and their clinical implications.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>NRTI Mutation</th>
<th>NRTI Resistance</th>
<th>NNRTI Mutation</th>
<th>NNRTI Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR-14-01</td>
<td>M184I</td>
<td>3TC, ABC, DDI</td>
<td>V90I, K103N,</td>
<td>EFV, NVP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Y181C, H221Y</td>
<td></td>
</tr>
<tr>
<td>AR-15-06</td>
<td>M184V, T215Y</td>
<td>3TC, ABC,</td>
<td>Y181C</td>
<td>EFV, NVP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AZT, D4T, DDI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR-15-09</td>
<td>M41L, D67N,</td>
<td>3TC, ABC,</td>
<td>V90I, K103N,</td>
<td>EFV, NVP</td>
</tr>
<tr>
<td></td>
<td>K70R, L74I,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M184V, T215F,</td>
<td>AZT, D4T,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K219E</td>
<td>DDI, TDF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EL-14-12</td>
<td>D67N, T69N</td>
<td>AZT, D4T, DDI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FG-15-02</td>
<td></td>
<td>K101H, G190A</td>
<td>EFV, NVP</td>
<td></td>
</tr>
<tr>
<td>FG-15-04</td>
<td>D67N, K70E,</td>
<td>3TC, ABC,</td>
<td>V106A, F227L</td>
<td>EFV, NVP</td>
</tr>
<tr>
<td></td>
<td>M184V</td>
<td>AZT, D4T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FK-15-15</td>
<td>D67N, M184V</td>
<td>3TC, ABC, DDI</td>
<td>V90I, K103N,</td>
<td>EFV, NVP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>K238T</td>
<td></td>
</tr>
<tr>
<td>FK-15-16</td>
<td>M184I</td>
<td>3TC, ABC, DDI</td>
<td>V106A, F227L</td>
<td>EFV, NVP</td>
</tr>
</tbody>
</table>

Green font indicates low level resistance, blue font intermediate level resistance and red font indicates high level resistance to NRTIs and NNRTIs in coloured font.
Figure 21: A pie-chart showing the distribution of RT inhibitor mutations. It shows the proportion of samples with no mutation, only Nucleotide Reverse Transcriptase Inhibitor (NRTI) mutations, only Non Nucleotide Reverse Transcriptase Inhibitor (NNRTI) mutations and both NRTI and NNRTI mutations.
Figure 22: Clustered column chart showing the number of samples with 1 or more than 1 intermediate or high level Nucleotide Reverse Transcriptase Inhibitor (NRTI) or Non Nucleotide Reverse Transcriptase Inhibitor (NNRTI) mutations.
CHAPTER FIVE

5. DISCUSSION AND CONCLUSION

5.1. DISCUSSION

The purpose of this study was to, first, identify the HIV-1 subtypes present in the sampled population in order to determine the predominantly circulating subtype and then to identify drug resistance mutations present, if any.

5.1.1. Immunologic and Virologic Markers

There were significant differences in the immunological markers between sample groups. Patients on first line therapy had the lowest median viral load and the highest median CD4 count (Table 4) which is an indication of comparatively better therapeutic effectiveness.

Despite being ART naïve and having the highest mean duration of infection, 10 years, the long term non-progressors (LTNPs) sample group had an average CD4 cell count of 603 cells/µl, comparable to the average of first line therapy sample group, 611 cells/µl. This is in line with their classification as LNTPs. These are HIV infected individuals who are able to maintain high CD4 and CD8 T cell counts over at least 8 years without the need for ART (Kumar, 2013). The fast progressor group however have a high average viral load (74230.7 copies/ml) despite therapy, even though they have the lowest mean duration of infection of 3 years. This is in line with the case definition of rapid disease progression (Langford et al., 2007).

