HIV-1 DRUG RESISTANCE PROFILES OF GHANAIAN WOMEN ON ANTIRETROVIRAL THERAPY AFTER PREVENTION OF MOTHER-TO-CHILD TRANSMISSION (PMTCT)

By

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This thesis is submitted to the University of Ghana, Legon in partial fulfillment of the requirement for the award of PhD Microbiology Degree.

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DECLARATION

I hereby declare that this thesis is a result of my own research work carried out under the supervision of Dr. Theophilus K. Adiku , Professor William Kwabena Ampofo, Professor Margaret Lartey, and Dr. Elena Delgado Blanco and that references made to other people's work have been duly acknowledged. I further declare that this work has not been submitted in part or whole to any other institution for the purpose of acquiring a degree.

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DEDICATION

I dedicate this thesis to my wife, Eunice Martin-Odoom, and my sons, Jasiel and Kadmiel Martin-Odoom.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS/ABBREVIATIONS

1. ABC:ABACAVIR
2. AIDS: ACQUIRED IMMUNE DEFICIENCY SYNDROME
3. ART: ANTIRETROVIRAL THERAPY
4. ARVs: ANTIRETROVIRALS(ANTIRETROVIRAL DRUGS)
5. CCR3 :C-C CHEMOKINE RECEPTOR TYPE 3
6. CCR5 :C-C CHEMOKINE RECEPTOR TYPE 5
7. CD4: CLUSTER OF DIFFERENTIATION TYPE 4
8. CDC : CENTERS FOR DISEASE CONTROL & PREVENTION
9. CRF: CIRCULATING RECOMBINANT FORMS
10. CXCR4: C-X-C CHEMOKINE RECEPTOR TYPE 4
11. D4T : STAVUDINE
12. DDI : DIDANOSINE
13. DNA:DEOXYRIBONUCLEIC ACID
14. EDTA: ETHYLENEDIAMINE TETRA-ACETIC ACID
15. EFV : EFAVIRENZ

16. eMTCT: ELIMINATION OF MOTHER-TO-CHILD TRANSMISSION

17. ETR ETRAVIRIDINE

18. FDA: FEDERAL DRUG AUTHORITY,USA

19. FTC :EMTRICITABINE

20. GP: GLYCOPROTEIN

21. HAART: HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

22. HIV-1:HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

23. HIV-2: HUMAN IMMUNODEFICIENCY VIRUS TYPE 2

24. IAS : INTERNATIONAL AIDS SOCIETY

25. 3TC: LAMIVUDINE

26. MTCT: MOTHER-TO-CHILD-TRANSMISSION

27. NACP: NATIONAL AIDS/STI CONTROL PROGRAMME

28. NASBA: NUCLEIC ACID SEQUENCE BASED AMPLIFICATION

29. NATS: NUCLEIC ACID BASED TESTS

30. NCBI: NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION

31. NNATS: NON-NUCLEIC ACID BASED TESTS

32. NFV : NELFINAVIR

33. NMIMR: NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH

34. NNRTIs: NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

35. NRTIs: NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

36. NtRTIs : NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITORS

37. PCR: POLYMERASE CHAIN REACTION

38. PIs : PROTEASE INHIBITORS

39. PLWHA: PEOPLE LIVING WITH HIV/AIDS

40. PMTCT: PREVENTION OF MOTHER-TO-CHILD-TRANSMISSION

41. *pol :* POLYMERASE GENE

42. PR: PROTEASE

43. RNA: RIBONUCLEIC ACID

44. RT-PCR: REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

45. RT: REVERSE TRANSCRIPTASE

46. TDF : TENOFOVIR

47. UNAIDS: UNITED NATIONS JOINT PROGRAMME ON HIV/AIDS

48. WHO: WORLD HEALTH ORGANIZATION

49. ZDV : ZIDOVUDINE

ABSTRACT

From 2007 to 2011 HIV-positive pregnant women in Ghana, were given a combination of Zidovudine and Lamivudine as prophylaxis during pregnancy and a single dose of Nevirapine upon the onset of labour towards the prevention of mother to child transmission (PMTCT) of HIV. Since these women were subsequently given the same antiretrovirals (ARVs) to manage their HIV infection, there was therefore the need to determine the HIV-1 drug resistance profiles in such patients in Ghana. This study sought to investigate HIV-1 drug resistance associated mutations present in mothers on ART who had previously received ART prophylaxis for PMTCT in selected centres in Ghana.

Genotypic Drug Resistance Testing for HIV-1 was carried out on study participants to identify any HIV-1 drug resistance associated mutations present and HIV viral load and CD4 counts were also measured.

There were three categories of HIV-1 patients encountered in this study- Concordant Responders (had increased CD4 with viral suppression to undetectable levels), Immunological Responders (had increased CD4 but with detectable viremia) and Immunological Non-Responders (had decreased CD4 with viral suppression to undetectable levels).

Participants who had prophylaxis before HAART, those who had HAART without prophylaxis and the drug-naïve participants had 32% (8), 5% (3) and 15% (4) HIV-1 drug resistance associated mutations (DRAMs) respectively.

Thirty-five percent (35%) had nucleoside reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance associated mutations(RAMs) in the RT gene and 1 (3%) had RAMs in the PR gene for PIs. The most common NRTI mutation found was M184V; K103N and A98G were the most common NNRTI mutation found in this study. Thymidine Analogue Mutations (TAMs) such as M41L, D67N, K70R, L210W, T215Y/F and K219E were found in all the groups; the most common of the TAMs found in the study is M41L and T215Y. The major resistance mutation to Protease Inhibitors (PIs) seen in the study was I84V. Two HIV-1 drug-naïve patients in the Eastern region had mutations that conferred resistance to both nucleoside reverse transcriptase inhibitors (M184V and L210W) and non-nucleoside reverse transcriptase inhibitors (K103N and V106A).

For the Reverse Transcriptase gene, 33(83%) of samples were of subtypes CRF02_AG, 2(5%) were subtype CRF01_AE, 1(3%) was subtype A, 2 (5%) were subtype B and 2 (5%) were subtype G. With the Protease gene, 32 samples (97%) were subtype CRF02_AG and 1(3%) was subtype A.

HIV-1 positive Ghanaian mothers who had prophylaxis prior to initiating ART had immunological, virological and HIV-1 drug resistance associated mutations profiles that have adverse effect on their clinical profiles, resulting in sub-optimal responses to ART. Emergence of transmitted HIV-1 drug resistance could be on the increase and needs to be monitored.

Drug resistance testing and early initiation of treatment upon diagnosis would help to produce a better treatment outcome for Ghanaian HIV-1 positive mothers and pregnant women.

The study has provided useful baseline information on the profiles of Ghanaian women on ART after prophylaxis.

CHAPTER 1

1.0 INTRODUCTION

1.1 Background

Antiretroviral therapy (ART) was started in Ghana in 2003 and has gone through a number of revisions to provide appropriate health care and support for HIV positive persons across the whole country (Ghana Health Service, 2008).

The National AIDS/STI Control Programme (NACP) of the Ghana Health Service implemented various research-backed interventions to monitor drug resistance known to arise in HIV patients through the use of the Antiretrovirals (ARVs) (Ghana Health Service, 2011). The emergence of HIV drug resistance viral strains is a major obstacle in the effective management of HIV infection and AIDS. Drug resistant strains may develop due to exposure to drugs but drug naïve persons could also be infected with drug-resistant strains (Clavel and Hance, 2004). The Ghana HIV Drug Resistance (HIVDR) Threshold Survey was initiated in 2007 (Bonney *et al*, 2013) to generate information on the presence of HIV drug-resistant strains in the locality where Ghana's ART for HIV was first introduced. It was also to seek information on active transmission of HIV drug-resistant strains in drug-naïve persons in the country so as to signal action to address transmitted HIV drug resistance (HIVDR) in Ghana. A Survey of Emergence of HIV Drug Resistance was also initiated by the NACP to monitor the emergence of HIV drug resistance in Ghana amongst patients initiating antiretroviral therapy (ART). These two surveys were designed to monitor the impact of HIV-1 drug resistance on the ART programme.

However, a group of patients fall in a grey area not covered directly by the two surveys. This group comprised HIV positive women given prophylaxis to prevent the transmission of HIV to their babies during pregnancy. Bearing in mind the emergence of drug resistance in the face of antiretroviral pressure, these women were taking the same ARVs they had been given during the phase of preventing the transmission of the infection from the mother to the infant. The effectiveness of the ARVs with such a background was becoming questionable in the absence of data on the resistance profiles of these women.

This study, "the HIV-1 Drug Resistance Profiles of Ghanaian women on Antiretroviral Therapy after Prevention of Mother-to-Child Transmission" was thus designed to provide data on the characterization of HIV-1 drug resistance present in Ghanaian women.

1.2 History of HIV Infection

Human Immunodeficiency Virus (HIV) infection has become a worldwide pandemic since the confirmation of HIV as the etiologic agent of AIDS in 1983; the first case of AIDS was reported in 1981 among a colony of homosexual men (CDC, 1982). Intense research activity carried out resulted in the development of serologic assays to test for HIV presence in asymptomatic people, to identify new infections and to screen blood donations by 1985 (Coffin *et al*, 1986). In 1987, the first anti-HIV drug, Zidovudine or Azidothymidine (AZT) was approved by the Food and Drugs Administration (FDA) in the United States of America (USA) for the treatment of AIDS (Fischl *et al*, 1987). Between 1988 and 1996, nucleoside reverse transcriptase inhibitors such as Stavudine (d4T) in 1994 and Lamivudine (3TC) in 1995 were also approved (aegis.com.2000).

Nevirapine, a non-nucleoside reverse transcriptase inhibitor was approved in 1996 to launch the era of a combination cocktail of three antiretroviral (ARV) drugs called Highly Active Antiretroviral Therapy (HAART) (aegis.com.2000). Despite considerable advances made in the virology of HIV and the clinical management of the infection, the AIDS pandemic claimed an estimated 3.1 million (between 2.4 and 3.3 million) lives in 2005 of which more than half a million (570,000) were children (UNAIDS, 2006) . In 2007, 30.8million (between 30.6 and 36.1 million) adults were believed to live with HIV which killed an estimated 2.1 million people that year, including 330,000 children (UNAIDS, 2013).

It has been reported that globally 35.3million people were living with HIV/AIDS at the end of the year 2012; 17.7million of these people were women (UNAIDS, 2013). These facts are detailed in Table 1 overleaf.

Table 1: Global Summary of the HIV/AIDS Epidemic from 2005 to 2012 (UNAIDS, 2013).

YEAR	2005	2006	2007	2008	2009	2010	2011	2012
PLWHA	32.5M	32.8M	33.2M	33.5M	34.0M	34.4M	34.9M	35.3M
Adults	30.2M	30.5M	30.7M	31.4M	31.5M	31.0M	31.6M	32.0M
Women	17.5M	17.7M	15.4M	15.7M	17.7M	16.3M	16.7M	17.7M
Children	2.3M	2.3M	2.5M	2.1M	2.5M	3.4M	3.3M	3.3M
NEW INFECTION	2.9M	2.8M	2.7M	2.6M	2.6M	2.5M	2.5M	2.3M
Adults	2.3M	2.3M	2.2M	2.2M	2.2M	2.2M	2.2M	2.0M
Children	540,000	520,000	480,000	450,000	400,000	360,000	310,000	260,000
DEATHS	2.3M	2.3M	2.2M	2.1M	2.0M	1.9M	1.8M	1.6M
Adults	2.6M	2.6M	1.7M	1.7M	1.7M	1.55M	1.5M	1.4M
Children	570,000	380,000	330,000	280,000	260,000	250,000	230,000	210,000

KEY: M= MILLION

HIV was first reported in Ghana in 1986 (Neeqyuaye *et al*, 1987). In 2008, the total number of Ghanaians infected by the virus was 236,151 rising to 267,069 in the year 2009 with a prevalence of 1.9%. There were 25,531 new infections in the country (NACP, 2010). In 2011, the estimated number of persons in Ghana living with HIV and AIDS was 225,478 comprising 125,141 females and 100,336 males. At the end of December, 2012 the total number of the HIV population was 208,248 comprising 120,724 females and 87,524 men (NACP, 2013).This indicates a decreasing trend in the number of HIV-infected persons.

The total number of deaths in 2012 among adults due to HIV/AIDS was 10,035 comprising 5,182 males and 4853 females (NACP, 2013). The HIV Sentinel Survey Report for 2013 indicates a decreasing trend in HIV infections and AIDS related deaths. The total number of people living with HIV/AIDS (PLWHA) at the end of 2013 for adults above 15 years was 189,932, comprising 75,023 males and 114,909 females. The total number of AIDS related deaths for 2013 was 7826, comprising 4,128 males and 3,698 females (NACP, 2014).

1.3 Treatment for HIV

1.3.1 Antiretroviral Therapy (ART)

Application of advanced knowledge and understanding of the biology of HIV and its pathogenesis has led to a significant reduction of the mortality and morbidity associated with the HIV/AIDS pandemic, making it a treatable, chronic disease. Though currently there is no available cure for HIV infection, a combination of antiretroviral (ARV) drugs used in treatment and referred to as 'highly active antiretroviral therapy' (HAART), is applied to maximally suppress the virus and halt the HIV infection progressing to AIDS (Moore *et al*, 2005; Piketty *et al*, 2001). The expanded access to ART globally has helped reduce the transmission of HIV at the population level, positively impacted on orphan-hood and helped to preserve more families with persons living with HIV/AIDS (PLWHA) (Tuboi *et al*, 2007). According to a WHO and UNAIDS Report in 2012, an estimated 34.4 million people were living with HIV in 2011, with at least 15 million of these people being in need of antiretroviral therapy out of which 8 million people had access to ART in low- and middle-income countries. Research has shown that when ART is initiated before the advanced disease stage of HIV, it reduces considerably plasma HIV RNA concentrations to undetectable levels in most patients who adhere to the treatment (Philips *et al*, 2007). Use of ARVs in treatment of HIV infection is intended to stop the replication of the virus, restore or maintain the patient's immune function, prevent mother-to-child transmission in pregnant women and to improve the quality of life for the patient. ART comprises different classes of ARVs which attack different stages of HIV replication; these are Nucleoside Reverse Transcriptase Inhibitors (NRTIs), Non-

Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), Protease Inhibitors (PIs), Fusion Inhibitors (FIs), CCR5 Inhibitors and Integrase Inhibitors (Clavel and Hance, 2004).

The combination of these drug classes prevents viral replication via more than one mechanism to minimize the occurrence of viral mutations which would escape the inhibitory action of the drugs. HIV-infected persons are not put on treatment with ARVs when their CD4+T cell count and clinical assessment indicate minimum risk of disease progression (Ghana Health Service, 2011). Such individuals are monitored for changes in their CD4 counts, which is a reflection of the person's immune status and necessitates initiation of ART. Antiretroviral Therapy is initiated in Ghana when the patients'CD4 count is less than 350 cells/µL (Ghana Health Service, 2011). The efficacy of an ART regimen should be monitored by determining the HIV viral load and CD4 count regularly. A decline in HIV viral load with an increase in the CD4 count is indicative of the success of the treatment (WHO HIV/AIDS Programme, 2006).

1.3.2 Prevention with ART

It is an accepted fact that ART has preventive effects in the transmission of HIV; it was reported in 2009 that universal voluntary HIV testing on an annual basis followed by immediate ART could reduce HIV incidence by about 95% within a decade (Granich *et al,* 2009)**.** Studies had proven that early treatment of HIV-infected people with antiretrovirals protected 96% of discordant partners from the infection (NIAID, 2011; Anglemyer *et al*, 2011).When post-exposure prophylaxis is initiated within 48-72 hours of exposure to the HIV using antiretrovirals, the risk of infection is significantly reduced for needle stick injuries and sexual assault from a known HIV positive person (Celum and Baeten, 2012; Linden, 2011). Reduction in vertical transmission of HIV is crucial in

the battle to control the infection. Findings from different studies have shown that prevention of mother-to-child transmission can reduce rates of HIV transmission by more than 90% (Coutsoudis *et al*, 2010). The use of combination antiretroviral therapy in HIV infection as opposed to mono-therapy has reduced the rates of mortality and morbidity associated with the infection. The use of combination ART has been recommended by the World Health Organization (WHO) for treatment and management of HIV infection because it presents less resistance problems and reduces Mother-To-Child Transmission rates (Ghana Health Service, 2011).

1.3.3 Antiretrovirals Therapy in Ghana

Antiretroviral therapy was initiated in Ghana in June 2003 and has proved to be a major life-saver for people living with HIV/AIDS. The country has since the initiation of ART continuously reviewed protocols and guidelines for managing people living with the infection (NACP Quarterly Technical Bulletin, 2012). The current ART regimen in use in Ghana is based on four principles of rational selection and sequencing of drug regimen, maximizing adherence to the selected regimen, preservation of future treatment options and the use of HIV drug resistance testing in selected clinical settings. Ghana has progressed from the use of mono-therapy in 2003 to the current triple combination therapy. By the end of the year 2012, 165 public and private facilities were providing ART in all the regions across the country. The number of persons accessing ART has increased from 2,017 adults and children in December 2004, to 73,339 by the end of 2012, including 3,461 children, It is estimated that 123,245 PLHIV (comprising 110,494 adults and

12,751 children) shall be on ART by the year 2015.The total number of HIV-infected persons on ART at the end of 2012 was 3,404, comprising 1,293 males and 2,111 females (HIV Sentinel Survey Report, 2012).

In Ghana, ART is initiated when the HIV positive person meets a laid down criteria as per the national guidelines (Ghana Health Service, 2011). These include a CD4 count less than 350cells/µL and /or a symptomatic HIV infection in the WHO clinical stage 3 or 4. Pregnant women who are HIV positive and the CD4 count is greater than 350cells/µL are put on ARV prophylaxis starting from 14 weeks of gestation to prevent mother-to-child transmission (MTCT) of HIV. Between the years 2007 and 2011, prophylaxis for PMTCT was initiated at 28 weeks gestation for pregnant HIV positive women. A thorough clinical evaluation was carried out on each HIV positive person prior to initiation of ART. The detailed clinical evaluation aimed at identifying past and current HIV related illnesses that will require treatment, is based on clarification of co-existing medical conditions and pregnancy, which may influence the choice of therapy and to assess the clinical stage of HIV infection in the person (Ghana Health Service, 2011). These goals require medical and social history, complete physical examination coupled with appropriate laboratory investigations.

Compelling new evidence for the use of antiretrovirals (ARVs) globally led to the revision of the guidelines for ART in Ghana in 2010. Currently, the recommended regimen of ARVs is based on combination of NRTIs, NtRTIs, NNRTIs and PI. The common triple ART regimen in use is a combination of two (2) Nucleoside/tide Reverse Transcriptase Inhibitors with one (1) Non-Nucleoside Reverse Transcriptase Inhibitor or two (2) NRTIs and one (1) boosted PI (Ghana Health Service, 2011). There are three

different lines of treatment options available for different categories of HIV positive patients. The first line regimen option is for treating ARV-naïve patients who meet the inclusion criteria for treatment. The second line regimen option is implemented when there is laboratory evidence of treatment failure with the first line option, normally by monitoring of CD4 count and viral load assay. A third line regimen called Salvage Therapy is utilized for heavily ARV-experienced patients and patients who have failed the second line treatment option (Ghana Health Service, 2011). The recommended ARVs and the associated drug regimen in use in Ghana are depicted in Table 2, Tables 3 and 4 at Pages 10, 11 and 12 respectively.

Table 2: Recommended Antiretrovirals for use in Ghana (Ghana Health Service, 2011)

Nucleoside Reverse Transcriptase Inhibitors (NRTI)	**Nucleotide Reverse Transcriptase Inhibitor (NtRTI)**	**Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI)**	**Protease Inhibitors (PI)**
Zidovudine (AZT/ZDV)	Tenofovir (TDF)	Nevirapine (NVP)	Ritonavir boosted Lopinavir (LPV/r)
Lamivudine (3TC)		Efavirenz (EFV)	Ritonavir boosted Atazanavir (ATV/r)
Abacavir (ABC)			
Emtricitabine (FTC)			

Table 3: FIRST LINE DRUGS RECOMMENDED FOR USE IN GHANA (Ghana Health Service, 2011)

	Drugs	**Contra-indications**	**Comments**
First choice drugs	**First Option** Zidovudine + Lamivudine + Nevirapine	Nevirapine is contraindicated in: o liver dysfunction o hypersensitivity Zidovudine is contraindicated in: o severe anaemia	Replace with Efavirenz Replace with Tenofovir
First choice drugs	**Second Option** Zidovudine + Lamivudine + Efavirenz	Efavirenz is contraindicated in: o First trimester Pregnancy o CNS presentations When there is Efavirenz related persistent adverse CNS effect	Replace with Nevirapine Replace with Nevirapine
Second Choice drugs	**First Option** Tenofovir +(Lamivudine or Emtricitabine) + Nevirapine		Tenofovir should be used when Zidovudine is contraindicated e.g. anaemia (Hb less than 8g/dl) When Hb drops significantly (more than a 25% drop from the baseline value) Tenofovir should replace zidovudine
Second Choice drugs	**Second Option** Tenofovir + (Lamivudine or Emtricitabine) + Efavirenz	Efavirenz in contraindicated in: o Efavirenz related Persistent adverse CNS effect o Pregnancy	Tenofovir should be used when Zidovudine is contraindicated e.g. anaemia When Efavirenz is contraindicated replace with Nevirapine

Table 4: SECOND LINE DRUGS RECOMMENDED FOR USE IN GHANA (Ghana Health Service, 2011)

	Drugs	Contra-indications	Comments
First Alternative	Tenofovir + (Emtricitabine or Lamivudine) + (Lopinavir/r or Atazanavir/r)		If AZT base first line. If LPV/r was used for HIV2 in first line, use ATV/r
Second Alternative	Zidovudine + Lamivudine +(Lopinavir/r or Atazanavir/r)		If TDF base first line. Consider Abacavir if patient has used both Tenofovir and Zidovudine

1.3.4 Emergence of HIV-1 Drug Resistance

The high rate of HIV replication combined with the equally high error rate of HIV-1 reverse transcriptase that occurs during each cycle of infection results in HIV infected persons having a complex and diverse mixture of viral quasispecies, each differing by one or more mutations (Virco, 2006). If any of these mutations can confer some selective advantage to the virus, such as a decrease in its susceptibility to an antiretroviral agent, the corresponding quasispecies which are the variants will overtake the wild type virus in the presence of drug pressure. If antiretrovirals do not suppress replication completely the

viral population evolves under specific selective drug pressure, and eventually develop resistance. The rapidity of this process depends on the level of the selective advantage conferred by the mutation, the prevalence of the mutant within the virus population, and the level of drug at the site of HIV replication. It is also known that single amino acids substitution could lead to emergence of high levels of resistance (Knipe and Howley, 2007). Over a period of time and with incomplete viral suppression the resistant strains become the dominant quasispecies in the infected person. The emergence of resistance to the specific drug is at a rate that is proportional to the frequency of pre-existing variants and their relative growth advantage in the presence of specific drugs (Virco, 2006).

1.4 Problem Statement

For HIV-positive mothers on ART after exposure to prophylaxis for MTCT, it is imperative to investigate the pattern of viral mutations since it may adversely affect treatment response. There should be an in-depth investigation of the HIV mutant variants at play to discern drug resistance and unveil the reasons for any lack of optimal response. There is the need to determine what resistance associated mutations are present in the mothers to fully understand the link between the class of drugs used in the prophylaxis and that used in the treatment regimen.

NNRTIs such as Nevirapine have been reported to cause reduction in future treatment response to the same class of drug whilst NRTIs such as Zidovudine generate resistance mutations in mothers after delivery (Jourdain *et al*, 2004). As the national ARV regimen for the prevention of mother-to-child-transmission as well as treatment includes these NNRTIs and NRTIs, the effect of the resistance associated mutations generated in the mothers as a result of the previous prophylaxis needs to be described. No studies in

Ghana have been done to analyse the profile of drug resistant mutations in the mothers and the possible effect this would have on their response to subsequent treatment. With combination ART scaled up nationally, the drug resistance profile of pregnant women could lead to suboptimal response to the drug with a possible future treatment failure since the same drugs are involved in the prophylaxis and the treatment phases.

It is of utmost importance to determine the resistance profiles in these patients and the possible source of the resistant strains in order to be in a position to handle suboptimal responses in such patients. Treatment options for women could be guided by the data obtained from this study which sought to generate specific information on the resistance of drug classes to HIV strains in pregnant women in Ghana.

1.5 Aim

The main aim of this study was to investigate HIV-1 drug resistance profiles in mothers who had previously received ART prophylaxis to prevent mother to child transmission of HIV.

The Specific Objectives were to:

- Determine the virological, immunological and clinical profile of these patients.
- Describe HIV-1 drug resistance mutations present in mothers on ART who had previously received prophylaxis.
- Characterize resistance mutations found as per the class of ARV administered.

1.6 Justification

For HIV-positive mothers on ART after previous exposure to prophylaxis to prevent MTCT, it is imperative to investigate the pattern of viral mutations since it may adversely affect treatment response as has been reported elsewhere. There is the need for local in-depth examination of the HIV variants to determine the extent of drug resistance and unveil the reasons for the lack of optimal response. As per the classes of ARV drugs in use in Ghana, there is the need to determine the resistance associated mutations present in the HIV positive mothers after delivery. This will enable us better understand the links between drugs used for prophylaxis and those used in the treatment regimen. This will facilitate appropriate management of suboptimal responses in such patients.

Prolonged suboptimal responses to ARVs would militate against the drive to prevent and eliminate the transmission of HIV to the unborn child. Treatment options for HIV positive women would be better informed by data obtained from this study, which will also generate additional information on the resistance of drug classes to HIV strains in Ghana.

CHAPTER 2

2.0 LITERATURE REVIEW

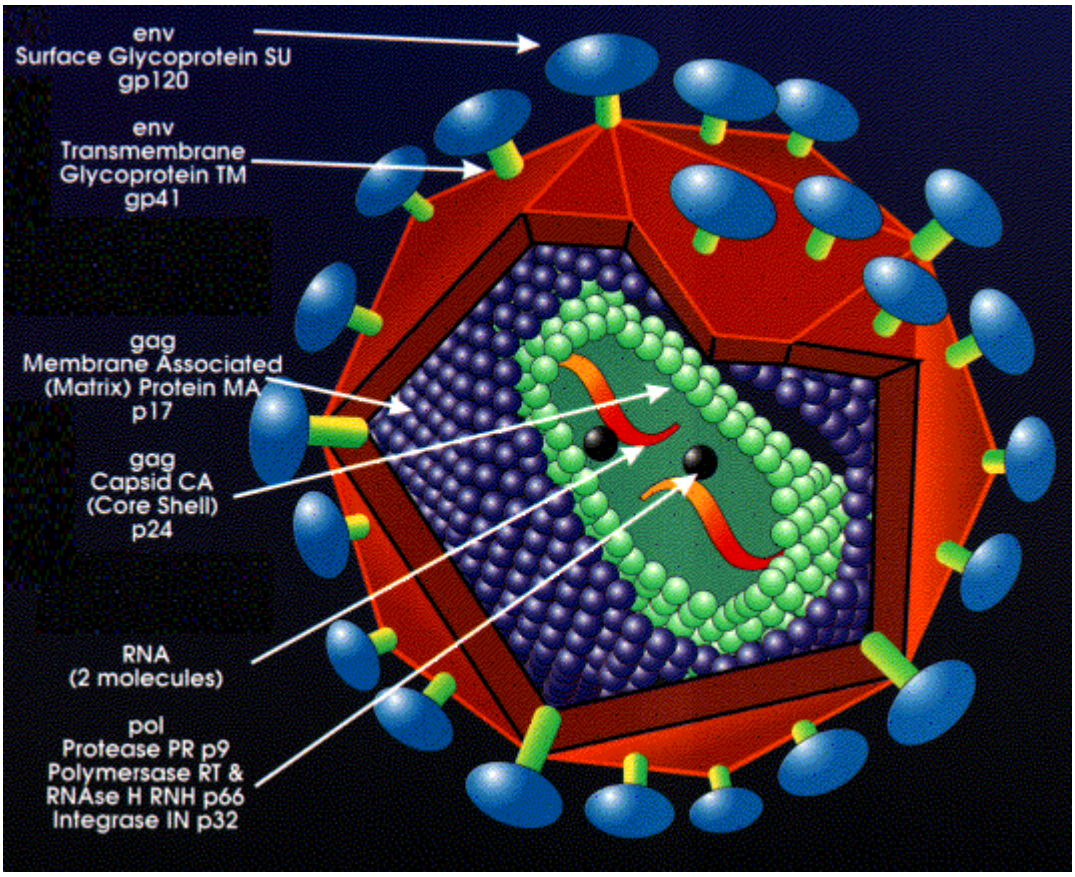
2.1 Human Immunodeficiency Virus (HIV)

Human Immunodeficiency Virus (HIV) is the causative agent of Acquired Immune Deficiency Syndrome (AIDS) (Levinson, 2008).The virus was formerly referred to as Human T-lymphotropic Virus type-3 (HTLV III) and also as Lymphadenopathy-Associated Virus (LAV).There are two known types of HIV; HIV-1 and HIV-2.Whilst HIV-1 is of worldwide distribution, HIV-2 has been found to be primarily in West Africa (Levinson, 2008). This virus targets the host immune system thus making it a very difficult virus for the targeted host to fight. AIDS is the condition caused by the HIV in humans in which there is progressive failure of the immune system leading to life-threatening opportunistic infections and cancers (Douek *et al*, 2009).

