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TRANSMITTED DRUG RESISTANCE MUTATIONS AND SUBTYPE DIVERSITY AMONG HIV-1 SERO-POSITIVE VOLUNTARY BLOOD DONORS IN ACCRA, GHANA

By

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This thesis is submitted to the University of Ghana, Legon in partial fulfilment of the requirements for the award of MPhil Medical Microbiology degree.

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DECLARATION

I declare that, , this thesis is the result of my own research work and that the material has not been presented either in whole or in part elsewhere for the purpose of acquiring another degree. References to other people's works have been duly cited. All works were performed by me under the supervision of Rev. Dr. Kwamena William Coleman Sagoe and Dr. (Mrs.) Evelyn Yayra Bonney.

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DEDICATION

With my heart filled with love as I write this section, I completely dedicate this thesis to my lovely parents (Mr. Musah Moro Obeng and Mama Eno), my uncle (H. H. Justice Yusif Assibey) and siblings (Eric, Aisha, Sadia, Mummy, Salima, Firdaus).

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Indeed! All praises and glory to God for His blessings, love, and protection to me and my family. He granted me life and strength to undertake and research successfully.

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LIST OF ABBREVIATIONS AND ACRONYMS

µl	Microlitre
3TC	Lamivudine
ABC	Abacavir
ABI	Applied Biosystems Inc.
AIDS	Acquired Immunodeficiency Syndrome
bp	Base pair
CCR	Chemokine Receptor
CD	Cluster of Differentiation
cDNA	Complementary DNA
CNS	Central Nervous System
CRF	Circulating Recombinant Form
D4T	Stavudine
DDI	Didanosine
EDTA	Ethylenediaminetetraacetic Acid
EFV	Efavirenz
EIA	Enzyme Immunoassay
env	Envelope
ETR	Etravirine
FI	Fusion Inhibitors
FPV	Fosamprenavir
FTC	Emtricitabine
gag	Group-specific antigen
gp	Glycoprotein
HIVdb	HIV Database
HIVDR	HIV Drug Resistance
IDV	Indinavir
IgG	Immunoglbulin G
II	Integrase Inhibitors

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KBTH	Korle-Bu Teaching Hospital
LIA	Line Immunoassay
LPV	Lopinavir
ml	Millilitres
mRNA	Messenger RNA
MSM	Men having sex with men
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitors
NRTI	Nucleoside Reverse Transcriptase Inhibitors
NVP	Nevirapine
PC	Positive Control
PCR	Polymerase Chain Reaction
PI	Protease Inhibitors
PR	Protease
rev	Regulator of expression of virion
RIA	Radio Immunoassay
RNA	Ribonucleic Acid
RPV	Rilpivirine
RT	Reverse Transcriptase
SABC	Southern Area Blood Centre
SD	Subtype Diversity
TAE	Tris-acetate EDTA
tat	Trans-activator
TDF	Tenofovir
TDR	Transmitted Drug Resistance
URF	Unique Recombinant Form
vif	Viral infectivity factor
vpr	Virus protein R
vpu	Virus protein U
W.H.O	World Health Organisation

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ABSTRACT

Background

Detection of HIV-1 transmitted drug resistance (TDR) and subtype diversity (SD) are public health strategies to assess current HIV-1 regimen and ensure effective therapeutic outcomes of ART among HIV-1 patients. Globally, limited data exist on transmitted drug resistance and subtype diversity among blood donors. In this study, drug resistance mutations and subtype diversity among HIV-1 sero-positive blood donors in Accra, Ghana was characterized.

Methods

Purposive sampling method was used to collect 81 blood samples from the Southern Area Blood Center, Korle-Bu that tested positive for HIV using the HIV Ag/Ab 4th gen (Fortress Diagnostics, U.K). Serology was used to confirm and discriminate between the presence of HIV-1 and HIV-2 antibodies in all samples using INNO-LIA HIV I/II score (Fujirebio, Belgium). Viral RNA was then extracted using QIAamp viral RNA Mini Kit (QIAGEN, Germany) from plasma samples confirmed as HIV-1 positive. Reverse transcription polymerase chain reaction (RT-PCR) was used to amplify the reverse transcriptase (RT) and protease (PR) genes of HIV-1 using Transcriptor High Fidelity cDNA synthesis kit for the reverse transcriptase (RT) step and Expand High Fidelity PCR System for the PCR step (Roche Diagnostics, Germany). Agarose gel electrophoresis was used to ascertain the presence of expected amplification products. The expected products were purified and cycle sequenced using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, U.S.A). Sequences were read out using the ABI genetic analyzer 3130xl (Hitachi, Japan) and data analyzed for drug resistance mutations and HIV-1

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subtypes using the Stanford University HIV drug resistance database and Los Alamos HIV database respectively.

Results

Out of the 81 plasma samples collected, 61 (75%) were confirmed as HIV-1 sero-positive by INNO-LIA HIVI/II Score kit with no HIV-2 and dual HIV-1/2 infections. The remaining samples (25%) were confirmed as HIV sero-negative. Of the 61 confirmed positive samples, 53% and 50% were successfully amplified in the RT and PR genes respectively. Nucleotide sequencing of amplified samples revealed the presence of major drug resistance mutations in two (2) samples; E138A in one sample and another with K65R. HIV-1 Subtypes including subtypes A, B, CRF02_AG and CRF09_cpx were found.

Conclusion

This study has found major drug resistance mutations, E138A and K65R in the RT gene that confer high level resistance to most NNRTIs and NRTI respectively. CRF02_AG was most predominant, the recorded percentage of subtype B and the evolutionary relationship inferred by phylogenetic analysis suggest possible subtype importation. The data will inform the selection of drugs for ART initiation to maximize therapeutic options in drug-naïve HIV-1 patients in Ghana.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Human Immunodeficiency virus (HIV) infection in Africa has recorded a greater percentage of the global prevalence of the pandemic (W.H.O, 2014). The virus belongs to the viral family, Retroviridae and the genus Lentivirus that progressively affects the human immune system. This results in the development of Acquired Immunodeficiency Syndrome (AIDS) characterized by various opportunistic infections (Antinori *et al.,* 2013). HIV-1 and HIV-2 are the types of HIV with the HIV-1 lineages being M, N and O. Majority of infection has been caused by the M lineage which has subtypes A-D, F-H, J and K with many Circulating Recombinant Forms (CRF) and Unique Recombinant forms (URF).

A major therapeutic intervention to the HIV pandemic is for infected persons to receive antiretroviral therapy (ART) (Kiptoo *et al.,* 2013). An estimated number of 13.6 million infected persons had access to ART globally as at June 2014. Current reports show that treatment coverage is 37% of all people living with HIV in Africa (W.H.O, 2014). In Ghana, ART was introduced in 2003 and has scaled up to about 58% of HIV patients (NACP, 2014). The introduction and widespread use of ART has reduced HIV related morbidity and mortality rates by inhibiting key viral replication steps in susceptible immune cells (Palella *et al.,* 1998). These ART drugs include Fusion Inhibitors (FI), Nucleotide and Non-nucleotide Reverse Transcriptase Inhibitors (NRTI and NNRTI respectively), Integrase Inhibitors (II) and Protease Inhibitors (PI).

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However, therapeutic success of these ARTs is reduced as a result of the emergence of HIV-1 drug resistance mutations (DRM) and subtype diversity (SD) in patients receiving ARTs (ART experienced) (Richman *et al.,* 2004), spontaneously generated polymorphisms due to immune pressure and the transmission of HIV drug resistant strains from treated persons to patients initiating ART (ART naïve) (Bonney *et al.,* 2013). Genotypic testing and subtype identification at the time of diagnosis provides the effective therapeutic intervention as resistance mutations are detected in the polymerase (*pol)* gene before initiating ART.

The polymerase (*pol)* gene has the reverse transcriptase (RT) and protease (PR) genes that code for the reverse transcriptase and protease enzymes respectively which are very important in the HIV viral replication. Although mutations could be detected in other genes of HIV-1, most available HIV-1 antiretroviral drugs target the regions in the *pol* gene, hence, higher possibility of detecting mutations conferring resistance to these drugs. HIV*Pol* gene has been recommended as a reliable gene for detecting transmitted HIV-1 drug resistance mutations (Hue *et al*., 2004; Njouom *et al*., 2003).

1.2 Problem Statement

The HIV global burden as at 2013 was estimated at 35 million of which 24.7 million lived in Sub-Saharan Africa (W.H.O, 2014). HIV-1 infection was first detected in Ghana in 1986 and has caused increased mortality rates among children, adults and pregnant women (UNAIDS/W.H.O, 2012). The prevalence of HIV-1 in Ghana was estimated to be 1.6% in 2014 (UNAIDS/W.H.O, 2015).

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The prevalence of HIV drug resistance transmission in patients initiating ART recorded nearly 11% in Europe, 13% in North America, 4% in Asia and 5% in Africa (Frentz *et al.,* 2012). In the Europe and North America, most subtypes identified were subtypes B and C compared to non-B subtypes in Asia and Africa. Most HIV research and the development of ARTs have been based on the subtype B which is predominant in the developed countries. However, majority of people are infected with non-B subtypes, hence making it imperative to elucidate the circulating mutants and subtypes especially in drug naïve patients to maximize treatment outcome.

1.3 Justification

The emergence of HIV-1 drug resistance and subtype diversity has been linked to the naturally faster error-prone replication machinery of HIV-1 in the presence of antiretroviral drug (Richman *et al.,* 2004). The HIV-1 subtype diversity has been found to influence the development of certain drug resistance mutations. In a study by Coutsinos *et al.* (2010), K65R, a major drug resistant mutation that confers resistance to many of the NRTIs, was associated with subtype C than in subtype B. G17E, a mutation that occurs within the protease gene of HIV-1 is associated with CRF02_AG that is predominant in West Africa (Santos *et al*., 2012). This mutation causes susceptibility to protease inhibitors such as indinavir and atazanavir. For effectiveness of ART programs, it is essential to assess HIV-1 drug resistance mutations (WHO, 2003) and subtype diversity among drug-naïve patients (Lessells *et al*., 2012). This could be achieved by genotypic testing and subtype identification at the time of initiating ART to detect any drug resistance mutation and HIV-1 subtype present prior to therapy. Thus, the detection of

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HIV-1 mutants and subtypes before administering a particular antiretroviral drug is an important step to successful antiretroviral therapy.

1.4 Aim

To determine transmitted drug resistance mutations and subtype diversity among HIV-1 sero-positive voluntary blood donors in Accra, Ghana.