Using data from Figure 12 and Figure 13, a fairly robust conclusion can be drawn about which group of patients are doing well and which are not. In this case, the evidence seems to suggest that the LTNP are doing quite well whereas the second line therapy group is not.
5.1.2. Nucleic Acid Extraction and Amplification

Results from the extraction and amplification of viral RNA (Table 5, Figure 14 and Figure 15) point to a low amplification success. Out of 63 plasma samples that viral RNA was extracted from, the PR and RT genes were only successfully amplified in 21 and 29 samples respectively. This corresponds to an overall PCR positive rate of 39.7%. This poor amplification was same despite a repeat of the procedure. Plausibly, this was due to a number of factors, the most likely of which is the storage and handling of the samples. Being archival samples, the viral RNA may have degraded over time due to the high sensitivity of RNA to degradation by abundant and ubiquitous RNAses (Elliott and Peakman, 2008). This was possibly worsened by the lack of continuous power supply during the storage period. There may be need therefore to take another look at the sample handling and storage protocols in our laboratories to ensure proper preservation and maintenance of nucleic acid integrity. Some samples also had extremely low or undetectable viral loads which may have accounted for the failure to amplify the target gene in those samples. It is puzzling however that in some cases, the targets of interest were successfully amplified in samples with low and/or non-detectable viral loads but not in those with high viral loads.

The difference in the amplification success of the PR gene (29) as compared to the RT gene (21) could be due to the difference in the size of the expected PCR products. The expected amplicon size of the RT gene is 887bp, about two times that of the PR gene (463bp) and shorter fragments are generally more easily amplified than longer fragments.
5.1.3. Sequencing of PR and RT Genes

The sequencing success of RT genes was generally better than PR genes. Efforts to improve the overall success of the process by repeating and also using a different batch of primers were unsuccessful. In a few of the failed cases, there were base calls but the quality was really poor. However, in most of the cases, there were no base calls at all. This could not have been due to the primers because the same sets of primers worked for other samples, and it could also not have been due to the absence of a template because an agarose gel electrophoresis was done to confirm the presence of the amplified targets in the purified PCR product. Thus sequencing failure could simply be due to variability at the primer binding sites of the amplified genes. In such instances, new primers that would bind within the region could be designed and explored for sequencing; however this was not possible in this study due to financial and time constraints.

5.1.4. Subtype Information

All the PR targets successfully sequenced were of the subtype CRF02_AG. Of the RT targets, as shown in Figure 18, 77% were of the CRF02_AG subtype, 13% were subtype G and 10% were of the CRF01_AE subtype. Thus, overall, 73.7% were classified as HIV-1 subtype CRF02_AG confirming earlier reports that it was the predominant subtype in West Africa and in Ghana as well (Buonaguro et al., 2007; Esbjörnsson et al., 2011; Fischetti et al., 2004; Nii-Trebi et al., 2013). From phylogenetic analysis of PR subtypes, Figure 19, it was seen that the study samples clustered around the two CRF02_AG reference subtypes although they cluster more with the Nigerian (Ref.02 AG.NG) reference subtype than the Cameroon reference subtype (Ref.02 AG.CM). This may be because Nigeria is a closer neighbour to Ghana and also because there is more human traffic to and from Nigeria than Cameroon. This
is the same with the RT CRF02_AG subtypes (Figure 20). The CRF01_AE subtype isolate seems to be equally distant from the reference Chinese and Afghan CRF01_AE subtypes (Figure 20). The fact that the subtype CRF01_AE isolate is not so closely related to either of the South East Asian reference subtype CRF01_AE sequences may point to the possibility that it is evolving. It is worthy to note that, the CRF01_AE subtype isolate had two amino acid insertions in the reverse transcriptase gene. At codon 44, there was a glutamate (nucleic acid GAA) and also a glutamic acid (nucleic acid GGG) insertion at codon 299. Of the G subtypes, samples clustered around the Nigerian reference subtype. These results suggest the need for a nationwide epidemiological project in order to establish if the distribution and proportions of the common circulating subtypes in Ghana are changing and also to ascertain if certain subtypes are evolving.