2.1.1 Structure and Genome Organization

The virus, HIV, belongs to the family Retroviridae, in the genus *Lentivirus* and classified under Group VI. It is composed of two copies of positive single-stranded RNA (ssRNA-RT) that codes for nine genes enclosed by a conical capsid composed of 2,000 copies of the viral protein p24. The single-stranded RNA is tightly bound to nucleocapsid proteins (p6 and p7) and enzymes needed for the development of the virion such as reverse transcriptase, proteases, ribonuclease and integrase. A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle, as shown in Figure 1 overleaf (HIV Compendium, 2008). The viral capsid is surrounded by a viral envelope composed of a lipid bilayer membrane which the virus acquires from the cellular membrane of the host cell as the new virion buds out of the host cell.

Figure 1: Structure of HIV: this sketch shows the positive single-stranded RNA (ssRNA-RT) genome and the Polymerase gene surrounded by the capsid.

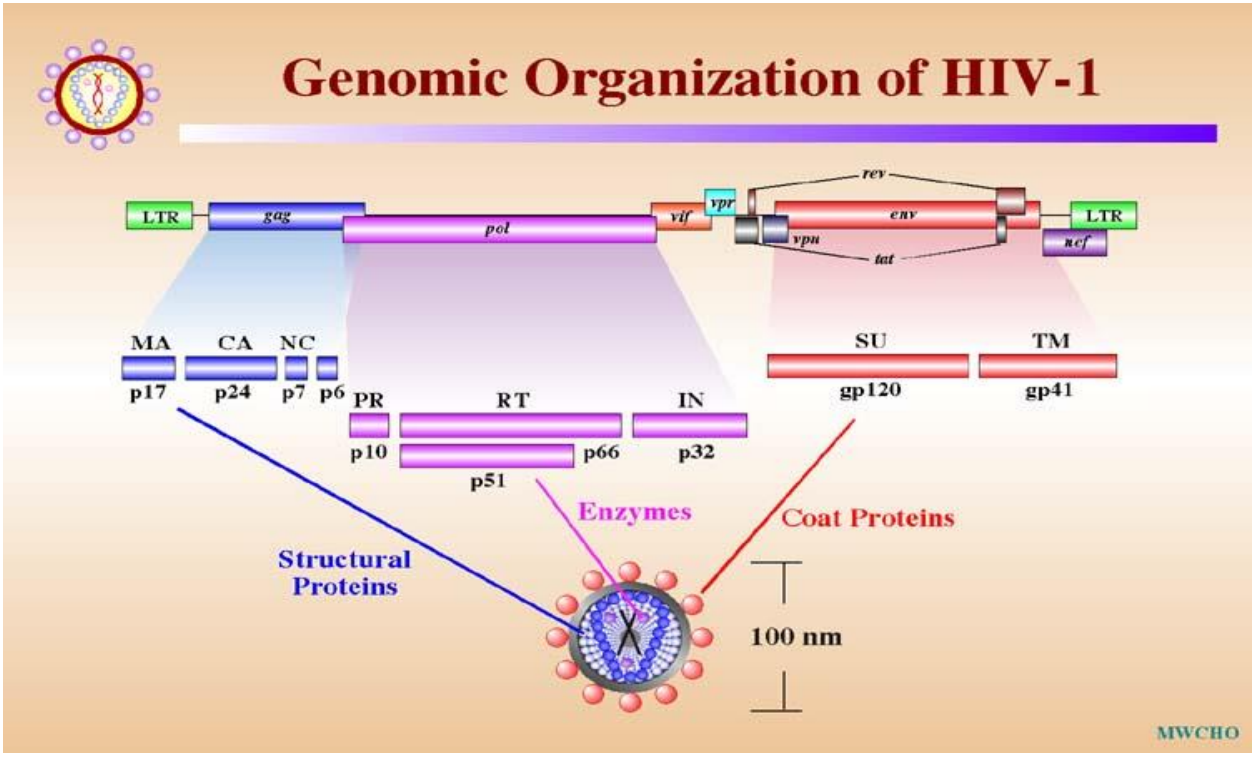


(Source: htpp://www. Kenyan.web.org)

The HIV genome is diploid and is 9 to 10 kilobases in length. There are three major viral proteins which encodes for its structural proteins; these viral proteins are the gag, pol and env genes (see Figure 2 overleaf). The genome also has six regulatory proteins, two of which are for replication called tat (transactivating transcription protein) and rev (regulator of expression of virus protein); the remaining four being accessory proteins called vpu (virus protein U), vpr (virus protein R), vif (virus infectivity factor) and nef (negative regulator factor) (Levinson, 2008). The proteins of the core of the virion are encoded for by the gag gene; the pol gene encodes the three enzymes in the nucleocapsid

crucial to viral replication. These enzymes are the Reverse Transcriptase (RT), Integrase (I) and Protease (PI). Reverse Transcriptase is a bi-functional enzyme carrying Ribonuclease H activity. The env gene encodes the precursor of the surface glycoproteins, gp 160 which is cleaved to give rise to gp120 and gp41 on the surface of the viral envelope (Levinson, 2008).

Figure 2: Genome of HIV-1: The Protease, Reverse Transcriptase and Integrase enzymes are needed for replication and encoded by the Polymerase Gene.



MA-Matrix;CA-Capsid; NC-Nucleocapsid; SU- Structural Unit; TM-Transmembrane PR-Protease; RT-Reverse Transcriptase; IN-Integrase; LTR-Long Terminal Repeat

(Source: http://www.stanford.edu/group/virus/retro/2005gongishmail/HIV-1b.jpg)

2.2 Life Cycle

The composite stages of the life cycle of HIV is detailed in subsections 2.2.1 to 2.2.3 and depicted in Figure 3 on page 23.

2.2.1 Viral Entry

Human Immunodeficiency Virus has an affinity for CD4+ T-cells, macrophages and monocytes and gains access to these cells by the adsorption of the glycoproteins on its surface to receptors on the target cells. This is followed by fusion of the viral envelope with the cell membrane with the subsequent release of the viral genome into the target cell (Stages 2 &3 in Figure 3 on Page 23). The viral glycoproteins that mediate the entry process are the trans-membrane proteins gp41 and gp120 (within the gp160 spike which contains domains for CD4 and chemokine co-receptors) (Clapham and Mcknight, 2001). The glycoprotein 120 is covalently linked to gp41 and recognizes the CD4 ligand on host cells. The co-receptors bind a host chemokine receptor – either CXCR4 or CCR5 depending on the type of HIV particle. M-tropic particles recognize CCR5, infect macrophages and primary T-cells, and have a low specificity for CD4+ T-cell lines. T-tropic particles recognize CXCR4, which is highly expressed in CD4+T-cells, and induce syncitia, the fusion of cells to create one large cell with many nuclei, a precursor to cell death (Moore and Caisson, 1999). After HIV gp120 binds to the CD4 receptor and the co-receptor, a conformational change in gp41 causes the insertion of the N-terminal hydrophobic fusion-peptide region into the target- cell membrane resulting in membrane fusion and the release of the contents of the viral particle, which includes copies of the viral genetic material and the Pol protein (Reverse Transcriptase), into the cytoplasm of

the target cell (Eckert and Kim, 2001). After this step the copying of the viral genetic material from RNA into DNA, ie, reverse transcription, then takes place.

2.2.2 Replication and Transcription

Immediately after entry of the host cell, an enzyme encoded by the virus called Reverse Transcriptase (RT) liberates the (+) single-stranded RNA genome from the attached viral proteins and transcribes the viral genome into a complementary DNA (cDNA) molecule, using a cellular lysine tRNA molecule as a primer (Zheng, *et al* ,2005). The reverse transcriptase has ribonuclease activity that degrades the viral RNA during the synthesis of cDNA, as well as DNA-dependent DNA polymerase activity that creates a sense DNA from the *antisense* cDNA. Together, the cDNA and its complement form a double-stranded viral DNA that then travels to the cell nucleus. Entry of the cell nucleus is mediated by Vpr and Vif accessory proteins, as well as by nuclear localization signals within the Vpr and p17 matrix sequences. Another viral encoded enzyme, Integrase, inserts the viral DNA into the host's chromosomal DNA (Garcia and Gaynor, 1994). The integrated form of the virus, known as the Provirus or Proviral DNA is replicated as part of the normal cell genome and may lie dormant for long periods during the latent phase of the infection (Zheng *et al*, 2005)**.** The proviral DNA has identical LTR copies flanking the coding regions. The 5' end of the LTR now functions as a promoter, regulating the production of RNA transcripts dependent on the presence of host-cell transcription factors (such as promoter-specific transcription factor, SP1, and nuclear factor - kappa beta) and the viral protein Tat (Nekhai and Jeang, 2006). The integrated DNA provirus is

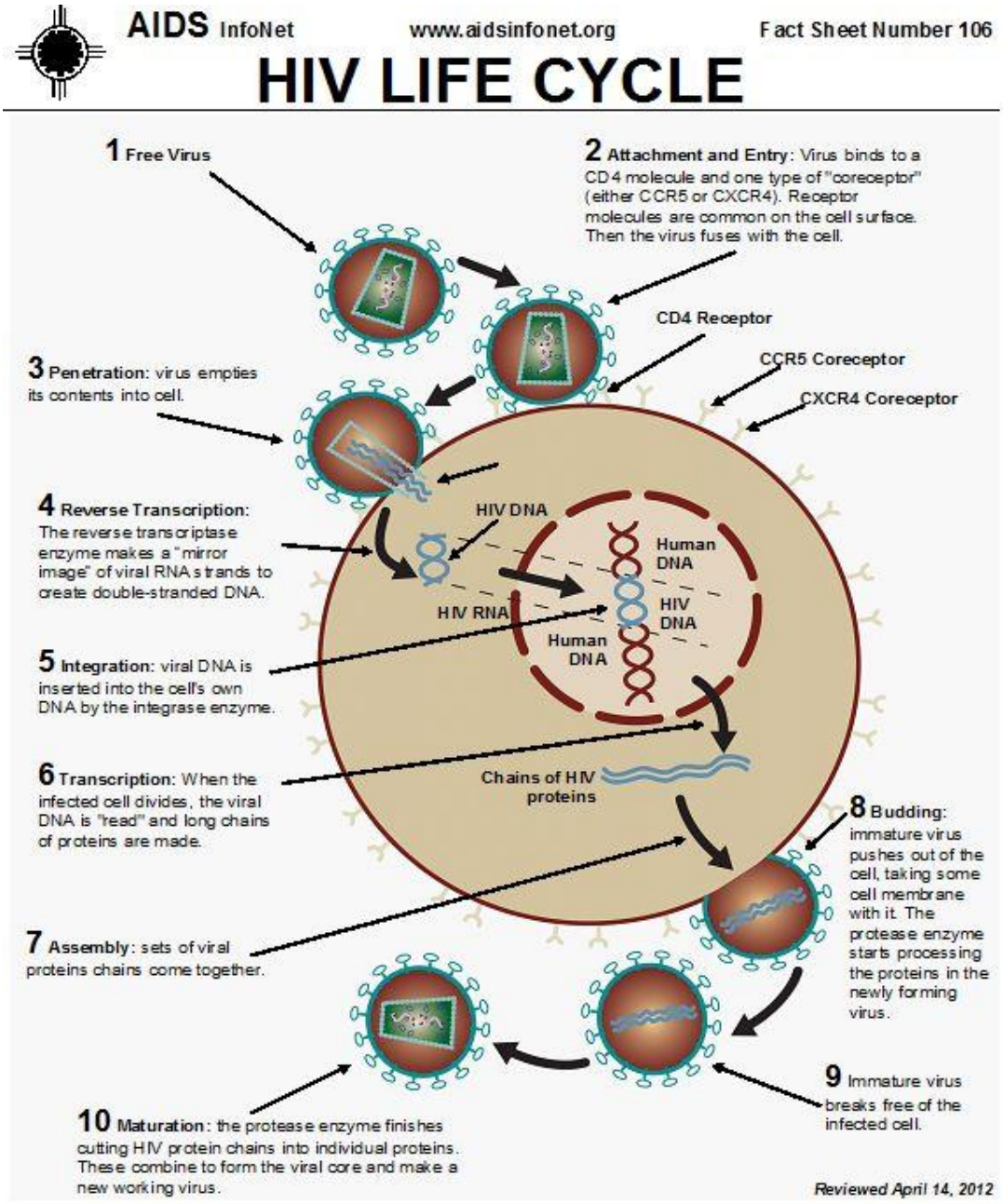
transcribed into mRNA molecules which may either be spliced in preparation for translation of viral proteins, or exported from the nucleus in an unspliced form for packaging into newly produced virions (See Stages 4, 5, 6 in Figure 3 at Page 23). Spliced mRNA pieces are exported from the nucleus into the cytoplasm to be translated into a regulatory protein Tat, which encourages production of new virions and Rev, an accessory protein. As the newly produced Rev protein accumulates in the nucleus, it binds to viral mRNAs and allows unspliced RNAs to leave the nucleus, where they would otherwise be retained until spliced. The structural proteins Gag and Env are produced from the full-length mRNA; the full length RNA is actually the virus genome and it binds to the Gag protein and is packaged into new virus particles (Nekhai *et al*, 2006, Pollard and Malim, 1998). The different length versions of the viral RNA (spliced and unspliced) direct the synthesis of different viral proteins by the host cell ribosomes. New viral particles are assembled at the host plasma membrane and incorporate Gag subunits, Pol, Nef, Env, Vpr and viral genomic RNA (Zimmerman and Klein, 2002).

2.2.3 Assembly and Release

The assembly of new HIV virons signifies the final step of the viral replication cycle and begins at the plasma membrane of the host cell. Viral proteins Gag, Gag-Pro-Pol and Env are needed for progeny virions formation and as they are synthesized they come together for this purpose (See Stages 7, 8, 9, 10 in Figure 3 at Page 23). The formation of the HIV virion is driven by the Gag precursor protein (Hunter, 1994; Sakalian and Hunter, 1998). The mRNA of the Env gene which codes for viral surface glycoproteins is translated in the endoplasmic reticulum (ER) to yield the gp160 pre-protein which is transported to the Golgi complex. During the formation of the virion the gp 160 is cleaved by Protease, a

viral enzyme, into the two HIV envelope glycoproteins- gp120 and gp41.These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. The Gag (p55) and Gag-Pol (p160) polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. Maturation occurs either in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV-encoded Proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion (Gelderblom, 1997**).** Thus immature viral polypeptides are processed into their functional forms by the enzyme Protease and assembled with full-length HIV RNA transcripts into nascent viral particles. During the budding from the plasma membrane maturation occurs and the new viral particle can infect another cell. The viral accessory protein, Vpu, facilitates virion release from the cell membrane in the late stage of the replication cycle by interacting with a host cell factor called Tetherin. Tetherin is an interferon-alpha-induced human protein, an endogenous membrane-associated protein that inhibits the release of viral particles from the plasma membrane. Without Vpu, HIV-1 particles would be tethered to the host cell membrane and cannot be released (Neil *et al*, 2008).

Figure 3: Life Cycle of HIV



(Source: AIDSINFO (2012) Available at http://www.aidsinfonet.org/factsheets/view/106. [Accessed: April,2012]. **Main stages are: viral entry (2 & 3), replication and transcription (4, 5,& 6) and assembly and release of new matured virion (7, 8, 9 &10).**

2.2.4 Genetic variability and subtypes of HIV-1

HIV has a very high genetic diversity as a result of several factors; its error-prone viral DNA synthesis during reverse transcription (generating $3x10^5$ mutations/nucleotide/replication cycle), the high levels or rapid rate of progeny virus production in vivo (10^9 virions/day; 150 to 300 replication cycles/year), and high recombinant frequencies accompanying reverse transcription (Rambaut et al, 2004). All this leads to the generation of many HIV variants in a single infected individual within a day. Recombination occurs when a single host cell is simultaneously infected by two or more different strains of the virus resulting in progeny virions with genomes composed of RNA strands from two different strains. The hybrid virion thus formed infects a new cell and undergoes replication during which the reverse transcriptase with two different RNA templates to work with generates a newly synthesized retroviral DNA sequence, a recombinant of the two parental genomes (Robertson *et al*, 1995).

The highly diverse genetic nature of HIV-1 has led to distinct genetic subtypes or clades which are classified into four major groups: Main (M), New (N), Outlier (O) and P on the basis of the differences in the envelope (env) region. Group M is the most prevalent and contains more than 95% of the global virus isolates and is subdivided into nine subtypes or clades, based on the whole genome (A, B, C, D, F, G, H, J and K); within this group there are also 61 variants of the virus called Circulating Recombinant Forms (CRFs) (Gao *et al*, 1998; Robertson *et al*, 1999). The subtypes have distinct geographical distribution; the most prevalent are subtypes C (found mainly in Africa and Asia), B (found mainly in North America and Europe), A and D (found mainly in Africa). In the year 2000, when an analysis of global subtype prevalence was made, 47.2% of infections

worldwide were of subtype C, 26.7% were of subtype A/CRF02_AG, 12.3% were of subtype B, 5.3% were of subtype D, 3.2% were of CRF01_AE, and the remaining 5.3% were composed of other subtypes and CRFs (Osmanov *et al*, 2002)**.**

In Ghana, studies have shown that the prevalent CRF is CRF02_ AG (Brandful *et al*, 2007) which related to strains circulating in Senegal and Cameroun (Sagoe *et al*, 2009; Delgado *et al*, 2008; Noble *et al*, 2006). Studies in Ghana have further shown that the number of unique recombinant forms (URFs) is high, accounting for 25% of the CRFs (Nii Trebi *et al*, 2013).

2.3 Natural history and disease progression

2.3.1 Primary Infection

Untreated HIV infection naturally progresses from primary infection (or the acute HIV syndrome phase), through a period of chronic or asymptomatic infection (though not viral latency) of a median duration of ten (10) years, to an AIDS-defining illness followed by the demise of the person from AIDS. The time period from the initial infection with HIV to the development of an antibody response detectable by standard tests is referred to as the primary infection stage. This stage is accompanied by relatively nonspecific symptoms of an acute viral illness in approximately 50% to 70% of infected persons (Kahn and Walker, 1998). The symptoms may present 2 weeks after exposure and commonly include fever (which is seen in over 75% of infected persons), fatigue, pharyngitis, myalgias, arthralgias, headache (Bozzette *et al*,1993; Tindall *et al* ,1988). In most cases, generalized lymphadenopathy is also a frequent finding with mucocutaneous ulceration and weight loss distinguishing between primary HIV-1 infection and other

viral syndromes (Hecht *et al*, 2002). Within a period of 3 to 4 weeks the symptoms of primary infection resolves spontaneously in most patients. Hematological disorders usually detected in primary HIV-1 infection are leukopenia and mild thrombocytosis, usually accompanied by a decrease in the absolute CD4+ T-lymphocyte count along with an increase in circulating activated CD8+ T cells (Gaines *et al*, 1988). A week after the onset of symptoms of HIV-1 infection plasma RNA titers generally peak averaging levels between 10^6 and 10^7copies/ml; within a post-infection period of two months, the RNA titers decline to levels between 10^3 and 10^5copies/ml (Lindback *et al*, 2000). The HIV patient is more infectious during the acute phase (Daar *et al*, 2001).

2.3.2 Chronic Infection

After the period of acute HIV infection, there follows a prolonged state of asymptomatic chronic infection. A strong host immune defense reduces the level of viremia, marking the start of the secondary or chronic HIV infection. The secondary/chronic stage of HIV infection can vary between two weeks and 20 years, averaging 10 years. This chronic infection state comes with rapid viral replication in the lymph nodes and results in large amounts of HIV particles being sequestered in the lymph nodes, with ongoing CD4+ T lymphocyte depletion (Levinson, 2008).During the chronic phase of HIV infection, HIV plasma RNA levels correlates with the rate of CD4 depletion; higher plasma viral RNA levels predict more rapid progression to AIDS-defining illness and death. During the chronic infection phase, early initiation of antiretroviral therapy can significantly improve the clinical profile or the survival of the patient, as compared with when therapy is deferred. Due to HIV being active within the lymph nodes, they typically become persistently swollen, in response to large amounts of virus that become trapped in the

follicular dendritic cells (FDC) network. (Kitahata *et al*, 2009). The surrounding tissues that are rich in $CD4^+$ T cells may also become infected, and viral particles accumulate both in infected cells and as free virus. Individuals who are in this phase are still infectious. In the prolonged chronic phase, a syndrome called AIDS-Related Complex(ARC) may occur, presenting as fatigue, persistent fevers, lymphadenopathy and weight loss in the untreated patient (Levinson,2008). In the chronic phase, wasting of the body (slim's disease) is prevalent in developing countries where full access to antiretroviral therapy is difficult to achieve (Grinspoon and Mulligan, 2003).

2.3.3 Advanced Disease

During the last phase of the progress of untreated HIV infection, cell-mediated immunity is lost due to the consistent decline of CD4 + T lymphocytes levels to below 400 cells/µL, and infections due to different opportunistic microorganisms appear, increasing in frequency and severity. The initial manifestation of such a situation include moderate and unexplained weight loss, recurring respiratory tract infections (such as sinusitis, bronchitis, pharyngitis, otitis media), skin rashes, oral ulcerations and prostatitis (in men). Infection by *Candida albicans* and *Mycobacterium tuberculosis* may also occur leading to increased susceptibility to the occurrence of other opportunistic infections. Pneumonia caused by the fungus *Pneumocystis jirovecii*, which often occurs with high fatality, and Kaposi's sarcoma are the two most characteristic manifestation of AIDS in the untreated HIV- infected person (Levinson,2008).

2.4 Epidemiology

2.4.1 Global Overview

The World Health Organization (WHO) in 2011 estimated that there were 34 million people living with HIV globally, 3.4 million being children less than 15years old. There were 2.7 million new HIV infections, 390,000 of these were children less than 15years old. Despite the global decline in the annual number of people newly infected with HIV, there are clear regional variations with sub-Saharan Africa still having the highest number of people living with HIV (WHO, 2011). The global prevalence of HIV-1 had stabilized at 0.8% with 2.7 million new infections in the last decade and there are signs of declining infection rates in some regions including some heavily infected countries in Africa, with ascending infection rates in some eastern European and central Asian countries (Kilmarx, 2009). These ascending infection rates were also seen in China; the Chinese Center for Disease Control and Prevention (CCDCP) issued figures showing that 4,800 new cases of HIV infection were recorded in 2010 of which 85% were sexually transmitted (CCDCP, 2010). In the United States of America, it was estimated in 2010, that 48,079 adults and adolescents were newly diagnosed with HIV infection; 79% of these new diagnoses were males and 21% were females (CDC, 2010).

A disproportionate share of the HIV burden globally is seen in sub-Saharan Africa where 68% of all people living with HIV/AIDS (PLWHA) resided though the region carries only 12% of the global human population. In 2012, there were 25million adults and children living with HIV/AIDS and 1.6 million people in sub-Saharan Africa who became newly infected with HIV, representing 70% of the number of people who became

infected with HIV (UNAIDS, 2013) .In sub-Saharan Africa, 1.2 million people died from AIDS related illness. Women infected with HIV made up 59% of the People Living with HIV (PLWHIV) in the sub-Saharan region where the majority of the people infected were through unprotected heterosexual intercourse. HIV prevalence in sub-Saharan Africa is higher among women than in men and also more in the urban areas than the rural settings (Mavedzenge, 2011; UNAIDS, 2013).

2.4.2 Ghana Overview

In 1986, HIV/AIDS was first identified in Ghana when a total of 115 people were reported to be seropositive (Neequaye *et al,* 1987). At the end of 2011, 217,428 people were living with HIV (PLWHIV), representing a prevalence of 1.42% of the adult population and comprising 124,111 females and 93,017 males. There were 31,576 children living with HIV and an estimated 2,933 newly infected children. The number of deaths due to HIV/AIDS related illness at the end of 2011 was 14,330**.** The HIV prevalence in Ghana at the end of 2011 was 2.1% (HIV Sentinel Survey Report, 2012) and was maintained at the end of 2012(HIV Sentinel Survey Report, 2013). The total number of new HIV infections had reduced to 7,991 of which 852 were children, boosting the prospects of eliminating mother-to-child transmission of HIV by 2015. At the end of 2012, the number of AIDS related deaths had been reduced from 14,330 in the previous year to 11655 (NACP, 2013; HIV Sentinel Survey Report, 2013). The national HIV prevalence dropped from 2.1% in 2012 to 1.9% in 2013, confirming the declining HIV prevalence in Ghana (HIV Sentinel Survey Report, 2014). The HIV epidemic in Ghana is described as generalized and the estimates of HIV prevalence are primarily based on sentinel surveillance among pregnant women who attend antenatal care clinics

and a population-based survey that includes HIV testing. The national HIV Sentinel Survey (HSS) is a cross-sectional survey initiated in selected antenatal care clinics in the country since 1992. The HSS report represents annual prevalence among pregnant women which is a good proxy indicator of the level of HIV infection in the country (NACP, 2014).

2.5 Laboratory Diagnosis of HIV Infection

The need to accurately screen for and confirm the presence of HIV in asymptomatic people has led to the availability of different assays for diagnosing HIV infection. These assays range from detection of antibody to confirmation of the presence of HIV antigens and nucleic acids. Specimens used for these diagnostic assays range from oral fluids to plasma or serum from whole blood (Alemnji *et al*, 2009; Fearon, 2005; FDA, 2004).

2.5.1 HIV Antibody Testing

Antibody tests for HIV are inexpensive, rapid and accurate tests methods specifically designed for routine diagnostic testing of adults; however, some limitations may be found with their sensitivity when false negative results may occur in spite of the presence of the virus. This may occur during the window period, an interval of three weeks to six months between the time of HIV infection and sero-conversion to the production of measurable antibodies to HIV. During the window period, an infected person can transmit HIV to others although their HIV infection may not be detectable with an antibody test. Antiretroviral therapy during the window period can delay the formation of antibodies and extend the window period beyond 12 months (Brooks *et al*, 2006; Hare *et al*, 2004). Diagnosis of HIV infection by the detection of HIV- 1 and/or -2 antibodies is possible by

the use of enzyme-linked immunosorbent assays (ELISA). Serological tests for detection of antibodies to HIV can be classified as initial screening, confirmatory or supplemental tests. Initial screening or tests gives a presumptive result whilst confirmatory tests affirm the reactive or non-reactive results obtained with the initial tests. Enzyme Linked Immunosorbent Assays (ELISAs) are commonly used for blood screening for blood transfusion. The Western Blot (WB) is a confirmatory test for HIV antibodies identification. Generic assays similar to WB are also available to confirm a presumptive HIV screening result. Some of these are the Line Immuno-Assays (LIA) such as the INNOLIA (from Innogenetics of Belgium).This is based on recombinant proteins and synthetic peptides from HIV-1 and HIV-2.This assay is capable of detecting antibodies to HIV-1 including group O and type 2 (HIV-2) proteins in human plasma and can also differentiate between HIV-1 and HIV-2 infections (Innogenetics, 2010).

As HIV infection progresses towards AIDS-defining illness,the pattern of the immune response changes. However, antibodies to the envelope glycoproteins (gp160 or gp120 and gp41) persist even to the advanced stages of the disease (Knipe, and Howley, 2007).

2.5.2 HIV Antigen Testing

Primary diagnosis of HIV-1 infection can be made before sero-conversion occurs in an infected person; this depends on the detection of HIV-1 capsid antigen (p24) or HIV-1 RNA in plasma. The viral protein p24 appears in the blood of infected individuals before sero-conversion to produce measurable quantities of HIV antibodies .The sensitivity (the percentage of the results that will be positive when HIV is present) and the specificity

(the percentage of the results that will be negative when HIV is not present) of various antigen assays range from 79% to 89% and 99% to 100% respectively; though HIV-1 RNA assays are highly sensitive (100%) their specificity is between 95 % to 97%. In identifying primary HIV infection, p24 is more specific than HIV-1 RNA in plasma assay but less sensitive than HIV-1 RNA assays such as PCR or the branched chain DNA (bDNA) determination (Hecht *et al*, 2002). Assaying for the capsid protein, p24, is useful in screening blood for transfusion and early infection since it appears before sero-conversion; it can be used in diagnosing infection in the newborn (Hecht *et al*, 2002).