1.5 Specific Objectives

1. To detect drug resistance mutations in the reverse transcriptase (RT) and protease (PR) genes of HIV-1.

2. To determine the HIV-1 subtype diversity among the study participants.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Structure of Human Immunodeficiency Virus -1

Human Immunodeficiency Virus type 1 (HIV-1) was initially described in 1983 at the Institut Pasteur in France by a group of scientist (Barre-Sinoussi *et al.,* 1983). The virus had been implicated as a causative agent of acquired immunodeficiency syndrome (AIDS) in homosexuals in 1981 (Gottlieb *et al.,* 1981). HIV-1 taxonomically belongs to the family Retroviridae and genus Lentivirus owing to the long term incubation period. The virus is approximately 100nm in diameter with a spherical lipid envelope and two identical single strands of RNA in its icosahedral capsid (figure 1). The two (2) single stranded RNA are positive sense in nature. The lipid envelope has a glycoprotein 120 (gp120) that binds to host $CD4^+$ receptors on Helper T-lymphocytes. The presence of an additional envelope fusion glycoprotein, gp41, promotes cell-to-cell adhesion (Cann, 2005; Zane, 2001).

The genome size is approximately 9 kb with three (3) major structural genes; group specific antigen (*gag*), polymerase (*pol)* and envelope (*env)* genes that are common to all retroviruses that code for the viral structural proteins (Rajarapu, 2014). The *gag* gene codes for p24, p18, p15 and p53 core proteins, the *env* gene also codes for gp120 and gp41 found on the envelope. The reverse transcriptase (RT), integrase (I) and protease (PR) are coded for by the *pol* gene (Abbas and Lichtman, 2005). Specific to all Lentiviruses of which HIV-1 belongs to, is the presence of two (2) auxiliary genes namely trans-activator (*tat)* and regulator of expression of virion *(rev)* genes responsible for transactivation of viral genes and regulation of RNA splicing respectively. Other

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accessory proteins responsible for disease pathogenesis and regulating viral expression are the negative regulation factor (*nef)*, virion infectivity factor (*vif)*, virus protein U *(vpu),* andvirus protein R *(vpr)* (figure 2).

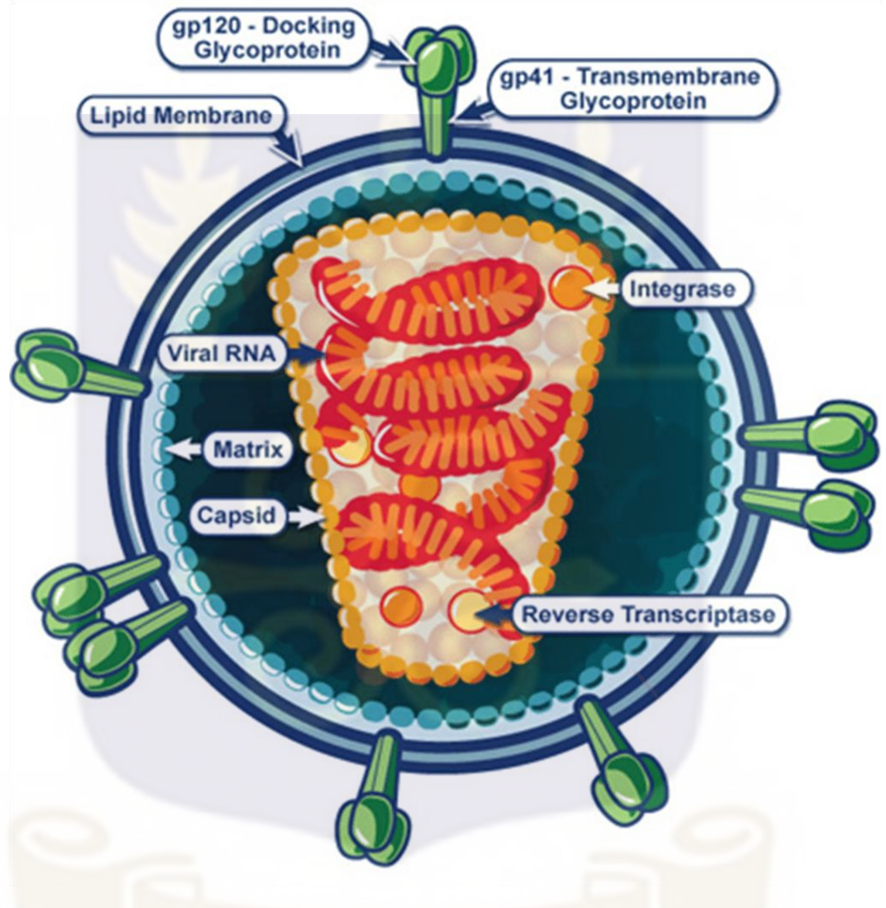

Figure 1: Structure of HIV-1

"HIV has a lipid envelope studded with a trans-membrane glycoprotein (gp41) and a docking glycoprotein, gp120 used for cell adhesion. Its RNA genome is contained in a protein capsid and has a reverse transcriptase to convert its RNA to DNA for integrating into the host genome by the aid of its viral encoded integrase."

(Source:http://microbeonline.com/wp-content/uploads/2010/04/HIV-Virus-Structure.png)

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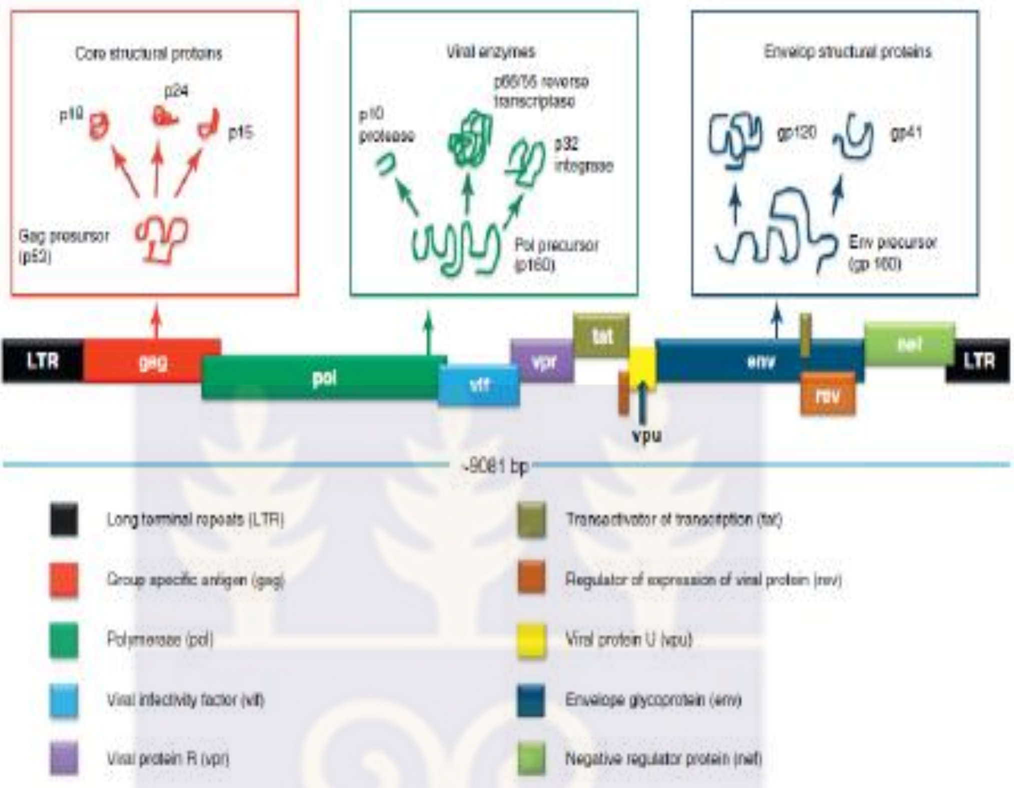

Figure 2: Genome Organization of HIV

*"The genome is approximately 9kb with three major structural genes; gag, pol and env. The gag encodes thecore proteins (p10, p24, p15), the pol encodes viral enzymes (protease, reverse transcriptase, integrase) and the env gene codes for envelope glycoproteins (gp41, gp120). Other accessory genes are the vif, vpr, tat, rev, vpu and nef (*Rajarapu, *2014)."*

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2.2 Replication of HIV-1

HIV infection is initiated by the binding of gp120 to the $CD4^+$ receptors or chemokine receptors such as CCR5 or CXCR4 on susceptible cells such as Helper T-lymphocytes, dendritic cells or macrophages (Talaro and Talaro, 2002). With successful fusion of the glycoprotein to host cell receptors, the single stranded viral RNA is released into the host cell and with the reverse transcriptase, a DNA copy is made from the viral RNA. The viral DNA is integrated into host genome as proviral DNA by the integrase (Cann, 2005). Transcription of proviral DNA to mRNA is facilitated by host's RNA polymerase and cleaved to several fragments of mRNAs by HIV protease. The cleaved mRNA molecules contain gene sequences which are later assembled into several virions that bud from the cell and infect other susceptible cells (Murray *et al.,* 2002). This cycle produces approximately 10 billion number of virus per day that affects new cells (Morse *et al*., 2004).

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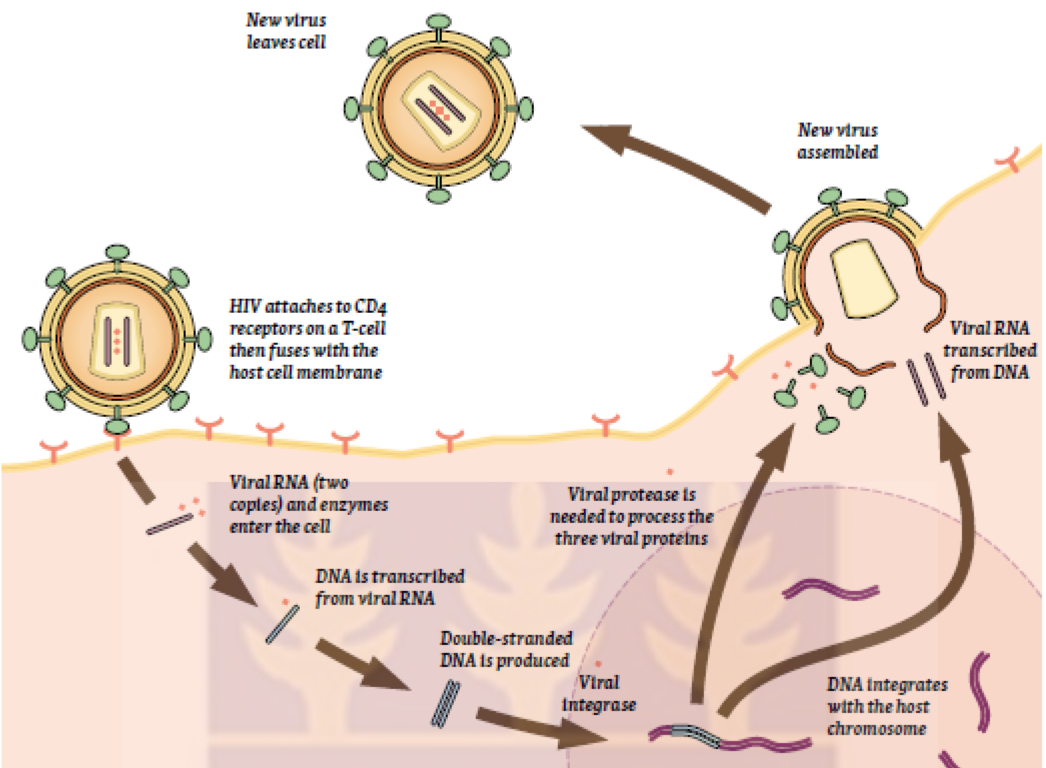

