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COLLEGE OF BASIC AND APPLIED SCIENCES

TRANSMITTED DRUG RESISTANCE AND CO-RECEPTOR TROPISM OF CIRCULATING HIV-1 SUBTYPES IN ART-NAÏVE HIV PATIENTS IN GHANA

BY

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(10701499)

THIS THESIS/DISSERTATION IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT FOR THE AWARD OF MPHIL IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASE DEGREE

JUNE 2021

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DECLARATION

I, Darius N.K Quansah, do hereby declare that except for the references to work done by other people, which have been duly acknowledged by referencing, this research was conducted by me in the Department of Biochemistry, Cell and Molecular Biology (DBCMB), West African Centre for Cell Biology of Infectious Pathogens (WACCBIP) and the Noguchi Memorial Institute for Medical Research (NMIMR), all of the University of Ghana, Legon. The work was carried out under the supervision of Dr. Evelyn Yayra Bonney, Dr. Osbourne Quaye and Dr. George Boateng Kyei.

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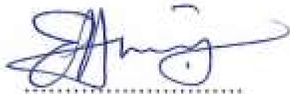
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DEDICATION

I dedicate this thesis to the Glory of the Lord Jesus Christ; my strength, providence and victory.

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'.... In all things, give thanks! Thessalonians 5:18'

This work has been possible and successful following the kind assistance from many, before and during the research and I deem it worthy to honour them with gratitude.

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LIST OF ABBREVIATIONS AND ACRONYMS

HIV- 1	Human Immunodeficiency Virus- 1
AIDS	Acquired Immunodeficiency Syndrome
CRF	Circulating Recombinant Form
PLHIV	People living with HIV
NRTI	Nucleoside Reverse Transcriptase Inhibitors
NNRTI	non-NRTI
ART	antiretroviral therapy
ARVS	antiretrovirals
Gp	glycoprotein
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
cDNA	complementary DNA
INSTIs	Integrase Strand Transfer Inhibitors
PIs	Protease Inhibitor
RT	Reverse transcriptase
PCR	Polymerase Chain Reaction
ARMS-PCR	Amplification-refractory mutation system PCR
TDR	Transmitted Drug resistance
WHO	World Health Organization
MVC	Maraviroc
FACS	Fluorescence Activated Cell Sorter
RLU	Relative Luminescence Units
ANOVA	Analysis of Variance

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DR	Drug Resistance
NVP	Nevirapine
EFV	Efavirenz
URF	Unique Recombinant Form
ETR	Etravirine
ATV	Atazanavir
LPV	Lopinavir

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ABSTRACT

Combination Anti-retroviral therapy (ART) has significantly reduced the burden of human immunodeficiency virus (HIV-1) infection, transforming it from a fatal disease into a manageable chronic one. The clinical benefits of ART are sustained viral suppression increase in CD4 T lymphocyte counts and improved general well-being. However, the emergence of drug resistance poses a strong challenge to the success of ART in managing HIV infection. Drug resistance (DR) to anti-retrovirals (ARVs) is caused by the high replication capacity of HIV, its error-prone reverse transcriptase and poor compliance by HIV-infected people on ART. In Ghana, reverse transcriptase (RT) and protease inhibitors (PIs) are the main drug combinations. While the transmission of multi-drug resistant variants, determined by genotypic tests, among other HIV-endemic populations informs the choice of drugs in their regimen, clinical management of HIV-1 in Ghana does not include genotypic tests. In this research, we hypothesized that transmitted drug resistant mutants present in treatment-naïve individuals are related to the co-receptors used for cell entry and may hasten ART failure.

Sixty-nine treatment-naive HIV-1 infected persons were enrolled from three hospitals in Accra and their clinical histories were obtained. CD4 lymphocytes and HIV-1 viral load were determined for all samples. HIV-1 RNA was extracted from patients' plasma and HIV *Pol* region was amplified by conventional PCR and sequenced. The assembled, edited and aligned contigs were used to phylogenetically characterise circulating HIV-1 subtypes. The sequences were submitted to the Stanford University HIV-DR database for analysis of drug resistance mutants. HIV co-receptor preference was measured using TZM-bl indicator cells.

The dominant HIV-1 subtype was CRF02_AG (64%), followed by subtype B (26%,) and others, CRF06_cpx, A, C and G, in smaller proportions. This confirms previous reports of CRF)2_AG as predominant subtype and indicates a growing population of the subtype B, which drives the HIV epidemic in Europe and America, in Ghana.

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Seven mutations on the WHO drug surveillance list were present in nine out of sixty-nine participants enrolled in this study. All seven of them were either non-nucleoside reverse transcriptase (NNRTI) or PI mutations. Quite surprisingly, no nucleoside reverse transcriptase (NRTI) mutations were found. The K103N, which strongly reduces susceptibility to nevirapine and efavirenz was detected in one individual. All PI mutations that were detected were accessory mutations. This was expected since protease inhibitors are not used on a large scale in Ghana. No one individual carried more than one mutation; indicating low levels of transmitted DR among the study population

The study participants had mean viral load and CD4 counts of 137,694copies/ml and 409cells/µl respectively and presented no symptoms of disease. Such attributes are indicative of rapid viral replication which is a major driver of drug resistance.

Preliminary analysis of the results from the phenotypic assays showed no association between co-receptor phenotypes and identified drug resistance mutations in individuals who carried these mutations. More experiments are needed to confirm these preliminary results. This study has confirmed the predominance of HIV-1 subtype CRF02_AG as the predominant subtype in Ghana while documenting an increase in the circulation of Subtype B. Transmitted HIV drug resistance is still low in Ghana, and this is good for the country's HIV control programmes. However, continuous surveillance for drug resistance strains among treatment-naïve HIV persons is highly recommended to inform treatment regimens going forward.

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background

Human immunodeficiency virus (HIV) infection occurs globally with an estimated 36.9 million people living with the virus. About 25.8 million, representing the largest fraction of people living with HIV (PLHIV), reside in sub-Saharan Africa (Abana *et al.*, 2019; Case *et al.*, 2019; Nichols *et al.*, 2018). Globally, HIV infections have declined and the UNAIDS projected by the year 2020, 90 % of people living with HIV must know their status, 90 % of the number who know their status should be on antiretroviral therapy (ART) and 90 % of those on ART having sustained viral suppression (Cao *et al.*, 2018; Case *et al.*, 2019).

Over the years, substantial advances have been recorded in HIV-1 clinical management. Anti-retroviral therapy (ART) is the most important intervention in the clinical management of HIV infection. ART includes the use of anti-retrovirals, counselling, monitoring of viral suppression as well as the immune recovery of HIV-infected individuals.

Anti-retrovirals (ARVs) have been effectively used to control viral replication in HIV-1 infected patients. The most used ARVs target protease, reverse-transcriptase (RT) and integrase enzymes. Drugs that target the protease are known as protease inhibitors while drugs that target the enzyme reverse transcriptase are classified into the nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). Currently, single-tablet combination anti-retroviral regimens are used, resulting in excellent virologic suppression and delayed progression to Acquired Immune Deficiency Syndrome (AIDS) disease (Zhabokritsky *et al.*, 2019). However, the emergence of drug resistance poses a major challenge to the effective management of HIV infection by ART. HIV-1 drug resistance mutations emerge due to selective pressure of drugs, poor drug compliance by patients on

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treatment and drug-drug interaction among others. These factors have varying impact on HIV patient populations from the different socio-demographic background. ((Luo *et al.*, 2019)

Recent studies among different HIV-infected populations have observed an increasing trend in the prevalence of HIV-1 transmitted drug resistance and the threat it poses to successful anti-retroviral therapy, with drug resistance mutations associated with individual differences (Luo *et al.*, 2019; RheeJordan*, et al.*, 2015).

Viral resistance to anti-retroviral therapy (ART) can emerge through selective pressure from anti-retrovirals (ARVs), spontaneously generated polymorphisms in the viral genome in a rare case or acquired at infection with an already resistant-virus. The latter phenomena are best described as transmitted drug resistance (Alexander Martin-Odoom *et al.*, 2017). Luo et al. show that, HIV-infected people showed different metabolic responses to anti-retrovirals (ARVs), which notably contributes to drug resistance among patients failing first-line ART. Globally, several drug mutations have been identified for many of the drugs that are used as anti-retrovirals. Some of the drug mutations confer resistance while others do not confer resistance. Clinically relevant mutations have been further described as major, minor and accessory mutations. All of these mutations have been captured in the WHO drug resistance surveillance list and the data is updated from time to time. Major drug resistant mutations drastically reduce susceptibility to the respective drug and directly contribute to failure. The major drug resistant mutations that have been identified include M814V, K65R and K103N. These mutations have been shown in studies to have different transmission patterns, which have several underlying reasons, including sub-type. Other factors that influence drug mutational patterns include age, co-receptor phenotype and random viral replication. The K65R and K103N mutations were more likely to occur in patients who aged over 50 years than in patients younger than 30 years. Interestingly, the K103N mutation was strongly associated with patients who were married than in unmarried patients (Luo *et al.*, 2019).

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In Ghana, treatment regimen follows the WHO guidelines. Treatment in all groups including children is a triple therapy and consists of 2 NRTIs and 1 NNRTI or 2 NRTI plus a boosted PI. Alternatively, patients are considered for regimen containing 2NRTI plus 1 integrase strand transfer inhibitor (INSTI) following the introduction of INSTIs. The regimens are changed in the event of virologic failure or adverse reactions. The use of protease inhibitors in Ghana is not without contentions as Kinomoto et al. have shown that HIV-1 proteases from drug-naïve West African HIV patients are less susceptible to protease inhibitors (Kinomoto *et al.*, 2005). These suggest that genotypic and pre-drug resistance tests are very important for the success of any ART.

Following the World Health Organization's (WHO) resolve to advance HIV therapy, newer generation of drugs have been adopted in HIV care, including integrase strand transfer inhibitors (INSTIs) and entry inhibitors (Malet *et al.*, 2019). The INSTIs are efficient at impairing retroviral integration while the Entry Inhibitors restrict HIV-1 entry. Approved INSTIs include raltegravir, dolutegravir and elvitegravir while maraviroc is a licensed CCR5 antagonist. The introduction to the use of entry inhibitors including maraviroc necessitates a look into the co-receptors that HIV-1 utilizes in host cell exploitation. During HIV entry into CD4+ T-lymphocyte, following attachment to the CD4 receptor, two co-receptors facilitate entry. These are the CXCR4 and CCR5 co-receptors (Agwu *et al.*, 2016). Viruses that utilize the CXCR4 receptor are known as X4-tropic viruses, while those that utilize the CCR5 are known as R5-tropic viruses.