Amplification assays (that depend on viral nucleic acid detection) such as RT-PCR, DNA PCR and bDNA are used to detect and measure the amount of viral RNA in clinical specimens. Such nucleic acid-based assays amplify and detect one or more of several target HIV genes such as HIV-1 env and pol genes (Defoort *et al*, 2000).

2.5.3 Viral Load Estimation

The viral load (VL) or the viral burden of HIV is a measure of the blood plasma HIV RNA concentration; it indicates the severity of the viral infection and is calculated by estimating the quantity of virus in RNA copies per milliliter of blood plasma. HIV quantification can be achieved either by use of nucleic acid based tests (NATS) or non-nucleic acid based tests (NNATS) (Puren *et al*, 2010). The NATS comprises amplification molecular methods which can be divided into different categories by virtue of the starting material used for amplification; these categories are:

- The target amplification method which uses the nucleic acid of the virus, exemplified by the Reverse Transcription Polymerase Chain Reaction (RT-PCR) and the Nucleic Acid Sequence Based Amplification (NASBA).
- There is also the Probe Specific amplification method which uses synthetic probes that preferentially bind to a target sequence with the probes being amplified.
- A third method for determining HIV viral load is the Signal Amplification method which makes use of large amounts of signal bound to an unamplified target originally present in the sample. An example is the branched chain DNA (bDNA) method.

An example of Non-Nucleic Acid Based tests for viral load estimation is the ExaVir™ Load assay from Cavidi AB (Puren *et al*, 2010).

2.5.3.1 Real Time Quantitative RT-PCR (Real Time qRT-PCR)

Polymerase Chain Reaction (PCR) is used to quantify integrated DNA, using a DNA template, polymerase, buffers, primers and nucleotides to amplify a specific region of the DNA target. PCR methods typically amplify DNA fragments up to ~10 kbs though some techniques allow for amplification of fragments up to 40 kbs. A variation of PCR termed Reverse Transcription Polymerase Chain Reaction (RT-PCR) is used to quantify viral RNA. In this method RNA is used as the starting material (RNA template) and is converted to double-stranded DNA using the enzyme Reverse Transcriptase (RT). In the RT-PCR technique, viral RNA is extracted from the patient's plasma and is treated with reverse transcriptase (RT) to convert the viral RNA into complementary DNA (cDNA). The polymerase chain reaction (PCR) process is then applied, using two primers unique

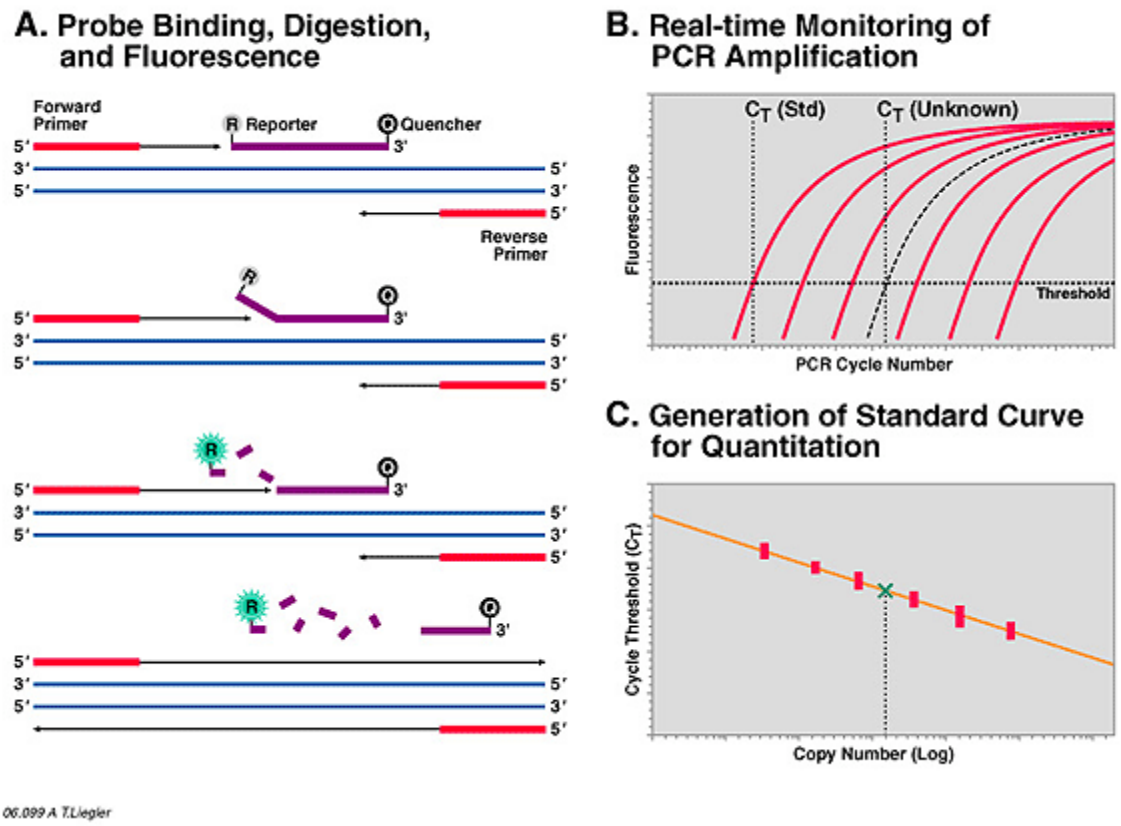
to the virus's genome. After PCR amplification is complete, the resulting DNA products are hybridized to specific oligonucleotides bound to the vessel wall, and then made visible with a probe bound to an enzyme. The amount of virus in the sample can be quantified as a result (Malmsten, 2005).

A recent major development is the Real Time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR**)** which enables reliable detection and measurement of products generated during each cycle of the PCR process. This technique became possible after introduction of an oligonucleotide probe**,** which was designed to hybridize within the target sequence. Cleavage of the probe during PCR because of the 5' nuclease activity of Taq polymerase is used to detect amplification of the target-specific product (Heid *et al*, 1996)**.** In contrast to end-point assays, real-time PCR monitors the accumulation of amplification products, and, when coupled with known standards, can be quantitative with a dynamic range of several orders of magnitude. One strategy for detection of the double-stranded DNA products during the course of amplification uses TaqMan chemistry (Malmsten, 2005).

As indicated by the schematic representation of the TaqMan Real-Time PCR at Page 36, in the Probe Binding, Digestion, and Fluorescence stage (Figure 4A), the target DNA sequences are amplified with forward and reverse primers. An additional target-specific probe is added for detection of amplicons, with a fluorescent reporter dye coupled to the 5' end and a quencher moiety coupled to the 3' end. When the oligonucleotide probe is intact (bound or unbound), there is no fluorescence. During the primer extension phase of PCR, the 5' nuclease activity of Taq DNA polymerase cleaves the bound probe, releasing the reporter dye from its quencher. This is repeated with each PCR cycle, resulting in

increased fluorescence that is proportional to the amount of amplicons produced. The probe binding, digestion and fluorescence stage is followed by the real-time monitoring of PCR amplification during which the amplification curve of the accumulating product tracks the amount of fluorescence at each PCR cycle number (Figure 4B). A predetermined threshold of fluorescence, usually chosen at a level expected to occur in the early phase of amplification, defines the C_T (threshold cycle) and is measured for quantitation standards (red curves in Figure 4B overleaf) and the unknown sample(s) (black hatched curve in Figure 4B overleaf). The more concentrated the target sequence, the earlier the fluorescent signal crosses the threshold, and the lower the C_T. In the final phase, in order to calculate the copy number of the unknown, a standard curve is generated by plotting the known (standard) copy number vs the C_T (Figure 4C overleaf). From the generated standard curve (or internal quantitation standard, in some cases), the copy number of the unknown is extrapolated from the observed C_T (unknown) (Leigler and Grant, 2006).

Figure 4: Schematic Representation of Real Time Quantitative RT-PCR



The TaqMan Real-Time PCR is based on the TaqMan chemistry (Malmsten, 2005) comprising the three stages of the quantitation process- the amplification of the target DNA sequences, the monitoring in real-time and the generation of the standard quantitation curve (Malmsten, 2005).

2.6 HIV Transmission

High titers of HIV in body fluids facilitate transmission; such high titers of the virus can be found in blood, semen and vaginal fluids. HIV is transmitted during sexual contact (including genital-oral sex), through parenteral exposure to contaminated blood and blood products, and from mother to child during the perinatal period (Knipe and Howley, 2007). The risk of transmission varies for each mode of transmission; being greater from male to female than from female to male, and the risk in male to male sexual transmission is greater than in heterosexual transmission, but highest in individuals who practice receptive anal intercourse ((Knipe and Howley, 2007).The risk of sexual transmission of HIV is increased by the presence of other sexually transmitted diseases (STDs) such as syphilis, gonorrhea and herpes simplex type 2 as a result of inflammations and sores which facilitate the transfer of the virus across mucosal barriers (Brooks *et al*, 2006). Sexually Transmitted Diseases increase the risk of HIV transmission and infection due to the associated disruption of the normal epithelial barrier by genital ulceration and/or micro-ulceration (STDs, 2011). Generally, the greatest risk of transmission of HIV is via transfusion of contaminated blood in which 95% of blood recipients become infected with the virus (Knipe and Howley, 2007). Sexual transmission of HIV has been found to be reliant on the infectivity of the index case and also on the susceptibility level of the uninfected partner, though infectivity varies among individuals and according to the duration of the illness (Dosekun and Fox, 2003). HIV transmission through blood-borne routes is common amongst intravenous drug users, patients who have been transfused with unscreened contaminated blood, acupuncture patients and

people who share invasive instruments like razors, sharp toothed combs, needles and shavers (Reid, 2009).

In North America, China and Eastern Europe, needle sharing is responsible for a third of all new HIV infections. In North America and Eastern Europe, most new infections are from intravenous drug use and also from tattoo instruments; acupuncture needles are thought to be responsible for such new infections in China where acupuncture healing is common and normal. The probability of being infected by a single prick already used by an HIV-infected person is just about 1 in 150, although post exposure prophylaxis with antiretroviral therapy can significantly reduce the associated risk (Reid, 2009).

In Africa, WHO estimates that 2.5% of all HIV infections in sub-Saharan Africa are transmitted through unsafe healthcare injections (UNAIDS/WHO, 2007). In more developed areas of the world, HIV infection through this route is less common. Also, due to improved donor selection and HIV screening performed on all transfused blood units, transmission through transfusion in the developed countries is rare. However, according to WHO, the economically-challenged overwhelming majority of the world's population does not have access to safe blood; about 15% of HIV infected people in less developed countries were infected through transfused blood and blood products (WHO, 2001).

In Ghana, the transmission distribution of HIV is 80% through heterosexual contact, 15% via mother-to-child-transmission and 5% due to other routes of transmission such as the use of contaminated sharp implements and shared needles among narcotic drug users (Ghana Health Service, 2011).

2.6.1 Mother -To-Child Transmission (MTCT) of HIV

Mother-To-Child-Transmission (MTCT) of HIV occurs when an HIV-infected pregnant woman passes on the virus to the infant during the pregnancy, labour, delivery or even post-delivery through breastfeeding (perinatal transmission). Perinatal transmission is the most common route for the infection in children. In young children, HIV infection is often acquired from the infected mother. Less than 7% of children now living with AIDS acquired the infection from other sources, including blood transfusion (as seen with blood products used to treat hemophilia or malaria) or sexual abuse. HIV can be transmitted through the breast milk of an infected mother; thus 12 to 14% of babies not infected at birth acquire HIV infection if they breastfeed from an HIV-infected mother (UNAIDS/WHO, 2007). Globally, the majority of the HIV-infected children under 5 years were infected during pregnancy, delivery and breastfeeding (UNAIDS, 2013). HIV is not transmitted through food, water, household articles, or social contact in a home, workplace, or school (Lallemant and Jourdain, 2010).

It has been surmised through research outcomes that without prophylaxis or treatment, 15-30% of expectant HIV-infected mothers would pass on the infection to their babies during pregnancy and delivery, and a further 5-20% will become infected during breastfeeding, especially in developing countries such as Ghana (Weinberg *et al*, 2009). Globally, according to UNAIDS, in 2012 , about 260,000 children under 15 years of age become infected with HIV mainly through MTCT with about 90% of these occurring in Africa where AIDS is threatening to set back decades of progress in containing the infection (UNAIDS,2013).

In order to make significant impact in the prevention of mother-to-child transmission of HIV in Ghana, there must be a reduction of the national level of MTCT to less than 5%. In the year 2011, WHO estimated that from the year 2009, between 12,000 and 16,000 pregnant women were HIV-positive and between 2,500 and 3,500 babies were born HIV-positive annually in Ghana. Though this translates into 33 pregnant women getting infected daily and 7 babies being born HIV-positive each day, UNAIDS reports that Ghana is among eight (8) African countries experiencing rapid declines in MTCT since the year 2009 (UNAIDS, 2011).

2.6.2 Prevention of Mother-To-Child Transmission (PMTCT) of HIV

Interventions to prevent the transmission of the HIV infection from a pregnant woman to her unborn baby follow a multi-pronged strategy involving (Duerr *et al*, 2004):

- Preventing HIV infection among prospective parents by making HIV testing and other related prevention interventions available in services related to sexual health such as antenatal and postpartum care.
- Avoiding unwanted pregnancies among HIV positive women through providing appropriate counseling and support to women living with HIV to enable them to make informed decisions about their reproductive lives.
- Preventing the transmission of HIV from HIV- positive mothers to their infants during pregnancy, labour, delivery and breastfeeding.

- Integration of HIV care, treatment and support for women found to be positive and their families, which can be achieved through the use of antiretroviral drugs and safer infant feeding practices

In Ghana HIV-positive pregnant women were given single dose nevirapine (sd NVP) from 2003 till 2007 at the onset of labour. From 2007, a combination therapy was initiated at 28 weeks of gestation with sd NVP at onset of labour (NACP, 2010) and currently since 2011 the prophylaxis programme is initiated at 14 weeks of gestation. The PMTCT programme has enabled a steady reduction in new HIV infections in children between 0 to 14 years. In 2010, this was 3,359 cases, in 2011 it came down to 1,707 and by March 2012, it was 1,083 (NACP, 2012). The use of combination ART has been recommended by the World Health Organization (WHO) for treatment and management of HIV infection (Ghana Health Service, 2011) because it presents less drug resistance problems and reduces MTCT rates.

The use of single dose nevirapine during pregnancy and for infants after delivery for PMTCT is of major concern due to the issue of drug resistance. Studies carried out on mothers who had been exposed to antiretroviral prophylaxis pointed to the emergence of drug resistance (Jourdain *et al,* 2004; UNAIDS, 2013).

2.6.3 PMTCT with Antiretroviral Therapy

It is an accepted fact that use of ART has preventive effects in the transmission of HIV; it was reported in 2009 that universal voluntary HIV testing on an annual basis followed by immediate ART could reduce HIV incidence by about 95% within a decade (Granich *et al,* 2009**).** Research has proven that early treatment of HIV-infected people with

antiretrovirals protected 96% of sero-discordant partners from the infection (NIAID, 2011; Anglemyer *et al*, 2011). It has been proven through research that when post-exposure prophylaxis is initiated within 48-72 hours of exposure to HIV using antiretrovirals, the risk of infection is significantly reduced for needle stick injuries and sexual assault from a known HIV-positive person (Anglemyer *et al*, 2011; Celum and Baeten, (2012). Reduction in vertical transmission of HIV is crucial to control the infection. It has been reported that prevention of mother-to-child transmission can reduce rates of HIV transmission by more than 90% (Linden, 2011). The use of combination antiretroviral therapy in HIV infection as opposed to mono-therapy has reduced rates of mortality and morbidity (UNAIDS, 2013).

2.6.4 Elimination of Mother-To-Child Transmission (eMTCT)

Due to the collective global efforts in expanding global access to HIV care and support, treatment and increased capacity at all levels, not only in the resource endowed nations but the resource-limited nations as well, new HIV infections are decreasing significantly as more people receive treatment. With the focus on vertical transmission of HIV, the world is getting closer to achieving complete care and support for HIV-positive pregnant women. There are now better chances to eliminate mother-to-child transmission of HIV. In 2011, global leaders at the United Nations General Assembly High Level Meeting on AIDS made a commitment to end HIV infections among children by 2015 and to keep the mothers alive. In the developed world, elimination of Mother-To-Child Transmission (MTCT) has virtually being achieved. Mother –To-Child Transmission of HIV has fallen from 11% in 2010 to under 4% in 2012 in Kenya as reported in Nairobi on the 19th December 2012 edition of Plus News (UNAIDS, 2013).

Ghana is among seven (7) African countries that had a rapid decline of 30% in new HIV infections among children between 2009 and 2011(UNAIDS, 2013). If this rate of decline is sustained, then the target of eliminating MTCT by 2015 in Ghana is indeed a realistic target. Interventions such as screening pregnant women for HIV on their first visit to the health centre enable early detection of HIV-positive pregnant women so they are put on the prophylaxis programme by the 14th week of gestation. HIV-positive pregnant women are initiated into the PMTCT programme when they receive maternity care at a different point of the health care. Stigmatization has led to some mothers or pregnant women falling out of the care and support programmes for HIV-positive persons. Integrating services in Ghana such that maternal and child health issues are provided for at the same care point in the facility as the PMTCT, would help increase the number of HIV positive pregnant women accessing the PMTCT and thus help to reduce MTCT further. In 2012, ~~T~~ the Ghana AIDS Commission put forward a modification of Ghana's HIV policy to include the WHO's Option B Plus for accelerated achievement of 50 percent reduction of new HIV infections by the year 2015 projection (Ghana News Agency, 2012). The Option B Plus Policy places HIV pregnant women on ART for life, rather than Option B, which had a therapy period between 14 weeks to 12 months. To be able to eliminate MTCT of HIV in Ghana by 2015, the multipronged programme advocated by Deurr and his co-researchers (Deurr *et al*, 2004) must be fully implemented in the Ghanaian setting.

2.7 HIV Drug Resistance

During the replication of HIV, the enzyme Reverse Transcriptase, which converts the RNA genome of the virus into cDNA for insertion into the host's chromosomes, makes mistakes in copying the RNA. During the replication process, HIV produces about 10^{10} virions a day and for each new genome (10,000 base pairs long) one mutation may occur as a result of these spontaneous errors. Consequently, every possible mutation of the virus occurs at least once daily (Virco, 2006). Nucleotide mutations in HIV genes can result in changes in HIV proteins which may confer on the virus an increased ability to replicate, infect cells or to resist drugs. Thus viral replication in the presence of drug pressure will select drug resistant viruses. The consequence of the mutations that emerge in the viral proteins targeted by antiretroviral agents is drug resistance (Clavel and Hance, 2004; Virco, 2006).

Nucleotide mutations may also decrease the fitness of the virus making it more susceptible to drugs; or they may have no impact on viral fitness since they do not significantly affect the structure or function of the protein they encode. Mutations that improve viral fitness confer an advantage on the virus and they become part of the population of genetically related viruses (Virco, 2006). HIV drug resistance can be put into two categories: transmitted resistance, which occurs when previously uninfected individuals are infected with a drug-resistant virus; and acquired resistance, which occurs when resistance mutations emerge because of drug-selective pressure in individuals receiving antiretroviral therapy (Karlsson et al, 2012; Richman *et al*, 2001). Patients who develop resistance to a drug in one class, will develop resistance to another

drug in the same class. For example, in the case of NNRTIs, if resistance to Nevirapine (NVP) develops, the patient will develop resistance to Efavirenz though the latter drug had not been administered to the patient. This is referred to as Cross-Resistance. This is because of similar biologic mechanism of resistance to different drugs of the same class. Drug-resistant HIV strains often exhibit resistance to several classes of antiretroviral drugs and because cross-resistance between drugs within a class is frequent, the emergence of resistance always complicates further efforts to control viral replication (Clavel and Hance, 2004).

Resistance mutations are mostly substitution of the location of an amino acid for that of another in an amino acid sequence leading to changes in the structure and function of the protein. Thus most of the changes are due to replacement mutations as seen with M184V, Q151M, K70R, etc. In HIV-1 mutations, the first letter identifies the amino acid in the wild-type HIV, the number represents the location of the amino acid in the RT genome sequence and the last letter represents the amino acid that replaced the first amino acid in the resistant strain. Thus K70R refers to a mutation in which Arginine (R) replaces Lysine (K) which is normally at amino acid position 70 of RT. There could be cases where there is a mixture of viruses carrying different mutations at a particular amino acid position; M184I/V typifies such a situation which shows the presence of strains of HIV with either an Isoleucine (I) or Valine (V) substituting Methionine at amino acid position 184 of the RT (Clavel and Hance, 2004; Zaccarelli *et al*, 2004).

Table 5 below shows the amino acids that may be replaced by substitutions that may lead to mutations and subsequent drug resistance.

Table 5: Amino Acid Abbreviations (Virco, 2006).

Abbreviation	Amino Acid	Abbreviation	Amino Acid
A	Alanine	N	Asparagine
C	Cysteine	P	Proline
D	Aspartic acid	Q	Glutamine
E	Glutamic acid	R	Arginine
F	Phenylalanine	S	Serine
G	Glycine	T	Threonine
H	Histidine	V	Valine
I	Isoleucine	W	Tryptophan
K	Lysine	Y	Tyrosine
L	Leucine	Z	Glutamine
M	Methionine		

Insertion mutations, though rare, occur when there is insertion of one or more amino acids into an amino acid sequence. An example is seen with the 69 Insertion Complex which consists of substitution at codon 69 and an insertion of two or more amino acids. This can confer high-level resistance to all nucleoside analogues (Johnson *et al*, 2013 Clavel and Hance, 2004; Virco, 2006). Insertion mutations are found in about 2% of viruses which are resistant to NRTIs (Shafer, 2002).

Mutations usually emerge gradually and spontaneously as deviations from the consensus wild-type virus. In the face of ART and incomplete suppression of viral replication, the mutations are selected because they afford HIV a survival advantage over the wild-type. Hence such quasispecies of the viral population are selected as the wild-type succumb to the antiretrovirals (ARVs). These mutant variants eventually become the dominant viruses in the infected person, presenting resistance to the ART. Though the wild-type virus may be in the minority due to the ART suppression, they stay in the patient ready to emerge once the ARV therapy is stopped because then they are usually more fit than the resistant viruses (Clavel and Hance, 2004; Virco,2006).

Mutations that induce specific drug resistance on their own by limiting the activity of ARVs are known as major mutations. Examples are M184Vand K70R for Lamivudine and Zidovudine respectively (both NRTIs). Mutations that do not effectively limit the activity of ARVs but mediate increased levels of resistance or induce drug resistance in the presence of other mutations are referred to as minor mutations. Examples are the thymidine analogue mutations (TAMS) D67N, K219Q and L210W for reverse transcriptase inhibitors. Minor mutations may be encountered before, during and after the

appearance of major mutations though both may persist for years after the patient has been taken off the ARVs selecting for them. Another type of mutations is the Multi-drug Resistance (MDR) mutations which occur as a single mutation or as a group of mutations and cause cross-resistance within an ARV drug class (Johnson *et al*, 2013 Virco, 2006; Clavel and Hance, 2004).

Studies have shown that patients receiving antiretroviral therapy are infected with viruses which express resistance to at least one of the available antiretroviral drugs, making the transmission of drug-resistant strains an additional complication in the fight against the HIV/AIDS pandemic. In the United States of America, as many as 50 percent of patients receiving antiretroviral therapy were found to be infected with viruses that express resistance to at least one of the available antiretroviral drugs (Karlsson et al,2012;Richman *et al*, 2001). The use of combination antiretroviral therapy (HAART) is able to block the rapid emergence of resistant strains because it is difficult for resistance to develop against all the drugs in the regimen, the possibility of new variants being able to resist all the drugs is highly unlikely. Mono-therapy was replaced throughout the world with the combination therapy (triple drug regimen) because the latter is able to suppress viral replication more effectively which consequently precludes the generation of new variants. Viral resistance also emerges when HIV continues to replicate in the face of levels of drugs that are insufficient to block viral replication but sufficient to exert a positive selective pressure on variants with decreased drug susceptibility. Under such conditions, viruses with resistance to all the components of the regimen in use will gradually emerge (Clavel and Hance, 2004; Zdanowicz, 2006).

2.7.1 Antiretroviral (ARV)-Resistant Virus Selection

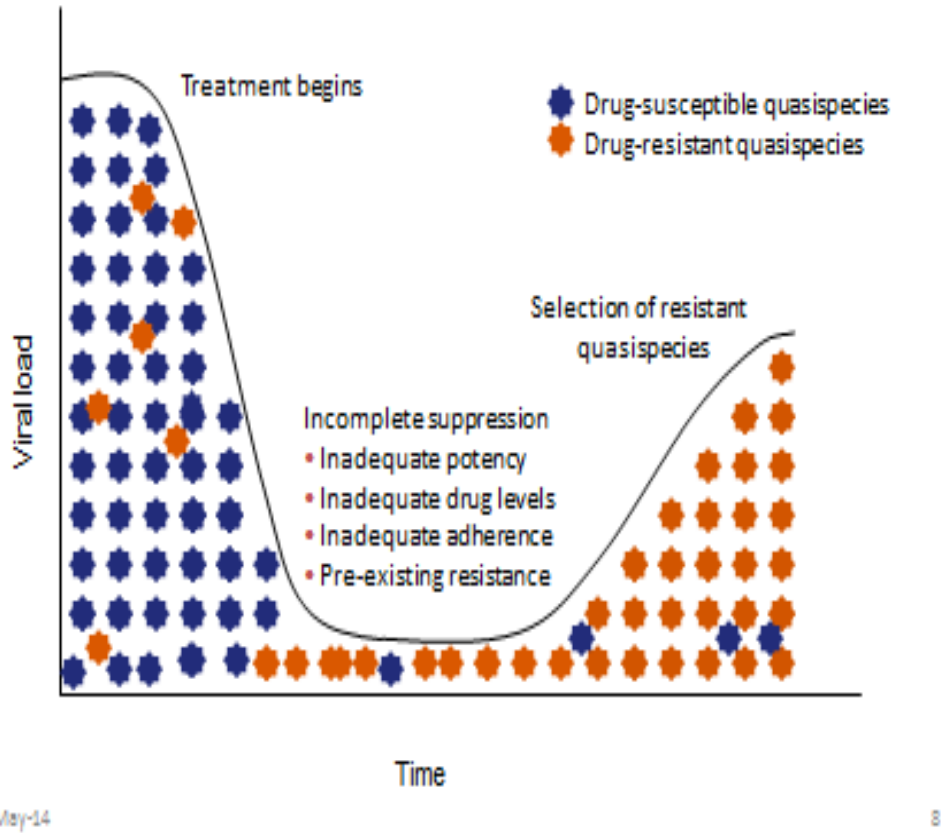
When ARVs are not able to completely suppress the replication of HIV, the virus population evolves under a specific selective pressure (the extent to which strains of the virus possessing certain particular characteristics are either eliminated or favoured by the prevailing drug controlled conditions) leading eventually to resistance. Two pointers to incomplete suppression of viral replication could be due to transmission of strains resistant to current regimen in use to the newly infected patient and inadequate adherence to ART as wells as inadequate potency or sub-therapeutic blood levels of the ARVs (Zdanowicz,2006; Clavel and Hance, 2004; Virco, 2006).

As a result of incomplete suppression of viral replication, ART preferentially selects quasispecies carrying mutations which confer survival advantage over the wild-type virus which still are sensitive to the existing drug regimen.

As depicted in Figure 5 on Page 50, during treatment with ARV drugs, the wild-type strains which are susceptible to the drugs become depleted whilst the mutant variant quasispecies increase in numbers , being resistant to the drugs and in time with sustained incomplete suppression of viral replication, the resistant HIV variants become the dominant quasispecies in the infected person (Figure 5 overleaf) who exhibits suboptimal response to the drug regimen and eventually fails therapy (Virco, 2006).

Figure 5: ARV –Resistant Virus Selection

(Source: http://www.vircolab.com/hiv-educational-forum/hivaids-learning modules)



2.8 Classes of Antiretrovirals (ARVs)

Each of the drugs in a combination regimen targets different points of the HIV-1 life cycle. Various combinations of HIV drugs are used in the drug regimen applied in HAART and have contributed immensely in controlling the development of drug resistance. Resistance mutations cause changes in the amino acid sequence, the structure and function of the HIV proteins, rendering the ARV agents ineffective against the virus. Most of these mutations are substitutions of one amino acid for another, and are named according to the position in the amino acid sequence and the change that occurred. The mechanisms by which HIV achieves resistance to the various ARVs differ by the class of the drug (Clavel and Hance, 2004; Virco, 2006).