5.1.5. Drug Resistance Information

The drug resistance profiles seen in the samples analysed were not unexpected. From the summary data in Table 8, it is seen that 3 of the sequenced PR targets had resistance conferring mutations in the PR gene. However, only one of those samples showed resistance to any of the PIs in use in Ghana. The major PI mutations found were M46I, V82A and L90M (Table 9). These are common PI mutations which select for Lopinavir and Nelfinavir (Liu and Shafer, 2006; Wensing et al., 2014). All the PI mutations were found only in patients who were on second line drug therapy. This is expected as these patients have been exposed to PIs.

There was a far higher frequency of drug resistance mutations in the RT gene than was seen in the PR gene. Error! Reference source not found. shows that almost half, 6%, of the samples had resistance conferring mutations in the RT gene that render
them resistant to both nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleotide reverse transcriptase inhibitors (NNRTI). All these mutations were found in both patients on first line and second line therapy. This is confirmed in Figure 22, by the number of samples (6) which show high level resistance to at least one NRTI and NNRTI. It is not surprising that there are more drug resistance mutations in the RT genes because reverse transcriptase inhibitors, particularly the NRTIs (the backbone of the antiretroviral therapy), are used in both the first- and second-line regimens.

The most common NRTI mutation identified is the M184V which accounted for 6 out of 7, 85.8% of all NRTI mutations found (Table 10). This confirms previously reported information that the M184V mutation is the dominant mutation found amongst patients on therapy (Bennett et al., 2009). The M184V mutation causes high level resistance to lamivudine. Its high frequency is therefore not unusual since lamivudine is the most common NRTI in use. Interestingly however, because M184V increases susceptibility to zidovudine, stavudine and tenofovir and is also associated to a clinically significant reduction in HIV-1 replication in vivo, patients with the mutation can still continue treatment with lamivudine (Liu and Shafer, 2006).

Other mutations that were detected which conferred resistance to NRTIs are M41L, D67N, K70R, T215Y/F and K219E (see Table 10). These mutations are thymidine analogue mutations (TAMs) which occur in patients being treated with thymidine based drugs; zidovudine and stavudine. T215Y/F and K219E are major TAMs which give rise to high level resistance to zidovudine and stavudine especially when they occur in concert with accessory TAMs like M41L, D67N and K70R (Liu and Shafer, 2006).
The mutations identified which give rise to NNRTI resistance included K103N and Y181C. At least one of these mutations occurs in 4 out of the 7 samples which had NNRTI resistance mutations. It is interesting to note that, all but one (85.7%) of the samples in which NNRTI resistance mutations were detected had high level resistance to both nevirapine and efavirenze and the other one having high level resistance to nevirapine and intermediate level resistance to efavirenz. This paints a dire picture with NVP and EFV being the two recommended NNRTIs for use by the Ghana Health Service. There is therefore need to use genotypic drug resistance mutation analysis in deciding drug regimens both at the national level and at the point-of-care.
5.2. Limitations

- Small sample size
- Poor amplification success
- Poor sequencing success

These limitations severely affected the successful completion of this study.
5.3. Conclusion

Despite its limitations, this study has shown that the subtypes in circulation in the study population include CRF02_AG, CRF01_AE and subtype G. It has evidently shown that subtype CRF02_AG remains the predominant circulating subtype of HIV-1 in the population sampled accounting for 73.7% of the subtypes identified in this study. It has also shown that the subtype CRF02_AG in circulation is more closely related to the Nigeria reference CRF02_AG subtype.

This study has also documented a high level of resistance to reverse transcriptase inhibitors. It has shown a high frequency of resistance to lamivudine and an even higher frequency of resistance to the two Non-Nucleotide Reverse Transcriptase Inhibitors (Nevirapine and Efavirenz) in use in Ghana.