During acute infection, most patients preferentially harbour R5-tropic viruses. However, the population of R5-tropic gradually decreases with time, leaving the more aggressive X4-tropic viruses to drive disease progression (Simmons et al., 2000).

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1.2 Rationale

Antiretroviral drug resistance limits treatment options, irrespective of how it develops (Agwu *et al.*, 2016). HIV, just like other RNA virus polymerases have high error rates that are not subjected to host replication proof reading activity mechanisms, leading to the evolution of HIV drug-resistant mutants. Studies done in HIV endemic regions and resource-developed regions globally have identified and established drug-resistant mutants as a cause of treatment failures (Gianella *et al.*, 2010; Mzingwane *et al.*, 2016; RheeJordan*, et al.*, 2015).

HIV-1 genotypic drug testing is done prior to initiation of ART, in developed countries, to guide physicians to select the most efficacious class of ART and effectively reduce the likelihood of virologic failure (Malet *et al.*, 2019; RheeJordan*, et al.*, 2015). However, pre-treatment resistance testing is not performed in Ghanaian HIV patients prior to the initiation of ART. It is therefore, possible that this gap in test significantly contributes to the reported cases of treatment failure (Abana *et al.*, 2019; Archampong *et al.*, 2017; A. Martin-Odoom *et al.*, 2018). In order to sustain ART gains in Ghana and it is important to conduct drug resistance surveys to inform ARV choices in the clinical management of HIV/AIDS.

HIV co-receptor tropism studies elucidate co-receptor use of circulating HIV-1 strains among people living with HIV. The principal role of tropism is to exclude the presence of detectable mixed-tropic viruses or X-4 viruses in patients being considered for treatment with a CCR5 antagonist, which has been introduced and approved for use in developed countries (Lin *et al.*, 2009). This study is relevant to highlight HIV-1 co-receptor tropism, which is necessary to decide on the use of CCR5 antagonists in the Ghanaian HIV populace.

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1.3 Hypotheses

It is hypothesized in this study that HIV-1 antiretroviral resistant mutants are present in infected patients before the initiation of anti-retroviral therapy (ART), and contributes to treatment failure in HIV-infected individuals.

This study further hypothesized that HIV-1 coreceptor phenotypes influence drug resistance patterns in HIV-1 infection.

1.4 Aim

To determine the incidence of drug resistance prior to ART initiation in HIV-1 infected patients at selected hospitals in Accra, Ghana and to determine co-receptor tropism in the viral strains.

1.5 Objectives

- To characterize circulating HIV sub-types from ART-naïve patients using molecular methods
- To determine transmitted HIVDR in HIV protease and reverse transcriptase sequences
- To characterise co-receptor phenotypes in ART-naïve HIV-infected people in Ghana

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biology and Pathogenesis of HIV

HIV is a ribonucleic acid (RNA) virus that causes HIV infection and subsequently acquired immunodeficiency syndrome (AIDS). The enveloped virus, belongs to the family retroviridae, and are named due to their ability to reverse transcribe their RNA.

HIV primarily infects CD4-positive T lymphocytes and macrophages in some cases, by utilising the CD4 protein which is the main receptor for entry and two identified co-receptors; CXCR4 and CCR5 co-receptors. Productive HIV infection begins with attachment of the viral particle to a host cell following interactions between viral envelope proteins gp120/gp41 and the CD4 protein. Viral membrane fusion, a key step for enveloped viruses to enter host cells then follows. Membrane fusion is an energetically favourable process for HIV, using its envelope (*Env*) glycoprotein (gp). The *env* polypeptide chain is produced as a precursor, gp160 and then undergoes cleavage to form two fragments gp120 and gp41. Gp120 is associated with HIV binding and attachment while gp41 facilitates fusion. Viral entry is often described as an explosion of the HIV capsid into the host cytosol, with its two encapsulated RNA. The RNA strands undergo reverse transcription to synthesize DNA using HIV reverse transcriptase. The DNA is then integrated into the host nucleus through the nuclear pore and the translocation is facilitated by the unique viral enzyme integrase. The virus then sheds its protein capsid to allow for the synthesized DNA to be spliced together with that of the host. Subsequently, viral proteins are synthesized from host cytosol to allow formation of a new viral protein which buds out and infects new cells (Chen, 2019; James, 2019). The summary of the replicative cycle of the virus is shown in figure 1 below.

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HIV infection reduces the immune abilities of the host, rendering the host susceptible to many opportunistic infections including tuberculosis (Kaminski *et al.*, 2016; Kitawi *et al.*, 2015) .

HIV replicates very fast, with an estimated 10^{10}-10^{11} virions being produced daily in treatment-naïve infected persons (Gianella *et al.*, 2010). However, the replication machinery is highly error-prone, resulting in the rapid evolution of variants, some of which become drug resistant. It has also been demonstrated that HIV-1 drug resistance mutations are mainly caused by selective pressure of drugs, poor drug compliance by patients on treatment and drug-drug interactions among others. These factors have varying impact on HIV patient populations from different socio-demographic backgrounds (Luo *et al.*, 2019).

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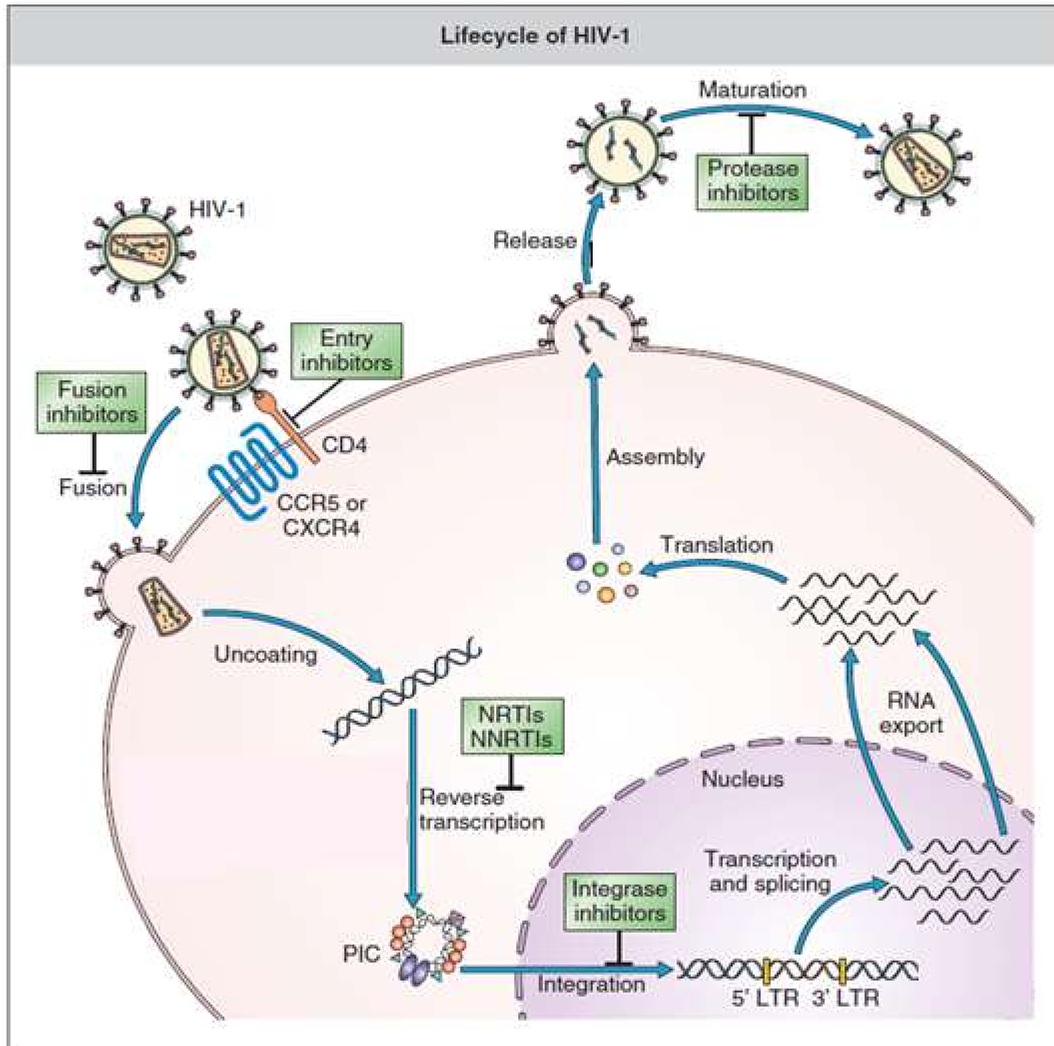

Figure 1: A diagram showing the stages of replication of HIV

(Barré-Sinoussi *et al.*, 2013)

2.1 HIV-1 Classification

HIV-1 is genetically diverse and characterised into sub-types that circulate in different parts of the world. The high genetic variability of the virus is due to its high replication rate, recombination and error-prone replication due to lack of proof-reading activity of the enzyme reverse transcriptase (Beloukas *et al.*, 2016).

HIV-1 is classified into four major groups, Group M, Group N and Group O and Group P. Group N (non-M and non-O) and Group P were observed in recent studies. Group M viruses

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are responsible for the HIV-1 epidemic and have been further classified into sub-types. The known M sub-types are A, B, C, D, F, G, H, J and K. In addition to these subtypes, Group M viruses have distinct Circulating Recombinant forms (CRFs) and Unique Recombinant Forms (URFs) that are found in different regions (Desire *et al.*, 2018).

CRFs are strains that develop from the recombination of the basic sub-types and are relevant to studies on molecular epidemiology, recombination, superinfection and vaccine development. Typically, CRFs are viruses that are identified in at least three epidemiologically unlinked individuals and characterised by full-length genome sequencing and account for about 18 % of HIV-1 infections globally. URFs, are recombinant forms detected in a single individual or a single epidemiologically-linked cluster. URFs are common in areas where more than two genetic forms of HIV-1 co-circulate in the same population. More than 20 CRFs have been identified in different HIV-endemic regions (Casado *et al.*, 2005; Hemelaar *et al.*, 2006). Common CRFs that have been reported are CRF01_AE, CRF02_AG, CRF18 and CRF19_cpx which is common in Africa. CRF01_AE, CRF02_AG and CRF03_AB have played important roles in regional epidemics. Figure 2 below from Hemelaar's report summarizes the distribution of HIV-1 sub-types and recombinant forms over the years.