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2.8.1 Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

Structurally NRTIs resemble nucleotides and are thus able to act at the Reverse Transcriptase (RT) phase of HIV life cycle to inhibit DNA synthesis. They achieve this by inserting themselves in the growing DNA strand, preventing any further addition of nucleotides to the DNA strand. DNA strand elongation is therefore terminated before the retro-transcription of the entire gene by the RT could be completed. However, resistance by the virus to the action of the NRTIs develops through two mechanisms- nucleotide excision and NRTI blockade. Nucleotide excision removes the inserted NRTI from the terminated DNA strand, clearing the way for the transcription of the gene to be

completed by the RT (Virco, 2006). The group of mutations that confer resistance on NRTI by the nucleotide excision mechanism is known as Thymidine Analogue Mutations (TAMs) - as seen with K70R, D67N and M41L for Zidovudine and Stavudine. TAMs are known to be highly cross-resistant and their accumulation reduces clinical responsiveness to almost all NRTIs (Clavel and Hance, 2004; World Health Organization, 2012).

Mutations that use the NRTI blockade mechanism enable the virus to prevent the incorporation of the NRTI into the DNA strand. Both M184V and Q151M mutations use this mechanism to prevent Lamivudine, Emtricitabine and Stavudine from binding the RT (Clavel and Hance, 2004; Zaccarelli *et al*, 2004; Domaoal and Demeter, 2004). M184V is located in the catalytic site of the RT (the active site of the enzyme that converts the substrate to a product or interacts with it) thus block the binding of the drugs. M184V is rapidly selected in the presence of Lamivudine and Emtricitabine because they have a low genetic barrier (the number of resistance mutations required to confer resistance to a particular anti-HIV drug) and so need only a single resistance mutation in RT to cause substantial loss of antiviral activity: M184V thus induces very high levels of resistance to Lamivudine and Emtricitabine. Q151M also works close to the catalytic site of the RT and prevents most NRTIs from binding, example Lamivudine and Tenofovir; it is observed in less than 5% of all NRTI resistant viruses (Domaoal and Demeter, 2004).

2.8.2 Non- Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Non-Nucleoside reverse transcriptase inhibitors (NNRTIs) are small molecules lacking a 3´hydroxyl group and thus have a strong affinity for a hydrophobic pocket located close to the catalytic domain of the reverse transcriptase (RT); they act at the RT phase of the

HIV life cycle by binding directly to the RT and thus affecting the flexibility of the enzyme and making it non-functional. This activity blocks the ability of the enzyme to synthesize DNA. Resistance to NNRTIs develops when amino acid substitutions change the position or the three-dimensional structure of the RT so NNRTIs can no longer bind to the enzyme (Miller, 2001).

2.8.3 Nucleotide Reverse Transcriptase Inhibitors (NtRTIs)

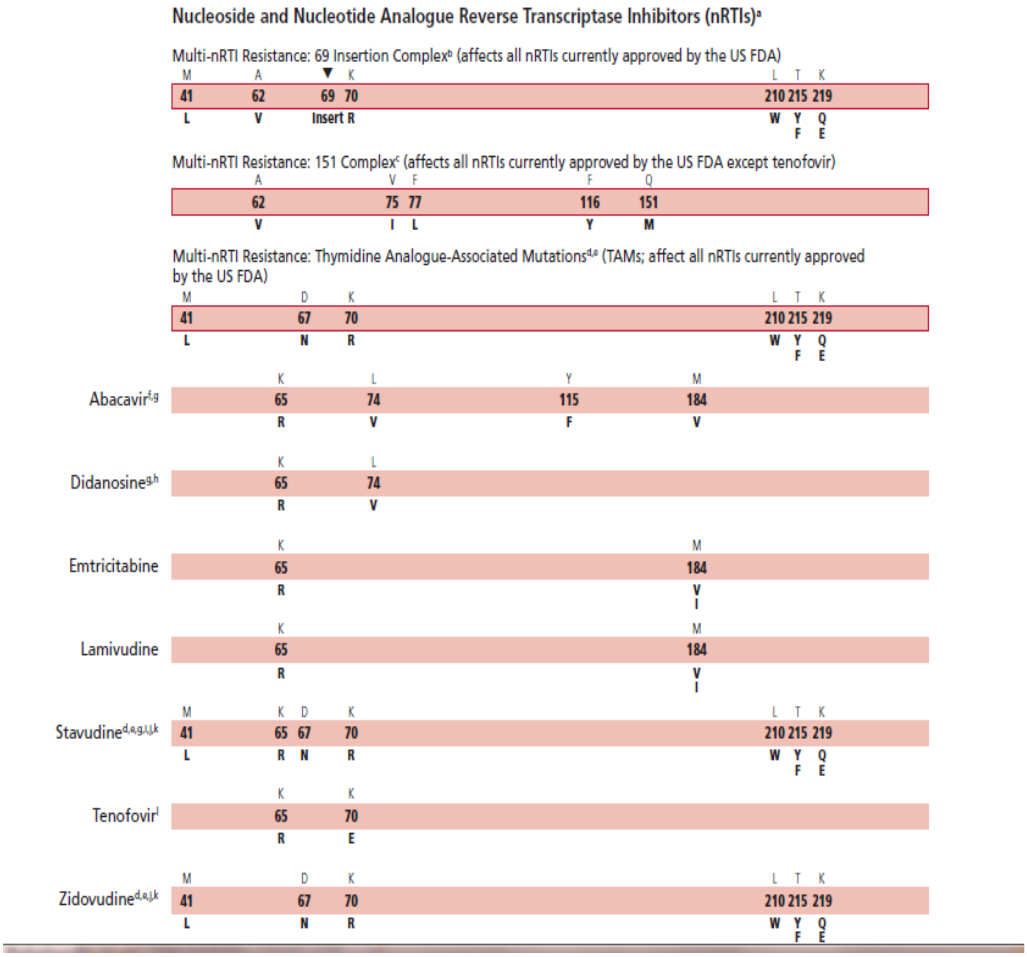
The Nucleotide Reverse Transcriptase Inhibitors *(NtRTIs)* are a class of antiretrovirals very similar to NRTIs and works by incorporation into the DNA strand, thus terminating the strand elongation prematurely. This class of antiretrovirals currently has Tenofovir (Viread) and a combination of Tenofovir with Emtricitabine (an NRTI) called Truvada. K65R mutation is able to prevent the incorporation of Tenofovir into DNA by using the nucleotide blockade mechanism; K65R mutation is seen with increasing frequency in HIV-1 patients in whom therapy with nucleoside or nucleotide analogues is failing especially if the regimen includes Tenofovir or Abacavir. The mutation appears to confer resistance to most analogues, with the exception of Zidovudine (Zaccarelli *et al,* 2004).

Figures 6 & 7 on Pages 54 and 55 show a list of mutations in the reverse transcriptase gene associated with clinical resistance to reverse transcriptase inhibitors which may contribute to a reduced virologic and immunologic response to an antiretroviral drug (Johnson *et al*, 2013).

This IAS-USA generated list of mutations have been identified by one or more of the following criteria: (1) in vitro passage experiments or validation of contribution to resistance by using site-directed mutagenesis; (2) susceptibility testing of laboratory or clinical isolates; (3) nucleotide sequencing of viruses from patients in whom the drug is failing; (4) association studies between genotype at baseline and virologic response in patients exposed to the drug (Johnson *et al*, 2013)..

Figure 6: IAS-USA-Recogniszed NRTI Mutations

(Source: http://www.iasusa.org/resistance_mutations/index.html)



Nucleoside and Nucleotide Analogue Reverse Transcriptase Inhibitors (nRTIs)[a]

Multi-nRTI Resistance: 69 Insertion Complex[b] (affects all nRTIs currently approved by the US FDA)

	M	A	▼	K								L	T	K
	41	62	69	70								210	215	219
	L	V	Insert	R								W	Y	Q
													F	E

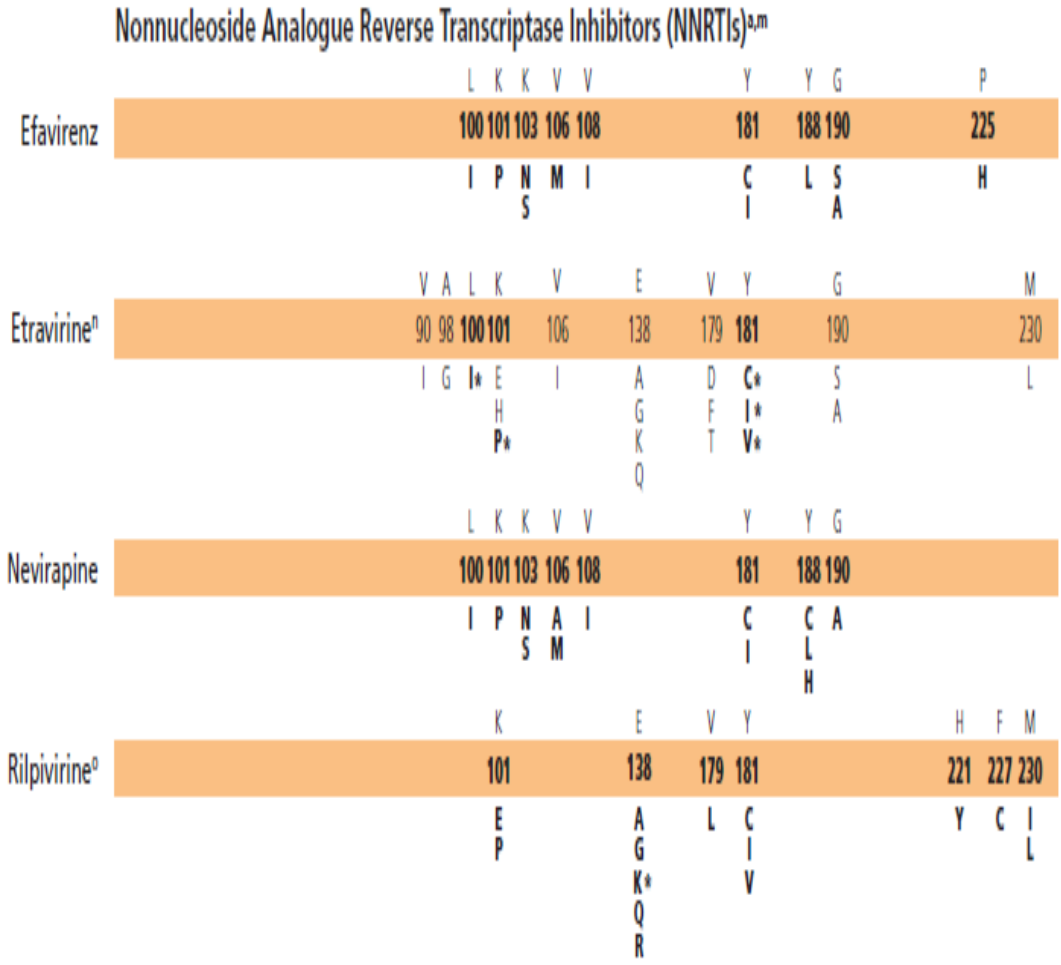
Multi-nRTI Resistance: 151 Complex[c] (affects all nRTIs currently approved by the US FDA except tenofovir)

	A		V	F			F		Q					
	62		75	77			116		151					
	V		I	L			Y		M					

Multi-nRTI Resistance: Thymidine Analogue-Associated Mutations[d,e] (TAMs; affect all nRTIs currently approved by the US FDA)

	M		D	K								L	T	K
	41		67	70								210	215	219
	L		N	R								W	Y	Q
													F	E
		K			L			Y		M				
Abacavir[f,g]		65			74			115		184				
		R			V			F		V				
		K			L									
Didanosine[g,h]		65			74									
		R			V									
		K								M				
Emtricitabine		65								184				
		R								V				
										I				
		K								M				
Lamivudine		65								184				
		R								V				
										I				
	M	K	D	K								L	T	K
Stavudine[d,e,g,i,j,k]	41	65	67	70								210	215	219
	L	R	N	R								W	Y	Q
													F	E
		K		K										
Tenofovir[l]		65		70										
		R		E										
	M		D	K								L	T	K
Zidovudine[d,e,j,k]	41		67	70								210	215	219
	L		N	R								W	Y	Q
													F	E

Figure 7: IAS-USA-recognized NNRTI Mutations



(Source: http://www.iasusa.org/resistance_mutations/index.html)

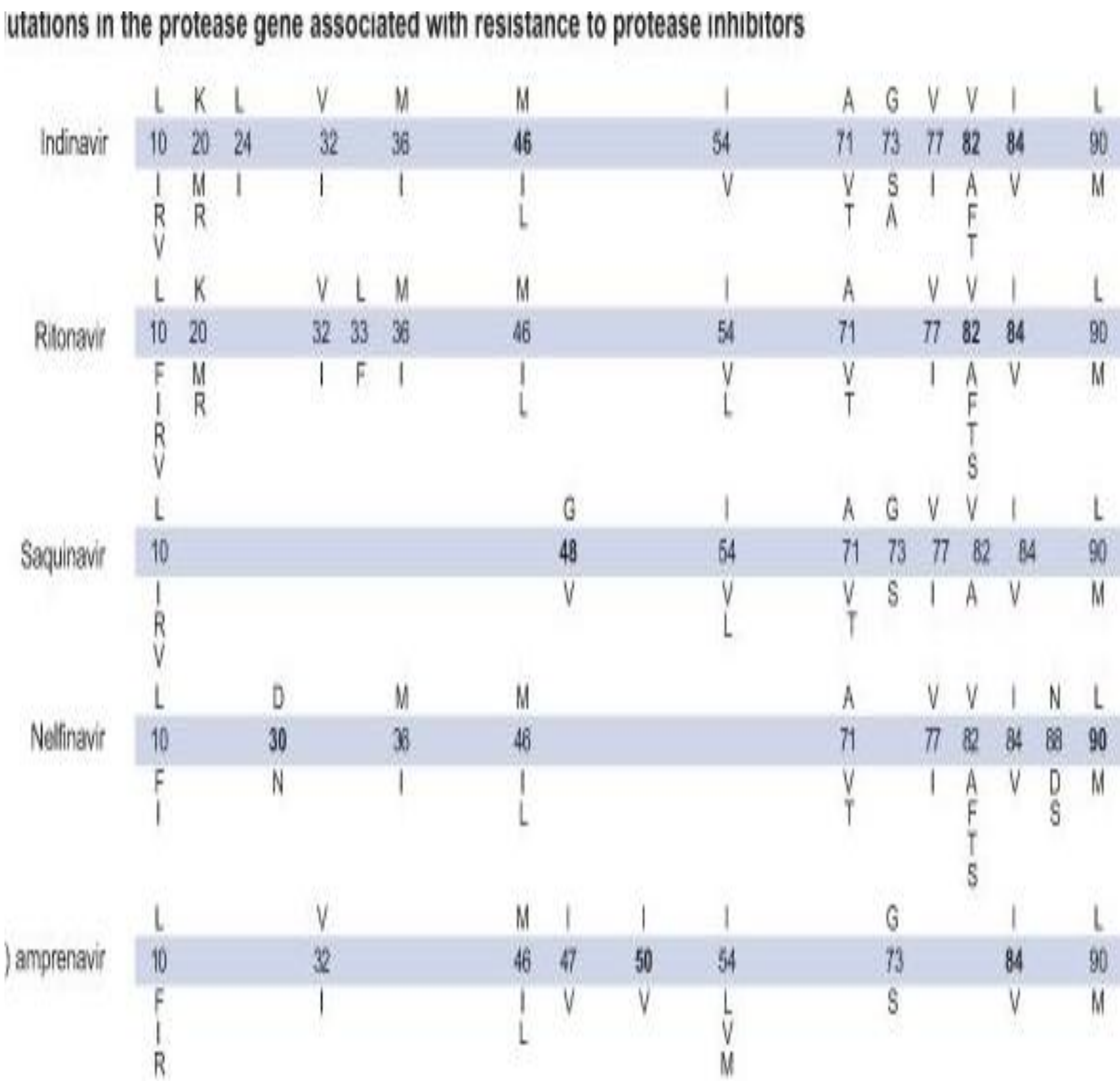
2.8.4 Protease Inhibitors (PIs)

Examples of Protease Inhibitors (PIs) are Nelfinavir, Ritonavir, Lopinavir and Indinavir. The action of PIs in the HIV life cycle is seen much later after the action of NRTIs and NNRTIs. The HIV protease enzyme cleaves polypeptides into structural and non-structural protein precursors for packaging into new virions (infectious viral particles).When this function is blocked, viral particles produced are not infectious. The HIV protease enzyme is functional when it is part of a symmetrical homo-dimer. The PIs bind to the substrate-binding site of this dimerized enzyme, preventing it from cleaving the non-functional polypeptide chains into functional individual HIV structural and non-structural proteins. In the absence of these functional proteins the new virions cannot mature and infect other cells. Resistance to the PIs develops when amino acid substitutions emerge either inside the substrate-binding domain of Protease or at distant sites (Clavel and Hance, 2004; Virco, 2006). This changes the structure of the substrate-binding site of the enzymes and thus reduces the affinity of the inhibitors for the enzymes as the inhibitor's binding capacity is decreased but allows the natural substrate to be processed (Bally *et al*, 2000). Examples of such mutations are V82A which frequently causes decreased susceptibility in Ritonavir and Indinavir therapy, D30N which is frequently selected for by Nelfinavir treatment, I84Vwhich is frequently found after prolonged ineffective therapy with PIs and is associated with high-level resistance to most PIs (Clavel and Hance, 2004; Johnson *et al*, 2013)**.** PIs select for mutations that tend to confer cross-resistance within the class such that resistance to one PI would usually lead to resistance in some other or most other PIs though it takes several mutations to produce high-level resistance to PIs (Clavel and Hance, 2004; Virco, 2006).

Gag and *Gag-Pol* polyproteins are large multiprotein chains that are cleaved by protease during HIV-1 replication. PI resistance may also be due to mutations at these cleavage sites in *gag* and *gag-pol* polyproteins. When such mutations occur, HIV protease can cleave *gag* and *gag-pol* polyproteins in the presence of protease inhibitors. Such mutations make up for the loss of cleavage capability of Protease in the presence of PIs, and are referred to as Compensatory Mutations; examples are A431V and L449F (Reeves *et al*, 2004).

Overleaf are some mutations associated with resistance to PIs (Johnson *et al*, 2013)**.**

Figure 8: IAS-USA Recognized PI Mutations



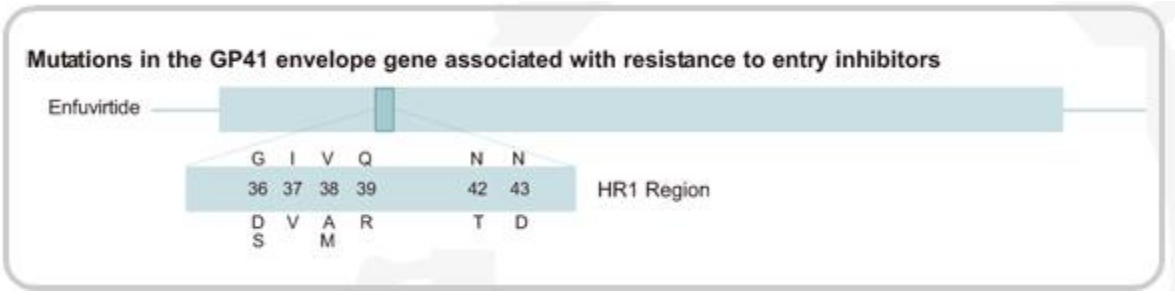
(Source: http://www.iasusa.org/resistance_mutations/index.html)

2.8.5 Entry Inhibitors: Fusion Inhibitors (FIs)

Fusion Inhibitors (FIs) act earlier in the life cycle of HIV-1 than NRTIs, NNRTIs and PIs. In a bid to prevent HIV-1 fusing with and subsequently gaining entry into the host cells, FIs target the glycoprotein 41(gp41) of the HIV envelope. In the gp41 two domains, the proximal helix (HR1) and the distal helix (HR2), must fold together to enable the gp41 to fuse with the target T-cell membrane (Virco, 2006). Enfuvirtide (T 20), which is a 36-amino acid peptide derived from HR2, binds to HR1 preventing the folding of HR1 and HR2 and therefore HIV-1 cannot fuse with the T-cell membrane of the target and cannot infect the cells. Viral resistance to enfuvirtide develops when mutations in a span of 10 amino acids- 36 to 45- of the gp41 in the HR1 domain reduce the ability of the FI to bind to it (Virco, 2006; PRN, 2008).

The figure below shows the mutations associated with resistance to FIs, in which resistance to Enfuvirtide is associated primarily with mutations in the first heptad repeat (HR1) region of the gp41 envelope gene (Johnson *et al*, 2013):

Figure 9: IAS_USA Recognized FI Mutations



(Source: http://www.iasusa.org/resistance_mutations/index.html)

2.8.6 Integrase and Co-Receptor Inhibitors

Integrase inhibitors are the newest class of antiretroviral agents developed to treat HIV-1 infection. The HIV-1 encoded enzyme, Integrase, integrates HIV-1 complementary DNA (cDNA) into the host genome. When this process is prevented, the establishment of viral latency within the host cell is blocked, replication and the subsequent infection of new cells by competent virions stops (Clavel and Hance, 2004; Virco, 2006). An example of Integrase Inhibitors is Raltegravir (RAL) which has proven to be a potent and well-tolerated antiretroviral (ARV) agent. It is yet to be used for the treatment of both ARV-experienced and ARV-naive patients in Ghana.

Co-receptor blockers such as CCR5 Inhibitors (Maraviroc) have been designed to stop HIV from locking onto immune system cells in the host (Masquelier *et al*, 2001).

2.9 Resistance Testing

Though there are potent and effective ART agents in use under the HAART programme, drug resistance continues to lower the long-term efficacy of HAART (Zaccarelli *et al*, 2004). Resistance testing is therefore essential to reveal the extent to which the susceptibility of the drug has been compromised, providing specific information on the resistance profile of the patient to guide appropriate regimen. Resistance testing also identifies ARVs to which the dominant quasispecies in an HIV positive person are susceptible or resistant. It can also be effectively used in monitoring the development of resistance and cross-resistance in an individual. Resistance testing is useful in understanding the mechanisms of viral resistance to ARVs and also for establishing reasons for viral rebound (Virco, 2006; Knipe and Howley, vol 1, 2007).

There are two resistance testing technologies in use presently; Genotypic Resistance Testing which reveals the presence of mutations in the viral genome that are known to be linked to drug resistance, and Phenotypic Resistance Testing which describes the level of susceptibility of a virus to antiretroviral drugs measured under *in vitro* conditions in the laboratory (Zdanowicz,2006).

2.9.1 Genotypic Resistance Testing

In genotypic testing, specific nucleotide sequences encoded by the virus genome are identified. The nucleotide sequence is then translated into amino acids and duly interpreted. Genotypic assays check for the genetic basis for resistant phenotypes, and also involve detection of restriction fragment length polymorphism and nucleotide sequencing. These assays are useful when the genetic basis for resistance is known and the resistance is accounted for by a limited number of genetic changes (Knipe and Howley, vol 1, 2007; Shafer, 2002). Genotypic testing can be done for persons with a viral load under 1,000 copies/ml because genotypic testing only involves comparing a person's specific HIV mutations to known drug resistant mutation points. In genotypic assays, using blood from the patients, the relevant parts of the viral genome-either of the protease gene or of the reverse transcriptase gene- are amplified by PCR and the sequence of nucleic acids determined and duly translated into amino acids. The amino acid sequence is aligned with the wild type virus (WT) sequence so as to identify amino acid substitutions or mutations. At the end of a genotypic resistance testing a list of all the amino acid substitutions that were identified is generated and mutations are interpreted using rules-based algorithms such as the Stanford HIV Drug Resistance Database

(http://hivdb.stanford.edu) which provides useful guidance in interpreting the genotypic results (Johnson *et al,* 2013;Virco,2006; Shafer,2002).

2.9.2 Phenotypic Resistance Testing

Phenotypic resistance testing measures the concentration of the drug required to inhibit HIV replication to a certain degree of an internationally defined amount of inhibition, either by 50% (IC_{50}) or 90% (IC_{90}), the concentration of drug that inhibits viral replication by the percentage indicated (Virco, 2006; Garcia-Lerma and Heneine, 2002) . In this test, the virus is either directly isolated from the plasma of the patient or generated from its genome and the genome of a wild type reference laboratory isolate. *In vitro* effects of the drugs on the replication of the virus or recombinant virus is evaluated. Replication of the virus is inhibited if susceptible to the ARV, but if resistant then replication will occur. The ICs are calculated for each ARV being tested against a wild type reference HIV isolate, expressing the value in concentration units or change in the IC indicated (Virco, 2006).

CHAPTER 3

3.0 METHODS

3.1 Study Design

This was a cross-sectional study that involved HIV-infected patients previously exposed to HIV drugs administered as prophylaxis to prevent transmission to the baby of the pregnant woman. These women were later treated with the same antiretrovirals in accordance with the national ART protocols. HIV-infected treatment-naïve pregnant women and treatment-experienced mothers were included as separate groups.

3.2 Study Participants & Sites

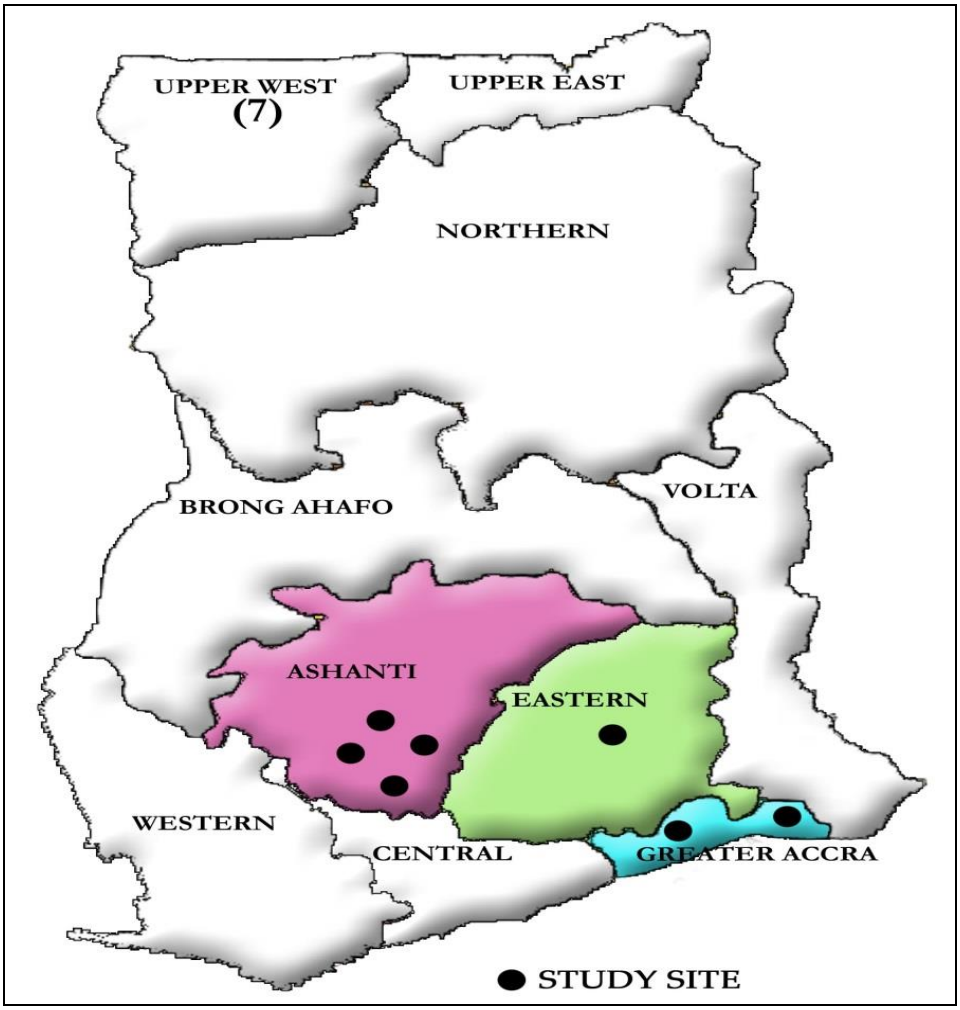
Patients accessing treatment, care and support at selected hospitals and clinics in three (3) regions in Ghana were recruited as participants in the study. These HIV seropositive women sought care at health facilities which are care and support sites of the National AIDS/STI Control Programme (NACP). The selected sites were the Korle -Bu Teaching Hospital in Accra, Tema General Hospital (both in the Greater Accra Region); Kumasi South Hospital, Suntreso Government Hospital, Kumasi, Bomso Clinic, Kumasi and Animwah Medical Centre, Kumasi (all in the Ashanti Region); and the Eastern Regional Hospital, Koforidua. Bomso Clinic and Animwah Medical Centre are private facilities in two suburbs of Kumasi, ie Bomso and Emena respectively (Figure 10 at Page 65). The other NACP sites in the study are government-owned hospitals covering wide catchment areas in the various cities. These health facilities have all participated in the national Prevention of Mother-To-Child Transmission (PMTCT) of HIV programme for at least three (3) years. The patients who consented to participate in the study were HIV positive mothers and HIV positive pregnant women at gestational periods less than 28 weeks. At

all the study sites, the patient folders at the Data Unit were used to select patients who met the following criteria.