Figure 3: Replication of HIV-1

*"HIV attaches to CD4 T-cell receptor using its glycoprotein, gp 120. It gains entry and finally releases its viral RNA and enzymes in the host cytoplasm. RNA is reverse transcribed to DNA by viral reverse transcription and integrated into host genome. Viral genes and proteins are produced by host transcriptional and translational machinery (*Fischer and Madden, 2011*)."*

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2.3 HIV-1 Subtypes and Recombinants

The error-prone replication machinery of the viral reverse transcriptase, the faster nature of replication of HIV-1 and the immune pressure causes several mutations. This has led to the emergence of several lineages namely; M (main), N (non), O (outer) with the M lineage most associated with the HIV-1 pandemic (Tebit and Arts, 2011). The subtypes associated with M lineage are A-D, F-H, J and K which have the tendency for recombination leading to most recombinant forms. The vast number of HIV-1 variants is associated with some epidemiological and biological factors (Tebit and Arts, 2011; Hemelaar, 2012). Some studies in Ghana found CRF02_AG as the most prevalent subtype circulating among some study groups and areas (Nii-Trebi *et al*., 2014; Bonney *et al*., 2013). Genetic variation among HIV-1 subtypes could impact disease progression, development of drug resistance mutations and hence the effectiveness to certain antiretroviral drugs.

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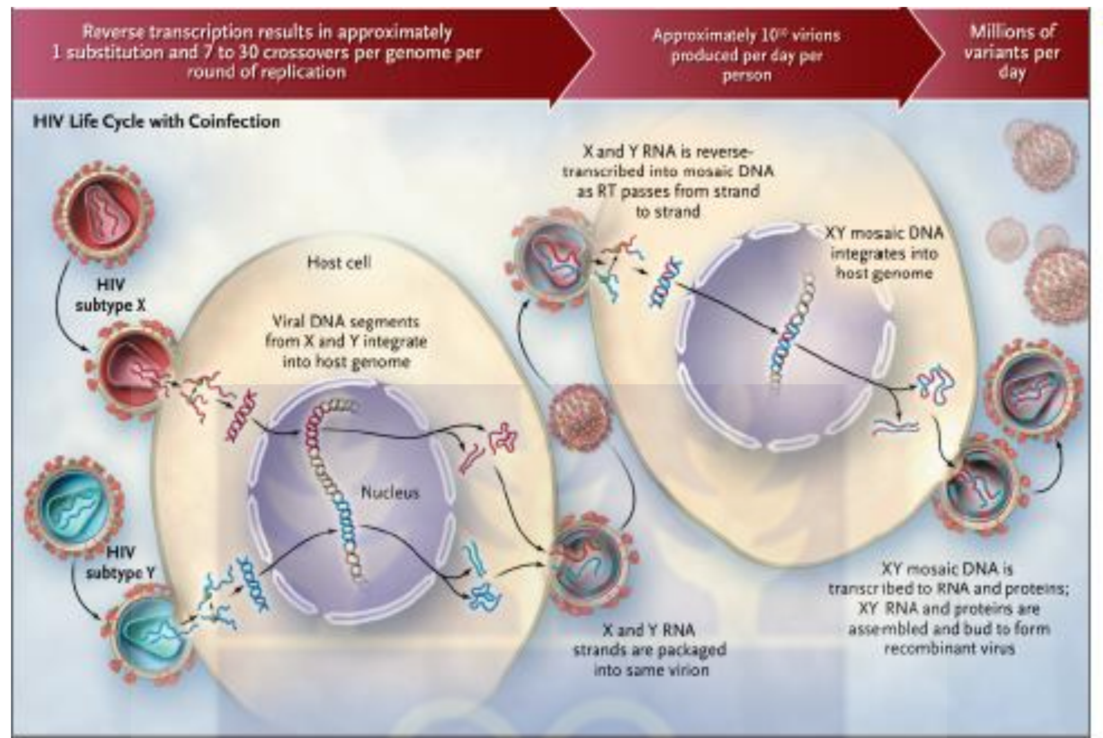

Figure 4: The Emergence of HIV-1 Subtypes and Recombinants

*"The error-prone nature of the reverse transcriptase causes substitutions in the viral genome during viral replication and hence different subtypes. Different subtypes may infect one cell and may eventually produce recombinants (*Taylor *et al.*, 2008*)."*

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2.4 Pathogenesis of HIV-1

Initial dissemination of the virus in lymphoid tissues is enhanced by the active replication of the virus within dendritic cells. This occurs within days after infection leading to viraemia and consequent dissemination to various target cells (Abbas and Lichtman, 2005).

At this phase, there is latency as a result of equilibrium achieved by HIV replication and suppression of the virus by the immune system. Immuno-competent cells at this point are very active and mount active immune response against most infectious pathogens including the circulating HIV-1 and other opportunistic microbes leading to no or few clinical manifestation (Abbas and Lichtman, 2005).

The steady destruction of target cells, CD4+ T cells occurs and the number circulating in the peripheral blood also declines. This is because the immune system at this point is unable to continually suppress viral replication. Active replication also occurs simultaneously in other reservoir cells resulting in increased viraemia (Murray *et al.*, 2002). The consequent depletion coupled to incompetent circulatory CD4+ T cells leads to various clinical manifestations referred to as AIDS.

However, disease progression has been linked to subtype differences (Taylor et al., 2008). In Southeast Asia, CRF 01-AE has been described to accelerate disease progression as compared to subtype B (Costello *et al*., 2005). Studies in East Africa indicate faster progression with subtype D than other subtypes recorded in the region (Kaleebu *et al*., 2002; Kiwanuka *et al*., 2008; Baeten *et al.,* 2007). In West Africa, higher rate of replication was associated with CRF02_AG than subtype B (Njai *et al*., 2006).

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Certain mutations within the gene coding for the chemokine receptors on host cells as well as over expression of chemokines among some infected individuals tend to slow down disease progression (Hunt, 2009).

2.5 Diagnosis of HIV-1 Infection

Different diagnostic methods for HIV infection is currently available with the application ranging from detecting HIV-1 antibodies, HIV-1 antigens and HIV-1 nucleic acid. Rapid methods for qualitatively detecting IgG antibodies in blood and other body fluids such as Oral Quick Advance HIV-1/2 (OraSure Technologies, U.S.A), Uni-Gold™ Recombigen HIV-1 Test (Trinity Biotech Plc, Ireland) and Multispot HIV-1/2 Rapid Test (Bio-Rad Laboratories, U.S.A) are available. These tests may take about 15-30 minutes for providing results but will need to be confirmed by other highly sensitive methods like enzyme immunoassay (EIA), radioimmunoassay (RIA) and nucleic acid testing using polymerase chain reaction (PCR) (Pesce *et al.*, 2006) .

Enzyme immunoassays such as ELISA are traditionally sensitive methods that qualitatively and quantitatively detect HIV antibodies and/ or HIV antigens (Pagana and Pagana, 2002). The method is based on antibody-antigen complex that are detected by enzyme-substrate detection system. In this technique specific antibody or specific antigen is immobilized in wells. Addition of patient's sample forms antigen-antibody complex that is confirmed or detected by the addition of enzyme-linked antibody (conjugate) and subsequent substrate to produce a colour change. Absorbance can be measured using spectrophotometric methods to quantitatively evaluate positivity. Enzyme immunoassays have been used to detect p24 antigen before sero-conversion in recently infected persons.

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Polymerase chain reaction (PCR) can qualitatively and quantitatively detect the presence of HIV nucleic acid even in minute quantities (Pagana and Pagana, 2002). In special cases, especially in infants of HIV positive mothers, PCR is important in confirming active infections by detecting HIV-1 nucleic acid even in the presence of HIV-1 antibodies.

Detecting only HIV antibodies in infants less than 13 months is not diagnostic of HIV infection since maternal antibodies would still be circulating (Fearon, 2005). Polymerase chain reaction (PCR) is very sensitive in evaluating the amount of viral particles (viral load) circulating in peripheral blood. This is an effective way to monitor circulating HIV-1 to inform therapeutic interventions before and after exposure to antiretroviral therapy (ART) (De'sire *et al.*, 2001). However, this is not readily and routinely available in patient care especially in most developing countries.

2.6 Antiretroviral Therapy

There is no doubt that the introduction of antiretroviral drug has altered the natural course of HIV /AIDS (Lataillade *et al.,* 2010). This has become possible since ARTs interrupt with various stages in the replication cycle of HIV-1 (Mills *et al.,* 2011). Gene products such as reverse transcriptase (RT), protease, integrase and fusion proteins involved in various stages of replication cycle have being the target for HIV-1 therapy. These drug classes include fusion/entry inhibitors (enfuvirtide, maraviroc), nucleoside/nucleotide reverse transcriptase inhibitors (zidovudine, emtricitabine, abacavir, lamivudine, tenofovir, didanosine, stavudine), non-nucleoside/non-nucleotide reverse transcriptase inhibitors (efavirenz, nevirapine), protease inhibitors (ritonavir boosted lopinavir,

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ritonavir boosted atazanavir, tipranavir, indinavir, nelfinavir) and integrase inhibitors (raltegravir).

In Ghana, drugs within the NRTI, NNRTI , PI and II are approved in the HIV-1 drug regimen for the management of HIV infected persons. Fusion inhibitors (FI) tend to block chemokine receptors such as CCR5 on $CD4^{+}$T cells and macrophages preventing fusion of the virus by its glycoproteins (Lobritz *et al*., 2013). Other fusion inhibitors may block gp 41 on the viral envelope producing the same effect. Reverse transcriptase inhibitors may also explore different mechanisms of action. In nucleoside or nucleoside reverse transcriptase inhibitors (NRTI), the DNA chain is truncated by the incorporation of the analogue since it lacks 3'OH group (Clavel and Hance, 2004).

Integrate inhibitors (II) also block viral integrase preventing incorporation of viral DNA into host genome (Garido *et al.,* 2010). Protease inhibitors (PI) block viral protease and interrupts with cleaving and assembly of viral proteins into mature virions that leads to release of premature virions that are ineffective to infect other competent immunological cells (Lobritz *et al*., 2013).

Initiating ART has been based on virologic status (viral load) and immunologic status ($CD4^{+}$T cell count) (Cater, 2013). In some developing countries where viral load testing is not readily available, it is recommended to check $CD4^{+}$T cell count and therapy introduced at $\leq$ 350 cell/ ul (WHO, 2009) or $\leq$ 500 cells/ul recommended by the international AIDS society (Thompson *et al*., 2013). These differences are based on several factors such as consequent emergence of drug resistance, benefits of early

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initiation of ART (better treatment outcomes) and cost of sustaining ART programs (Cater, 2013).