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Region of the world	A	B	C	D	F	G	H	J	K	CRF01_AE	CRF02_AG	CRF03_AB	Other recombinants	CRFs & other recombinants (%)[a]
North America	0.26	98.42	0.45	0.06	0.03	0.00	0.00	0.00	0.00	0.58	0.10	0.00	0.10	0.77
Caribbean	0.00	94.07	0.97	0.03	0.03	0.13	0.03	0.03	0.00	0.00	0.00	0.00	4.72	4.72
Latin America	0.00	74.50	12.60	0.15	4.35	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.40	8.40
Western Europe	1.69	87.62	2.15	0.46	0.35	2.43	0.00	0.00	0.02	0.62	2.05	0.00	2.60	5.27
Eastern Europe and central Asia	78.88	14.54	1.86	0.00	1.18	0.01	0.00	0.00	0.00	0.60	0.27	2.66	0.00	3.53
India	1.17	0.23	96.95	0.00	0.00	0.00	0.00	0.00	0.00	0.47	0.00	0.00	1.17	1.64
South and south-East Asia (excl. India)	0.00	8.09	3.28	0.00	0.00	0.00	0.00	0.00	0.00	84.28	0.00	0.00	4.36	88.63
East Asia	1.28	38.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	15.53	0.31	0.00	44.85	60.69
Oceania	0.32	88.06	5.01	0.20	0.12	0.00	0.00	0.00	0.00	4.58	1.22	0.00	0.49	6.29
North Africa and middle east	6.21	7.46	28.66	47.03	0.00	0.00	0.00	0.14	0.00	0.00	1.05	0.00	9.45	10.50
West Africa	20.63	0.01	0.59	0.54	0.41	34.94	0.05	0.22	0.01	0.25	27.87	0.00	14.48	42.60
East Africa (excl. Ethiopia)	34.97	0.11	25.09	10.93	0.11	0.24	0.00	0.00	0.00	0.00	0.00	0.00	28.55	28.55
Ethiopia	1.40	0.00	98.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Central Africa	37.60	0.23	11.64	11.08	4.02	11.14	3.14	1.77	0.84	3.99	3.98	0.00	10.57	18.55
Southern Africa	0.47	0.21	98.30	0.39	0.21	0.13	0.00	0.03	0.00	0.00	0.00	0.00	0.25	0.25
Global	12.30	10.42	49.91	2.53	0.59	6.32	0.17	0.14	0.04	4.69	4.77	0.10	8.02	17.59

The proportions of HIV-1 subtypes and recombinants within each region and the world. The countries comprising each region are specified below. Underlined are the countries for which HIV-1 subtype distribution data were obtained.

[a]: The combined proportions of CRF01_AE, CRF02_AG, CRF03_AB and other recombinants.

1. Americas.

North America: Canada, USA;

Caribbean: Bahamas, Barbados, Cuba, Dominican Republic, Haiti, Jamaica, Trinidad and Tobago;

Latin America: Argentina, Belize, Bolivia, Brazil, Chile, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Guyana, Honduras, Mexico, Nicaragua, Panama, Paraguay, Peru, Suriname, Uruguay, Venezuela.

2. Europe.

Western Europe: Albania, Austria, Belgium, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Serbia and Montenegro, Slovenia, Spain, Sweden, Switzerland, The Former Yugoslav Republic Macedonia, the United Kingdom;

Eastern Europe & Central Asia: Armenia, Azerbaijan, Belarus, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, Estonia, Georgia, Hungary, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Poland, Republic of Moldova, Romania, Russian Federation, Slovakia, Tajikistan, Turkmenistan, Ukraine, Uzbekistan.

3. Asia.

India;

South & South-East Asia (excl. India): Afghanistan, Bangladesh, Bhutan, Brunei Darussalam, Cambodia, Indonesia, Iran, Lao People's Democratic Republic, Malaysia, Maldives, Myanmar, Nepal, Pakistan, Philippines, Singapore, Sri Lanka, Thailand, Viet Nam.

East Asia: China, Democratic People's Republic of Korea, Hong Kong Special Administrative Region, Japan, Mongolia, Republic of Korea;

Oceania: Australia, Fiji, New Zealand, Papua New Guinea.

4. North Africa & Middle East.

Algeria, Bahrain, Cyprus, Egypt, Iraq, Israel, Jordan, Kuwait, Lebanon, Libyan Arab Jamahiriya, Morocco, Oman, Qatar, Saudi Arabia, Sudan, Syrian Arab Republic, Tunisia, Turkey, United Arab Emirates, West Bank and Gaza Strip, Yemen.

5. Sub-Saharan Africa.

West Africa: Benin, Burkina Faso, Cameroon, Côte d'Ivoire, Equatorial Guinea, Gambia, Ghana, Guinea, Guinea-Bissau, Liberia, Mali, Mauritania, Niger, Nigeria, Senegal, Sierra Leone, Togo;

East Africa (excl. Ethiopia): Burundi, Djibouti, Eritrea, Kenya, Mauritius, Rwanda, Somalia, Uganda, United Republic of Tanzania;

Ethiopia;

Central Africa: Angola, Central African Republic, Chad, Democratic Republic of the Congo, Gabon, Republic of the Congo;

Southern Africa: Botswana, Comoros, Lesotho, Madagascar, Malawi, Mozambique, Namibia, South Africa, Zambia, Zimbabwe.

Figure 2: Global and Regional Distribution of HIV-1 sub-types and Recombinant forms (Hemelaar *et al.*, 2006)

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2.2.0 Anti-retroviral therapy

Anti-retroviral therapy (ART) is multi-factorial plan, including anti-retroviral drugs (ARVs) that are used to manage HIV. The development of combination ART (cART), which is a cocktail of anti-retroviral drugs has been described as one of the greatest achievements of modern medicine. With proper adherence and monitoring, cART significantly suppresses the level of viremia, allowing the host immune system to recover much of its lost function. The level of suppression achieved by an adherent cART is so great that viral evolution and the emergence of drug-resistant mutations are less likely (Deeks *et al.*, 2015). cART over the years have motivated the gains of HIV management, resulting in the decline of the numbers of AIDS-related deaths from a peak of 1.7 million in 2004 to 770,000 deaths in 2018. In addition to the use of anti-retroviral drugs, non-medicinal approach to HIV prevention, including behavioural, biomedical and policy formulation have accounted for the steep reduction of new infections, ART initiation and virologic suppression (Case *et al.*, 2019).

Approximately 25 unique ARVs have been approved for use in HIV-1 endemic countries. The drugs are first developed for adults and following successful implementation, the drugs are suited for paediatric populations. The WHO recommendation for antiretroviral therapy has been sub-divided into three regimens namely first-line therapy, second-line therapy and third-line or salvage therapy. The first-line therapy consists of 2 nucleoside reverse transcriptase inhibitors (NRTI) and 1 non-nucleoside reverse transcriptase inhibitor. Recently, integrase inhibitor-based combinations have been introduced as first-line options (Cao *et al.*, 2018). The second-line therapy is based on a protease inhibitor boost with two NRTIs, which must include one new NRTI. ART plans are changed when there is adverse reaction by patients to the drugs, in the event of virologic failure and or when the attending physician deems it fit, based on laboratory examination.

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2.2.1 Nucleoside reverse transcriptase inhibitors (NRTIs)

NRTIs are analogues of natural nucleosides and nucleotides that act by blocking HIV reverse transcriptase. They are preferentially incorporated into HIV DNA, and thereby terminates DNA synthesis due to the absence of the 3' hydroxyl end. NRTIs, which include abacavir, lamivudine, zidovudine, tenofovir, stavudine are an important component of ART regimens. Despite the importance of NRTIs, reports of drug toxicities and drug resistance have been observed in many instances. Potential complications include cardiovascular disease in the case of abacavir, anaemia, neuropathy and lactic acidiosis (Deeks *et al.*, 2015). The persistent concerns of the subtle toxicities of some NRTIs have led to calls for their withdrawal in many developed countries. A typical example, zidovudine, has been discontinued in many countries and has been replaced with less toxic options such as emtricitabine.

Nucleoside/nucleotide associated mutations (NAMs) have explained the mechanism of resistance to NRTIs. NAMs prevent NRTI incorporation into the nascent DNA chain, while Thymidine analogue mutations (TAMs) utilize ATP-dependent pyro-phosphorolysis to remove the NRTI from the DNA chain thus reversing chain termination. The table below summarizes commonly reported NRTI mutations and their respective ARV effects.

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Table 1: NRTI mutations and their susceptibility effects

NRTI Mutation	Drug Resistance and Implication
M184 V	3TC, FTC, DDI, abacavir
K65R	All NRTIs except zidovudine
Q151M	All NRTIs affected, although tenofovir retains activity
TAMs:M41L, L210W, T215Y, D67Y, K70R, K219Q	4 or more TAMS induce resistance to all NRTIs
L74V	Increased susceptibility to TDF and zidovudine
T69S-XX insertion mutations	Resistant to all NRTIs

(Cohen *et al.*, 2016; Deeks *et al.*, 2015)

2.2.2 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs inhibit HIV reverse transcriptase activity by binding to a pocket near the active site, causing a conformational change of the enzyme. Critical HIV amino acids that facilitate the inhibition activity are Y181 and K103 (Cohen *et al.*, 2016). Amino acid substitutions such as K103N or Y181C prevent NNTIs binding. HIV-2 and HIV-1 O types have a wild type Y181C that induce non-specific drug resistant mutations to NNRTIs. NNRTIs are potent and well tolerated, even though they have been reported to cause central nervous toxicities (Deeks *et al.*, 2015). They form part of first line and second line of anti-retroviral therapy and are the commonest class of ARVs. Due to this NNRTI resistance have been widely reported in many HIV DR studies (Abana *et al.*, 2019; Deletsu *et al.*, 2020; Hamers *et al.*, 2011; A. Martin-Odoom *et al.*, 2018).

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Table 2: NNRTI mutations and their drug implications

NNRTI mutation	Drug Implication
K103N	High-level resistance efavirenz and nevirapine, etravirine retains activity
Y181C	High-level resistance for efavirenz and nevirapine. Provides the foundation for etravirine resistance though it may have activity
E138 A/G/K/Q/R	Rilpivirine

(Cohen *et al.*, 2016; Deeks *et al.*, 2015)

2.2.3 Protease Inhibitors

Virion maturation is an important step in the replicative cycle of HIV. As HIV matures and buds from the host cell, long polypeptide chains are enzymatically cleaved into mature functional proteins by HIV protease. Protease inhibitors bind to the active site of HIV protease and prevents processing of gag and pol. Boosting nucleoside analogues with PIs, such as ritonavir reduces the likelihood of resistance. Drug susceptibility to PI is reduced when mutations occur in the protease gene and inhibits protease binding. Also, mutations in major cleavage sites in gag and pol have been shown to cause DR to PIs. Multiple mutations in the protease gene are usually required before resistance emerges with ritonavir-boosted PI and this explains the very low prevalence of PI mutations among HIV populations.