3.2.1 Study Group (Group1) and ART

The Study Group was made up of HIV positive mothers who had been on antiretroviral prophylaxis for prevention of transmission of the virus to the foetus when they were pregnant and had subsequently been put on full ART for their own health needs. According to the national protocol, some of the women had received single dose Nevirapine (sd NVP) as prophylaxis under the PMTCT programme. Others had received Combivir, which is a combination of Lamivudine (3TC) and Zidovudine (AZT), at week 28 of pregnancy plus the sd NVP at the onset of labour. These women were taken off the prophylactic drug regimen after delivery if their CD4 count was more than 350 cells /µL. These women were later deemed eligible for ART for their own health. The period since the prophylaxis ranged from 1 to 44 months and their CD4 counts had dropped to less than 350 cells /µL. The ART regimen given to these women contained the combination of 3TC and AZT, and Nevirapine (NVP), or Efavirenz (EFV) if the Nevirapine was contraindicated; in some cases Stavudine was given in place of Zidovudine.

Figure 10: Map of Ghana Showing the Regional Distribution of the Study Sites



- Greater Accra Region: Tema General Hospital, Tema & Korle Bu Teaching Hospital, Accra.
- Eastern Region: Eastern Regional Hospital ,Koforidua
- Ashanti Region: Kumasi South Hosptal , Suntreso Government Hospital, Bomso Clinic, & Animwah Medical Centre.

3.2.2 Drug-Naïve Control Group (Group 2)

This group comprised HIV-positive pregnant women who had not had any prior exposure to ARVs. The CD4 counts for patients in this group were above 350cells/µL. During the period of the study, the national policy on PMTCT stipulated that such women initiate prophylaxis at 28 weeks of gestation. For the purposes of the study, it was affirmed that these women had not had any prior initiation with antiretrovirals. The majority of them were pregnant for the first time. Those who consented had their blood samples taken before the date for initiation into the prophylactic drug regimen.

3.2.3 Drug-Experienced Control Group (Group 3)

This group was made up of mothers who had initiated ART when they were pregnant without prophylaxis because their CD4 count levels were below 350 cells /µL at the time of ARVs initiation. This group needed ART for their own health as well as for prevention of transmission of HIV to their babies. The drug regimen that was given to this category of HIV positive women was either a combination of Zidovudine (AZT), Lamivudine (3TC) and Nevirapine (NVP) or Stavudine (D4T) replacing AZT. Some of these women had been on ART before they became pregnant. These women were still on ARVs during the study period and needed to continue with the regimen for their own health.

3.2.4 Inclusion and Exclusion Criteria

- Inclusion Criteria:
  - ✓HIV positive mothers on ART after previous prophylaxis
  - ✓HIV positive mothers who had complete treatment records available
  - ✓Drug-naïve HIV positive mothers yet to initiate prophylaxis
  - ✓Drug-experienced HIV positive mothers who initiated ART without prophylaxis
- Exclusion Criteria:
  - ✓ HIV negative pregnant women
  - ✓ HIV positive non-pregnant drug-naïve women
  - ✓ HIV Type 2 infected women were excluded since the study was on HIV Type 1 infections only.

3.3 Sample Size and Clinical Details

The hospital attendance records of the patients at the health facilities were used to identify a total of 221 patients who satisfied the inclusion criteria for the study; hence the Convenient Sampling method was applied to recruit participants at the selected study sites. Both cases and controls were enrolled after written informed consent had been obtained. Antiretroviral history including drugs administered as prophylaxis, initiation date, adherence to drug regimen, confirmation of prophylaxis or full treatment during the first pregnancy were accessed from patient charts or electronic databases. The details of the ART regimen in use at the time of the study, the WHO HIV Clinical Stage, trends

of CD4 counts, and their adherence status were extracted from their hospital folders and recorded.

3.3.1 Sample Collection & Processing

A structured questionnaire was used to obtain basic socio-demographic and clinical data from cases and controls.

After explanation of the study and obtaining written informed consent from the patients at the study sites, the patients were seated comfortably and ten (10) mls of whole blood was taken from the antecubital vein of each participant into Ethylenediamine tetra-acetic acid (EDTA) treated tubes. At each sampling site, 1ml of the whole blood was pipetted into a CD4 tube for the CD4 + T cell count measurement on site, since this was done routinely at these sites.

The rest of the blood samples were placed in an ice chest with ice packs and transported to the Virology Department of Noguchi Memorial Institute of Medical Research (NMIMR) at Legon, Accra. At the NMIMR, plasma was separated from the whole blood through centrifugation at 2000*g* for 10 minutes and three aliquots of 1.8 mls in two vials and 0.5 mls in one vial were stored at minus (-)70^{o}C until analyzed.

3.4 Laboratory Analyses

3.4.1 CD4 +T Cell Measurement

The CD4 +T cell count was determined by flow cytometry using BD FACSCount Cytofluorometer and cell-quest software (~~m~~ Becton-Dickinson, Franklin Lakes, New Jersey, USA). During the procedure, 50µl of a well-mixed sample was added to the CD4

reagent in a tube, mixed and kept at room temperature in the dark for 1 hour. Then, 50µl of a fixative solution was added to stabilize and stop the reaction. The FACScount machine was used to measure the CD4 + T cell count.

3.4.2 Determination of HIV Type

HIV typing of the samples was done using a Line Immuno Assay, INNO-LIA HIV I/II Score kit (by INNOGENETICS, BELGIUM). The manufacturer's instructions for preparing the reagents and the test procedure were followed in carrying out the assay to identify HIV-1 and HIV-2 infections.

For each test run, the needed number of test troughs was labeled with an assay identity that corresponded to the coded sample identity; controls (positive and negative) were assayed with each test run. Into each test trough, 1ml of a sample diluent was placed and 10 microlitres (µl) of specimen and controls added to the appropriate test trough. One test strip was then placed into each trough with the membrane side upward, ensuring that the strip was submerged in the fluid in the trough.

The troughs were covered with an adhesive seal, and the test tray placed on a shaker and incubated at room temperature with rocking for 16 hours. After the incubation the adhesive was carefully removed to avoid cross-contamination. Each test strip was washed three times with 1ml of prepared wash solution each time and then 1ml of a conjugate solution was added to each test trough with the strip still membrane-side up. The test tray was placed back on the shaker and incubated at room temperature with rocking for 30minutes. After the second incubation period, the washing was repeated after which 1ml of a substrate solution was added to each trough and the tray placed on the shaker and incubated for another 30minutes at room temperature with rocking. The fluid in each

trough was carefully aspirated after this incubation process and then 1ml of the stop solution was added to each test trough. The troughs were incubated on the shaker for 15minutes with the stop solution. After the 15minutes incubation the stop solution was completely aspirated and the test trays left on the shaker to thoroughly dry. The dried test strips were then pasted membrane side-up on the score sheet provided with the kit for interpretation of the test results using the manufacturer's interpretation criteria to differentiate HIV-1 from HIV-2.

3.4.3 HIV RNA Extraction

RNA was extracted from the plasma samples and purified using QIAamp Viral RNA Mini kit (QIAGEN, USA). The RNA extraction procedures were all carried out in the Biosafety Class IIA Hood (Air Tech Services, India). The plasma samples were allowed to thaw and equilibrate to the room temperature (15-25^{o}C).Two sets of the required number of 1.5ml microcentrifuge tubes were labelled with specific identity numbers. The Carrier RNA from the test kit was added to the Buffer AVL to obtain the Buffer AVL-Carrier RNA. Then, 560µl of Buffer AVL-carrier RNA mixture was dispensed into one set of labeled microcentrifuge tubes, followed by 140 µl plasma being added. This mixture of plasma samples and Buffer AVL-carrier RNA was vortexed for 15seconds and incubated at room temperature (15–25°C) for 10 minutes. Each tube was briefly centrifuged to remove drops from the inside of the lid and 560 µl of absolute ethanol added and mixed by pulse-vortexing for 15 seconds, again taking the precaution of briefly centrifuging each tube to remove drops from inside the lid after pulse-vortexing. From this solution, 630 µl was carefully applied to previously labeled QIAamp Mini

columns in a 2ml collection tube without wetting the rim. The caps were closed and centrifuged at 8000 rpm for 1 minute, placing the QIAamp Mini columns into a clean 2 ml collection tubes, and discarding the tubes containing the filtrate. The mini columns were carefully opened and the step repeated with the remaining of the solution. With new collection tubes in place, the caps of the mini columns were carefully opened and 500 µl of Buffer AW1 added. The caps were then closed and centrifuged at 8000 rpm for 1 minute. After the centrifugation, the QIAamp Mini columns were placed in clean 2 ml collection tubes, discarding the tube containing the filtrate.

After this step, the caps of the Mini columns were carefully opened and 500 µl of Buffer AW2 was added; the caps closed and centrifuged at maximum speed for 3 minutes. After the centrifugation, the Mini columns were placed in new 2 ml collection tubes, discarding the old collection tube with the filtrate. The spin columns with the new collection tubes were centrifuged without any added material at maximum speed for 1 minute. This ensures that no remnant Buffer AW2 is carried over into the next step. The mini columns were then placed in the other previously labeled batch of clean 1.5 ml microcentrifuge tubes, having discarded the old collection tube containing the filtrate. The caps of the mini columns were carefully opened and 60 µl of Buffer AVE equilibrated to room temperature was added. The caps were closed and incubated at room temperature for 1 minute and centrifuged at 8000 rpm for 1 minute. The extraction produced a final RNA elution volume of 60µl. The RNA extracts were stored at -70^{o}C till further analysis.

3.4.4 Viral Load Determination

For each sample, plasma HIV-1 RNA was quantified using Taqman-based Real-Time RT-PCR and HIV-1 LTR primers according to an in-house protocol (Barnor *et al*, 2014) in the HIV Genotyping Laboratory of the Virology Department of the Noguchi Memorial Institute for Medical Research (NMIMR). The PCR reaction mixture was prepared in the PCR Clean Hood designated for Master Mixture preparation and a plate map designed to show the wells in which the samples would be analyzed. The following Primers were used:

F1 (sense) 5'GCCTCAATAAAGCTTGCCTTGA-3',

R1 (antisense) 5'GGCGCCACTGCTAGAGATTTT3' and

p1LNA probe 5' FAM -CAGTACATGCAGGGCCTATTCCACCAG-TAMRA 3'

An aliquot of 22µl of master mixture (MM) was dispensed into the wells of the plate and 3µl of the RNA extract was added to the wells according to the sample plate map previously designed. Each RNA template (RNA extract) was mixed thoroughly with the micro-pipettor as each was added and the wells covered with optical caps.

Using the Real-Time PCR System 7300 (ABI USA), a plate map was created in the software of the ABI 7300 system. The samples were then placed in the ABI 7300 system and the run started for quantification under the cycling conditions of the in-house protocol used (Barnor *et al*, 2014). The plasma HIV RNA copy was calculated by feeding the Ct values obtained into an in-house formula to generate the viral load copy numbers for the samples (Barnor *et al*, 2014).

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

Plasmid copy number for plasmid with concentration of 5 µg/µl = $1.14X10^4$

For samples with undetectable Ct values, the Ct value of 40 (indicating the maximum number of cycles for the PCR run) was used. This set the lower detection limit of the assay.

3.4.5 Reverse-Transcription Polymerase Chain Reaction (RT-PCR) for Reverse Transcriptase and Protease genes from RNA Extracts

Using RT-PCR and nested PCR techniques, the Reverse Transcriptase (RT) and Protease (PR) genes of HIV-1 in each sample extract was amplified. For the RT-PCR process, the QIAGEN One-Step RT-PCR Kit was used for the amplification, according to the manufacturer's protocol (Handbook, QIAGEN One-Step RT-PCR, 2010). Primers used for the RT gene and the PR gene were DRRT1L/DRRT4L and DRPRO5/DRPRO2L respectively (shown at Page 76), as has previously been described (Fujisaki *et al*, 2007). The thermal cycling conditions applied were described previously (Villahermosa *et al*, 2000).

For further amplification of the round 1 products by nested PCR, AmpliTaq Gold Master Mix Kit (ABI, USA) was used with primers DRRT7L/DRRT6L and DRPRO1M/DRPRO6 for RT and PR genes respectively (shown at Page 75), as previously described (Fujisaki *et al*, 2007). The thermal cycling conditions used were as previously described (Villahermosa *et al*, 2000).

Each batch of tests (run) included a known positive HIV-1 control sample and a negative sample. For the Round 1 PCR process, PCR tubes were labeled with the sample numbers, the gene of interest and date of assay. Then 20 µl of the prepared master mix was dispensed into the previously labeled PCR tubes and 5µl of the RNA extracts was added to corresponding tubes. These were properly mixed and placed in the thermal cycler, the cycling conditions set and the run started.

For the nested PCR (Round 2) run, the master mix reaction was prepared according to the manufacturer's instructions. Then 20 µl of the master mix was dispensed into the previously labeled PCR tubes (Ambion,USA) then 5µL of the Round 1 PCR products were added to the PCR tubes as template, properly mixed and placed in the thermal cycler (ABI, USA) with the appropriate cycling conditions and the run started.

Primers used in the RT-PCR (Round 1) and nested PCR (Round2) steps as previously described (Fujisaki *et al*, 2007) are shown below; the name of the primer, its location on the HXB2 sequence and the primer sequence from 5'to 3' is shown. HXB2 is a reference HIV-1 subtype B sequence.

RT OUTER SENSE 5' ATGATAGGGGGAATTGGAGGTTT 3'DRRT1L (HXB2 POSITIONS 2388-2410)

RT OUTER ANTISENSE):5' TACTTCTGTTAGTGCTTTGGTTCC 3'DRRT4L (HXB2 POSITIONS 3425-3402)

RT INNER SENSE: 5' GACCTACACCTGTCAACATAATTGG 3' DRRT7L (HXB2 POSITIONS 2485-2509)

RT INNER ANTISENSE: 5' TAATCCCTGCATAAATCTGACTTGC 3' DRRT6L (HXB2 POSITIONS 3372-3348) UY

PR OUTER SENSE: 5'AGACAGGYTAATTTTTTAGGGA 3' DRPRO5 (HXB2 POSITIONS 2074-2095)

PR OUTER ANTISENSE: 5'TATGGATTTTCAGGCCCAATTTTTGA 3'DRPRO2L (HXB2 POSITIONS 2716-2691)

PR INNER SENSE: 5' AGAGCCAACAGCCCCACCAG 3' DRPRO1M (HXB2 POSITIONS 2148-2167)

PR INNER ANTISENSE: 5' ACTTTTGGGCCATCCATTCC 3' (DRPRO6 (HXB2 POSITIONS 2611-2592))

3.4.5.1 Agarose Gel Electrophoresis

To verify and analyze the products of RT and PR genes from the nested PCR assay, two concentrations (1.5% and 2%) of Agarose Gel (from SIGMA, USA) in Tris–acetate-EDTA (TAE) buffer (1X) (from AMBION, USA) were prepared. For the products of both RT and PR genes, positive and negative controls and a 100bp DNA ladder (Invitrogen, USA) were incorporated in each run.

The bands in the gel were visualized using a Gel Documentation system (GEL-LOGIC 100 Imaging System from Kodak) with a High Performance Ultraviolet-Transilluminator (UVP, UK). The samples with positive bands for the RT gene (at 887bp) and PR gene (at 467bp) were recorded. These amplicons were purified for further analysis.

3.4.5.2 Purification of PCR products

The verified nested PCR products were cleaned using QIAquick PCR Purification Kit from QIAGEN. Each sample was prepared by adding 100 µl of Buffer PB (from the Kit) to 20 µl of the sample in small flat top PCR tubes. To bind DNA, the total volume was transferred to a combination of a QIAquick column and a 2ml collection tube and centrifuged at 13,000rpm for 60seconds. The flow-through was discarded and the column placed back in the same collection tube. Washing was done by adding 0.75ml of wash Buffer PE (from Kit) to the QIAquick column and centrifuged for 60seconds at 13,000rpm. The flow-through was again discarded and the column placed back in the same tube. The combination was centrifuged again without any additions for 1 minute at the same speed to remove residual wash buffer. Each QIAquick column was then placed

in previously labeled clean 1.5ml microcentrifuge tube. For the elution of DNA, 50 µl of the elution Buffer EB (10mMTris-Cl, pH 8.5 from Kit) was added carefully to the center of the QIAquick membrane and the column centrifuged for 60 seconds at 13,000rpm. The end product was stored at -20^{o}C until use. This provided the DNA required for sequencing.

3.4.6 Cycle Sequencing

Cycle sequencing was performed on the purified PCR products using the Big Dye Terminator Cycle Sequencing Kit vs 3.1 from Applied Biosystems Inc (ABI), USA. The master mixture contained 2µL each of nuclease-free water, 5X sequencing buffer, forward and reverse primers and Big Dye Terminator Mix. Master mixture (8µL) was dispensed into each PCR tube labeled with the appropriate sample ID, followed by dispensing 2µl of the template to the mix in the tube with a corresponding sample ID obtaining a total reaction mix of 10µL (Villahermosa *et al*, 2000) in the PCR tubes was well mixed using the pipette. The tubes were then transferred into a thermal cycler (ABI 2720) and the cycler programmed with the following cycling conditions

94^{o}C 2min/ (94 ^{o}C 30sec; 50 ^{o}C 15sec; 60 ^{o}C 4min) X 25 cycles/4 ^{o} C hold. For the RT gene the forward and reverse primers used were A2 and PRSec2A respectively whilst the primer used for the PR gene was P3G.The cycle sequencing run was then started.

The Primers used at this stage (Villahermosa *et al*, 2000; Fujisaki *et al*, 2007; Villahermosa et al,2000):

Primer	**Region**	**Sequence(**5'-3'**)**	**Gene of Interest**
A2	2583-2601	TTAAAGCCAGGAATGGATG	RT gene (sense)
PRSec2A	2811-2838	CTGGGAAGTTCAATTAGGAATACCACA	RT gene (antisense)
P3G	2198-2217	CAACTCCCTCTCAGAAGCAG	PR gene (sense)

3.4.7 Post-Cycle Sequencing Purification & Automatic Sequences Generation

The sequenced DNA obtained were purified using the CentriSep Column Purification Method (Princeton Separations, Inc, Adelphia, NJ, USA). This procedure was effective for cleaning up cycle sequencing products in order to remove excess reagents and dyes used in the purification. Following the manufacturer's instructions, three steps were carried out: the Column Hydration step, Removal of Interstitial fluid and the Sample processing. The first two steps primed the dry gel product from the manufacturer and allowed the sample to be effectively cleaned of any excess dyedeoxy terminators from completed DNA sequencing reactions. The column gel was hydrated with reagent-grade water (Ambion,USA) and spun in a microcentrifuge (Eppendorf,USA) for 2minutes at 3,000rpm. The cycle sequenced DNA samples were then carefully applied to the top of

the gel directly onto the centre of the gel bed and spun in the microcentrifuge at 3,000rpm for 2 minutes. The purified DNA samples were loaded into the ABI Genetic Analyzer 3130 and the manufacturer's instructions followed (Applied Biosystems 3130/3130xl Genetic Analysers, Getting Started Guide, 2004) for the automatic analysis of the HIV-1 sequences generated.

3. 5 Editing of Sequences

The Align IR version 2.0 software (from LI-COR Inc., Michigan Technology University, 2001) was used for assemblying and aligning all the sequences generated for each sample. The process included visual sequence-by-sequence inspection of all generated sequences for missing and/or inadequate nucleotides, wrong deletions and insertions and ambiguous mixtures using the chromatograms of the sequences as a comparative window. For each sequence a consensus sequence was obtained at the end of the editing.

3.6 Drug Resistance Analysis

The consensus sequences in their FASTA format were submitted online to the Stanford University HIV Database Programme (http://hivdb.stanford.edu) to generate the resistance data and to assign subtypes for each sample as well as for the interpretation of any resistance data elicited. The Stanford University HIV Database Programme is generally accurate though misclassification of some strains may occur. This also generated the impact of any resistance associated mutation on the drugs in use by the participant through mutation scores assigned to the resistance associated mutations which classified the mutations either as major or minor. Resistance associated mutations with such mutation score grading were considered for analysis with regards to the 2013 IAS-

USA recognized mutations for NRTIs, NNRTIs and PIs (see Figures 6, 7 & 8 at Pages 54, 55 and 58 respectively).

3.6.1 Phylogenetic Analysis

The edited sequences were submitted to the standard nucleotide-nucleotide BLAST database search programme (BLASTN 2.2.29+) of the NCBI website (Zhang *et al*., 2000; Morgulis *et al*., 2008) to search for closely related sequences in the non-redundant GenBank database. The default parameter settings were used. Sequences homologous to the sequences were retrieved from the DNA databanks for comparisons. The sequence data were aligned using the CLUSTAL W package (Thompson *et al*., 1994) integrated into the Bioedit 7.25 software suite (Hall, 1999).

In building the phylogenetic tree, the evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches (Figure 14 at Page 105). Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 49 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 533 positions in the final dataset. The evolutionary analyses were conducted in MEGA 6 (Tamura *et al*, 2013).

3.7 Statistical Analysis

All the data was entered into an Excel database and then exported into SPSS version 16.0 software (SPSS Inc, Chicago, Illinois) for the statistical analysis. Variables were summarized as frequencies, percentages, charts, means and standard deviation. Pearson's Chi-Square was used in comparing associations. The variables germane to the analysis included age, WHO Clinical Staging, adherence to treatment regimen, baseline and study CD4 values, study viral load values, time spent on HAART, HIV types in circulation and HIV-1 resistance mutations.

CHAPTER 4

4.0 RESULTS

4.1 Study Population

Out of 221 eligible women for the study from all the study sites, 116 (52.5%) participated, the others having being lost to follow-up (Table 6). The proportion of defaulters found in this study was 47.5%.

Table 6: Study Population Distribution

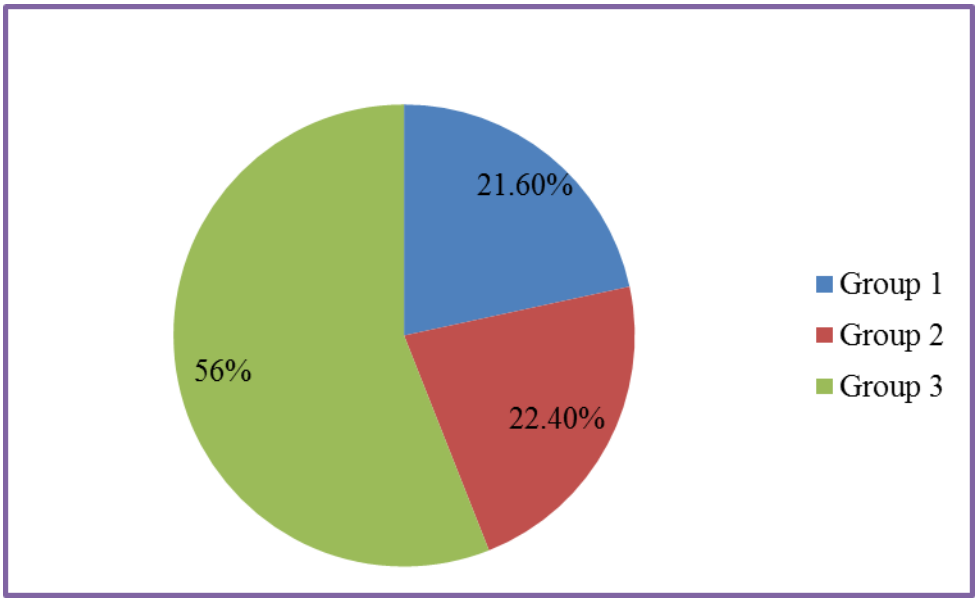
Study Site	Expected Subjects	Available Subjects	Defaulters N (%)
Korle Bu Teaching Hospital	51	31	20(39.2)
Tema General Hospital	10	5	5(50)
Eastern Regional Hospital	61	35	26(42.6)
Kumasi South Hospital	45	19	26(57.8)
Suntreso Government Hospital	10	1	9(90)
Bomso Clinic	29	16	13(44.8)
Animwah Medical Foundation	15	9	6(40)
TOTAL	**221**	**116**	105(47.5)

4.1.1 Characteristics of Study Participants

Out of 116 participants, 25 (21.6%) were mothers who were on HAART after previous PMTCT prophylaxis (Group 1), 26 (22.4%) were pregnant HIV-positive drug-naïve participants (Group 2), and 65 (56.0%) were mothers who had been put directly on HAART without prophylaxis as a result of low CD4 + T cell count at the time they were pregnant (Group 3).

The age range of the participants was 20 to 46 years with the mean ages (in years) being 33.1(±5.7), 30.7(±5.6) and 33.4(±4.7) for Group 1, Group 2 and Group 3 respectively.

Figure 11: Distribution of the Participating Groups



Group 1: HIV positive mothers on ART after previous prophylaxis

Group 2: HIV positive Drug-naïve pregnant women yet to initiate prophylaxis

Group 3: HIV positive mothers who initiated ART without prophylaxis

Table 7: Characteristics of the Study Participants

Characteristics		Group 1, N=25	Group 2, N=26	Group 3, N=65	P-Value*
Age(Mean) Years		33.1±5.7	30.7±5.6	33.4±4.7	0.104
Marital Status	Married	19(76%)	19(73%)	54(83%)	0.511
	Unmarried	6(24%)	7(27%)	11(17%)	
WHO Clinical Staging	1	11(44%)	15(57.7%)	12(18.5%)	0.001
	2	13(52%)	11(42.3%)	24(36.9%)	
	3	1(4%)	0(0%)	27(41.5%)	
	4	0(0%)	0(0%)	2(3.1%)	
Adherence to ART(Proportion of Patients on ART)[a]		22(88%)	__	61(93.8%)	0.353
Interval(months) between HAART initiation and Study(Median)		9	__	29	___
Baseline CD4 Count (Median cells/µL		440	454	217	___
Study CD4 Count(Median cells/ µL		583	454	591	___
Median Increase from Baseline CD4		144	__	374	___
Median Study HIV RNA Level		731	692	660	___

***Signinificant at 5%; a: Defined by self-report and drug collection schedule in hospital folders; Group 1-Prophylaxis with ART; Group 2- Drug-Naïve; Group 3-ART Only**

The study showed that the majority of the total number of participants, 92 (79.3%), were married, and 24(20.7%) were not married; 76% of Group 1 participants, 73.1% of Group 2 participants and 83.1% of Group 3 participants respectively, were married.

The study also showed that the participants were at different stages of disease progression according to the WHO Clinical Staging (Table 7 at page 84). For Group 1 participants, 44% were at Stage I, 52% at Stage II, 4% at Stage III and none at Stage IV of the infection. Of the participants in Group 2, 57.7% were at Stage I and 42.3% were at Stage II of the HIV infection with no one at Stages III and IV. With the mothers in Group 3, 18.5% were at Stage I of the infection, 36.9% at Stage II, 41.5% at Stage III and 3.1% at Stage IV of the infection.