2.7 HIV Drug Resistance

2.7.1 Overview of HIV Drug Resistance

Mortality due to HIV infection has reduced drastically since the inception of antiretroviral therapy (Kiptoo *et al.*, 2013). However, viral acquisition of resistance to one or more drugs consequently leads to treatment failure. Thus, there is steady rise in the viral load (virologic failure) and depletion of $CD4^{+}$T cells (immunologic failure) (Cater, 2013). Emergence of drug resistance has been reported to be partly due to pre-existing mutations due to viral error-prone replication machinery or mutations arising from drug therapy (Balley and Fisher, 2008; Pennings, 2012).

In treatment-experienced groups (exposed to ART) such as sero-positive pregnant women, a dose of nevirapine is administered to prevent mother-to-child transmission of HIV. However, drug resistance mutations were detected after several weeks of treatment in approximately 40% of the nevirapine-treated pregnant women (Arrive *et al.*, 2007). Treatment interruption is one key factor that leads to faster accumulation of drug resistance mutations as indicated by several cohort studies and clinical trials (Tam *et al.*, 2008; Gardner *et al.*, 2010; Yerly *et al.*, 2003; Danel *et al.*, 2009). This could result from patient's forgetfulness, inability to purchase drug or unavailability of drug at the time required. This leads to sub-therapeutic levels and consequent emergence of drug resistant variants (Gardner *et al.*, 2010).

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2.7.2 Transmitted Drug Resistance

Transmitted drug resistance is an important public health concern due to increased risk of virologic failure when ART is initiated (Wittkop *et al*., 2011). The phenomenon was first observed in patients resistant to zidovudine or stavudine which are reverse transcriptase inhibitors used in the management of HIV-1 infected persons (Mayers *et al*., 1993; Conway *et al*., 1999). To achieve the effectiveness of ART, it is recommended to test for HIV drug resistance in treatment-naïve individuals before initiating ART (WHO, 2003; European ART guidelines, 2006; DHHS ART guidelines, 2012).

Several studies have been conducted on transmitted drug resistance across the world (Weinstock *et al*., 2004; Wensing *et al*., 2005). Different population groups were used in the estimates with most studies on men having sex with men (MSM), sex workers, pregnant women and injection drug users (UNAIDS/WHO, 2009). Similar trends were observed in America and Australia with an overall prevalence of 13% and 23% respectively. Most studies in Africa recorded prevalence of <5% and this could be due to the relatively limited access to ART as compared to other parts of the world (UNAIDS/WHO, 2009). In all, prevalence rates range from 0% to 30% with lowest rates in resource limited countries.

Efficacy of antiretroviral therapy is limited by emergence of HIV-1 drug resistance and subsequent transmission of drug resistant variants to newly infected individuals (treatment-naïve individuals).This study focuses on transmitted drug resistance using a population of sero-positive voluntary blood donors in Accra, Ghana to inform initiation of ART.

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2.7.3 HIV Drug Resistance and subtype patterns among Voluntary Blood Donors

With the large data on transmitted HIV-1 drug resistance (TDR) across the world, few studies have focused on voluntary blood donors (Frentz *et al*., 2012). Different studies conducted in different areas among blood donors in Brazil have found high prevalence of HIV-1 TDR (Pessoa *et al.,* 2014; Alencar *et al*., 2013) with one recording low prevalence (Barreto *et al.*, 2006). In all the studies, the most prevalent form was subtype B followed by subtype F. Evidence of resistance mutations to protease inhibitors (M46I, L33F), NRTI (M184I) and NNRTI (M230I) were identified among blood donors in Pernambuco, Brazil (Pessoa *et al*., 2014). In this study, there was high rate of HIV-1 transmitted drug resistance among the study population with two novel recombinant forms CRF70_BF1 and CRF71_BF1 found. In a case-control study of HIV-1 TDR conducted from January 2007- March 2011 in four (4) dispersed blood centers in Brazil, HIV-1 TDR was recorded to be in the range of 8.2% - 19.4% (Alencar *et al*., 2013).

Lower prevalence (3.8%) was recorded in a nationwide survey conducted in China from 2004 to 2005 (Liao *et al*., 2010). Most resistance mutations recorded was K103N (2.1%) to NRTI followed by M184V/I (1.6%) to NNRTI and V82A, M46I, N88D (0.4%) to PI. The most prevalent HIV-1 subtype was subtype B (73.5%) followed by circulating recombinant form, CRF01_AE (13.9%). However, from 2007 to 2011, a similar study found CRF08_BC as the most prevalent (41.6%) followed by CRF01_AE (32.7%) with subtype B found to be 2.7%. Lower overall prevalence (4.4%) of TDR was also found (Zeng *et al*., 2010).

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One study in the U.S.A found TDR to be 12% (moderate) among voluntary blood donors. The most prevalent HIV-1 subtype was the subtype B with low non-subtype B (2.5%). The study showed resistance mutations directed to PI (M46I, I84V, L90M) and RTI (Y181C, T215D, M41L) to be 2% and 11% respectively (Delwart *et al*., 2012). Few studies have described trends in transmitted HIV-1 drug resistance among voluntary blood donors in Africa (Frentz *et al*., 2012). One study found key resistance mutations to ARTs (NRTI, NNRTI, PI) with an overall TDR prevalence of 5.6% (Toni *et al.,* 2003). A similar study from 2002 to 2006 found an overall prevalence of HIV-1 TDR to be 6% with most subtype being CRF02_AG in the same area (Toni *et al.,* 2007). Continuous monitoring of HIV-1 transmitted drug resistance (TDR) among different population groups located at different areas is recommended due to the increased access to ARTs (NACP, 2014).

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CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Site

The Southern Area Blood Center (SABC) situated at Korle-Bu Teaching Hospital (KBTH) was the site for sample collection. The center is a satellite facility of the National Blood Service (NBS) of Ghana mandated in the collection of blood, screening and distribution of safe blood to various hospitals and clinics in the southern part of Ghana. The NBS recruits voluntary blood donors using a questionnaire to first assess the behavioral risk factors and health status of people ready to donate voluntarily. Upon successful recruitment and blood donation, the blood is screened for hepatitis B surface antigen (HBsAG), Hepatitis C virus (HCV), Human immunodeficiency virus (HIV) and *Treponema pallidium*, the causative agent of syphilis. Any donated blood that tests positive for any one of these pathogens is autoclaved and disposed appropriately.

3.2 Study Design and Population

The study was cross-sectional using purposive sampling method to select eighty-one (81) donated blood samples from voluntary blood donors. Data extraction sheet was used in obtaining information on age and gender from the donors’ records upon approval from the SABC. All blood samples used in this study were screened as positive for HIV using the HIV Ag/Ab 4th gen (Fortress Diagnostics, U.K).

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3.3 Sample Size Determination

The prevalence of transmitted HIV-1 drug resistance mutations among voluntary blood donors in Abidjan, Cote D'Ivoire was found to be 5.6% (Toni *et al.,* 2003). With Z value of 1.96 at 95% confidence level and allowable error of 5%, the minimum sample size is 81.

$$N = \frac{Z^2 X P(1-P)}{E^2}$$

Where;

N=required sample size

Z=Confidence level at 95% (standard value of 1.96)

P=Prevalence of transmitted HIV-1 drug resistance

E=Allowable error at 5%

3.4 Materials

The following materials were used: cryovials (Wheaton, Millville, New Jersey, U.S.A), filter tips (Neptune, U.S.A), blood bag centrifuge (Thermo-scientific, U.S.A), powder-free nitrile gloves (BM polyco, Crown Road, U.K), blood bag expressor and sealer (Thermo-scientific, U.S.A), multi-channel and single channel pipettes (Gilson, France), centrifuge (Kokusan corporation, Japan), vortex mixer (Fisher Scientific, U.S.A), platform rocker (Stuart Scientific, U.K), UVP BioDoc-It 220 Imaging system (Life Sciences, U.S.A).

INNO-LIA™ HIV I/II score kit (Fujirebio, Belgium), QIAmp viral RNA mini-kit (QIAGEN, Germany), absolute ethanol (Sigma-Aldrich, Germany), Ultrapure 10X TAE buffer (Thermo-scientific, U.S.A), Agarose LE (Thermo-scientific, U.S.A), cDNA

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synthesis and PCR kits (Roche Diagnostics, Germany), cycle sequencing kit (Applied Biosystems, U.S.A), Agencourt® CleanSEQ® Dye-Terminator Removal system (Agencourt Bioscience Corporation, U.S.A), ABI 3130xl genetic analyzer for sequence reading (Applied Biosystems, U.S.A), 100bp DNA marker (Thermo-scientific, U.S.A).

3.5 Plasma Sample Collection

Three (3) aliquots (500µl, 500µl and 1000µl) of the plasma portion of each blood sample was pipetted into cryovials and transported in cold boxes with ice packs to the Virology Department of Noguchi Memorial Institute for Medical Research (NMIMR) and stored at -30°C until further processing.

3.6 Laboratory Procedures

3.6.1 Confirmation of HIV-1 Infection

A confirmatory test (INNO-LIA™ HIV-I/II score, Fujirebio, Belgium) was done on all eighty-one (81) plasma samples collected from voluntary blood donors and previously found HIV positive by sandwich ELISA (HIV Ag/Ab 4th gen, Fortress Diagnostics, U.K.). The test procedure was based on manufacturer's protocol.