PIs are rapidly metabolized and very tolerable in patients, with only a few experiencing mild symptoms of dyslipidaemia and cardiovascular diseases. Owing to their high manufacturing costs, protease inhibitors are generally only used in second-line and third-line regimens (Cohen *et al.*, 2016).

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Table 3: Protease Inhibitor mutations and their drug implications

PI mutation	Drug Implications
D30N	Nelfinavir with no cross-resistance
G48V	Saquinavir, no cross-resistance
L76V	When combined with other PI mutations (>3), decreases susceptibility to lopinavir/ritonavir and darunavir/ritonavir
L90M	Nelfinavir, saquinavir

(Cohen *et al.*, 2016; Deeks *et al.*, 2015)

2.2.4 Integrase Strand Transfer Inhibitors (INSTIs)

INSTIs prevent the HIV genome from being integrated into the host genome. INSTIs bind specifically to the part of the integrase that interacts with the ends of the viral DNA and chelates essential MG2+ at the enzyme's active site. Generally, INSTIs are efficacious and known to have less toxicities to patients. Drug resistance to INSTIs occur when primary mutations occur at the active site, accompanied by specific secondary mutations (Deeks *et al.*, 2015).

Table 4: Integrase mutations and their Drug Implications

INSTI mutation	Implication
Q148H/K/R	Raltegravir and elvitegravir resistance
N155H	Raltegravir and elvitegravir
Y143C	Raltegravir
T66I	Elvitegravir
Combination of Q148H, G140S and other integrase mutations	Decreased susceptibility to dolutegravir

(Cohen *et al.*, 2016; Deeks *et al.*, 2015)

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2.3 HIV-Drug Resistance Assays

Drug-resistant variants frequently exist in nearly-all HIV infected people but may be present as minor variants, resulting in undiagnosed cases of resistance in many instances (Gianella *et al.*, 2010). Resistance testing of protease and reverse transcriptase started years ago, with testing of ART-naïve individuals becoming widespread about a fifteen years ago, starting from the UK (Tostevin *et al.*, 2017).

HIV-1 drug resistance testing can be phenotypic or genotypic (Dana S. Clutter *et al.*, 2016). The phenotypic tests are *in vitro* assays that measure the susceptibility of virus to drugs in a cell culture. Susceptibility is usually reported as the ARV concentration that inhibits HIV-1 replication by 50% (IC_{50}). The IC_{50} of a patient-harboured virus is compared to a drug-susceptible reference strain and expressed as a ratio referred to as a foldchange (Dana S. Clutter *et al.*, 2016).

Drug resistance in HIV infection have been studied to be self-acquired or transmitted. Resistance testing of protease and reverse transcriptase genes began in the UK in 1998 following which sequencing of the integrase was introduced in 2007 (Tostevin *et al.*, 2017).

Diagnosing and understanding drug resistance remains an important part of successful HIV care. Due to the complication of opportunistic infections that bedevil HIV-positive people, special attention is needed to reduce drug resistance to the barest minimum. In many HIV-endemic countries, HIV care and treatment are given through specialist HIV outpatients clinics and are often sponsored by the government (Curtis *et al.*, 2015).

Regular care and continuous quality improvement are important for the well-being of people living with HIV in order to strengthen the effect of the use of anti-retroviral therapy (Curtis *et al.*, 2015). Many clinical tests accompany HIV clinical management, including ones done at the initiation of therapy and during routine clinical examinations. At diagnosis, people who test

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positive are examined for opportunistic infections including tuberculosis, which is a global cause of mortality and other chronic medical conditions (Ahonkhai *et al.*, 2017). Even though these practices occur in all HIV-endemic regions, there are discrepancies in the testing capacities due to the availability of resources and improvements in technical knowledge (Crowley *et al.*, 2014; Parker *et al.*, 2017).

In order to bridge the gap, the WHO employs a concerted framework to guide care delivery and the policy strongly models care delivery in resource-limited settings after effective systems that have been adopted in resource-rich environments, introducing important elements of patient care and monitoring (WHO 2019).

Generally, resistance testing in HIV-endemic regions rely on genotypic tests. Even though this test is not common especially for ART initiators in clinical management of HIV-1, it is widely used in research settings (Dana S. Clutter *et al.*, 2016; Song *et al.*, 2018; Steegen *et al.*, 2016).

2.3.1 Sequencing Approaches

Drug-resistance mutations have been identified and classified based on the WHO Drug Resistance Surveillance programme. The list extensively contains mutations that have been reported to confer resistance to the ART classes used in managing HIV infection (Hamers *et al.*, 2011). Successful sequencing approaches of HIV-DR include the identification of mutations that cause or contribute to drug resistance. The mutations basically exist in three or more of well-established expert networks, including the ANRS drug resistance interpretation algorithm, the Stanford HIV database, IAS-USA Mutations Associated with Drug Resistance, Los Alamos National Laboratories HIV Sequence database or Rega Institute Drug Resistance Interpretation Algorithm. A comprehensive list of mutations associated with each of the lists can be found on the Surveillance Drug Resistance Mutation (SDRM).

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In identifying the mutations, viral RNA is extracted from patient's plasma and reverse transcribed to cDNA, which is used in downstream analysis (Lee *et al.*, 2014; Ssemwanga *et al.*, 2012). HIV pol gene is a 1.8kb region that spans the protease gene and an approximate majority of the reverse transcriptase gene. Successful amplification of these genes is followed by Sanger sequencing of the amplicons.

Several sequencing strategies have been used to identify HIV -DR. Most of the sequence-based approaches target the *pol* region, which encompass protease, reverse transcriptase and integrase coding sequences of HIV. The different methods have advantages as well as disadvantages and these approaches have been commonly used by studies, and still an efficient method for drug-resistance searches. Examples of sequencing approaches that have been used in HIV-DR include Sanger and Next-generation sequencing platforms.

2.3.2 Non-Sequencing Approaches

Point mutation assays are generally PCR-based assays which detect specific genomic point mutations that confer drug resistance and are an excellent alternative to the traditional genotyping assays. The amplification-refractory mutation system-PCR (ARMS-PCR) is a well utilized point mutation assay in HIVDR studies. While several studies have resorted to sequencing approaches, a couple of studies have also considered the use of ARMS-PCR to comparatively analyse the occurrence of transmitted drug resistance (TDR) (Nanfack *et al.*, 2017). ARMS-PCR uses the difference in extension efficiency between primers with matched and mismatched 3' bases to identify mutations based solely on the presence of PCR products visualized on a gel electropherogram.

Nanfack et al in a multi-longitudinal study, optimized the premier 1989 protocol to identify HIVDR mutations with high performance, comparatively to the standard sequencing (Sanger) and Next generation sequencing (NGS) to detect HIVDR mutations in ART-naïve patients

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sampled from a high genetically diverse HIV region. The longitudinal design adequately catered for the monitoring of reversion as well as acquisition of HIVDR mutations in the absence of selection pressure (Nanfack *et al.*, 2017).

2.5 Transmitted Drug Resistance

Drug resistance (DR) is a term used to describe the presence of one or more mutations that reduce susceptibility to ARVs (Song *et al.*, 2018; Tostevin *et al.*, 2017). Transmission of drug-resistant HIV strains is associated with sub-optimal virologic response to initial ART, in ART-naïve individuals. Most of the reported DR mutations have been relevant to protease and reverse transcriptase, with resistance mutations to integrase inhibitors gradually increasing among many HIV populations (Tostevin *et al.*, 2017).

Current updates on clinical management of HIV infection in HIV-endemic African countries suggest that, drug resistance persists if not increasing (Nanfack *et al.*, 2017). The propensity of the HIV-1 genome to recombine coupled with the fallibility of the replicative machinery of the virus explains the many novel recombinants that have been discovered since the viral subtypes were characterized. Novel recombinants and second-generation Recombinants (SGRs), which are indicative of superinfection have been reported in West African countries including Cameroon. SGRs are strains composed of at least a part of the conventional identified CRFs, especially CRF02_AG (Fokam *et al.*, 2011; Nanfack *et al.*, 2017; RheeBlanco*, et al.*, 2015) .

Superinfections occur when an individual infected with a primary strain of HIV-1 is infected again with another strain other than the primary genotype identified at seroconversion. The role of superinfections in HIV clinical care cannot be overlooked. The sustained effort of ART intensification may be ruined by superinfections, which often lead to the temporary masking of a resistant virus strain and hence complicating the treatment (Nanfack *et al.*, 2017).

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It is noteworthy that, the ART plan in the African region is close in similarity as most of the countries have limited resource (Fokam *et al.*, 2011). The selected countries follow the WHO approach for HIV care, recommending two nucleoside reverse transcriptase inhibitors (NRTIs) plus one non-NRTI (NNRTIs) for first line option. The second line options are a combination of 2 NRTIs plus a protease inhibitor (PI), preferably ritonavir. The third line, also known as salvage therapy usually contains a regimen of a booster PI with an integrase inhibitor. While developed countries include highly efficient drugs like maraviroc, an entry inhibitor in their treatment plan, it is not so in limited resource countries due to high costs (RheeBlanco*, et al.*, 2015).

The diversity and recombination provide opportunities to study and monitor the evolution of HIV-1 strains circulating HIV-endemic countries. Though ART has been quite successful, one obstacle that remains is the emergence of HIVDR (Fokam *et al.*, 2011).

Rhee et al., conducted a meta-analytical study of 287 independent studies published between 2000 and 2013 from 111 countries, identifying reverse transcriptase sequences (with or without protease) of HIV submitted from 50,870 ARV naïve, HIV-positive individuals. They analysed each virus sequence for the presence of drug-resistant mutations which are indicative of clinical virologic failure. Their study showed an overall prevalence of Transmitted Drug resistance (TDR) of 8% in sub-Saharan Africa with about 11.5% in North America (RheeBlanco*, et al.*, 2015; Rhee *et al.*, 2020).

Their study attributed an unusual increase of about 1.09-fold per year of TDR to the high surge in the use of NRTIs and NNRTIs. TDR however remained unchanged during the period in context, in low- and middle-income countries (LMICs) south and south-east Asia (RheeBlanco*, et al.*, 2015).