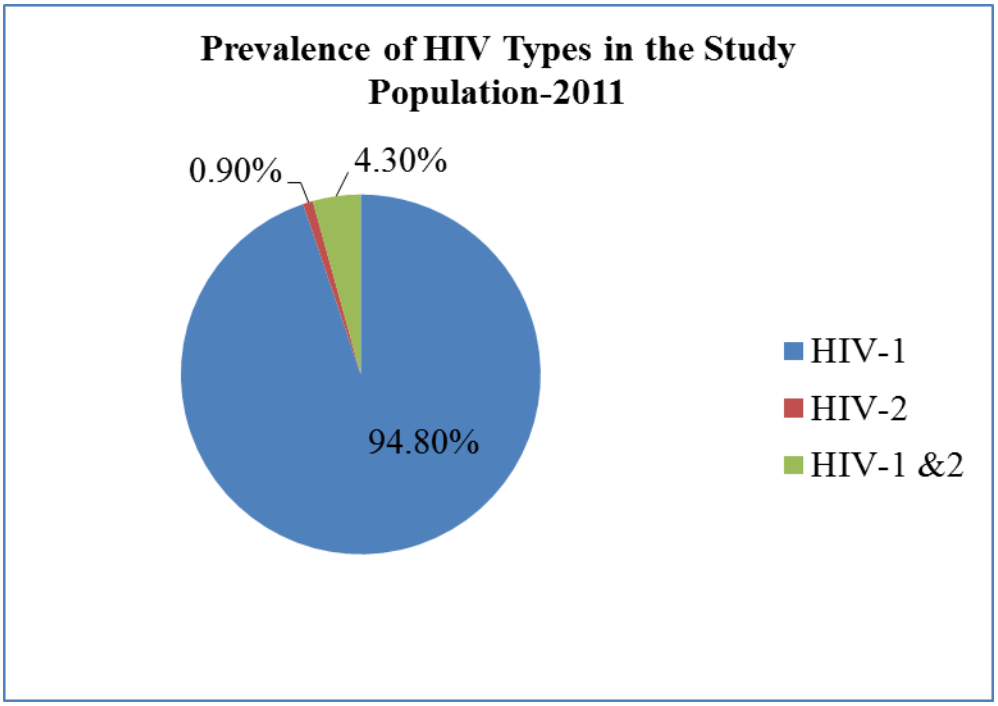
Considering adherence to ART, 88.0% and 92.3% respectively of the mothers in Groups 1 and 3, had adhered to their treatment regimen. Group 2 participants had not been given any ARV at the time of the study. The median study HIV RNA level was highest in Group 1 (731copies/ml) followed by that of Group 2 (692 copies/ml) then by Group 3 (660copies/ml). For Group 1, at the time of the study, there was a median increase in CD4 of 144cells/µL from baseline whilst there was a median increase in CD4 values of 374cells/µL from baseline for Group 3 participants (Table 7 at page 84).

4.2 Types of HIV Infection

Both HIV Type 1 and HIV Type 2 were detected among the study participants. Of the total number of participants, 110(94.8%) were infected by HIV-1, one (1) person (0.9%) was infected by HIV-2 and 5 (4.3%) were infected by both HIV-1 & 2 types (dual

infection). This pattern of HIV types in circulation among the study population is depicted by Figure 12.

Figure 12: Prevalence of HIV Types



Results of serological typing of samples using INNO-LIA HIV 1/2 Score assay from INNOGENETICS, Belgium.

4.3. Immunologic & Virologic Responses

Study participants in the different groups showed varying responses both immunologically and virologically.

4.3.1 Changes in CD4 Counts and Viral Load

The WHO Clinical Stage at the time of the initial attendance of the patients at the Care Centres, the Baseline CD4 counts and the Previous CD4 count (at least 6 months before the study time) were extracted from the hospital folders. For each of the groups of participants the Mean CD4 count at Baseline, Previous and Study time are shown in Figure 13 overleaf. The Viral Load for all participants were determined at the time of the study, as baseline viral load figures were not available since they are not determined as part of the care and support programme in Ghana.

The proportions of detectable and undetectable viral load in each group are depicted in Table 8 at Page 89. The quantitative real-time PCR assay used in determining the Viral Loads had a detection limit of 153copies/ml. The majority of the participants, 110 (94.8%) had undetectable levels (<153copies/ml) with 6 (5.2%) showing detectable levels (>153copies/ml) which ranged from 200 to < 1000 copies/ml.

SEE APPENDIX IV FOR THE DETAILED RESULTS OF CD4 AND VIRAL LOAD.

Figure 13: Comparison of Mean CD4 Counts at Baseline, Previous and Study Time

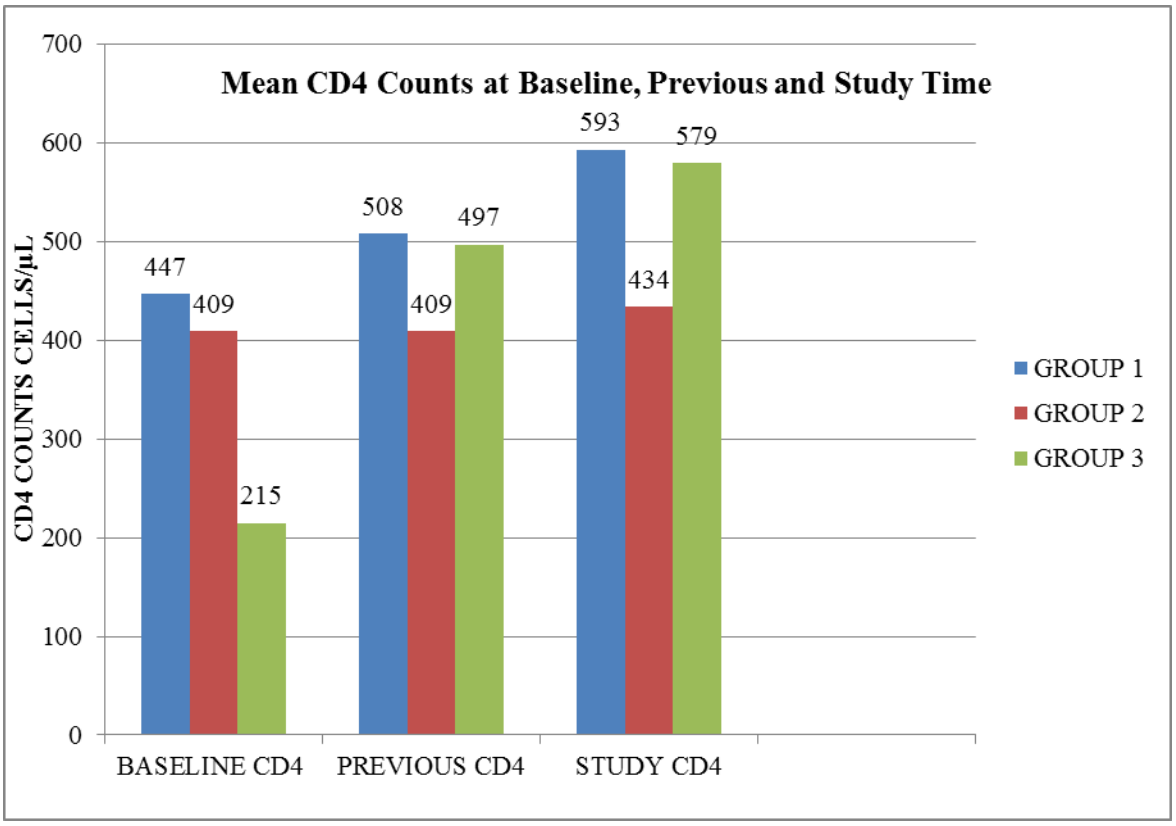


Table 8: Comparison of Viral Load Values at Study Time

GROUPS	STUDY VIRAL LOAD N=116		TOTAL N (%)
	Undetectable N (%)	Detectable N (%)	
1	24(20.7)	1(0.9)	25**(**21.6)
2	23(19.8)	3(2.6)	26(22.4)
3	63(54.3)	2(1.7)	65(56.0)
TOTAL	110(94.8%)	6(5.2%)	116(100)

The mean levels of CD4 at baseline (p=0.02), previous count before study time (p=0.04) and at study time (p=0.02) among participants who adhered to treatment from the time of PMTCT with prophylaxis and without prophylaxis when HAART was initiated directly until the study time and those who did not adhere to treatment during the same period are shown in Table 9 with Table 10 showing the difference in the mean CD4 values. WHO stipulates Immunologic Response as an increase in CD4 levels equal or more than 100cells/µL above the baseline CD4 count (World Health Organization, 2010).

Table 9: Comparison of Mean CD4 levels among Drug Adherent and Non-Adherent Participants.

	ADHERENCE	N	Mean	S.D	P-value	95% C.I.	
BLCD4	Adherence	83	280.6	182.7			
	Non-adherence	33	378.3	206.3	0.02*	-180.18	-15.16
PRCD4	Adherence	83	514.4	245.2			
	Non-adherence	33	415.4	225.9	0.04*	3.24	194.70
STDCD4	Adherence	83	581.35	231.7			
	Non-adherence	33	469.0	234.7	0.02*	15.839	208.49

***Significant at 5%**

BL CD4 means Baseline CD4; PR CD4 means Previous CD4 before the study-at least 6months prior to the study; STD CD4 means Study CD4(CD4 obtained during the study); N represents the number of patients which included the drug-naïve group who were automatically considered non-adherent to treatment for this analysis.

Table 10: Differences in the Mean CD4 counts between adherent and non-adherent participants.

PERIOD OF CD4 ASSAY	INCREASES IN MEAN CD4	
	ADHERENT PATIENTS	NON-ADHERENT PATIENTS
PREVIOUS AND BASELINE COUNT	233	37
STUDY TIME AND BASELINE COUNT	301	91

4.3.2 Responses to ART

Table 11 depicts the immunologic and virologic responses to ART by the women on treatment. The increases in the CD4 counts are from the baseline assessment prior to initiating prophylaxis or HAART and the CD4 counts at the time of the study.

Table 11: Immunologic-Virologic Responses to ART

GROUPS	PATIENTS WITH INCREASED CD4, UNDETECABLE VIRAL LOAD, N (%)	PATIENTS WITH INCREASED CD4, DETECTABLE VIRAL LOAD, N (%)	PATIENTS WITH DECREASED CD4, UNDETECTABLE VIRAL LOAD N (%)	TOTAL STUDY PATIENTS N (%)
GROUP 1	19 (76)	1(4)	5(20)	25(21.6)
GROUP 3	59(91)	2(3)	4(6)	65(56)

Group 1 initiated prophylaxis and continued later with ART.

Group 3 initiated ART without prior prophylaxis.

Increases in CD4 refer to differences between Baseline and Study Time values.

Viral Load was determined at Study Time with Undetectable levels being <153copies/ml and Detectable levels being > 153copies/ml.

Table 12 refers to the baseline immunologic status of the HIV positive drug-naïve participants of Group 2 prior to initiating PMTCT and their viral load as at the time of sampling. Study participants in this group who had CD4 values higher than the 350cells/µL barrier and showed suppressed or undetectable viremia made up 57.6% of the group. On the other hand, 7.7% of the participants in this group had detectable viremia though CD4 counts were more than the 350cells/µL limit (Table12).One (1) person (3.9%) out of the participants in this drug-naïve group had CD4 count less than 350cells/ µL and detectable viral load of 444 copies/ml. Eight (8) participants (30.8%) registered undetectable viral loads but had CD4 counts less than 350cell/ µL, the threshold for initiation of ART under the existing ARVs option for PMTCT programmes.

Table 12: Immunologic and Virologic Patterns of Group 2 Participants (ART-NAÏVE)

IMMUNOLOGIC AND VIROLOGIC PARAMETERS	**GROUP TWO (DRUG-NAÏVE) N (%)**
CD4<350, VL UNDETECTABLE	8 (31%)
CD4 < 350, VL DETECTABLE	1 (4%)
CD4>350, VL UNDETECTABLE	15 (57%)
CD4>350,VL DETECTABLE	2 (8%)

4.3.3 Antiretroviral Regimen under PMTCT

During the study, the PMTCT programme in Ghana administered a combination of Zidovudine (AZT) and Lamivudine (3TC),both NRTIs, (known as Combivir) to the patients from 28 weeks of pregnancy as prophylaxis until labour onset when a single dose Nevirapine (sd NVP), a NNRTI, was added. For some patients, Stavudine (D4T), an NRTI, replaced AZT. In post-PMTCT periods when these mothers needed antiretrovirals (ARVs) for their own health, they were given the same drugs as during the prophylaxis phase- 3TC, AZT and NVP or Efavirenz (EFV), another NNRTI but this time as a triple therapy. For some of these participants, Didanosine (DDI) or Abacavir (ABC)–both NRTIs, or Tenofovir (TDF), a Nucleotide Reverse Transcriptase Inhibitor (NtRTI), substituted AZT. For pregnant HIV positive women who reported at the Care and Support Centres and had CD4 levels below 350cells/µL, they were given the triple therapy without a prophylactic phase.

4.3.4 Presence of Resistance Associated Mutations

The study detected drug resistance associated mutations (DRAMs) with participants from each group; Table 13 below depicts the presence of DRAMs and the ARVs the participants were on, if any. Participants of Group 2 (total of 26) were drug-naïve at the time of the study and 4 (15%) had resistance associated mutations, whilst participants of Group 1 (totaling 25) had 8 (32%) patients with resistance associated mutations and Group 3 (65 patients) had 3 (5%) showing DRAMs.

Table 13: Presence of Resistance Associated Mutations with ARVs

PARTICIPATING GROUPS	STUDY PARTICIPANTS N (%)	RESISTANCE ASSOCIATED MUTATIONS, N (%)	MEAN HAART PERIOD (MONTHS)	HAART REGIMEN USED
GROUP 1 (PROPHYLAXIS WITH ART)	25 (21.6)	8(32)	11	AZT/D4T+3TC +NVP/EFV
GROUP 2 (DRUG-NAÏVE)	26 (22.4)	4 (15)	NO ARVs	NO ARVs
GROUP 3 (ART WITH NO PROPHYLAXIS)	65 (56.0)	3 (5)	34	AZT/D4T+ 3TC +NVP/EFV
TOTAL	116(100)	15(13)		

4.4 Emergence of HIV-1 Drug Resistance Associated Mutations

Out of the total of 116 samples analyzed for the reverse transcriptase and protease genes, 53(46%) and 61(53%) respectively were successfully amplified. A representative gel photograph for the successfully amplified products of the RT gene is shown at Page 110. Sequencing was successfully done for 40 (75%) and 33 (54%) of the reverse transcriptase and protease genes respectively. All the sequences generated for the Reverse Transcriptase Gene and the Protease Gene of the *pol* region of the HIV-1 genome were submitted to the Stanford HIV Database Programme for drug resistance analysis and interpretation as well as subtyping.

A total of fifteen (15/116) samples (13%) showed the presence of drug resistance associated mutations (DRAMs), the distribution varying across the three groups involved in the study. There were 14 (35%) sequences that had resistance associated mutations with the reverse transcriptase gene for both NRTIs and NNRTIs but only 1 (3%) that had RAMs in the protease gene for PIs.

The proportions of DRAMs seen in the study as a result of the impact of duration on ART, WHO clinical staging, study viral load, difference between study and baseline CD4 counts and adherence to treatment, on the emergence of such DRAMs is shown in Table 14 overleaf at page 97.

Table 14: Association between the study Variables and the Emergence of HIV Drug Resistance

STUDY VARIABLES		HIV DRUG RESISTANCE			P VALUE
		DRAMs, N (%)	NO DRAMs, N (%)	TOTAL N (%)	
GROUPS	1 (ART with prophylaxis)	8(32)	17(68)	25(21.6)	.002*
	2 (Drug-Naïve)	4(15)	22(85)	26(22.4)	
	3 (ART with no prophylaxis)	3(5)	62(95)	65(56.0)	
DURATION ON ART (GROUPS 1 & 3)	< 1 Year	4(16.7)	20(83.3)	24(26.7)	.624*
	1-2 Years	5(12.5)	35(87.5)	40(44.4)	
	≥ 3Years	2(7.7)	24(92.3)	26(28.9)	
WHO CLINICAL STAGING	1	9(23.7)	29(76.3)	38(32.8)	.113*
	2	6(12.5)	42(87.5)	48(41.4)	
	3	1(3.6)	27(96.4)	28(24.1)	
	4	0(0)	2(100)	2(1.7)	
VIRAL LOAD	DETECTABLE	1(16.7)	5(83.3)	6(5.2)	.834*
	UNDETECTABLE	15(13.6)	95(86.4)	110(94.8)	
CD4 DIFFERENCE BETWEEN BASELINE & STUDYCOUNTS	≥100cells/ul increase	15(14.3)	90(85.7)	105(90.5)	.635*
	<100cells/ul increase	1(9.1)	10(90.9)	11(9.5)	
ADHERENCE	YES	10 (12.0)	73 (88.0)	83(92.2)	.560*
	NO	1 (14.3)	6 (85.7)	7(7.8)	
TOTAL		11 (12.2)	79 (87.8)	90	

***Significant at 5%**

There were major DRAMs to both the NRTIs and the NNRTIs from the 35% RT sequences obtained. Tables 15-17 at Pages 100-101 depict the DRAMs in each group and the ARVs given the patient, if any.

For Group 1(Prophylaxis plus ART Group) all the participants were given the same combination of ARVs as prophylaxis (AZT, 3TC and NVP) with the exception of one patient who was given only NVP as prophylaxis. The subsequent ART regimen for all Group 1 mothers was the same (Table 15 at Page 100).There was no drug resistant associated mutations (DRAM) to the Protease Inhibitors (PIs) in this group.

M41L, M184V, M184MV, L74V and T215Y were the major drug resistance associated mutations to NRTIs seen among the participants; there were no minor drug resistant associated mutations to NRTIs in this group. The most commonly seen drug resistant associated mutations to NRTIs in this group were M184V, T215Y and M41L.

Major DRAMs to NNRTIs seen in Group 1 were K103N, Y181C, M230L and L100IL and the minor DRAMs to NNRTIs seen was A98G.The most common HIV-1 drug resistance associated mutations seen with the NNRTIs were K103N, M230L and A98G. There were no resistance associated mutations with regards to the Protease Inhibitors (PIs) and no participants in this Group had been treated with a Protease Inhibitor.

In the drug-naïve participants (Group 2), there were no drug resistance associated mutations with Protease Inhibitors. However, there were four (4) participants (15%) showing DRAMs to NRTIs and NNRTIs (Table 16 at Page 101). Two major HIV-1 drug resistance associated mutations for NRTIs, M184V and L210W, were seen in two of the participants with one minor DRAMs to NRTIs, V75S, seen in one patient; one patient did

not have any drug resistance mutation to NRTIs. Three major DRAMs to NNRTIs were seen in 3 patients in this group; these DRAMs were K103N, V106A and E138A. One minor drug resistance associated mutation, A98G, was seen in one patient in this group.

In Group 3 where participants had received ART but no Prophylaxis for PMTCT, 3 patients (5%) showed drug resistance associated mutations to NRTIs, NNRTIs and PIs. Major and minor DRAMs to NRTIs, NNRTIs and PIs were seen in one patient who had been given NRTIs and a PI (Nelfinavir) initially, followed by other NRTIs and an NtRTI. The major DRAMs to NRTIs seen in this group were M184V, Y115F, K70R, K219E and M41LM; the minor DRAMs to NRTIs seen were T215S, T215I and D67G. There were no major DRAMs to NNRTIs in this group though the minor drug resistance mutation, A98G, was seen in two of the patients. The major DRAMs to PI seen in the group was I84V; the minor drug resistance mutations seen were A71V, L89V and M46MV (Table 17 at Page 101).

M184V mutation was found to be the most common among this group of mothers.

Thymidine analogue mutations (TAMs) which confer resistance to nucleoside analogues were seen among the DRAMs in all the 3 different groups; TAMs seen in this study were M41L, K70R, L210W, T215Y and K219E. The most common TAM seen in this study were M41L and T215Y; these appeared in 7 (88%) out of the 8 participants with DRAMs in Group1 which had prophylaxis followed by HAART (Tables 15, 16 and 17 at Pages 100-101).

Table 15: Drug Resistance Mutations in Group 1 Participants (HAART WITH PROPHYLAXIS).

SUBJECTS	PROPHY-LAXIS DRUGS	HAART REGIMEN	REVERSE TRANSCRIPTASE RAMs		PROTEASE RAMs
			NRTIs	NNRTIs	
KBSG/1	AZT+3TC + NVP	AZT+3TC+ NVP	M184V	K103N, Y181C	NONE
KBSG/2	AZT+3TC +NVP	AZT+3TC+ NVP	M41L, M184V, T215Y	A98G, K103N, M230L	NONE
KBSG/3	AZT+3TC +NVP	AZT+3TC+ NVP	M41L, M184V, T215Y	A98G, K103N	NONE
KBSG/4	AZT+3TC +NVP	AZT+3TC+ NVP	M41L, M184V, T215Y	A98G, K103N	NONE
KBSG/5	AZT+3TC +NVP	AZT+3TC+ NVP	M41L, M184MV, T215Y	A98G, K103N, M230LM	NONE
KBSG/6	AZT+3TC +NVP	AZT+3TC+ NVP/EFV	M41L, M184MV, T215Y	A98G, K103N,M230LM	NONE
KBSG/7	NVP	AZT+3TC+ NVP	M41L, L74V, T215Y	K103N A98G, L100IL, ,M230L	NONE
KDSG/5	AZT+3TC +NVP	AZT+3TC+ NVP	K219KR	G190EG	NONE

Table 16: HIV-1 Drug Resistance Mutations in Group 2 Participants (Drug-Naïve).

SUBJECTS	PROPHY-LAXIS DRUGS	HAART REGIMEN	REVERSE TRANSCRIPTASE RAMs		PROTEASE RAMs
			NRTIs	NNRTIs	
KBC1/5	NONE	NONE	V75S	E138A	NONE
KDC1/1	NONE	NONE	NONE	A98G	NONE
KDC1/6	NONE	NONE	M184V	K103N	NONE
KDC1/10	NONE	NONE	L210W	V106A	NONE

Table 17: Drug Resistance Mutations in Group 3 Participants (ART WITH NO PROPHYLAXIS).

SUBJECTS	PROPHYLAXIS DRUG	HAART REGIMEN	REVERSE TRANSCRIPTASE RAMs		PROTEASE RAMs	
			NRTIs	NNRTIs	MAJOR-	MINOR
KBC2/4	NONE	AZT+3TC + NVP	M184V, Y115F, T215S	A98G	NONE	NONE
KBC2/13	NONE	AZT+3TC + NFV; ABC+ DDI+TDF ; 3TC+TDF + ABC.	M41LM, D67G, K70R, K219E, T215I, M184V	A98G	I84V;	M46MV ,A71V, L89V
KDC2/20	NONE	D4T+3TC + EFV/NVP	M184V	NONE	NONE	NONE

4.5 Distribution of HIV-1 Subtypes

All the sequences obtained were subtyped using the Stanford HIV database drug resistance programme (www.hivdb.stanford.edu), which produced 33 (83%) CRF02_AG subtypes, 2 (5%) being subtype CRF01_AE, 1 (3%) subtype A, 2 (5%) subtype B and 2 (5%) subtype G for the RT gene (Table 18) whilst the PR gene had 32samples (97%) being subtype CRF02_AG and 1(3%) subtype A (Table 19). Subtyping by phylogenetic analysis was also performed though some of the sequences for the RT gene were excluded from the phylogenetic tree-building (see Figure 14 at Page 105) because they were relatively shorter fragments. The Phylogenetic analyses were conducted in MEGA 6. Majority of study sequences clustered with the circulating recombinant form CRF02_AG. However, three samples were classified differently by phylogenetic relationships compared to the Stanford database programme; KBC2/4 classified as CRF02_AG, KBC1/6 as subtype B and KDSG/10 as CRF02_AG by phylogenetic analysis as compared to subtype B, CRF02_AG and CRF01_AE respectively ,by the Stanford database hiv drug resistance programme. This difference could be due to the different models used by the two tools.

Of the 8 mothers in Group 1(Prophylaxis with ART) with HIV-1 drug resistance associated mutations, 7 (88%) were of subtype CRF02_AG and 1(13%) was of CRF01_AE. Out of the 4 mothers (15%) in Group 2 (Drug-Naïve patients) with HIV-1 drug resistance associated mutations, 3 (75%) were of subtype CRF02_AG and 1 (25%) was of subtype A. Two of the HIV-1 strains showing drug resistance associated mutations in group 3 were of Subtype CRF02_AG and one was of Subtype B (Table 18 overleaf).

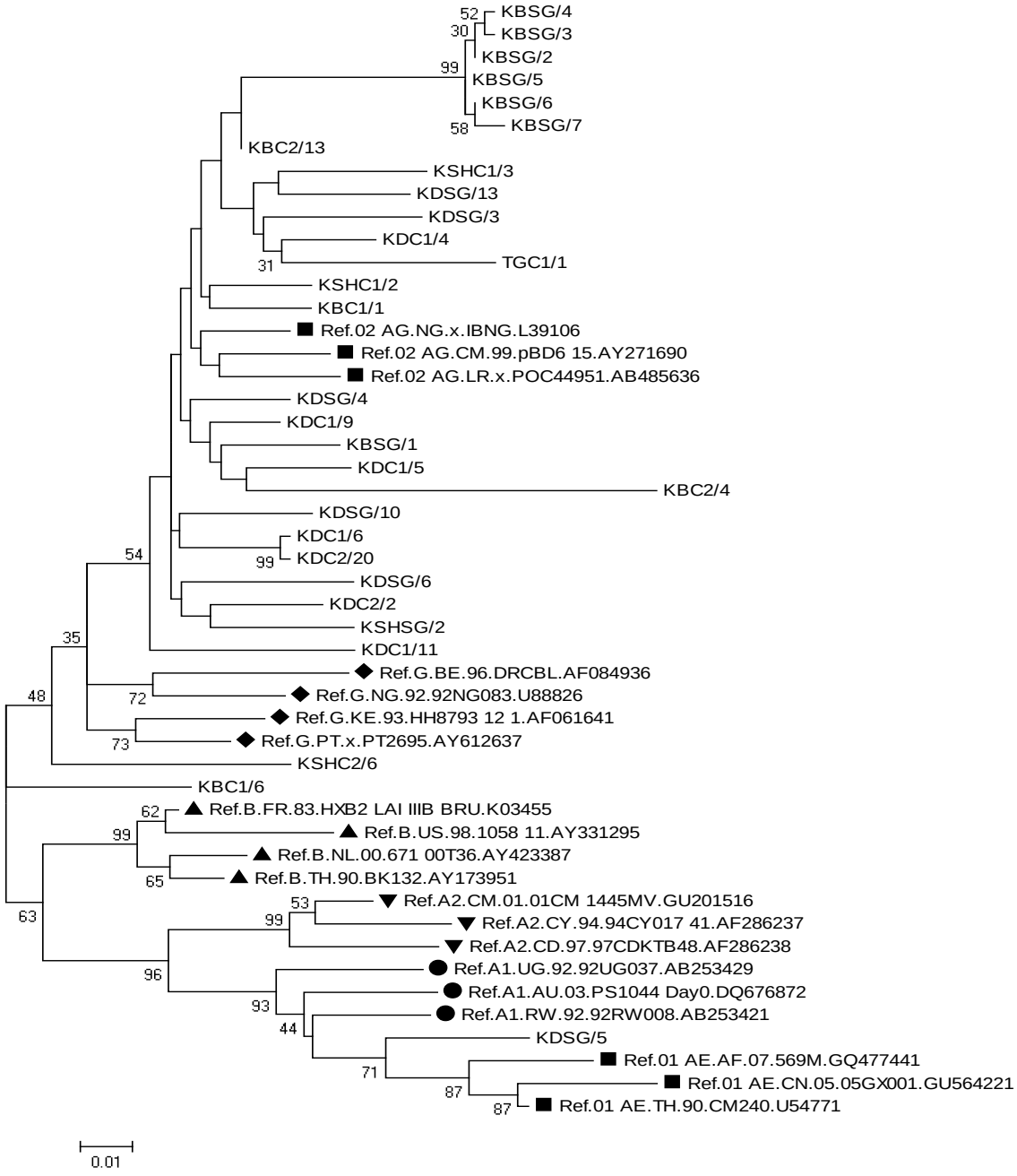
Table 18: HIV-1 Subtypes and Drug Resistance Mutations for RT Gene

		HIV-1 DRUG RESISTANCE ASSOCIATED MUTATIONS	
SUBJECTS	**SUBTYPES**	**NRTIs**	**NNRTIs**
KBSG/1	CRF02_AG	M184V	K103N, Y181C
KBSG/2	CRF02_AG	M41L, M184V, T215Y	A98G, K103N, M230L
KBSG/3	CRF02_AG	M41L, M184V, T215Y	A98G, K103N
KBSG/4	CRF02_AG	M41L, M184V, T215Y	A98G, K103N,
KBSG/5	CRF02_AG	M41L, M184V, T215Y	A98G, K103N, M230LM
KBSG/6	CRF02_AG	M41L, M184V, T215Y	A98G, K103N, M230LM
KBSG/7	CRF02_AG	M41L, L74V,T215Y	A98G, L100IL, K103N, M230L
KDSG/3	CRF02_AG	NONE	NONE
KDSG/4	CRF02_AG	NONE	NONE
KDSG/5	CRF01_AE	K219KR	G190EG
KDSG/6	CRF02_AG	NONE	NONE
KDSG/10	CRF01_AE	NONE	NONE
KDSG/12	G	NONE	NONE
KDSG/13	CRF02_AG	NONE	NONE
KSHSG/2	CRF02_AG	NONE	NONE
KBC1/1	CRF02_AG	NONE	NONE
KBC1/5	CRF02_AG	V75S	E138A
KBC1/6	CRF02_AG	NONE	NONE
TGC1/1	CRF02_AG	NONE	NONE
TGC1/2	CRF02_AG	NONE	NONE

TABLE 18 HIV-1 Subtypes and Drug Resistance Mutations for RT Gene (continued)

		HIV-1 DRUG RESISTANCE ASSOCIATED MUTATIONS	
SUBJECTS	**SUBTYPES**	**NRTIs**	**NNRTIs**
KDC1/1	CRF02_AG	NONE	A98G
KDC1/2	CRF02_AG	NONE	NONE
KDC1/4	CRF02_AG	NONE	NONE
KDC1/5	CRF02_AG	NONE	NONE
KDC1/6	CRF02_AG	M184V	K103N
KDC1/7	CRF02_AG	NONE	NONE
KDC1/9	CRF02_AG	NONE	NONE
KDC1/10	A	L210W	V106A
KDC1/11	B	NONE	NONE
KSHC1/1	CRF02_AG	NONE	NONE
KSHC1/2	CRF02_AG	NONE	NONE
KSHC1/4	CRF02_AG	NONE	NONE
KBC1/3	CRF02_AG	NONE	NONE
KBC2/4	B	M184V, Y115F, T215S	A98G
KBC2/13	CRF02_AG	M41LM, D67G,K70R,M184V,T215I, K219E	A98G
KDC2/1	CRF02_AG	NONE	NONE
KDC2/2	CRF02_AG	NONE	NONE
KDC2/20	CRF02_AG	M184V	NONE
KSHC2/3	CRF02_AG	NONE	NONE
KSHC2/6	G	NONE	NONE

Figure 14: Molecular Phylogenetic analyses for RT sequences and selected HIV-1 subtype references by Maximum Likelihood method.