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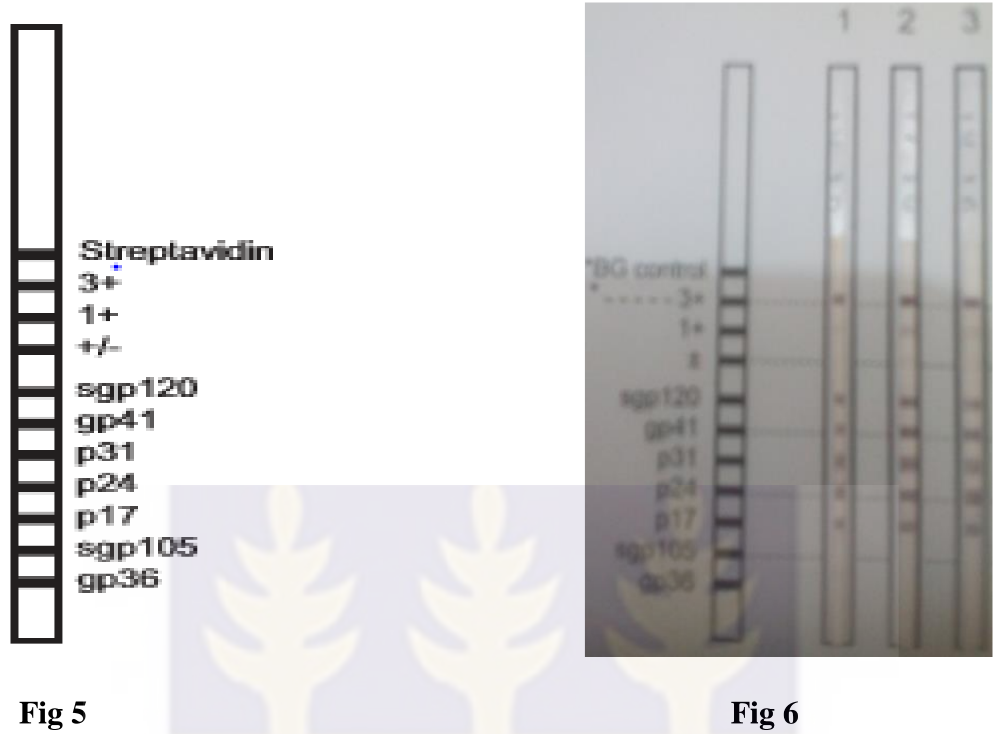

Fig 5 **Fig 6**

Figure 5: INNO-LIA HIV I/II Score reference strip

Figure 6: INNO-LIA results of study samples compared with reference strip

"*Figure 5 shows an INNO-LIA™ HIV I/II Score test strip with control bands (3+, 1+, +/) and test bands for HIV-1 (sgp 120, gp 41) and HIV-2 (sgp 105, gp 36). p31, p24, p17 may be cross-reacted in both HIV-1 and HIV-2. A line is considered as positive for an HIV type (HIV-1 or HIV-2) if a minimum score of 1+ is observed. In figure 6, samples 1, 2 and 3 were all confirmed as HIV-1 after comparing to the bands on the reference strip.*

(search.cosmobio.co.jp/cosmo_search_p/search_gate2/docs/IGT_/80540.20070926.pdf on November 11, 2016)."

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3.6.2 Viral Ribonucleic Acid (RNA) Extraction

Viral RNA was extracted using the QIAamp® viral RNA mini kit (QIAGEN, Germany) following manufacture's protocol. Sixty microlitres (60ul) of the RNA was eluted and stored at -30°c until use.

3.6.3 Complementary DNA (cDNA) Synthesis

A two-round reverse transcriptase (RT) method was used to generate complementary DNA (cDNA) of reverse transcriptase (RT) and protease (PR) genes of HIV-1 from extracted RNA using Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics, Germany). The reaction and cycling conditions were previously published with few modifications (Brandful, 2009).

3.6.4 Polymerase Chain Reaction (PCR) Amplification

Nested PCR was done to separately amplify the PR and RT genes from the cDNA synthesized using the Expand High Fidelity^{plus} PCR kit (Roche Diagnostics, Germany). The reaction mix was previously published with few modifications (Brandful, 2009).

Cycling conditions were as shown in table 2 below.

A fragment of 463 base pairs (bp) and 887 bp for the protease (PR) and reverse transcriptase (RT) genes respectively were generated after nested PCR and confirmed by agarose gel electrophoresis. Details of primers used in nested PCR are shown in Table 1.

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3.6.5 Agarose Gel Electrophoresis

PCR amplification products (amplicons) were evaluated on 2% agarose gel prepared with 1X Tris-acetate EDTA (TAE) buffer and ethidium bromide (Thermo-scientific, U.S.A). A total of 5µl PCR product in 6X loading dye (Thermo-scientific, U.S.A) for each sample was loaded alongside a 100bp DNA ladder, positive and negative controls for PR and RT genes. The electrophoresis run was set at 100 volts for 20 minutes.

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Table 1: Primers used to amplify and sequence the PR and RT genes of HIV-1

Name	Position/ Direction	Sequence (5'-3')	Purpose/ Target gene
DRPRO5	2074–2095 Forward	AGACAGGYTAATTTTTTAGGGA	Round 1 PCR / PR gene
DRPRO2L	2716-2691 Reverse	TATGGATTTTCAGGCCCAATTTTT GA	Round 1 PCR / PR gene
DRPRO1M	2148–2167 Forward	AGAGCCAACAGCCCCACCAG	Round 2 PCR / PR gene
DRPRO6	2611-2592 Reverse	ACTTTTGGGCCATCCATTCC	Round 2 PCR / PR gene
DRRT1L	2388-2410 Forward	ATGATAGGGGGAATTGGAGGTTT	Round 1 PCR / RT gene
DRRT4L	3425-3402 Reverse	TACTTCTGTTAGTGCTTTGGTTCC	Round 1 PCR / RT gene
DRRT7L	2485-2509 Forward	GACCTACACCTGTCAACATAATTG G	Round 2 PCR / RT gene
DRRT6L	3372-3348 Reverse	TAATCCCTGCATAAATCTGACTTG C	Round 2 PCR / RT gene
PRTS	2157–2177 Forward	AGCCCCACCAGAAGAGAGCTT	Sequencing/ PR gene
P3G	2198-2217 Forward	CAACTCCCTCTCAGAAGCAG	Sequencing/ PR gene
A2	2583–2601 Forward	TTAAAGCCAGGAATGGATG	Sequencing/ RT gene
RTSec1s	2692-2716 Forward	CAAAAATTGGGCCTGAAAATCCA TA	Sequencing/ RT gene
PRSec2A	2838-2811 Reverse	TGGGAAGTTCAATTAGGAATACC ACATC	Sequencing/ PR and RT genes

NB: "Primer positions are based on the reference HXB2 sequence of HIV-1 subtype B."

Primer sequences (Fujisaki *et al*., 2007; Villahermosa *et al.,* 2000).

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Table 2: Cycling conditions for PCR amplification of PR and RT genes of HIV-1

ROUND 1		ROUND 2	
Temperature/time	**Cycle**	**Temperature/time**	**Cycle**
94°C 4min	-	94°C 5min	-
94°C 30s,45°C 30s, 68°C 1min	10	94°C 30s,50°C30s,72°C 30s	35
94°C 30s, 50°C 30s, 68°C 2mins	10		
94°C 30s,58°C 30s,68°C 3mins	15		
68°C 7mins	-	72°C 7mins	-
Hold at 4°C	-	Hold at 4°C	-

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3.6.6 Purification of PCR Amplicons

The purification of nested PCR amplicons was done using QIAquick PCR purification kit (QIAGEN, Germany) following manufacturer's protocol. Purified amplicons were eluted in 50µl of elution buffer for cycle sequencing.

3.6.7 Cycle Sequencing

The BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, U.S.A) was used for the sequencing of PR and RT genes of HIV-1. A total reaction volume of 10µl comprising of 2µl each of a primer (P3G, PRTS for the PR gene and A2, RTsec1s, PRsec2A for the RT gene), BigDye terminator, BigDye terminator buffer, nuclease free water (NFW) and purified PCR product was used. The cycling conditions used for amplification were 94°C for 2 minutes followed by 25 cycles of 94°C for 30sec, 50°C for 15sec, and 60°C for 4 mins and cooled to 4°C. Details of primers used for sequencing are shown in Table 1.

3.6.8 Purification of cycle-sequenced products

The BigDye terminator mix used for cycle sequencing was removed with the Agencourt® CleanSEQ® Dye-Terminator Removal system (Agencourt Bioscience Corporation, U.S.A). The protocol used is based on manufacturer's instructions. The plate was loaded onto a genetic analyzer, ABI 3130xl (Applied Biosystems, U.S.A) to generate sequence data for HIV-1 drug resistance mutations and HIV subtype analysis.

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3.7 Sequence and Phylogenetic Analysis

Nucleotide sequences generated by the genetic analyzer for each sample were assembled using SeqManPro 13 (DNASTAR Incorporation, U.S.A). Consensus sequence was generated and aligned with a reference sequence (B-HXB2-PRT_2253-3700) in BioEdit (http://www.mbio.ncsu.edu/Bioedit/bioedit.html). Sequences were submitted to the Stanford University HIV Drug Resistance Database (https://hivdb.stanford.edu/hivdb/by-sequences/). This is an online database to store and analyze HIV drug resistance data for possible HIV drug resistance mutations and subtypes. The subtypes were confirmed with the Los Alamos National Laboratory HIV Database (http://www.hiv.lanl.gov). Neighbor-joining tree with the Kimura's 2-parameter distances was generated with the RT sequences using Molecular Evolutionary Genetic Analysis version 7.0 (MEGA7).

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CHAPTER FOUR

4.0 RESULTS

4.1 Study Population

A total of eighty-one (81) blood samples from voluntary blood donors that were sero-positive for HIV were obtained from October 2016 to April 2017 at the Southern Area Blood Center (SABC), Ghana.

4.2 Donor Information

Donor information such as age and gender were obtained from donor records upon approval from SABC (Table 3). Study numbers were assigned to anonymize the blood samples of the voluntary blood donors.

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Table 3: Demographic Characteristics of the Study Population

Variable	Frequency (%)
Age	
≤25	19 (23)
26-35	32 (40)
36-45	12 (15)
46-56	8 (10)
N/A	10 (12)
Gender	
Male	60 (74)
Female	11 (14)
N/A	10 (12)
Total	81 (100)

Table 3 shows the age and gender distribution of blood donors in this study. Most (40%) were between the ages of 26 to 35 years. Majority (74%) were males whilst 14% were females. Demographics for 12% of the study population were not available.

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4.3 Confirmation of HIV-1 in Study Samples

Out of 81 samples collected, 61 (75%) were confirmed as HIV-1 positive by INNO-LIA HIV I/II Score (Fujirebio, Belgium). Twenty samples representing 25% were found to be negative for HIV-1, HIV-2 or dual HIV-1/2 infection (figure 7). All HIV negative samples were stored at -80°C.

4.4 Nested PCR of Protease and Reverse Transcriptase Genes

Nested PCR was used to separately amplify the protease (PR) and reverse transcriptase (RT) genes of HIV-1 in each sample. Of the 61 samples confirmed, PR and RT genes were successfully amplified from 28 (50%) and 32 (53%) respectively. Figures 8 and 9 show electrophoregrams of amplification products of PR and RT genes respectively.