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2.6 Transmitted Drug Resistance in Ghana

The first case of HIV infection was reported and diagnosed in Ghana in 1986 following which the HIV situation in Ghana is managed by the National AIDS/STI Control Programme and the Ghana Health Service. Currently, about 1.7% of the estimated 30 million Ghanaians live with HIV-1 infection, with a minority of that population harbouring the less frequent HIV-2 in mixed infections (Dwomoh *et al.*, 2018). The HIV statistics in Ghana is well represented among all gender and age groups, with women being the most affected.

Antiretroviral therapy was started in Ghana in 2003, following the first reported case of HIV-1 infection, to provide appropriate healthcare and support for people living with HIV in Ghana (A. Martin-Odoom *et al.*, 2018). In order to assess the impact of ART, several studies have considered the accessibility to ART, compliance to drugs and reported cases of HIV/AIDS-caused mortalities (Dwomoh *et al.*, 2018; Alexander Martin-Odoom *et al.*, 2017; A. Martin-Odoom *et al.*, 2018).

Nii-Trebi et al. in a 33-sample sized study who were ART-experienced, reported on drug resistant mutations in circulation in the HIV population in Ghana. The study found M184V and K103N, which are major NRTI and NNRTI mutations respectively. Less threatening drug mutations such as A62V, F116Y, Q151M, H221Y and Y181C. They also reported the circulation of K65R and E138A mutations. (Nii-Trebi *et al.*, 2017).

Subsequently, a study by Abana et al which was not limited to ART-naïve individuals in Ghana also did not find any drug resistance, despite reporting similar drug resistant mutations as done already (Abana *et al.*, 2019).

Limited studies on ART-naïve patients have been done in Ghana, with the most prominent being the Ghana HIVDR threshold survey. The authors found only two major drug resistant mutations in 60 individuals who were sampled, prior to ART initiation. The participants were

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sampled over a period of two years (2007 to 2009). One protease associated mutation and a reverse transcriptase-associated mutation were found by the authors, suggesting a prevalence of less than 5 % as at February 2009 (Bonney *et al.*, 2013). No such work has been done thereafter, which makes this purposeful study very important.

It is important to conduct surveillance studies since genetic diversity of circulating strains presents a serious challenge for designing and developing broadly effective HIV-1 vaccines (Ndongmo *et al.*, 2006).

2.7 HIV Tropism

HIV-1 typically infects lymphocytes, by attaching to cell membrane receptors preferentially expressed on lymphocytes and known as CD4 receptor. HIV-1 entry into CD4+T cells is a multiple-step process (Simmons, 2000). First, the external envelope glycoprotein (gp) 120 of HIV-1 binds to the CD4 receptor on the membrane of the T cell and subsequently, a conformational change in gp120 allows interaction. In addition to the CD4 receptor, the viral *envelope (env)* machinery, comprising glycoprotein gp120 exploit other cell membrane chemokines known as co-receptors to penetrate the host cell. Binding of the chemokine receptor leads to conformational changes in HIV-1 gp41, followed by fusion of the viral membrane and the host cellular membrane. The viral particles are then released into the cytoplasm of the host cell (Lin *et al.*, 2009; Wilkin *et al.*, 2012).

Two major different co-receptors have been identified namely C-C chemokine Receptor type 5 (CCR5) and C-X-C chemokine Receptor type 4 (CXCR4), as shown in figure 3. These cellular molecules belong to the G-protein coupled receptors superfamily and are expressed independently but preferentially on different cell types. (Nascimento-Brito *et al.*, 2015; Wilkin *et al.*, 2012). The glycoprotein 120 is a virus surface protein encoded by the *env* gene and it is

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the viral binding component to the cellular receptor. It contains conserved regions labelled C1 to C5 and variable regions labelled V1 to V5. Amino acids which are dispersed in the primary sequence of conserved regions are directly implicated in the contact to CD4 receptor while changes in the V3 loop of gp120 explains that phenotypic changes in the co-receptor usage (James, 2019; Nascimento-Brito *et al.*, 2015).

Regarding the type of coreceptor used, three different phenotypes are recognized in HIV-1 infection. Viruses that exclusively employ CCR5 are known as R5-tropic viruses while viruses that exclusively employ CXCR4 receptor are known as X4-tropic viruses. Viruses that utilize both coreceptors are known as dual/mixed tropic. It is important to note that the coreceptor used during infection determines the cellular outcomes of the infected host cells (Nascimento-Brito *et al.*, 2015).

2.7.1 CXCR4 and CCR5 are HIV co-receptors

HIV-1 enters host cells by binding initially to the CD4 receptor on the surface of CD4+ T lymphocytes, and this induces a change in conformation of the glycoprotein (gp)-120, allowing a secondary interaction with one of seven transmembrane G-protein-coupled receptor (GPCR) superfamily (Chen, 2019; James, 2019).

CXCR4 is an integral membrane protein belonging to the G-protein coupled receptors family of receptors and its signalling is induced by the binding of its natural ligand, stromal-derived-factor-1 (SDF-1), leading to physiological effects such as stem cell homing of the bone marrow and lymphocyte chemotaxis (Wilkinson *et al.*, 2013).

CCR5, a chemokine, is a co-receptor that HIV uses to infect CD4+ T lymphocytes. CCR5-tropic HIV-1 is more commonly found in the beginning of infection and has been described to be the most efficiently transmitted variant, responsible for the maintenance and progression of the HIV-1 epidemic.

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Several years after the discovery of the HIV-1 coreceptor, several CCR5 antagonists have been developed and tested as antiretroviral agents in clinical trials. They are available for use in adults and are under evaluation in children (Agwu *et al.*, 2016). One of the most successful; maraviroc (MVC) was approved by the U.S Food and Drug Administration in 2007 for the treatment of HIV infection in individuals with R5-tropic HIV, demonstrating safety and high efficacy (Wilkin *et al.*, 2012). Additionally, gene therapy has been exploited in coreceptor-based therapeutic strategies, with a genetic approach to depleting coreceptors from the surface of the target cell.

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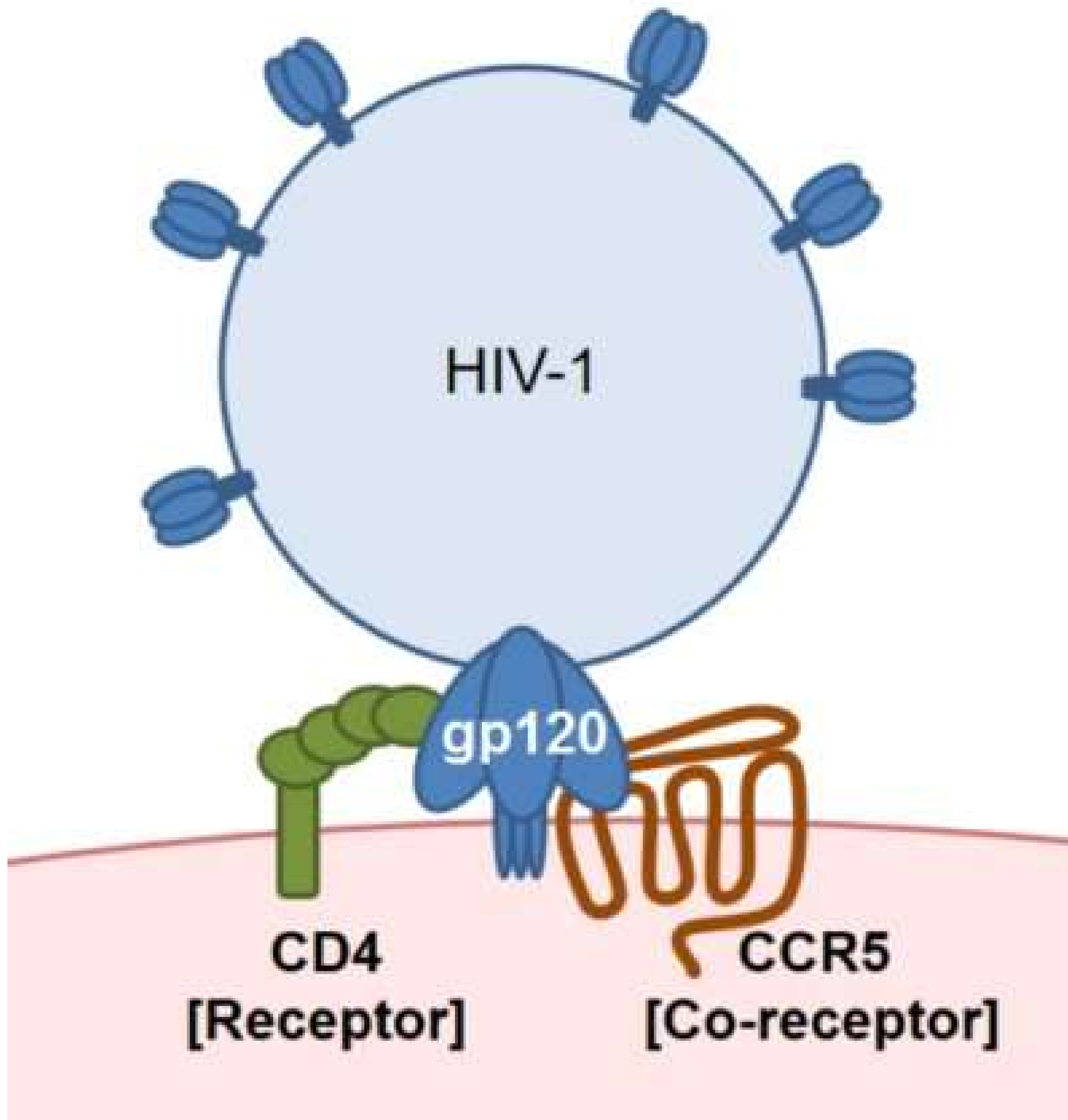

Figure 3: A cartoon diagram showing the interaction between HIV-1 and the co-receptors of a CD4 positive T lymphocyte.

(Chen, 2019)

2.8 HIV Tropism and ART

HIV tropism is the prediction of selected chemokines (co-receptors) that are used by HIV in attachment and infection of target host cells. The co-receptors (CXCR4 and CCR5) put a molecular face on the complex phenomenon of HIV-1 disease. As described in the earlier chapters of this report have been novel alternative targets in anti-retroviral therapy (Lin *et al.*, 2009). The principal clinical role of tropism testing is to exclude the presence of detectable

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dual/mixed or X4 viruses in patients who are being considered for treatment with CCR5 antagonists (Lin *et al.*, 2009; Wilkin *et al.*, 2012).