Markers indicate reference sequences: ▲ Ref B, ▼ Ref A2, ● Ref A1, ♦ Ref G, ■ Circulating recombinant forms (CRFs)

Table 19: HIV-1 Subtypes and Drug Resistance Mutations for the PR Gene

SUBJECT	SUBTYPES	HIV-1 DRUG RESISTANCE ASSOCIATED MUTATIONS TO PIs	
		MAJOR	MINOR
KBC2/12	A	NONE	L10I
KBC2/13	CRF02_AG	I84V	M46MV,A71V,L89V

All the other 29 samples were Subtype CRF02_AG. The minor mutations to PIs seen were L10V, F53Y and L10I although there was no clinical history of Protease Inhibitors use in these patients.

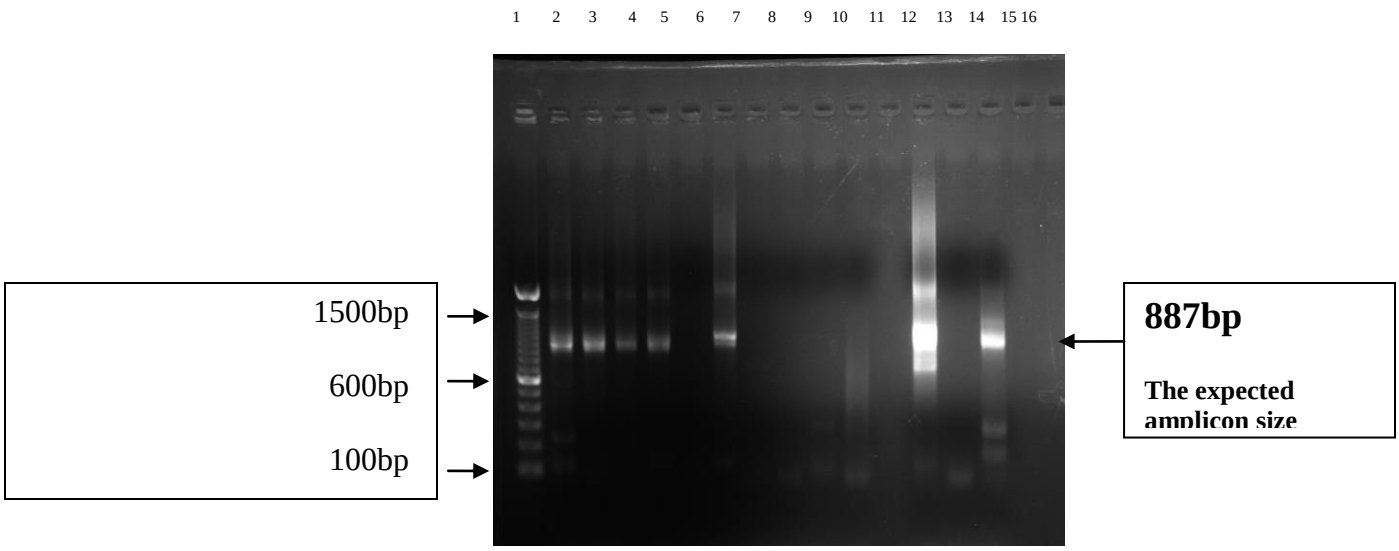


Figure 15: A representative gel photograph showing results of the amplification of the RT gene. Lane 1 contained Trackit^{TM} 100bp DNA ladder(from Invitrogen,USA). The band sizes of the moleclar weight marker increased from 100 bp,at the top of the gel, towards the sample wells in steps of 100. Lanes 2 to 14 contained samples from participants, Lane 15 contained a positive control and Lane 16 contained a negative control.The expected size of the positive product was 887bp . Samples in Lanes 6, 8-12, and 14 were considered as failed amplifications whiles samples in Lanes 2-5,7 and 13 were successful amplifications.

CHAPTER 5

5.0 DISCUSSION AND CONCLUSION

5.1 Characteristics of Study Participants

The study found that the defaulting rate among HIV-positive mothers reporting to the care and support facilities was high (47.5%) as shown in Table 6. This could have a negative impact on the outcome of the PMTCT programme being implemented at the Care and Support Centres. This could be one of the reasons for the observed differences between the total number of mothers needing PMTCT and the number who actually received PMTCT in the country from 2005 to 2012 as recorded by the 2013 National AIDS/STI Control Programme (NACP) report (NACP, 2013). Though PMTCT coverage in Ghana is expected to increase after 2012 (NACP, 2013), if the default rate among HIV positive mothers is not reduced, the Elimination of Mother-To-Child Transmission (eMTCT) of HIV plan may not be achieved.

The mean age for the total number of participants was 32.7 ±5.5 years (Table 7). The mean age range for the different groups of participants was between 30 and 34 years (Table 7). This agrees with NACP reports that the age group 30-34 years had the highest number of HIV-positive females in 2012 (NACP, 2013). In all the three groups ,the proportion of HIV positive unmarried women were less than those married; there was no significant association between the women in the different groups and their marital status ($p>0.05$) as shown in Table 7.

The study found that there was a significant association between participating groups and the WHO clinical staging, $p < 0.05$. The WHO guidelines underlying the clinical staging of HIV positive individuals gives an indication of the clinical progression of HIV infection in an individual infected by the virus (World Health Organization,2010). The majority of the participants in Groups 1and 2 were in the moderate stages of the clinical staging, Stages 1 and 2, with none in the more severe stages of 3 and 4. Out of the patients in Group 3 (ART without prophylaxis), the majority (41.5%) were at the Stage III (a severe stage of AIDS). These patients had been on ART for longer than 24 months and were at Stage 3 from the point they initiated ART to the time of the study. The study found out that these patients are continuously reassessed clinically as part of the care and support programme in Ghana to enable early detection of treatment failure in the absence of viral load testing as seen in other resource-constrained country such as Ghana as reported by Mee *et al* in 2008.

This study found that an adequate level of adherence to treatment had been attained in the two groups of mothers, Group 1 (prophylaxis with ART), 88% and Group 3 (ART without prophylaxis) 93.8% during pregnancy and after delivery. This was a better outcome of adherence obtained when compared to a pooled meta-analysis of 51 studies in 2012, involving over 20,000 women from the United States, Kenya, South Africa and Zambia, in which 73.5% of pregnant women with HIV attained adequate adherence to ART (defined as equal to or greater than 80%), during and after pregnancy (Nachega *et al*, 2012).

An adequate level of adherence has a positive impact on PMTCT and will help attain the goal of eliminating mother-to- child transmission (eMTCT) of HIV in Ghana by the year 2015.

5.2 Co-circulation of HIV-1 and HIV-2

The study found that HIV-1 was the predominant type in circulation in Ghana during 2011, with HIV-2 infecting only a small proportion (0.9%) of the study participants. This HIV prevalence pattern was also seen in the findings of the NACP in its 2011 and 2012 HIV Sentinel Survey Reports for Ghana (HIV Sentinel Survey Report, 2011; HIV Sentinel Survey Report, 2012). These reports gave 96.4% of HIV-1, 0.7% of HIV-2 and 2.9% of HIV-1 & 2 and 95.1% of HIV-1, 0 % of HIV-2 and 4.9% of HIV-1 &2 respectively, as against 94.8% of HIV-1, 0.9% of HIV-2 and 4.3% of HIV-1 & 2 reported in 2011 in this study. The overall HIV trend from the year 2005 to 2012 by the NACP Sentinel Surveys confirmed HIV type 1 as predominant. HIV type 2 co-circulated though with minimal variations over the period (HIV Sentinel Survey Report, 2012). The presence of HIV-2 has implications for the treatment of HIV infected persons. Studies have described suboptimal responses to treatment as HIV-2 RNA in plasma of such individuals remained unaffected or increased, though HIV-1 RNA was suppressed (Schutten *et al*, 2000)**.**

In Ghana, the ART first line regimen comprises two Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and one Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs); however, studies have shown HIV-2 to be naturally resistant to NNRTIs (Schutten *et al*, 2000; Nkengasong *et al*, 2004). In a study in Accra, Ghana in 2005, it was found that dual

seropositivity for HIV was common (Sagoe *et al*, 2005) and therefore there was the need to identify patients dually infected before initiating ART since the existing ART regimen included NNRTIs which have been shown to be ineffective against HIV-2. Thus for such patients, the overall response to the ART regimen in Ghana could be suboptimal with immune function impaired despite long periods of treatment. Discrimination of HIV-1 and HIV-2 types of infection in infected pregnant Ghanaian women should be carried out before treatment initiation since HIV-2 present will not be affected by NNRTIs administered.

5.3 Immunologic, Virological and Clinical Profiles

Figure 13 shows that for both Groups 1 and 3(ART after prophylaxis and ART without prophylaxis respectively), there is an increasing trend in the CD4 values from initial evaluation at the Care and Support Centre till the time of the Study. There is a mean increase of 374 cells/µL and 146 cells/µL respectively for Group 1 and 3. By the WHO guidelines an increase of ≥ 100cells/µL from baseline levels indicate an immunologic response. Thus, immunologically these patients were doing well. However, there were three kinds of immuno-virological responders to ART encountered in this study- Concordant Responders who showed increases in CD4+T cell counts with viral suppression below detectable limits; Immunological Responders with increases in CD4+ T cell counts despite detectable viral loads; and Immunological Non-Responders who presented with decreases in CD4+ T cell counts though there was viral suppression to undetectable levels (Table 11). Other studies have found Immunological Responders and Immunological Non-Responders represented suboptimal responses to the ART regimen (Gilson *et al*, 2010; Schecter and Tuboi, 2006).

The majority of HIV infected women who initiated ART after prophylaxis, 76%, (Group 1) and HIV infected women who initiated ART without prophylaxis, 91%, (Group 3), in this study were Concordant Responders. These persons were at minimal risk of clinical progression to AIDS-related illness or death. Their immune systems were functional, as seen in the increases in CD4 count from pretreatment levels, and viremia had been suppressed below level of detection of the assay. This met the primary goal of ART, which is to suppress plasma HIV-1 RNA levels lower than the detection level of the assay within 3 to 6 months of treatment. ART also seeks to restore immunologic function, to reduce morbidity and mortality, to reduce vertical transmission, and improve quality of life (Volberding and Deeks, 2010). The Concordant Responders outcome seen in this study was in agreement with reports from Ethiopia (Misgena, 2011). Treatment with triple ART for pregnant women after diagnosis without prophylaxis had a better outcome than initiation of prophylaxis followed subsequently by ART.

The study found that 4% and 3% of participants of Group 1 and Group 3 respectively, had increases in CD4+T cell counts despite detectable viral loads. These participants were at a high potential risk of clinical progression to AIDS-related illness or death due to the lack of virologic suppression despite the long-term immunologic success. Findings from various studies are in support of the result of this finding (Schecter and Tuboi, 2006; Grabar *et al*, 2000; Nicastri *et al*, 2005). These studies confirm that in response to ART some patients experience a discordant response, characterized by a number of factors-sustained CD4+ T cell count rise despite persistent viraemia, HIV-1 RNA plasma levels below the limit of detection accompanied by a blunted CD4+ T cell count response and patients who exhibited only a virologic response without an immunologic response and

were at intermediate risk for clinical progression to AIDS-related illness or death whilst complete non-responders had the least favorable prognosis.

Immunological Non-Responders encountered in this study constituted 20% of Group 1 participants (who had prophylaxis before ART) and 6% of Group 3 participants (who had no prophylaxis before ART). These participants are at a high risk of the HIV infection progressing clinically to AIDS-related illness or death as a result of their impaired immunity despite viral suppression. Such patients should be monitored closely until their immunity is sufficiently restored due to their higher risk of AIDS-related death. These findings by this study are consistent with studies carried out by different workers in 2003 and 2010 (Florence *et al*, 2003; Gilson *et al*, 2010).

HIV infected mothers in the Immunological Responders and Immunological Non-Responders categories in Ghana are at risk and merit targeted medical care and support. However, they are likely to be missed as a result of the clinico-immunological mode of assessment of the disease progression as practiced in Ghana. These groups of HIV-infected patients are at risk in other low-income countries too as seen in a study in Rwanda in 2009 (Griensven *et al*, 2009).

Among the drug-naïve group, 57% had a functional immune system (CD4 +T cell count ranging from 400 to 900 cells/µL) and viremia suppressed as per their initial hospital attendance (time of the study). These women were at minimal risk of AIDS-related illness or death and were enrolled into the PMTCT programme. Some of the drug-naïve participants (8%) had an immuno-virological status similar to Immunological Responders

since they had a functional immune system with detectable viremia. Outside the study these pregnant HIV-infected women would have initiated PMTCT without viral load assessment. Such pregnant women would have a high potential risk of AIDS-related illness or death without antiretrovirals.

Two groups of drug-naïve participants had CD4+ T cell counts less than the threshold of 350cells/µL for PMTCT initiation. These were qualified for ART without prophylaxis but their viral load status was not determined by the Care and Support Centre. One group had undetectable viremia (31% of Group Two participants) and the other group had detectable viremia (4% of Group Two participants) .Both of these groups were at risk of progressing to AIDS-related illness or death. They also missed out on relevant clinical management needed because viral load was not determined at initiation of care and support nor was this used in assessment with CD4+T cells count. In 2013, the NACP established that 7,730 HIV-positive pregnant Ghanaian women with CD4 counts less than 350 cells/µL needed to initiate PMTCT (NACP, 2013).

In this study viral loads less than 153copies/ml was classified as Undetectable. According to the WHO guidelines of 2006, CD4+ T cells count of less than pre-treatment levels or persistently less than 100cells/µL was classified as Immunologic Failure whilst viral loads more than 10,000 copies/ml was classified as Virologic Failure (World Health Organization HIV/AIDS Programme, 2006). Based on these guidelines, two participants (3%) of Group Three, KBC2/4 and BCC2/1, had failed immunologically after five years of ART since their CD4+T cells counts were persistently less than 100cells/µL (APPENDIX IV). Going by the same guidelines, none of the participants had failed

virologically though there was incomplete viral suppression seen in 5% of participants (Virologic failure: > 1,000copies/ml), (World Health Organization, 2012).

The lack of significant difference in mean CD4 counts between Groups 1 and 3 whose members had experienced ARVs (Table 14) makes a good case for Ghana to switch the current treatment policy to the Option B Plus advocated by World Health Organization for pregnant women (World Health Organization Programmatic Update, 2012). The Option B Plus recommends that regardless of CD4 count, triple ARVs regimen should be started as soon as the pregnant woman is diagnosed with HIV infection and this should be continued for life (World Health Organization Programmatic Update, 2012). Participants in Group 3 had been put on ART in line with the Option B Plus –they had initiated ART when pregnant and diagnosed with HIV and had low CD4 counts; they were still on this regimen at the time of this study.

This study has confirmed that CD4+ T cells count and viral load testing together would be more effective for the assessment of the clinical profiles of Ghanaian HIV-positive mothers and pregnant women because of the different responses elicited by antiretroviral therapy which is linked to the risk levels of progression to AIDS-related illness or death.

5.4 Emergence of HIV-1 Drug Resistance

This study showed that there is a significant association between the emergence of HIV-1 drug resistance and the various groups of participants. The groups of the study consisted of the different groups of HIV-infected women in the PMTCT programme in Ghana.

HIV-1 drug resistance will therefore be encountered among the general populace. Under the PMTCT programme, the mothers who were on ART after prophylaxis were the most affected (32% DRAMs out of the total in Group One). Mothers who were put on ART without ARV prophylaxis showed the least level of HIV-1 drug resistance (5% of the total in Group Three) though these participants (Group 3) had been on ART for a longer time than participants in Group 1; a mean of 34 months in the former compared to 11 months in the latter group (Table 13). Hence there was no significant difference among the participating groups with regards to the impact of duration on ART on the emergence of HIV-1 DRAMs.

Administration of ART upon HIV diagnosis without prophylaxis has a better outcome in preventing the emergence of HIV-1 drug resistance. The study has provided data to support the adoption of the new Option B Plus proposed by the WHO for all HIV-1 infected pregnant women regardless of the CD4 counts (Volberding and Deeks, 2010; Schouten *et al,* 2011). This study shows that the target to eliminate mother-to-child transmission of HIV-1 in Ghana by the year 2015 would be enhanced by giving ART upon diagnosis without prophylaxis. The study has also provided initial evidence for Ghana that addresses concerns about long term use of ART when initiated in early HIV disease and the emergence of HIV-1 drug resistance. In Table 14, participants who had been longest on ART (≥3years) presented the least level of HIV-1 drug resistance as compared to those who had been on HAART for lesser periods (1 to 2 years). Adherence to treatment was a contributory factor in this situation as shown by high adherence levels achieved in this study (Table 11).

When an individual is infected with an HIV-1 strain harbouring drug resistant mutations, this is described as Transmitted Drug Resistance (TDR). This causes restricted drug options and suboptimal treatment outcomes for patients with HIV-1 (Booth and Geretti, 2007). In 2013, 8,907 HIV-1 positive pregnant women in Ghana were estimated to require PMTCT (NACP, 2013). Of the drug-naïve group in this study, 4 (15%) of the participants showed the presence of HIV-1 drug resistance associated mutations. Some of these mutations reduce the susceptibility of NRTIs and others reduce the effect of NNRTIs (Johnson *et al*, 2013). These Ghanaian women were found to be harbouring strains of the virus resistant to the drug regimen available to them even before initiating PMTCT. Hence the effect of the drugs would be suboptimal.

This study has re-emphasized the need to carry out genotypic resistance testing for pregnant HIV-1 positive women before initiating PMTCT.

5.5 HIV-1 Drug Resistance Mutations and ART

5.5.1 Acquired HIV-1 Drug Resistance

Acquired drug resistance occurs when resistance mutations emerge because of drug-selective pressure in individuals receiving antiretroviral therapy (Clavel and Hance, 2004). This study determined that HIV-1 drug resistant associated mutations had emerged in mothers who had received ART for their own health after previous exposure to prophylaxis to prevent the transmission of HIV-1 to the baby. The HIV-1 drug resistance associated mutations (DRAMs) encountered in the study have different effects on the susceptibility of the ART administered to the patients enrolled in the study groups. For the mothers who were on ART after prophylaxis in the PMTCT programme, the HIV-1 drug resistance associated mutations seen were dominated by M184V and Thymidine

Analogue-Associated Mutations (TAMS) including M41L and T215Y for NRTIs and K103N with A98G for NNRTIs. Generally, it is known that mutations selected by TAMS confer resistance to internationally approved NRTIs; such TAMS are M41L, D67N, K70R, L210W, T215Y/F and K219Q/E (Johnson *et al*, 2013).

It has been established that M184V and M184MV lead to high-level resistance to Lamivudine (3TC) and Emtricitabine (FTC) and low-level resistance to Didanosine (DDI) and Abacavir (ABC). When the M184V mutation is selected with TAMS, it leads to increased resistance to Abacavir (ABC). It is also known that mutations selected by TAMS, as seen in these mothers, confer reduced susceptibility to the currently approved NRTIs used in Ghana (Whitcomb *et al*, 2003). Study participants had been previously given a combination of Zidovudine (AZT) and Lamivudine (3TC) which are both NRTIs with Nevirapine (NVP) or Efavirenz (EFV) (both NNRTIs). The mutations in the reverse transcriptase gene associated with resistance to NRTIs are related to and have reduced susceptibility effects on the ARVs (AZT and 3TC) used by the participants during and after PMTCT. Though none of the mothers had been given ABC, TDF, DDI or D4T, resistance to these ARVs had emerged in these participants. This could be due to the effect of cross-resistance in drug classes (Virco, 2006; Clavel and Hance, 2004). M41L in combination with T215Y (as seen in this study) is known to confer high level resistance to AZT and D4T, whilst causing low resistance to DDI, ABC and TDF. On its own, T215Y causes AZT and D4T resistance and reduces susceptibility to other NRTIs (ABC, DDI and TDF). L74V was also encountered in this study and causes high-level resistance to DDI and intermediate resistance to ABC. A mutation that is associated with heavily-

treated NRTI patients, K219KR, was seen in this study (the prophylaxis with ART group); this mutation confers intermediate reduced susceptibility (resistance) to ABC, AZT, D4T, DDI and TDF with no effect on 3TC and FTC. All the NRTIs used in Ghana are affected by this mutation. It makes it imperative to institute genotypic resistance testing before initiating ART after the prophylaxis for PMTCT.

HIV-1 drug resistance mutations to NNRTIs seen in this study all work against the susceptibility of HIV to the ARVs recommended for use in Ghana. K103N was seen in all the participants in this group and causes high-level resistance to NVP and EFV, the two NNRTIs widely used in Ghana. When K103N is seen in combination with L100I as seen in this group, it causes high-level resistance to both NVP and EFV, leaving the patient with only the AZT and 3TC combination to be effective against the virus. Also, A98G found in the group also causes reduction in susceptibility to NVP by 5-fold and to EFV by 3-fold and has the ability to cause reduction in other members of the NNRTIs not in use in Ghana such as Etravirine (ETR) and Rilpivirine (RPV). G190EG was another mutation found in this study that causes high-level resistance to NVP and EFV. NNRTIs resistance mutations Y181C and M230L are known to confer high-level and intermediate resistance to NVP and EFV (Johnson *et al*, 2013). These were also found to be present in the participants in Group 1. The presence of Y181C, M230L and M230LM posed resistance to all the NNRTIs used in the country.

HIV-1 drug resistance seen in the group of participants who had been given ART without prophylaxis was only 5% (3 out of 65 participants). The resistance associated mutation in the RT gene to NNRTIs (NVP & EFV) seen in this group was A98G; this was found in

two of the participants who had been on treatment for less than 6 years. No mutation with resistance to NNRTIs was seen with the third participant, KDC2/20, who had been on the ARVs for only ten months. The presence of A98G in these mothers causes high-level resistance to NVP and EFV. Resistance mutation to NRTIs seen in these mothers was M184V which confers high-level resistance to 3TC and FTC, and low-level resistance to DDI and ABC. However, M184V without other mutations is not contraindicated for continuous treatment with 3TC or FTC because then it rather causes increased susceptibility to AZT, TDF and D4T. M184V as a stand-alone mutation results in a clinically significant reduction in HIV-1 replication in the patient (Turner *et al*, 2003). This situation was seen with participant KDC2/20 who had been on treatment for less than 12 months as at study time and had achieved viral suppression (viral load <153copies/ml). These findings support the recommendation by WHO in the Drug Resistance Report of 2012 that virological testing as an additional early warning indicator should be carried out at 12 months after initiating treatment, for better prevention of emergence of drug resistance mutations (World Health Organization, 2012). However, in the other mothers in this group, M184V occurred with Thymidine Analogue-Associated Mutations (K70R, K219E, M41LM and T215I); it therefore produced a synergistic effect that led to different levels of reduction in susceptibility (Johnson *et al*, 2013; Clavel and Hance, 2004). T215S and Y115F mutations, seen in participant KBC2/4, are associated with low-level resistance to ABC, AZT, DDI and D4T and intermediate resistance to ABC and TDF respectively. Participant KBC2/13 had begun treatment on ARV regimen which did not conform to the recommended options of First Line choice of the drugs in use at the time of the study (Ghana Health Service, 2011; NACP Quarterly Technical

Bulletin, 2012) as seen in Tables 2 and 3. The HIV-1 drug resistance mutations for NRTIs seen in this participant was a combination of M184V and TAMs indicating resistance ranging from low-level to intermediate level for all the NRTIs in use in Ghana. For this patient, there was no alternative NRTIs that she could be given under the First Line options. She had begun treatment on a Second Line Regimen with Combivir (a combination of AZT and 3TC) together with Nelfinavir (NFV) which is a Protease Inhibitor (PI). Mutations emerged in the Protease gene associated with resistance to PIs recommended for use in Ghana, ie NFV and Ritonavir boosted Lopinavir (LPV/r). Mutation I84V was seen in participant KBC2/13. This is a major resistance mutation to PIs and causes intermediate- to high-level resistance to NFV and LPV/r (Johnson *et al,* 2013). PI minor resistance mutations found in this participant were M46MV, A71V and L89V; these may cause low-level resistance, though on their own, do not cause significant reduction in susceptibility to NFV or LPV/r. This participant had been switched to a First Line Regimen comprising 3TC, ABC and TDF at the time of the study.

This study set out to establish that the ARVs given during prophylaxis for PMTCT and also during treatment of HIV positive mothers were associated with the emergence of HIV-1 drug resistance mutations. According to the WHO HIV drug resistance report released in the year 2012, among individuals receiving antiretroviral therapy in resource-challenged countries, acquired drug resistance continues to hamper treatment effectiveness (World Health Organization, 2012). This study has also confirmed this assertion.

5.5.2 Transmitted HIV-1 Drug Resistance

Transmitted drug resistance of HIV-1 (tHIVDR) occurs when previously uninfected individuals are infected with a drug-resistant variant of the virus (World Health Organization, 2012). Mutations associated with NRTIs and NNRTIs emerged in 4 participants in the drug-naïve group of the study. V75E and T69S mutations in the RT gene detected in the study were not associated with drug resistance in any of the NRTIs currently in use in Ghana and outside Ghana. Their assigned mutation scores from the Stanford HIV database against NRTIs were zero (0) (Stanford University HIV Drug Resistance Database, 2011). One participant had K103EK, a transition mutation in the NNRTIs but not associated with drug resistance to current NNRTIs and also with an assigned mutation score of zero against the NNRTIs. However, the E138A mutation in the RT gene seen in this group (though did not cause reduction in susceptibility to NVP and EFV) causes low-level resistance to RPV and ETR which were not used in Ghana.

Mutations in the RT gene which are associated with drug resistance to NRTIs and NNRTIs emerged in the drug-naïve group. The mutations associated with NRTIs seen were M184V, V75S and L210W and with NNRTIs were A98G, K103N and V106A. Patient KDC1/6 had M184V mutation which is a major mutation in the RT gene associated with NRTIs and is known to cause high-level resistance to 3TC and FTC, low-level resistance to DDI and ABC. However, M184V on its own is not a contraindication to continued treatment with 3TC and FTC because it increases susceptibility to AZT, TDF and D4T and also leads to a significant decrease in HIV-1 replication (Stanford University HIV Drug Resistance Database, 2011). The V75S mutation is weakly selected by NRTIs and thus causes a low-level resistance to DDI and D4T.

L210W is a TAM, a major mutation detected in one participant in this group (KDC1/10); it causes low-level resistance to all NRTIs in use in Ghana except 3TC and FTC.

K103N and V106A are major mutations associated with NNRTIs conferring high-level HIV-1 drug resistance to NVP and EFV. A98G is a minor mutation found in one drug-naïve participant (KDC1/1) and causes 5 fold and 3 fold reduced susceptibility to NVP and EFV respectively. Though both patients KDC1/6 and KDC1/10 were HIV-1 drug-naïve, they had major mutations (transmitted HIV-1 drug resistance) for both NRTI and NNRTI (Johnson *et al*, 2013).

This study detected the presence of transmitted HIV-1 drug resistance (tHIVDR) associated mutations in two participants of the drug-naïve group, KDC1/6 and KDC1/10. Out of the total of 26 participants in the drug-naïve group, these 2 (8%) women had major mutations in the RT gene that were associated with transmitted HIV-1 drug resistance to NRTIs and NNRTIs. These two participants were part of the eleven (18%) from Koforidua in the Eastern Region of Ghana. This finding is not surprising since ARVs had been available in the region since the pilot stage of initiating HAART in Ghana in May, 2003 (Ghana Health Service, 2011).