This study has also highlighted the need to improve the sample collection, handling and storage protocols in our laboratories.
5.4. Recommendations

- A nationwide epidemiological study of the prevalence and evolution of both HIV-1 and HIV-2 subtypes
- A longitudinal study of a seroincident HIV-1 cohort to determine the effect of CRF02_AG and other subtypes on disease progression in Ghana
- Increase in the use of genotypic drug resistance data to determine therapy policy and regimen
- Develop and enforce an efficient protocol for the collection, handling and storage of samples in our laboratories
REFERENCES


UNAIDS (2014). AIDSinfo | UNAIDS.


APPENDIX I

MATERIALS

A. Reagents

Absolute ethanol (molecular biology grade) [SIGMA, USA]
Nuclease-free water (Ambion, USA)
QIAamp viral RNA kit (QIAGEN, USA)
OneTaq 2X Master Mix with Standard Buffer (NEB, England)
One Step RT-PCR Kit (QIAGEN, USA)
AmpliTaq Gold Master Mix Reagents (ABI, USA)
Agarose (SIGMA, USA)
Ethidium bromide (SIGMA, USA)
Tris-Acetate-EDTA (TAE) [Ambion, USA]
DNA molecular weight 100bp ladder (Invitrogen, USA)
QIAquick PCR purification kit (QIAGEN, USA)
Big Dye Terminator Cycle Sequencing Kit vs. 3.1 (ABI, USA)
AgenCourt CleanSeq Dye Terminator Removal kit (Beckman Coulter, USA)
Sequencing Buffer with EDTA 5X (ABI, USA)
Performance Optimized Polymer-POP 7 (ABI, USA)
Primers (NACP, Ghana)

B. Laboratory Equipment

Biosafety Cabinet Class IIA (AirTech Services, India)
Biological Safety Cabinet Class II (LABGARD, USA)
Centrifuge (H-900) (Kokusan, Japan)
Autoclave SS-325 (Tomy, Japan)
Vortex Genie-2 (Scientific Industries, USA)
Microcentrifuge 5415D (Eppendorf, USA)

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

AirClean 600 PCR Workstation (AirClean Systems, USA)

GeneAmp PCR System 2700 and 2720 (ABI, USA)

Microwave oven (LG Electronics Inc., Ghana)

Electrophoresis system (Mupid-2 Plus) [Japan]

Geldoc Imaging System (UPV, USA)

Genetic Analyser 3130 (ABI, USA)

C. Consumables

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

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

Nalgene 1.8ml cryovials (Nalge Nunc, USA)

Nalgene cryoboxes (Nalge Nunc, USA)

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

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

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

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

D. Software

SeqmanPro (DNASTar, Madison, WI)

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

MEGA 5 (http://www.megasoftware.net/)

Finch TV (GeoSpiza, USA)

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

Sequence Analyses Software (ABI, USA)
Los Alamos HIV Sequence Database

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

Statistics/Data Analysis, STATA 13 (http://www.stata.com)
### APPENDIX II

AMINO ACID NAMES, ONE LETTER CODES AND ACRONYMS

<table>
<thead>
<tr>
<th>Name</th>
<th>One Letter Code</th>
<th>Acronym</th>
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<tbody>
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</tr>
<tr>
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<td>C</td>
<td>Cys</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>D</td>
<td>Asp</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>E</td>
<td>Glu</td>
</tr>
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<td>Phenylalanine</td>
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<td>Tyrosine</td>
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<td>Tyr</td>
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</table>
APPENDIX III

ETHICAL CLEARANCE

2nd March, 2016

FEDERALWIDE ASSURANCE FWA 00001824
NMIMR-IRB CPN 089/15-16
IRB 00001276
IORG 0000908

On 2nd March 2016, 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: Genotypic characterization of HIV 1 subtypes from positive patients in Ghana

PRINCIPAL INVESTIGATOR: Deletsu Dennis Selase, 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 1st March, 2017. You are to submit annual reports for continuing review.

Signature of Chair: 

Mrs. Chris Dadzie
(NMIMR – IRB, Chair)