HIV tropism largely affects the time of initiation and the choice of regimen of ART. The aggressive nature of X4-tropic viruses has been shown in studies resulting in the early initiation of ART by patients harbouring X4-tropic viruses, compared to their colleagues who at the time of diagnosis harboured R5-tropic viruses (Waters *et al.*, 2008).

Research has shown that X4-tropic viruses are more aggressive and occur at the later stages of infection. The devastating effect of X4-tropic viruses have been extended to their presence in dual/mixed tropic isolates (Agwu *et al.*, 2016; Waters *et al.*, 2008). Waters et al. showed this observation, explaining that the presence of dual/mixed tropic HIV is associated with accelerated immunological and clinical progression to disease (Waters *et al.*, 2008). In their study, HIV patients who harboured X4-tropic or dual/mixed -tropic HIV were in the minority population. However, they had a significantly decreased CD4 cell count over 12 months and more clinical events including Atypical *Mycobacterium tuberculosis* infection, Lymphoma, Cryptococcal disease, Kaposi sarcoma, Oesophageal *Candida* infection, *Pneumocystis carinii* pneumonia among others. Despite this observation, both classes of patients had a similar virologic response to ART over a two year period regardless of baseline tropism (Waters *et al.*, 2008).

The appearance of HIV-1 X4 complicate infections and has been shown to be strongly associated with disease progression with further reported cases of drug resistance. Drug resistance is more linked to X4 than to R5 HIV-1 (Wilkinson *et al.*, 2013). It is however unclear whether the impact of tropism in ART-naïve HIV individuals on decrease in CD4 cell counts differs from the observation of dual/mixed or X4-tropic virus during coreceptor antagonist therapy (Waters *et al.*, 2008).

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In determining tropism, several methods have been adopted both for clinical and research methods. The different methods have individual advantages including cost, turn-over time, precision and efforts. However, for clinical trials, phenotypic tests using HIV-1 adapted cell lines remain the optimum choice (Lin *et al.*, 2009). The relevance of HIV-1 tropism and co-receptor usage have resulted in the development of cell lines that express the important cell membrane receptors. The cell lines are able to support replication of primary HIV-1 isolates, infectious lab-adapted clones and pseudotyped HIV-1.

2.8.1 Phenotypic Tropism

Phenotypic-based tropism studies utilize cells lines that are able to support HIV-1 replication. Most of these cells have been engineered to express any or all of the receptors (CD4 receptor, CCR5 co-receptor and CXCR4 co-receptor) that facilitate HIV attachment and entry (Lin *et al.*, 2009). HIV replication in these cells can then be measured by p24 antigen production or by expression of a reporter gene. Typical phenotypic assays use human glioma cell lines such as U87, U373 or NP2 cells. All of these cells stably express CD4 and either of the co-receptors (Lin *et al.*, 2009).

Cell lines

TZM-bl cell lines are derived from a HeLa cell clone that were modified to express the CD4 and HIV co-receptors CCR5 and CXCR4 and contain integrated reporter genes for firefly luciferase under the control of an HIV-1 long terminal repeat. These attributes allow sensitive and precise measurements of infection of nearly all strains of HIV and SIV, including primary isolates and or molecularly-cloned Env-pseudotyped viruses (Xing *et al.*, 2016).

Tzm-bl cell lines have been widely used in HIV infectivity assays due to their abundant expression of the HIV receptors and co-receptors (Luo *et al.*, 2019; Xing *et al.*, 2016).

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2.8.2 Genotypic Tropism

Genotypic approach to studying HIV tropism is the use of gp120 V3 sequence reads amplified from primary HIV isolates and interpreted using bioinformatic algorithms. Genotypic approaches to viral tropism are based on the principle that the V3 loop is the major determinant in the selection of coreceptor during HIV attachment and entry (Lin *et al.*, 2009). The bioinformatic algorithms also known as geno2pheno, that are adopted in genotypic testing are based on known genotype and phenotype pairs and have been shown to perform well when compared to phenotypic set-ups (Lin *et al.*, 2009).

The genotypic approach to study HIV has been used by several studies, with majority of the studies suggesting that X4-tropic viruses averagely prevail at rates between 19% and 33% (Wilkin *et al.*, 2012) . Despite the widespread use of this approach, a number of limitations have been raised, including no phenotypic confirmation, an inability to detect minority HIV populations and determinants of X4 tropism residing outside of the V3 region (Agwu *et al.*, 2016). However, this approach is beneficial in many instances where it is cost efficient, shorter turn-around time and much standardized than phenotypic tests (Lin *et al.*, 2009).

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CHAPTER THREE

3.0 Methods

3.1 Study Design

The study was purposive and cross-sectional. Sixty-nine (69) ART-naïve HIV-1 sero-positive individuals were enrolled for the study, following willingness to participate and formal consent.

3.2 Study site selection and participant enrolment

Participants for the study were enrolled at the HIV care centres at the Korle-Bu Teaching Hospital, the Greater Accra Regional Hospital, and the La General Hospital, all in the Greater Accra Region of Ghana. These hospitals are well-structured healthcare Centres that provide antiretroviral therapy and care for people living with HIV. The Korle-Bu Teaching hospital also serves as a referral centre for patients across the country and even overseas. The protocol for this study was approved by the Institutional Review Boards of the Noguchi Memorial Institute for Medical Research (NMIMR) and the Ghana Health Service (GHS).

3.3 Demographic and clinical data

Following successful consenting, baseline socio-demographic and clinical data were accessed from clinical records and oral interviews, and recorded with a well-structured questionnaire (Appendix 1). Information that was collected included age, gender, date of HIV-1 diagnosis, risk behaviours, history of any sexually transmitted diseases (STIs), and any diagnosed co-infections, among others. It is important to note that, some participants refused to disclose some of the above information and that was allowed, as part of ethical considerations.

3.4 Specimen collection and processing

About 6 ml of venous blood was collected by a certified Medical Laboratory Scientist into an appropriately labelled, sterile EDTA tube. The tubes were inverted for about 6 to 10 times and immediately transported in cold chain to the Virology Department of Noguchi Memorial

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Institute for Medical Research for further analyses. An aliquot of 50 µl of whole blood was taken aside for CD4+ T cell count estimation as described in section 3.4.1.

The remaining whole blood was centrifuged at 2500 rpm for 10 minutes to separate plasma. Two aliquots of 1.5 ml and 3 ml of plasma were taken into separate cryovials and stored at -80°C for viral load quantification and cell culture assays respectively. Peripheral Blood Mononuclear Cells (PBMCs) were then prepared from the remaining whole blood as described below.

Whole blood was transferred into a 15 ml falcon tube and diluted in equal volume of Phosphate Buffered Solution (PBS) prepared from dissolution of PBS tablets (Invitrogen) as specified by the manufacturer, in sterile distilled water.

The diluted blood was layered on 5 ml of lymphoprep medium from Stem Cell Technologies in a new 15ml falcon tube and centrifuged at 2500 rpm for 15 mins. After centrifugation, the buffy coat containing the PBMCs was carefully pipetted into a new 15 ml falcon tube containing 5 ml PBS. The PBMCs were centrifuged at 2000 rpm for 10 minutes and the washing step repeated. After the final wash, the PBMCs were stored in Bambanker freezing medium in cryovials and kept at -80 C.

3.4.1 CD4+ T cell measurement

CD4+ T lymphocytes count were measured from whole blood, using a fluorescence activated cell sorter (FACS)Count assay set-up from Becton Dickinson (BD) and Company Biosciences, San Jose, CA 95131 USA. In this assay, reagents (BD FACSCount™ CD4/CD3 Reagent LOT 70672121) and controls (BD FACSCount Controls LOT 69101721) were provided by the manufacturer. The reagent tubes are pre-filled with CD4/CD3 conjugated monoclonal antibodies and beads in buffer. Assay controls are run weekly following a similar protocol described here. The reagent tubes were labelled with control IDs or sample IDs respectively

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and vortexed for 6 seconds. The tubes were there de-sealed using a Coring workstation. Using a calibrated pipette, 50 µl of whole blood was pipetted into the pre-filled reagent and control-labelled tubes. The tubes were then vortexed for 6 seconds and then incubated at room temperature for 1 hour in a dark environment. After incubation, the control reagent beads were added to the tubes and vortexed, following which a fixative reagent supplied with the reagent tubes are added to both sample and control tubes. The tubes are then vortexed for 6 seconds and then ready to be acquired on a FACSCount platform. The tubes containing the samples are vortexed again for 6 seconds just before acquisition.

3.4.2 HIV viral load quantification

Viral load was determined for all samples using previously stored plasma and the commercial CobasAmplicor HIV-1 Monitor™ Test, from Roche Diagnostics Corporation, Indianapolis, USA. HIV-1 RNA was extracted, from which cDNA is synthesized. The cDNA was then used in qPCR procedure as described by the manufacturer's protocol. The assay was performed at the Immunology department of the Central Laboratory at the Korle-bu Teaching Hospital (KBTH) in Ghana.

3.4.3 HIV Drug Resistant Genotyping

3.4.3.1 HIV RNA purification

HIV-1 RNA was purified from plasma using nucleic acid purification reagent (Quick-RNA Viral Kit-R1034) supplied by Zymo Research. Two hundred microlitres of plasma was aliquoted into a clean, labelled 2 ml micro-centrifuge tube. Two hundred microlitres of 2X concentrate RNA shield was added to the plasma sample in the 2 ml micro-centrifuge tube. 400 µl of Viral RNA buffer was added to the mixture and vortexed for the 10 seconds. The mixture was transferred into a Zymo-Spin IC Column in a Collection Tube and centrifuged. All centrifugations were done at 15,000 g in a high speed refrigerated micro centrifuge (TOMY

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MX-207). Five hundred microlitres of Viral Wash Buffer was added to the column and centrifuged for 30 seconds. The flow-through was discarded and the wash was repeated. 500 µl of absolute ethanol was added to the column and centrifuged for 1 minute. It was ensured that the preceding centrifugation was thoroughly done to completely dry the column by removing any remnants of the Wash Buffer. The column was transferred into a nuclease-free microcentrifuge tube. Fifteen microlitres of nuclease-free water was added directly to the column matrix and centrifuged for 30 seconds at room temperature. The eluted RNA was immediately stored at -20°C temporarily, pending immediate PCR and at -80°C when the PCR was to be done later than 24 hours.