In view of the presence of tHIVDR encountered in the drug-naïve group, genotypic resistance testing before initiation of PMTCT would contribute significantly to eliminate mother-to-child transmission of HIV in Ghana. These HIV-1 drug-naïve pregnant women would need the NRTIs and the NNRTIs when they initiate HAART subsequently for their own health and such resistance mutations would not allow optimal response to ART.

No major mutation in the Protease gene that was associated with PIs for the drug-naïve group was seen in this study though a minor mutation, L10V, was seen in one participant, KDC1/2. L10V is a mutation that is seen in 5-10% of untreated persons and confers no resistance to the PIs in use in Ghana. Different studies carried out in Ghana (Bonney *et al,* 2013; Sagoe *et al,* 2007) also showed the presence of L10V in the drug-naïve subjects of those studies.

5.6 Characterization of HIV-1 Subtypes and Drug Resistance Mutations

The major resistance associated mutations found in the study were related to the ART regimen in use in Ghana. The HIV-1 strains were predominantly subtype CRF02_AG of HIV-1, confirming the findings of other studies in Ghana that HIV-1 CRF02_AG is the prevalent subtype in the country (Brandful *et al,* 2007; Fischetti *et al,* 2004**).** One subject, KDSG/5, had K219KR resistance-associated mutations for NRTI and G190EQ for NNRTI and was subtyped CRF01_AE using both the Stanford HIV database programme and phylogenetic analysis (Figure 14). Subject KDC1/10 had L210W, a resistance associated mutation for NRTI and V106A, a major resistance mutation for NNRTI was classified as HIV-1 Subtype A. Subject KBC2/4 with M184V, Y115Y and T215S mutations for NRTI and A98G for NNRTI was classified as HIV-1 Subtype B using the Stanford HIV database programme.

In the PMTCT plus ART group (Group 1), the majority of the RAMs for the RT gene 7(88%) were labeled as subtype CRF02_AG. The M184V, M41L and L74V mutations against NRTIs along with the TAM T215Y were also determined to be subtype

CRF02_AG. For these patients, major resistance associated mutations for NNRTIs (K103N, Y181C, M230L) as well as A98G and M230LM were also found with subtype CRF02_AG. The subtype CRF02_AG (Ghana) was related closely to CRF02_AG from Nigeria, Cameroun and Liberia (Figure14) confirming strain related mutations and the genetic complexity of HIV-1 infection in the west coast of Africa as shown by other studies (Delgado *et al*, 2008; Sagoe *et al*, 2007).

For the drug-naïve patients, (Group 2), a thymidine associated mutation, L210W, for NRTIs was observed in subject, KDC1/10, classified as HIV-1 Subtype A. A major resistance mutation for NNRTI, V106A, was present in this patient conferring high level resistance to Nevirapine.

5.7 Conclusions

The virologic and immunologic patterns of HIV-1 positive mothers on ART are factors that influence the clinical profiles of these mothers during treatment after the prophylaxis that was administered to prevent mother-to-child transmission. Concordant Responders to ART were described as being at minimal risk of progression to AIDS-related illness or death based on the limited range of markers used to monitor treatment. However, 32% of mothers on ART after prophylaxis had major resistance mutations to NRTIs and NNRTIs though they were Concordant Responders.

The immunologic and virologic patterns coupled with the presence of HIV-1 drug resistance mutations contributed significantly to suboptimal responses to ART among such patients with a history of prophylaxis. The proportion of patients with HIV-1 drug

resistance mutations was found to be higher in the mothers with a history of prophylaxis before initiation of treatment compared to mothers who initiated treatment without prophylaxis. Consequently the study concludes that mothers who had prophylaxis and are on ART are more likely to develop drug resistance mutations than those on ART without prophylaxis. Therefore, this endorses the maintenance of all HIV positive pregnant women on ART irrespective of the level of their CD4 counts.

Major acquired HIV-1 resistance mutations to NRTIs and NNRTIs found across the groups of this study, along with Thymidine Analogue Mutations, tend to deepen the resistance of HIV-1 to the ARVs used in Ghana.

The presence of HIV-1 transmitted drug resistance mutations detected in drug-naïve pregnant women will militate against progress to control and to eliminate mother-to-child transmission of HIV-1 in Ghana.

The study has provided further evidence that HIV-1 and HIV-2 infections are co-circulating in Ghana with HIV-1 dominating and subtype CRF02_AG being the most dominant HIV-1 subtype in the country.

5.8 Recommendations

1. In the light of the low levels of HIV-1 drug resistance mutations encountered among the patients on ART without prophylaxis in this study, antiretroviral treatment Option B Plus recommended by the WHO for treating HIV in pregnant women should be adopted for implementation in Ghana.

2. It would be of immense benefit to the effective management of the HIV infection and the plan for the elimination of mother-to-child transmission (eMTCT) in Ghana for further work to be done among ART-naïve pregnant women in the Eastern Region of Ghana to monitor the possible increase in the emergence of transmitted HIV-1 drug resistance since ART has been available in the region for the longest duration in Ghana.

3. As an inference from this study, there is the need to institute HIV serotyping in Ghana before the initiation of PMTCT in the light of the documented co-circulation of HIV-1 & 2 in the country since HIV-2 has been shown by other studies to respond poorly to the type of NNRTIs used in the drug regimen in the country.

5.9 Study Limitations

The non-existence of baseline viral load data (viral load determined prior to treatment) for HIV-1 at all the study sites at the time of the study created the limitation of an inability to describe trends of virologic responses to the drug regimen in use. Relating baseline viral load to baseline CD4 counts would have enabled an early indication of suboptimal response by these patients to the ART. There were also difficulties in recruiting HIV positive mothers at the study sites since contact telephone numbers provided in the patient hospital folders were unreachable and in some cases incorrect. This posed a limitation to the sample size due to the large number of defaulting patients who however, met the study criteria.

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APPENDICES

APPENDIX I: CONSENT FORM FOR SPECIMEN

RESEARCH TOPIC: HIV DRUG RESISTANCE PROFILES IN GHANAIAN WOMEN ON ANTIRETROVIRAL THERAPY AFTER PREVENTION OF MOTHER-TO-CHILD TRANSMISSION.

Principal Investigator (Student): **Alexander Martin-Odoom**

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University of Ghana Medical School

College Of Health Sciences

University of Ghana, Korle-Bu, Accra.

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Email: siel.miel@yahoo.com

General Information:

This study seeks to determine the different kinds of the virus present in HIV positive women who were given prophylaxis during the pregnancy, and are now on full antiretroviral treatment. This knowledge will be very useful in the kind of drug regimen such patients are placed on.

You are one of the people needed to be part of this research in order to help determine the usefulness of the medication in the future. If you agree to participate in this research, it would entail the following:

- You would have to provide us with some personal information such as your name, age, occupation, ART regimen you are on, how long you have been on the medication, and how well you take your drug.

- We would need to collect the equivalent of two (2) teaspoons of blood samples from you for the running of the laboratory work in the research.

Possible Risks and Discomforts

The research will not pose any risks to you though you may experience a minor discomfort during the sample collection due to the prick of the needle used.

Possible Benefits

You may not personally benefit immediately from this research but the findings would benefit Ghanaians in general because of its potential to guide your doctor and caregivers to choose the best combination of drugs to reduce the presence of the virus in your body.

Confidentiality

All the information obtained from you and from the analysis of the blood sample would be handled confidentially and used for the purpose indicated for the study.

Compensation

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

Choice of Participation

You are free to choose to participate or not to participate. If you decide not to participate, this will not affect the care you receive in any way.

If you agree to participate, you are free to decide to end your participation at any time.

Contact Numbers

If you have any questions or problems relating to your participation in the study, please call:

Alexander Martin-Odoom on 0208136778

VOLUNTEER AGREEMENT

The document detailing the risks, discomforts, benefits and procedures involved in the research work entitled "HIV Drug Resistance Profiles in Ghanaian Women on Antiretroviral Therapy Following Prophylaxis " has been read and adequately explained to me. I have been given ample opportunity to have any questions I may have answered to my satisfaction. Consequently, I agree to participate as a volunteer.

Signature or Thumbprint of Volunteer Date

If a volunteer cannot read the document, then a Witness is needed:

I was present during the reading and explanation of the consent document to the volunteer; all questions from the volunteer were duly answered and the volunteer agreed to participate in the study.

Signature of Witness Date

I certify that the purpose and nature of the research, the potential benefits and possible discomforts associated with participating in this research have been explained to the volunteer who has agreed to voluntarily participate.

Signature of Person Who Obtained Consent Date

APPENDIX II: QUESTIONNAIRE FOR STUDY PARTICIPANTS

TOPIC: HIV DRUG RESISTANCE PROFILES IN GHANAIAN WOMEN ON ANTIRETROVIRAL THERAPY AFTER PREVENTION OF MOTHER-TO-CHILD-TRANSMISSION(PMTCT)

NAME:…… AGE: ……..

MARRIED: YES /NO

OCCUPATION:……

LOCATION: ……..

ATTENDING HEALTH FACILITY:…… ……

ADHERENCE STATUS BY i. SELF-REPORT:……

ii. FROM FOLDER……

DATA FROM PATIENT FOLDER

WHO CLINICAL HIV/AIDS STAGE:……

CD4 COUNT AT LAST CHECK AND DATE:……....

DATE 1ST PMTCT INITIATED: …… ……

DATE PATIENT MOVED FROM PROPHYLAXIS TO TREATMENT:……..

DRUG REGIMEN FOR TREATMENT AFTER PROPHYLAXIS:……..

……

…….

ARV TREATMENT REGIMEN PRIOR TO CYESIS:……..

……

…….

DEFAULT HISTORY: YES /NO IF YES, DURATION:……

<u>RESEARCHER'S USE ONLY</u>

CODE NAME OF PARTICIPANT:…….

CONFIRMED CD4 COUNT:…….

COMMENTS:

APPENDIX III: MASTER MIX PREPARATION GUIDE

Reagents	**Stock conc.**	**Final conc.**	**µl /tube**	**X N + 1/2**
Nuclease-free water (Ambion,USA)	-	-	1.375	
2X Taqman One-Step RT-PCR Master Mix (ABI,USA)	2X	1X	12.5	
40X MultiScribe™ and RNase Inhibitor Mix	40X	1X	0.625	
Primer (F1)*	3µM	0.3µM	2.5	
Primer (R1)*	3µM	0.3µM	2.5	
p1LNA Probe	2µM	0.2µM	2.5	
Volume of Master Mix			**22.0**	
Template RNA			3.0	
Total Reaction Volume			**25.0**	

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

R1 (antisense) 5'GGCGCCACTGCTAGAGATTTT3'

Probe: p1LNA probe:

5' FAM -CAGTACATGCAGGGCCTATTCCACCAG-TAMRA 3'

Cycling conditions

	Temp/Time	No of cycle
Stage 1 Reverse Transcription	48°C / 30 min	1
Stage 2 Hot start	95°C / 10 min	1
Stage 3 Denaturation Annealing & Extension*	95 °C / 15sec 60 °C / 1 min	40
	4/Hold	

Plasma HIV RNA copy number calculation:

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

Plasmid copy number for plasmid with concentration of 5 µg/µl = $1.14X10^4$

APPENDIX IV: CD4 AND VIRAL LOAD MEASUREMENTS FOR STUDY PARTICIPANTS

Determination of CD4 count and Viral load for the participants in Group 1.

Patient ID	Baseline CD4 cells/μL	Study CD4 cells/μL	Viral Load[a] copies/ml	WHO Clinical Stage	Pre-Study HAART Period(mths)
KBSG/1	419	569	<153	I	23
KBSG/2	452	867	<153	I	18
KBSG/3	426	580	<153	I	6
KBSG/4	455	588	<153	II	8
KBSG/5	860	193	<153	II	33
KBSG/6	386	936	<153	I	12
KBSG/7	379	431	<153	I	1
KBSG/8	770	467	<153	II	15
KBSG/9	746	492	<153	II	13
TGSG/1	568	935	<153	II	3
TGSG/2	417	690	<153	II	3
KDSG/1	532	765	<153	II	9
KDSG/4	546	753	<153	I	9
KDSG/5	191	307	<153	I	25
KDSG/9	401	561	<153	I	1
KDSG/11	574	383	<153	II	16
KSHSG/1	968	1054	<153	II	7
KSHSG/2	420	359	<153	II	1
KSHSG/3	134	386	510	III	4
BCSG/1	330	586	<153	II	2
BCSG/2	243	483	<153	I	27

Determination of CD4 count and Viral load for the participants in Goup 1 (cont'd).

Patient ID	**Baseline CD4 cells/µL**	**Study CD4 cells/µL**	**Viral Load[a] copies/ml**	**WHO Clinical Stage**	**Pre-Study HAART Period(mths)**
BCSG/3	532	404	<153	I	2
BCSG/4	366	744	<153	II	7
BCSG/5	427	534	<153	I	10
AMFSG/1	397	782	<153	II	19

[a] Viral Load of <153copies is the Study Undetectable Level.

Determination of CD4 count and Viral load for the participants in Group 2

Patient ID	Baseline CD4 cells/µL	Study CD4 cells/µL	Viral Load copies/ml	WHO Clinical Stage
KBC1/1	283	283	<153	II
KBC1/2	608	608	<153	II
KBC1/3	195	195	444	II
KBC1/4	73	73	<153	II
KBC1/5	503	503	<153	II
KBC1/6	410	410	<153	II
KBC1/8	408	408	<153	II
TGC1/1	468	468	<153	I
TGC1/2	217	217	<153	II
KDC1/1	577	710	<153	I
KDC1/2	138	138	<153	I
KDC1/3	526	469	<153	I
KDC1/4	437	439	<153	I
KDC1/5	483	483	933	I
KDC1/6	761	805	<153	I
KDC1/7	51	51	<153	II
KDC1/8	335	553	<153	I
KDC1/9	582	610	692	I
KDC1/10	246	320	<153	I
KDC1/11	360	411	<153	I
KSHC1/1	526	526	<153	II
KSHC1/2	63	63	<153	II
KSHC1/4	759	759	<153	I
BCC1/1	658	658	<153	I
AMFC1/1	674	843	<153	I
AMFC1/2	279	279	<153	I

Determination of CD4 count and Viral load for the participants in Group 3

Patient ID	Baseline CD4	Study CD4	Viral Load	WHO Clinical Stage	Pre-Study HAART Period(mths)
KBC2/1	215	1102	<153	II	80
KBC2/2	162	625	<153	II	58
KBC2/3	192	594	<153	III	43
KBC2/4	63	85	<153	III	65
KBC2/5	123	237	<153	II	36
KBC2/6	409	842	<153	I	50
KBC2/7	172	783	<153	II	68
KBC2/8	194	911	<153	III	60
KBC2/9	113	492	660	II	61
KBC2/10	80	619	<153	III	71
KBC2/11	260	443	<153	II	2
KBC2/12	107	443	238	II	86
KBC2/13	405	782	<153	II	83
KBC2/14	182	1005	<153	II	39
KBC2/15	524	524	<153	II	0
TGC2/1	65	508	<153	IV	16
KDC2/1	150	360	<153	II	84
KDC2/2	781	523	<153	I	5
KDC2/3	136	390	<153	II	10
KDC2/4	215	402	<153	I	10
KDC2/5	102	496	<153	II	32
KDC2/6	199	803	<153	I	17
KDC2/8	82	352	<153	III	16
KDC2/9	300	982	<153	I	60

Patient ID	Baseline CD4 cells/µL	Study CD4 cells/µL	Viral Load[a] copies/ml	WHO Clinical Stage	Pre-Study HAART Period(mths)
KDC2/10	294	664	<153	II	27
KDC2/11	294	1051	<153	II	13
KDC2/12	67	367	<153	III	26
KDC2/13	266	270	<153	II	27
KDC2/14	212	763	<153	III	22
KDC2/15	249	357	<153	I	23
KDC2/16	325	862	<153	I	13
KDC2/17	257	588	<153	I	23
KDC2/18	61	705	<153	II	32
KDC2/19	102	415	<153	II	12
KDC2/20	136	390	<153	II	10
KSHC2/1	219	288	<153	III	4
KSHC2/2	267	889	<153	III	15
KSHC2/3	189	590	<153	III	61
KSHC2/4	131	504	<153	I	29
KSHC2/5	287	431	<153	III	36
KSHC2/6	151	630	<153	III	29
KSHC2/7	182	480	<153	III	32
KSHC2/8	279	952	<153	III	29
KSHC2/9	258	487	<153	III	21
KSHC2/10	60	262	<153	III	10
KSHC2/11	260	661	<153	III	7
KSHC2/12	319	897	<153	III	16
KSHC2/13	297	561	<153	III	37
BCC2/1	158	85	<153	III	64

Determination of CD4 count and Viral load for the participants in Group 3 (cont'd)

Patient ID	Baseline CD4 cells/µL	Study CD4 cells/µL	Viral Load[a] copies/ml	WHO Clinical Stage	Pre-Study HAART Period(mths)
BCC2/2	52	592	<153	IV	60
BCC2/3	319	299	<153	I	44
BCC2/4	139	382	<153	II	34
BCC2/5	58	439	<153	II	57
BCC2/6	192	814	<153	II	45
BCC2/7	338	585	<153	I	39
BCC2/8	73	595	<153	III	45
BCC2/9	158	415	<153	II	28
BCC2/10	250	693	<153	III	47
AMFC2/1	68	329	<153	III	29
AMFC2/2	537	900	<153	III	29
AMFC2/3	51	400	<153	III	37
AMFC2/4	472	400	<153	III	15
AMFC2/5	459	875	<153	III	27
AMFC2/6	249	834	<153	II	33
SU/1	22	607	<153	I	9

APPENDIX V: DETERMINATION OF HIV-1 AND HIV-2 INFECTION TYPE BY INNOLIA ASSAY KIT (INNOGENETICS,BELGIUM)

KORLE BU TEACHING HOSPITAL,ACCRA & TEMA GENERAL HOSPITAL

SAMPLE ID	Strep	sgp 120	gp41	p31	p24	p17	sgp105	gp36	Final Results
KBSG/1		3+	3+	3+	3+	3+	_	_	HIV 1
KBSG/2		2+	3+	2+	3+	3+	_	_	HIV 1
KBSG/3		2+	2+	2+	3+	2+	_	_	HIV 1
KBSG/4		2+	3+	2+	3+	3+	_	_	HIV 1
KBSG/5		3+	3+	3+	3+	3+	_	_	HIV 1
KBSG/6		3+	3+	2+	3+	3+	_	_	HIV 1
KBSG/7		3+	3+	3+	2+	2+	_	_	HIV 1
KBSG/8		2+	3+	3+	3+	3+	_	_	HIV 1
KBSG/9		2+	3+	3+	3+	3+	_	_	HIV 1
KBC1/1		1+	2+	1+	2+	2+	_	_	HIV 1
KBC1/2		3+	3+	2+	3+	2+	_	_	HIV 1
KBC1/3		3+	3+	3+	3+	3+	_	_	HIV 1
KBC1/4		2+	3+	2+	3+	3+	_	2+	HIV 1& 2
KBC1/5		3+	3+	2+	3+	3+	_	_	HIV 1
KBC1/6		3+	3+	3+	3+	3+	_	_	HIV 1
KBC2/1		2+	3+	2+	1+	2+	_	_	HIV 1
KBC2/2		3+	3+	1+	3+	3+	_	_	HIV 1
KBC2/3		3+	3+	1+	3+	3+	_	_	HIV-1
KBC2/4		3+	3+	3+	3+	3+	_	_	HIV-1
KBC2/5		3+	3+	3+	3+	3+	_	_	HIV 1
KBC2/6		3+	3+	2+	3+	3+	_	_	HIV 1
KBC2/7		3+	3+	1+	3+	3+	_	_	HIV 1
KBC2/8		3+	3+	2+	3+	3+	_	_	HIV 1
KBC2/9		1+	2+	1+	2+	1+	_	_	HIV 1
KBC2/10		2+	3+	2+	2+	1+	_	_	HIV 1
KBC2/11		3+	3+	2+	3+	2+	_	_	HIV 1
KBC2/12		3+	3+	2+	3+	2+	_	_	HIV 1
KBC2/13		3+	3+	3+	3+	3+	_	_	HIV 1
KBC2/14		3+	3+	2+	3+	3+	_	_	HIV 1
KBC2/15		3+	3+	2+	3+	3+	_	_	HIV 1
KBC1/8		3+	3+	3+	3+	3+	_	_	HIV 1
TGSG/1		3+	3+	2+	3+	2+	_	_	HIV 1
TGSG/2		3+	3+	3+	3+	3+	_	_	HIV 1

TGC1/1	3+	3+	3+	3+	2+	_	_	HIV 1
TGC1/2	3+	3+	3+	3+	3+	_	_	HIV 1
TGC2/1	3+	3+	2+	3+	2+	_	_	HIV 1

KOFORIDUA REGIONAL HOSPITAL

SAMPLE ID	Strep	sgp 120	gp41	p31	p24	p17	sgp105	gp36	Final Results
KDSG/1		3+	3+	3+	3+	2+	_	_	HIV 1
KDSG/2		3+	3+	2+	3+	2+	_	_	HIV 1
KDSG/3		3+	3+	3+	3+	3+	_	_	HIV 1
KDSG/4		3+	3+	3+	3+	3+	_	_	HIV 1
KDSG/5		3+	3+	3+	3+	3+	_	_	HIV 1
KDSG/6		3+	3+	3+	3+	3+	_	_	HIV 1
KDSG/7		2+	3+	2+	3+	3+	_	_	HIV 1
KDSG/8		1+	2+	1+	3+	2+	_	_	HIV 1
KDSG/9		3+	3+	3+	3+	3+	_	_	HIV 1
KDSG/10		3+	3+	3+	3+	3+	_	_	HIV 1
KDSG/11		3+	3+	2+	3+	2+	_	1+	HIV 1 & 2
KDSG/12		3+	3+	3+	3+	3+	_	_	HIV 1
KDSG/13		3+	3+	3+	3+	2+	_		HIV 1
KDC1/1		3+	3+	3+	3+	3+	_	_	HIV 1
KDC1/2		3+	3+	3+	3+	1+	_	_	HIV 1
KDC1/3		2+	2+	2+	3+	3+	_	_	HIV 1
KDC1/4		3+	3+	2+	3+	3+	_	_	HIV 1
KDC1/5		3+	3+	2+	3+	3+	_	_	HIV-1
KDC1/6		3+	3+	2+	3+	3+	_	_	HIV-1
KDC1/7		3+	3+	2+	3+	3+	_	_	HIV 1
KDC1/8		2+	2+	2+	2+	2+	_	_	HIV 1
KDC1/9		2+	3+	1+	3+	2+	_	_	HIV 1
KDC1/10		2+	2+	2+	2+	2+	_	_	HIV 1
KDC1/11		3+	3+	2+	3+	3+	_	_	HIV 1
KDC2/1		2+	3+	3+	3+	3+	_	_	HIV 1
KDC2/2		2+	3+	2+	3+	3+	_	_	HIV 1
KDC2/3		2+	3+	2+	3+	3+	_	_	HIV 1
KDC2/4		2+	2+	2+	2+	2+	_	_	HIV 1
KDC2/5		3+	3+	2+	3+	2+	_	_	HIV 1
KDC2/6		3+	3+	2+	3+	2+	_	_	HIV 1
KDC2/7		3+	3+	3+	3+	2+	_	_	HIV 1
KDC2/8		2+	2+	2+	2+	2+	_	_	HIV 1
KDC2/9		3+	3+	2+	3+	3+	_	_	HIV 1
KDC2/10		2+	3+	1+	1+	1+	_	_	HIV 1

KDC2/11	3+	3+	2+	2+	1+	_	_	HIV 1
KDC2/12	2+	3+	1+	3+	3+	_	_	HIV 1
KDC2/13	3+	3+	3+	3+	3+	_	_	HIV 1
KDC2/14	3+	3+	3+	3+	3+	_	_	HIV 1
KDC2/15	3+	3+	1+	3+	3+	_	_	HIV 1
KDC2/16	2+	3+	2+	3+	3+	_	_	HIV 1
KDC2/17	3+	3+	2+	3+	3+	_	_	HIV 1
KDC2/18	3+	3+	2+	3+	3+	_	_	HIV 1
KDPPNH/8	3+	3+	3+	3+	3+	_	_	HIV 1

KUMASI SOUTH HOSPITAL

SAMPLE ID	**Strep**	**sgp 120**	**gp41**	**p31**	**p24**	**p17**	**sgp105**	**gp36**	**Final Results**
KSHSG/1		2+	3+	2+	3+	3+	_	_	HIV 1
KSHSG/2		2+	2+	2+	2+	2+	_	_	HIV 1
KSHSG/3		3+	3+	3+	3+	3+	_	_	HIV 1
KSHC1/1		3+	3+	3+	3+	3+	_	_	HIV 1
KSHC1/2		3+	3+	3+	3+	3+	_	_	HIV 1
KSHC1/3		3+	3+	3+	3+	3+	_	_	HIV 1
KSHC1/4		1+	1+	2+	2+	2+	_	_	HIV 1
KSHC2/1		_	_	_	1+	1+	3+	3+	HIV 2
KSHC2/2		2+	3+	2+	1+	1+	_	_	HIV 1
KSHC2/3		3+	3+	3+	3+	3+	_	_	HIV 1
KSHC2/4		2+	2+	2+	2+	2+	_	_	HIV 1
KSHC2/5		2+	2+	2+	3+	2+	2+	2+	HIV 1 & 2
KSHC2/6		3+	3+	3+	3+	3+	_	_	HIV 1
KSHC2/7		3+	3+	3+	3+	3+	_	_	HIV 1
KSHC2/8		3+	3+	1+	3+	3+	_	_	HIV 1
KSHC2/9		2+	3+	3+	3+	3+	_	2+	HIV 1 & 2
KSHC2/10		3+	3+	3+	3+	3+	_	_	HIV 1
KSHC2/11		2+	2+	2+	3+	2+	_	_	HIV-1
KSHC2/12		3+	3+	3+	3+	3+	_	_	HIV-1
KSHC2/13		3+	3+	2+	3+	3+	1+	_	HIV 1

BOMSO CLINIC, KUMASI

SAMPLE ID	Strep	sgp 120	gp41	p31	p24	p17	sgp105	gp36	Final Results
BCSG/1		3+	3+	3+	3+	3+	_	_	HIV 1
BCSG/2		3+	3+	3+	3+	3+	_	_	HIV 1
BCSG/3		3+	3+	3+	3+	3+	_	_	HIV 1
BCSG/4		3+	3+	3+	3+	3+	_	_	HIV 1
BCSG/5		2+	3+	3+	3+	3+	_	_	HIV 1
BCC1/1		2+	2+	1+	2+	2+	_	_	HIV 1
BCC2/1		3+	2+	3+	3+	3+	_	_	HIV 1
BCC2/2		3+	3+	2+	3+	3+	_	_	HIV 1
BCC2/3		2+	3+	2+	3+	3+	_	_	HIV 1
BCC2/4		3+	3+	3+	3+	3+	_	_	HIV 1
BCC2/5		3+	3+	3+	3+	3+	_	_	HIV 1
BCC2/6		3+	3+	2+	3+	3+	_	_	HIV 1
BCC2/7		3+	3+	3+	3+	3+	_	_	HIV 1
BCC2/8		3+	3+	3+	3+	3+	_	_	HIV 1
BCC2/9		2+	2+	2+	3+	2+	_	_	HIV 1
BCC2/10		2+	3+	3+	3+	3+	_	_	HIV 1

ANIMWAH MEDICAL FOUNDATION, KUMASI

SAMPLE ID	Strep	sgp 120	gp41	p31	p24	p17	sgp105	gp36	Final Results
AMFSG/1		3+	3+	3+	3+	3+	_	_	HIV 1
AMFC1/1		2+	2+	2+	2+	2+	_	_	HIV 1
AMFC1/2		2+	2+	2+	3+	2+	_	_	HIV 1
AMFC2/1		3+	3+	3+	3+	3+	_	_	HIV 1
AMFC2/2		3+	3+	3+	3+	3+	_	_	HIV 1
AMFC2/3		3+	3+	2+	3+	3+	_	_	HIV 1
AMFC2/4		2+	2+	2+	3+	2+	_	_	HIV 1
AMFC2/5		1+	3+	2+	3+	3+	_	_	HIV 1
AMFC2/6		1+	3+	2+	3+	3+	_	_	HIV 1

SUNTRESO GOVERNMENT HOSPITAL,KUMASI

SAMPLE ID	Strep	sgp 120	gp41	p31	p24	p17	sgp105	gp36	Final Results
SU/1		1+	2+	3+	3+	_	3+	3+	HIV 1 & 2