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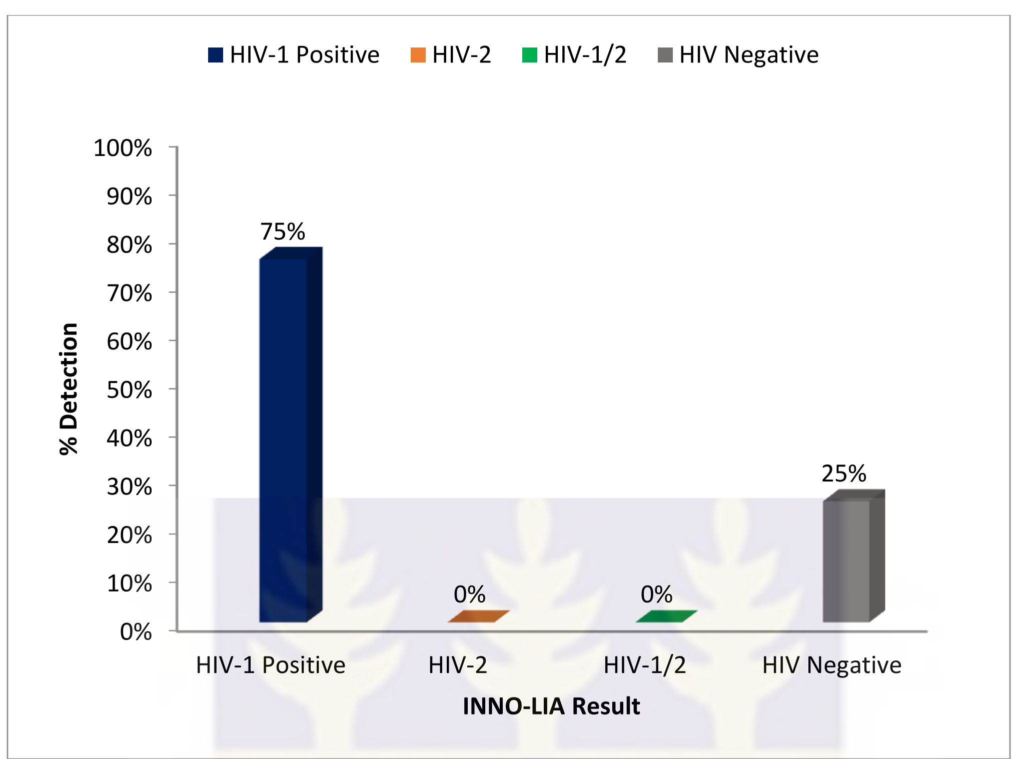

Figure 7: HIV-1 confirmation using INNO-LIA HIV-I/II score kit

"HIV-1 was confirmed in 61 (75%) of the samples whilst 25% were negative for HIV. There were no detection of HIV-2 and dual HIV-1/2 infections."

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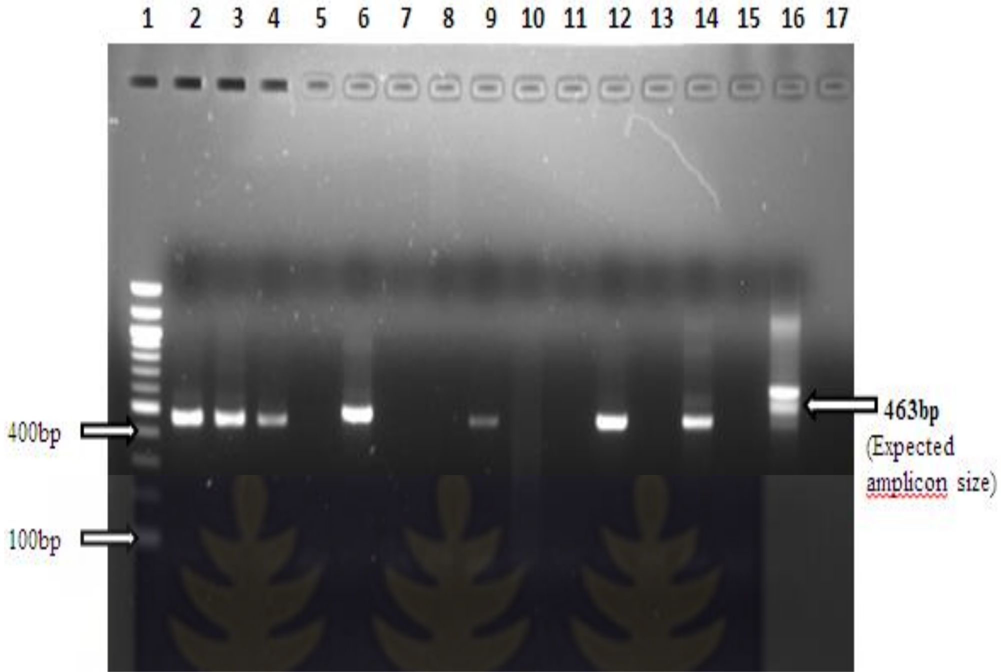

Figure 8: An Electrophoregram of Samples Amplified in the PR Gene

"2% Agarose gel was run using 1X TAE buffer. Lane 1 contained the 100bp DNA marker (Thermo-scientific, U.S.A). Lanes 2 to 15 contained study samples; Lane 16 contained a positive control and Lane 17 contained a negative control. The expected amplicon size was 463bp. Samples in lanes 2, 3, 4, 6, 9, 12 and 14 were successfully amplified for the PR gene. Failed amplification was observed in lanes 5, 7, 8, 10, 11, 13 and 15.Successful amplification of the PR gene was recorded to be 50.0%."

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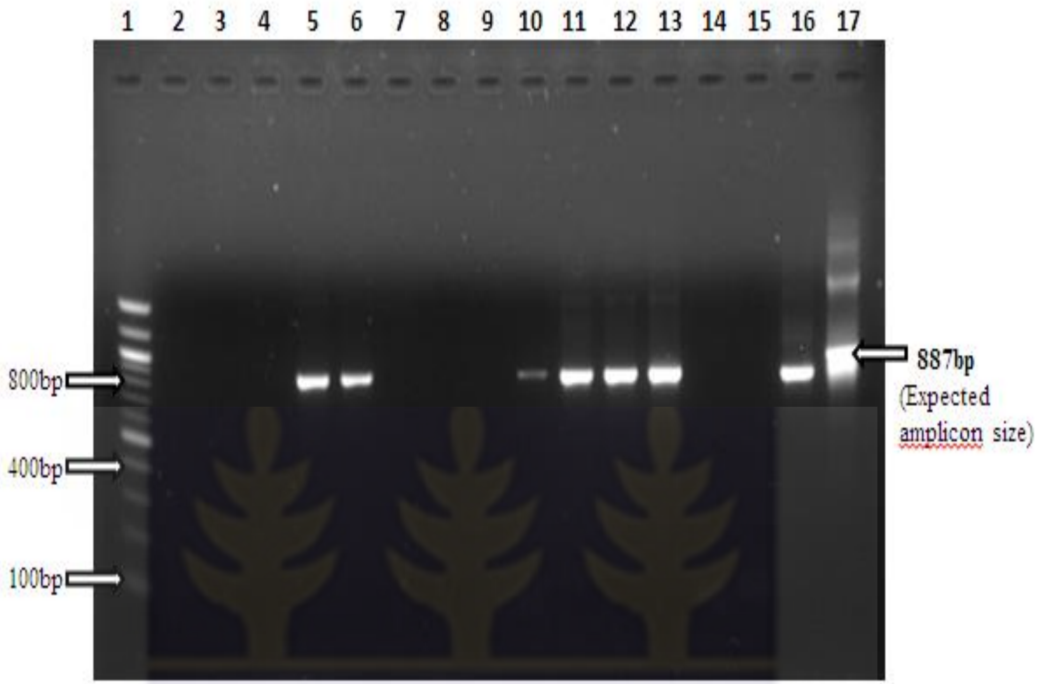

Figure 9: An Electrophoregram of Samples Amplified in the RT Gene

"2% Agarose gel was run using 1X TAE buffer. Lane 1 contained the 100bp DNA marker (Thermo-scientific, U.S.A). Lane 2 contained the negative control; Lanes 3 to 16 contained the study samples; Lane 17 contained a positive control. The expected amplicon size was 887bp. Samples in lanes 5, 6, 10, 11, 12, 13 and 16 were successfully amplified for the RT gene. Failed amplification was observed in lanes 3, 4, 7, 8, 9, 14 and 15. Successful amplification of the RT gene was recorded to be 53.0%."

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4.5 Sequencing

Out of the samples successfully amplified, 13/28 (46%) and 20/32 (63%) were successfully sequenced for the PR and RT genes respectively (Table 4). The sequences were edited and submitted to the Stanford University HIV Drug Resistance Database (HIVdb) to provide information on any HIV drug resistance mutation (DRM) present. The number of sequences with drug resistance mutations for the available antiretroviral drug classes (PI, NRTI, NNRTI) is shown in Table 4.

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Table 4: Drug Resistance Mutations in Successfully Sequenced Samples

Gene	Samples Sequenced	DRMs Present
PR	13	PI= 1
RT	20	NRTI= 3 NNRTI= 2

Direct sequencing was done for all the positive amplicons of PR and RT. The number of successful sequencing for PR and RT are shown. Only one (1) sample had minor drug resistance mutation (DRM) in the PR gene whilst a total of five (5) had DRM in the RT gene.

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4.6 Mutational Analysis for PR and RT-Related Drug Resistance

Of the samples sequenced in the PR and RT genes, minor PR-related DRM was observed in only one (1) sample whilst major drug resistance mutations in two (2) samples were found; E138A in one sample and another with K65R. Drug resistance implications of these mutations are shown for each participant in Table 5.

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Table 5: Drug Resistance Mutations (DRMs) and Implications on Available Drug Regimen

Study ID	Subtype	DRM		ARV
		PR	RT	
BD 003	CRF02_AG	-	F77L	ABC, AZT, D4T, DDI, TDF, 3TC, FTC
BD 030	CRF02_AG	L10F	-	FPV/r, IDV/r, NFV, LPV/r, DRV/r
BD 034	B	-	D67E	ABC, AZT, D4T, DDI, TDF
BD 039	CRF02_AG	-	**E138A**	ETR, RPV
BD 063	CRF02_AG	-	V179E	NVP,EFV, ETR, RPV
BD 077	B		**K65R**	ABC, AZT, D4T, DDI, TDF

NB: A: Alanine; D: Aspartate; E: Glutamate; F: Phenylalanine; L: Leucine; V: Valine; K: Lysine; R: Arginine; Boldened DRMs (E138A, K65R) indicate major drug resistance mutations.

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4.7 HIV-1 Subtype Analysis

HIV-1 subtypes were analyzed in both the PR and RT genes. Out of the 13 PR sequences, 1 (8%) was subtype A, 2 (15%) each were subtype B and 10 (77%) were CRF02_AG. Of the 20 RT sequences, 2 (10%) were subtype A, 8 (40%) were subtype B, 9 (45%) were CRF02_AG and 1 (5%) was CRF09_cpx (Figure 10).