3.4.3.2 cDNA (complementary DNA) synthesis

Complementary DNA was synthesized for all samples using the ProtoScript II First Strand cDNA Synthesis Kit supplied by New England Biolabs Inc. The reagent kit contained individual reagent tubes of a Random Primer Mix [60 µM], ProtoScript II Reaction Mix [2X], ProtoScript II Enzyme Mix [10X] and Nuclease-free water. The reactions were carried out in 200 µl sterile polypropylene PCR tubes, free of DNAse, RNAse and endotoxins.

All components of the kit were thawed on ice and inverted several times to properly mix. The PCR tubes were appropriately labelled with sample identification numbers with a control tube also labelled for a control set-up. Two microlitres of the Random Primer Mix was mixed with 4 µl of RNA sample and topped up with nuclease-free water to make a total volume of 8 µl. The samples were incubated at 5minutes for 65 °C, span briefly to mix components and immediately put on an ice rack.

Ten microlitres of ProtoScript II Reaction Mix [2X] was added to each microfuge tube followed by an addition of 2 µl of ProtoScript Enzyme Mix [10X] to make a total volume of

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20 µl. The reaction was first incubated at 25 °C, and a second incubation at 42 °C for one hour and a further inactivation of the enzyme at 80 °C at 5 minutes to complete the cDNA synthesis. In the control set-up, all components were added as described above without the ProtoScript II Enzyme Mix (10X). The cDNA was stored at -20 °C until ready for use.

3.4.3.3 HIV Protease and Reverse transcriptase amplification

HIV protease and reverse transcriptase were amplified in the samples using a nested Polymerase Chain reaction adopted in the HIV Genotyping Laboratory at the Noguchi Memorial Institute for Medical Research. The detailed protocol is described in the sections below.

3.4.3.3.1 Primers

HIV-1 protease and reverse transcriptase coding sequences were amplified using the following primer pairs, in a two-round nested PCR. The tables below identify the primer names, the primer sequence and the primer targets used in the PCR.

Table 5: Primer names and respective targets, that were used in the amplification HIV-1 Protease

HIV-1 PROTEASE		
Primer Name	**Primer Sequence (5' to 3')**	**Primer description**
DRPRO5 (2074-2095)	AGACAGGTTAATTTTTTAGGGA	PR OUTER SENSE
DRPRO2L (2716-2691)	TATGGATTTTCAGGCCCAATTTTTGA	PR OUTER ANTISENSE
DRPRO1M (2148-2167)	AGAGCCAACAGCCCCACCAG	
DRPRO6 (2611-2592)	ACTTTTGGGCCATCCATTCC	PR INNER ANTISENSE

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Table 6: Name and sequences of primers that were used to amplify HIV-1 reverse transcriptase

HIV-1 REVERSE TRANSCRIPTASE		
Primer Name	**Primer Sequence (5' to 3')**	**Primer description**
DRRT1L (2388-2410)	ATGATAGGGGGAATTGGAGGTTT	RT OUTER SENSE
DRRT4L (3425-3402)	TACTTCTGTTAGTGCTTTGGTTCC	RT OUTER ANTISENSE
DRRT7L (2485-2509)	GACCTACACCTGTCAACATAATTGG	RT INNER SENSE
DRRT6L (3372-3348)	TAATCCCTGCATAAATCTGACTTGC	RT INNER ANTISENSE

3.4.3.4 HIV-1 Protease and Reverse Transcriptase PCR Amplification

The amplification of HIV protease and reverse transcriptase genes was done using the Phusion Master Mix supplied by New England Biolabs. The individual reaction mixture was constituted in the proportions described below. The amplifications were done using a SimpliAmp Thermal Cycler, Applied Biosystems, Thermo Fischer Scientific.

Round 1 PCR

Table 7 shows the master mix composition used in the round one amplification of HIV-1 protease and reverse transcriptase

Reagents	Working concentration	Volume (µl/ reaction)
Fusion master mix	1X	12.5
Forward primer	1X	1.25
Reverse primer	1X	1.25
Nuclease-free water		5
Template cDNA		5
Total		**25 µl**

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Round 2 PCR

Table 8: Master mix composition for the second nest amplification of HIV-1 protease and reverse transcriptase

Reagents	Working concentration	Volume (µl/ reaction)
Fusion master mix	1X	12.5
Forward primer	1X	1.25
Reverse primer	1X	1.25
Nuclease-free water		5
Round 1 product		5 µl
Total		**25 µl**

Cycling conditions

In both reactions, the cycling conditions used are described in the table shown below.

Table 9: Cycling conditions used for the amplification of HIV-1 protease and reverse transcriptase

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	98	30 seconds	1
Denaturation	98	10 seconds	30
Annealing	58	30 seconds	
Extension	72	90 seconds	
Final extension	72	10 minutes	1
Hold	4	∞	

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3.4.3.5 Gel Electrophoresis

The PCR products were resolved on a 1.5% agarose gel in electrophoresis. In the preparation of the gel, 1.5 g of agarose powder was dissolved in 100ml of 1X TAE buffer and heated for about 3 minutes. 5 µl of E-Z vision dye was added per 100 µl of gel prepared. The gel was allowed to cool and cast in a gel tray. Five microlitre amplicons were diluted in 2 µl of Trackit Orange loading dye prior to loading into the wells. A 100bp TrackIt molecular ladder (marker), a positive control and negative control were loaded in the first three lanes respectively. A constant 100 V electric current was applied for 30 minutes after which the gel was visualized under ultra-violet (UV) light, in a UV imager (UVP BioDoc-It^2 Imager, Analytikjena).

3.4.3.6 Sequencing

The amplicons from the second round were sequenced using Sanger Sequencing methods. The primer sequences and methods used in generating the sequences were adopted from (Fujisaki *et al.*, 2007; Villahermosa *et al.*, 2000), and are shown in table 10 below.

Table 10: Primer names and sequences that were used in sequencing HIV-1 protease and reverse transcriptase.

Gene	Primer name	Position on HXB2	Sequence (5'-3')
Protease	Fw PRTS	2157-2177	AGCCCCACCAGAAGAGAGCTT
	Fw P3G	2198-2217	CAACTCCCTCTCAGAAGCAG
	DRPRO6	2611-2592	ACTTTTGGGCCATCCATTCC
Reverse	Fw A2	2583-2601	TTAAAGCCAGGAATGGATG
Transcriptase	Fw RT SEC 1S	2692-2716	CAAAAATTGGGCCTGAAAATCCATA
	Rv PR SEC 2A	2692-2716	TGGGAAGTTCAATTAGGAATACCACATC
	DRRT6L	3372-3348	TAATCCCTGCATAAATCTGACTTGC

Fw; forward, Rv: reverse, HXB2: HIV-1 reference strain

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The primers used in sequencing the regions were designed such that they overlap at any given position of the amplicon. This was done to allow for a quality check for every sequence read.

The raw sequences were then assembled in Seqman Pro software version 13 to form a contig that was manually edited by removing gaps and correcting mistakes in base calling. A consensus sequence was saved and aligned with the reference HIV-1 HXB2 strain sequence, in BioEdit version 7 software to ascertain the quality of the sequence editing and the strain diversity.

To identify HIV-1 drug resistant mutants, the consensus sequences were analysed in the Stanford University HIV Drug Resistance Database. The database reports on the subtype of the sequence submitted, the drug mutations present and the score of susceptibility of the respective class of anti-retroviral drugs. The subtypes recorded from the Stanford HIV database was confirmed using COMET, an HIV subtyping tool developed by the Luxembourg Institute of Health. For every mutation identified, the Stanford HIV-database and previously published literature were used to interpret observations. Statistical computations for each data set were applied, where necessary, in Graphpad Prism version 8.

3.5 Phenotypic tropism

In describing the coreceptor phenotypes of the circulating viruses, a modified form of the Trofile test was adopted. The Phenotypic Trofile is a common bench assay that utilizes specific HIV-1 cell lines that stably express CD4 and the co-receptors (CXCR4 and CCR5), to determine HIV-1 infection (Lin *et al.*, 2009). Patient samples were then incubated with the cells to ascertain entry and infection.

The experiment was controlled with established lab strains of X4 and R5 tropic viruses, HIV P50 and P125 respectively. HIV P50 and P125 plasmids were acquired from a donor lab from

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Washington University in St. Louis and verified using the Tzm-bl assay described in subsequent section. The plasmids were then transfected into mammalian 293T cells to produce the P50 and P125 lentiviruses.

3.5.2 Transfection

Transfection experiments were carried out to produce lentiviruses from the earlier produced plasmids. The plasmids used in this experiment were HIV-P125, HIV-P50 and HIV-NL4-3 plasmids. The plasmids, were expanded in the procedure, described in section 3.5.1

293T cells, which are an adherent group of mammalian cells and easily transfectable were used in this assay. The cells were previously maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 5 % each of L-glutamine, penicillin-streptomycin antibiotic, sodium pyruvate and 10 % fetal bovine serum (FBS) with a constant supply of 5% CO_2 in a humidified CO_2 chamber (Hirayama). The cells were passaged at confluence after microscopy and just before seeding.

About 800,000 to 1,000,000 live, healthy cells were counted using an automated cell counter (Countess Invitrogen). The cells were seeded into 6 well treated cell culture plates, in 2 ml of OPTIMEM media, supplemented with 10 % fetal bovine serum (FBS), 5% L-glutamine, 5%penicillin-streptomycin and 5% sodium pyruvate. The cells were incubated in a humidified chamber with a constant supply of 5% CO2. The cells were observed after 24 hours for attachment and morphology (as shown in figure 4). Following successful attachment and at about 70-90% confluence, the spent media was replaced with 1 ml of fresh complete OPTIMEM. Lipofectamine 3000 reagent (Invitrogen, ThermoFischer Scientific) was used in transfection. It is a proprietary formulation for transfecting nucleic acids into a wide range of

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eukaryotic cells. It contained two individual components of Lipofectamine 3000 (A) and lipofectamine P3000 (B)-a lipid reagent to which the nucleic acid is complexed.

For each well of a 6-well plate, the transfection reagent was set up as follows. 3.75 µl of Reagent A was diluted in 125 µl of OPTIMEM media in a sterile, labelled microcentrifuge tube. 10 µl of Reagent B was complexed with 7 µg of the plasmid and further diluted in 250 µl of complete OPTIMEM media. The diluted reagents were then put together as a master-mix (DNA-lipid complex) in an equal ratio and totalling a volume of 250 µl. The master mix was incubated at room temperature for 15 minutes and added to the cells. After 6-hours, the packaging medium was removed and replaced with 2 ml of OPTIMEM media. The cells were returned to humidified CO2 incubation at 37 °C for 48 hours, during which the media containing viable viruses were harvested at every 24-hour interval. The harvested virus was filtered through a 0.22-micron filter and stored at -80 °C until use.