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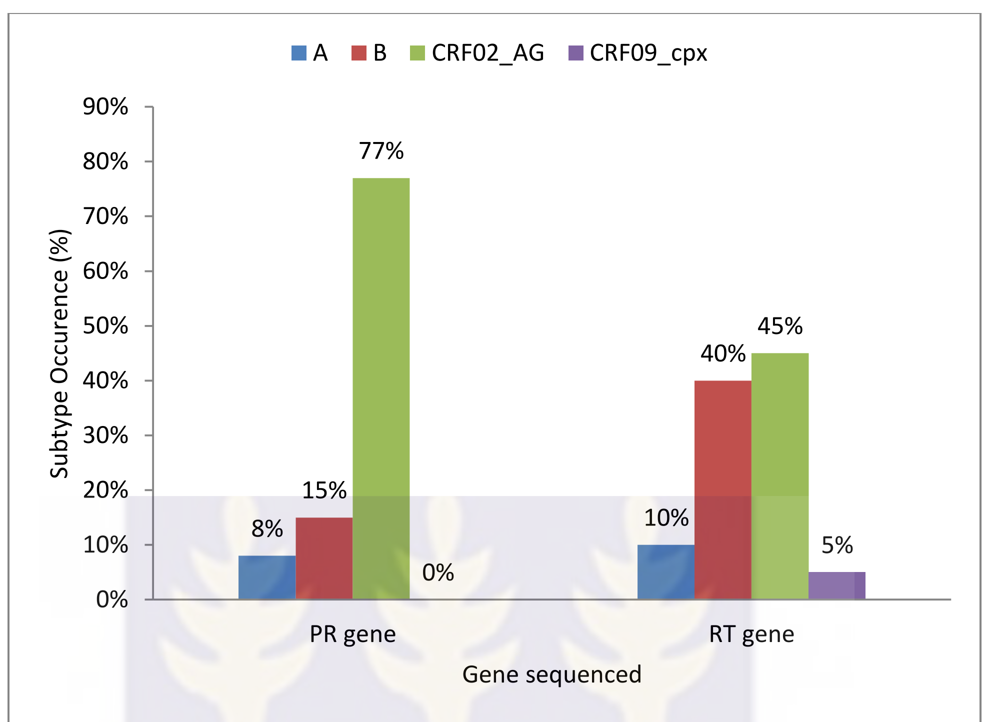

Figure 10: HIV-1 Subtype Pattern in the PR and RT Genes

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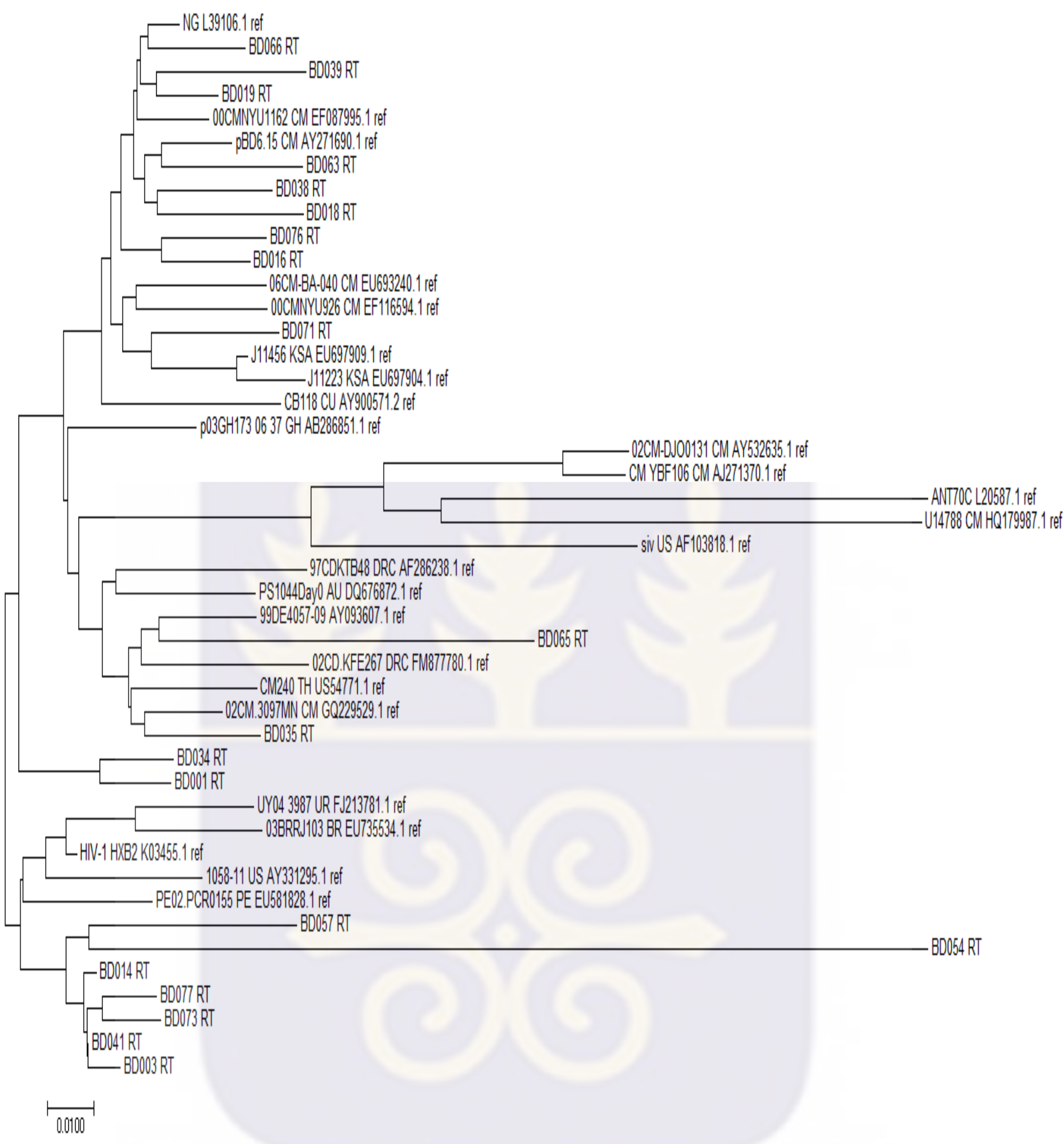

Figure 11: Phylogenetic analysis of RT sequences

"Neighbor-joining tree with the Kimura's 2-parameter distances was generated with the RT sequences using Molecular Evolutionary Genetic Analysis version 7.0 (MEGA7). IDs with suffix 'RT' are study samples and those with suffix 'ref' are reference sequences used to infer evolutionary relationships. Study sequences clustered around reference sequences from Nigeria, Cameroon, Kingdom of Saudi Arabia, Democratic Republic of Congo, United States of America, Peru, Uruguay and Britain."

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CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

5.1.1 General Information

This study investigated transmitted drug resistance mutations (TDR) and subtype diversity (SD) among voluntary blood donors. Donated blood that was found to be HIV-1 sero-positive by HIV Ag/Ab 4th gen (Fortress Diagnostics, U.K) was selected for this study.

Transmitted drug resistance (TDR) studies encourage recruiting recently infected (asymptomatic) individuals other than long-term infected persons. This is because resistant viruses may become minor quasispecies in long-term infections and not detected by Sanger sequencing (Booth and Garetti, 2007). Unlike developed countries where serological and nucleic acid testing is readily available to differentiate recent and long-term infections, parameters such as age and first pregnancy status is recommended in selecting pregnant women; voluntary donations and asymptomatic conditions are considered as proxies for recent infections and in recruiting donors for TDR studies in resource-limited countries (W.H.O., 2003). In Ghana, persons eligible to donate blood satisfy an extensive medical and physical examination to rule-out risk factors and other clinical features of infectious diseases including HIV. Due to exclusion criteria used, people at risk may be discouraged from voluntary donation and hence a possibility of recruiting greater proportion of recently infected persons in this study.

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With reference to the demographics, the proportion of males to females with HIV-1 in this study does not suggest a similar proportion of HIV-1 nationwide. The same could be said for the distribution of HIV infection across the age groups. Although, the number of females infected with HIV nationwide are more than males; voluntary blood donors are usually males. Females are usually disqualified due to relatively lower haemoglobin (HB) level that arises partly because of monthly blood loss during menstruation and the toll of pregnancy. Also, it is active men usually between the ages of 25- 50 who volunteer to donate blood. Thus, these reasons could have accounted for the pattern seen.

Of the eighty one (81) samples screened as HIV-1 by ELISA, sixty one (61) samples were confirmed as HIV-1 with no HIV-2 or dual HIV-1/2 infections. This correlates with studies reporting HIV-1 infection as the predominant HIV type in Ghana (Brandful *et al.,* 1997; Bonney *et al.,* 2013). The twenty (20) samples that were later confirmed to be HIV negative implicates the need for diagnostic methods with higher sensititivity to minimize the probability of transfusing infected blood. (Pesce *et al*., 2006).

Amplification of a 463bp and 887bp fragments of the PR and RT genes respectively was achieved by nested PCR. The nested PCR tends to further amplify the gene fragments from the first round PCR thereby increasing the amplicon numbers for successful downstream sequencing. In addition, it increases the specificity by eliminating non-specific fragments that are due to random primer binding.

The amplification success of PR and RT genes were 50.0% and 53.0% respectively. The low amplification rate may be due to the low viral load observed for some samples. However, it is not entirely the reason for low amplification rate as samples with low viral loads were successfully amplified whilst others with higher loads not amplified. Previous

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research shows that samples from patients with persistently low viral load could be genotyped by a nested PCR method (Mackie *et al.*, 2004).

The inability to obtain peripheral blood mononuclear cells (PBMC) to amplify alongside the plasma could also account for a lower amplification rate. In a study by Kabamba-Mukadi *et al*. (2009), amplification success with proviral DNA from PBMC was relatively higher than viral RNA from plasma. Genotyping from plasma RNA and proviral DNA concurrently could have increased amplification success since some plasma samples may be amplified and not their PBMC samples and vice versa (Bonney *et al.*, 2013).

Nucleotide sequencing of amplified genes was done to determine any HIV-1 drug resistance mutations and assess the subtype diversity. Not all amplified samples for PR and RT were successfully sequenced. In some cases, sequence data was bad with a lot of background "noise" and made it difficult to edit and analyze. Using different sequencing platforms such as the next generation sequencing other than the Sanger method of sequencing used in this study could have produced sequences with better base calling.

5.1.2 Drug Resistance Mutations

The first objective of this study was to document the presence of any mutations that confer resistance to available antiretroviral drug regimen among the voluntary blood donors studied. HIV-1 transmitted drug resistance (TDR) occurs when recently infected individuals who are not exposed to antiretroviral drugs habour drug resistant viruses. In

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this study, HIV-1 TDR is defined at least the presence of one major HIV-1 drug resistance mutation (DRM) in a study participant.

The E138A mutation is a polymorphic mutation that occurs in an appreciable number of drug-naïve patients such as the population studied in this work and confers resistance to etravirine (ETR) and rilpivirine (RPV) which are NNRTIs (Haddad *et al.*, 2011; Tambuyzer *et al.,* 2011). In a similar study, E138A was found in ART-naïve pregnant women attending antenatal care in a teaching hospital in Accra, Ghana (Martin-Odoom *et al.,* 2017). The mutation is due to a substitution of alanine (A) for glutamic acid (E) at position 138 in the RT gene. The mutation has been found to reduce ETR and RPV susceptibility by 2-folds.

The K65R mutation is found to reduce the susceptibility of most NRTIs. It is due to a substitution of lysine (K) for arginine (R) at position 65 of the RT gene. It reduces tenofovir (TDF), abacavir (ABC), didanosine (DDI) susceptibility by 2-fold and stavudine (d4T) by 1.5 fold (Petropoulos *et al.,* 2000). However, K65R increases zidovudine (AZT) susceptibility and hence reduces viral replication (Stephan *et al.*, 2010).

These results are similar to other studies previously conducted among drug naïve populations in Ghana where no PR-related DRM was found (Delgado *et al.,* 2008; Martin-Odoom *et al.,* 2017). However, two other similar studies found only one participant in the respective studies with PR-related DRM (Brandful *et al.,* 2012; Bonney *et al.,* 2013). Generally, low level of protease inhibitor related DRMs has been found in the Ghanaian population. This could be attributed to the high genetic barrier of protease inhibitors and that the virus would have to mutate several times to develop resistance to

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such drugs. Also, sparing use of protease inhibitors since they are reserved for use mostly in the second-line therapy and majority of those on treatment are on first-line drugs. Thus PI mutant strains are less frequently acquired and so rarely passed on during virus transmission.

5.1.3 Subtype Diversity and Phylogenetic Analysis

To determine the predominant HIV-1 subtype among the drug-naïve voluntary blood donors was the second study objective. The overall percentage of subtypes in the samples sequenced in both the PR and RT genes revealed the circulating recombinant form, CRF02_AG as most predominant subtype in the study population followed by subtypes B, A and CRF09_cpx. This result is similar to some studies conducted in West Africa (Mamadou *et al.,* 2002; Njai *et al.,* 2006) and in Ghana (Delgado *et al.,* 2008; Brandful *et al.,* 2012; Bonney *et al.,* 2013; Sagoe *et al.,* 2016). The CRF02_AG has subtypes A and G as parent subtypes. Its predominance has been associated with high viral infectivity and productivity (Nii-Trebi *et al.,* 2016), possible replicative fitness (Njai *et al.,* 2006) and high viral loads which favour viral transmission (Fischetti *et al.,* 2004).

Subtype B was the next predominant subtype and was relatively frequent compared to a previous studies in Ghana (Delgado *et al*., 2008). Subtype B is most predominant in the Americas (Louwagie *et al.,* 1994; Louwagie *et al.,* 1995) and Western Europe (Deroo *et al.,* 2002; Tatt *et al.,* 2004). Although, travel history of the voluntary donors in this study were not obtained in their donor records, some may have travelled to the Americas and Europe and have led to the importation of subtype B.

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Subtypes A and CRF09_cpx share ancestors with the predominant CRF02_AG subtype in West Africa. Subtype A is a parental subtype of CRF02_AG whilst CRF09_cpx is a recombinant of subtypes A, F and G (McCutchan *et al.,* 2004). Their presence as less frequent subtypes in the West African region including Ghana is therefore not surprising.

The dynamic nature of subtype predominance in different geographical areas has been linked to socio-epidemiologic factors such as mobility and migration (Tebit and Arts, 2011; Hemelaar, 2012). These factors, however, cannot be confirmed for this population due to lack of data on residence and citizenship among the participants studied. Phylogenetic analysis of study sequences using the Neighbour-joining method revealed evolutionary relationship with reference sequences from Nigeria, Cameroon, Kingdom of Saudi Arabia, Peru, Uruguay and Britain.

5.1.4 Study Limitations

- Low amplification and sequencing rate made it difficult to draw conclusions on all 61 samples.
- Inability to obtain extensive information such as residence, citizenship, and travel history on study participants limits possible association with subtype pattern.

5.2 CONCLUSION

This study has found major drug resistance mutations, E138A and K65R in the RT gene that confer high level resistance to most NNRTIs and NRTI respectively. These mutations, found in 10% of the study population, indicate an increasing trend in TDR in

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Ghana. Although, CRF02_AG was most predominant, the recorded percentage of subtype B and the evolutionary relationship inferred by phylogenetic analysis suggest possible subtype importation. The data will inform the selection of drugs for ART initiation to maximize therapeutic options in drug-naïve HIV-1 patients in Ghana.

5.3 RECOMMENDATIONS

- Surveillance of drug resistance mutations and subtype diversity should be continued for effective ART outcomes.
- Ghana should include more population groups such as voluntary blood donors in the HIV sentinel survey to maximize the success of antiretroviral treatment.

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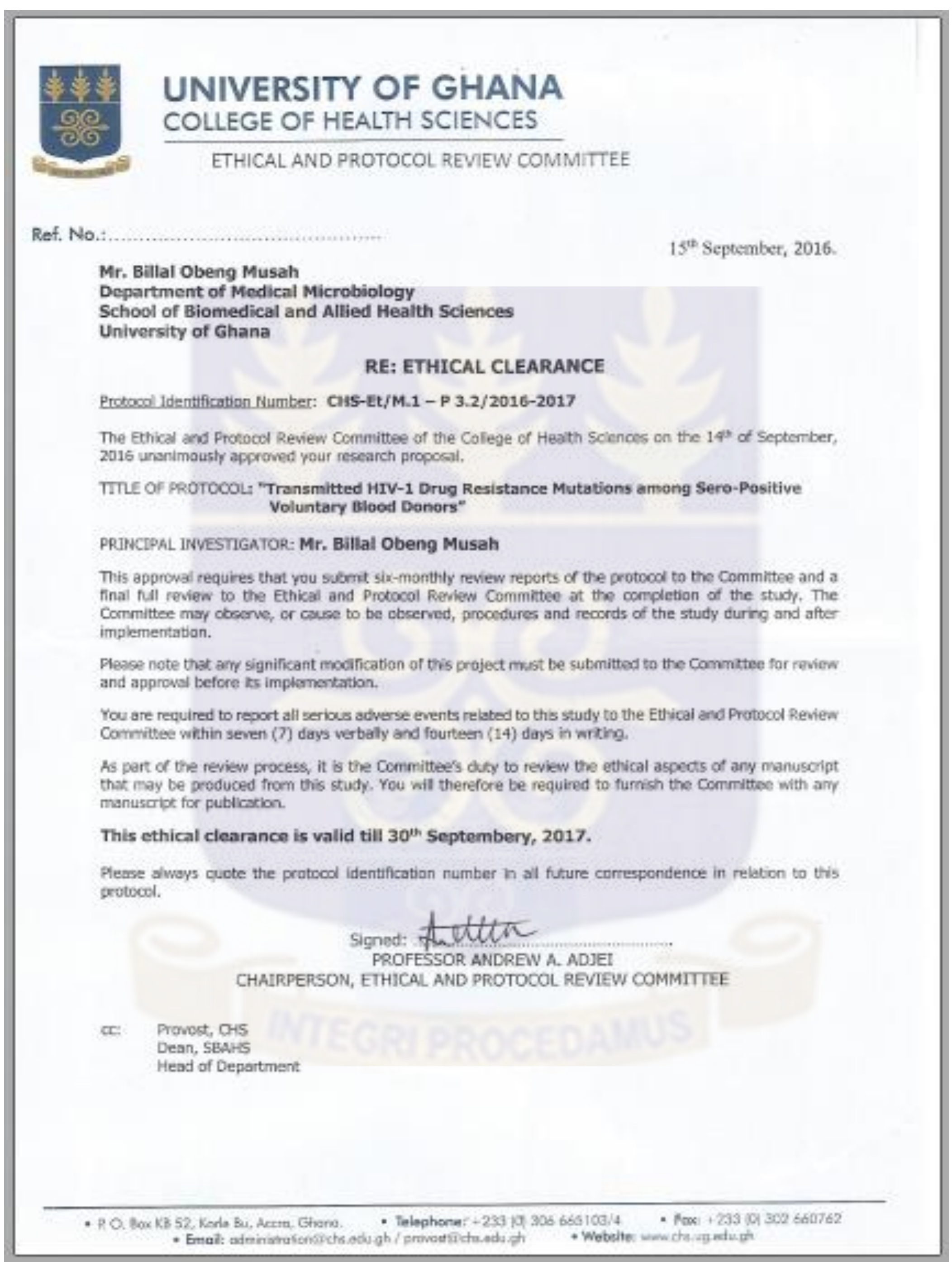
APPENDICES

Appendix A: Ethical Clearance Certificate from the Ethical and Protocol Review Committee (EPRC), College of Health Sciences, University of Ghana.



UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

Ref. No.:..

15th September, 2016.

Mr. Billal Obeng Musah
Department of Medical Microbiology
School of Biomedical and Allied Health Sciences
University of Ghana

RE: ETHICAL CLEARANCE

Protocol Identification Number: **CHS-Et/M.1 – P 3.2/2016-2017**

The Ethical and Protocol Review Committee of the College of Health Sciences on the 14th of September, 2016 unanimously approved your research proposal.

TITLE OF PROTOCOL: **"Transmitted HIV-1 Drug Resistance Mutations among Sero-Positive Voluntary Blood Donors"**

PRINCIPAL INVESTIGATOR: **Mr. Billal Obeng Musah**

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 30th Septembery, 2017.

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

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

cc: Provost, CHS
Dean, SBAHS
Head of Department

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University of Ghana http://ugspace.ug.edu.gh

Appendix B: Institutional Approval Certificate from the National Blood Transfusion Service, Ghana.

NATIONAL BLOOD TRANSFUSION SERVICE

Republic of Ghana

Post Office Box KB 78
Korle-Bu, Accra

Our Ref: NBTS/RES-76/RDAL-02

Your Ref: ....

Date: 12TH OCTOBER, 2016

Mr. Billal Obeng Musah
School of Biomedical & Allied Health Science
Department of Medical Microbiology
University of Ghana

Dear Mr. Billal Obeng Musah,

Re: Research Protocol (NBSGRD/160929/01) "Transmitted HIV-1 Drug Resistance Mutations among Sero-Positive Voluntary Blood Donors"

Thank you for your letter seeking approval to conduct the above research at the Southern Area Blood Centre. You completed the online registration form and provided the following documents for consideration;

- Project Proposal
- Ethical Clearance
- Data Collection Sheet

These documents have been considered and your project has been approved for the collection of Five (5) ml stored HIV-1 positive blood donor samples and anonymized donor data from the Blood Centre by 31st December, 2016.

Approval is conditional upon:

- Continued adherence to NBSG approved operating procedures.
- Adherence to all ethical requirements.
- Provision of notification of when the sample collection commences and ends.

You are required to submit a copy of the final report once the study is completed.

Yours sincerely,

Dr. Lucy Asamoah-Akuoko
Head, Research & Development
E-mail: lucyasamoah@yahoo.com

Cc: Ag. Head, SABC
Laboratory Services Manager
Research Officer, R&D

INTEGRI PROCEDAMUS

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