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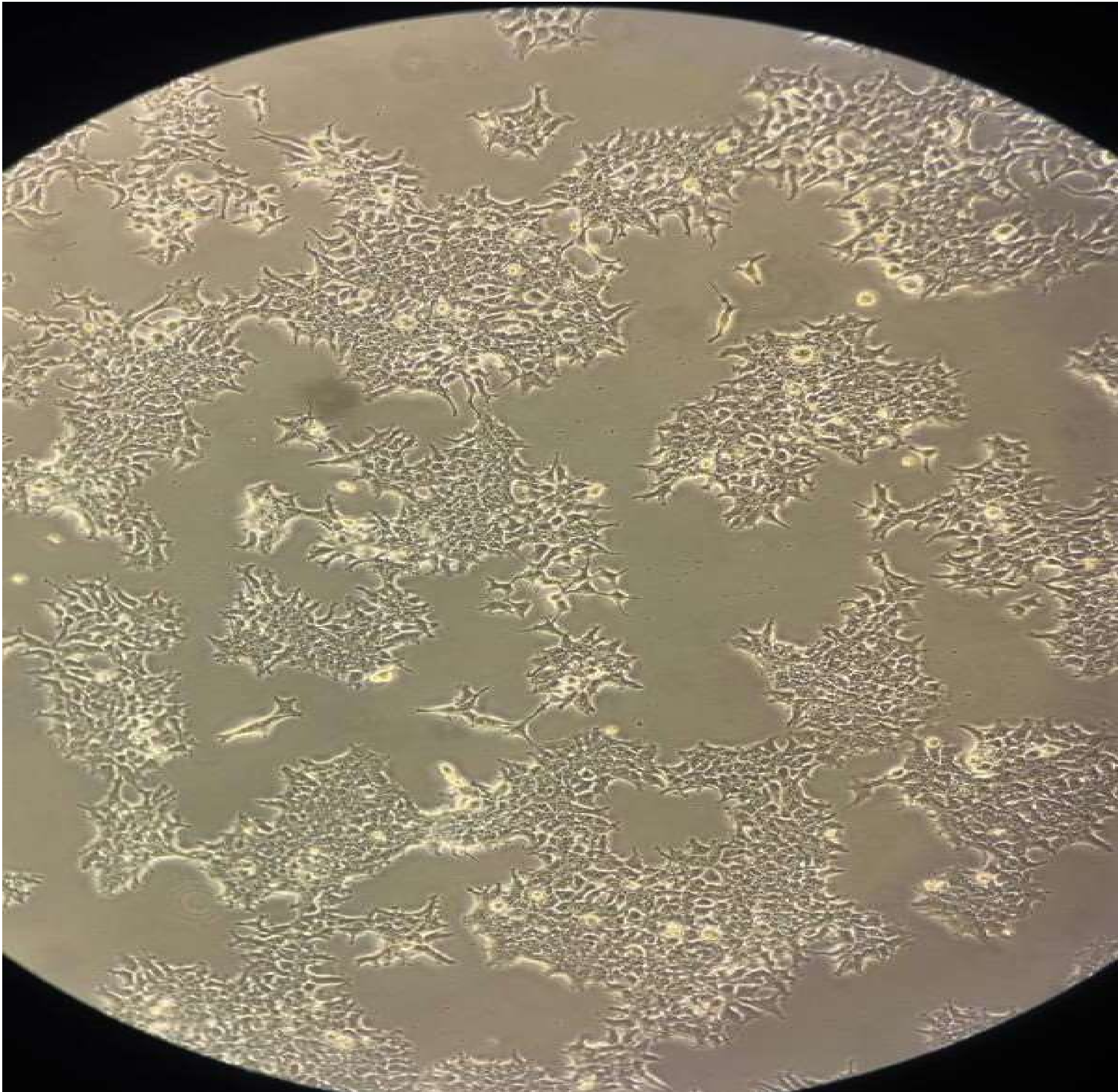

Figure 4: A field image of attached 293T cells using an inverted microscope at a magnification of x100

3.5.3 Infection assay

Tzm-bl cell lines were used for the infection assays. The assay procedure was modified from methods adopted from previously published work (Wilkinson *et al.*, 2013; Xing *et al.*, 2016).

In the infection experiment, 100,000 to 150,000 freshly trypsinized Tzm-bl cells in 1 ml complete DMEM were plated overnight in 48-well tissue culture plates (NUNC). The cells

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were at passage 10 or earlier and live cells count for each experiment was greater than 80%. After 24 hours, the cells were observed for successful attachment and the spent media was replaced with fresh media. Patient virus isolates in plasma were used in the infection experiments. It was determined after several optimizations, that 40 µl of patient plasma showed sufficient infection and replication in the absence of any entry antagonists.

The attached Tzm-bl cells were incubated with 40 µl of patient plasma at 37.0 °C and 5% humidified CO_2 in a CO_2 chamber for 6 hours. After the period, the media was topped up to 1 ml and the cells were incubated for 48 hours during which the media was replaced after 24 hours. Two distinct controls were set-up on every plate. The first set of controls included non-infected cells. These non-infected cells were used to measure the background of luciferase in the Tzm-bl cells, comparable to the infected cells. At the end of the 48-hour incubation period, the cells were observed under an inverted microscope. The spent media was replaced and the attached cells were washed gently with pre-warmed Phosphate Buffered Saline (PBS). The cells were then lysed with 200 µl of a 2 % of Triton-X in PBS and the plate was gently swirled on a rocking platform for 10 minutes, to allow complete lysing. The cells were observed under an inverted microscope to ensure that lysis was complete.

One hundred microlitres of the lysed cells were aliquoted into an opaque 96-well cell culture plate and the relative luciferase unit was determined after addition of equal volume of luciferin.

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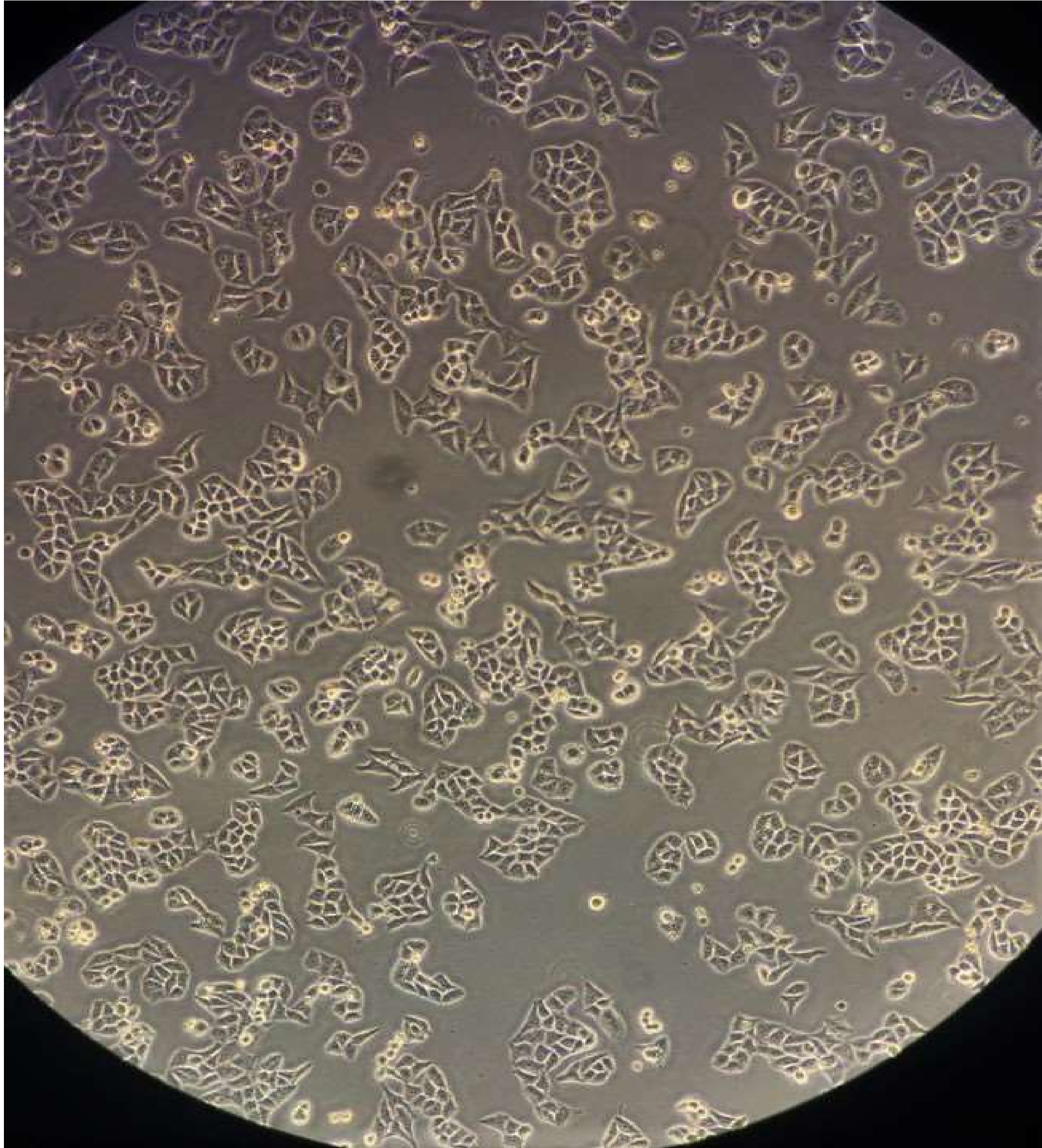

Figure 5: A field image of attached Tzm-bl cells in culture, captured from an inverted microscope at a magnification of X100

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3.5.4 Infection inhibition assay

Following a first successful infection, infection with the sample was repeated to determine co-receptor usage.

Maraviroc and AMD-3100 (plerixafor), which are known antagonists to HIV-1 entry were used in the drug inhibition assay, at concentration of 5 µg/ml. The effective drug concentration was determined in an inhibitor challenge test using previously described methods. (Wilkinson *et al.*, 2013) (Xing *et al.*, 2016).

Virus-infected cells without inhibitors were used as the infection control set-up in the drug assay. Each plate set-up included non-infected cells, infected cells without inhibitors and infected cells with each inhibitor in separate wells. The assay for each patient isolate, for each parameter was conducted in duplicates and a representation of the set-up is shown in figure 6 below.

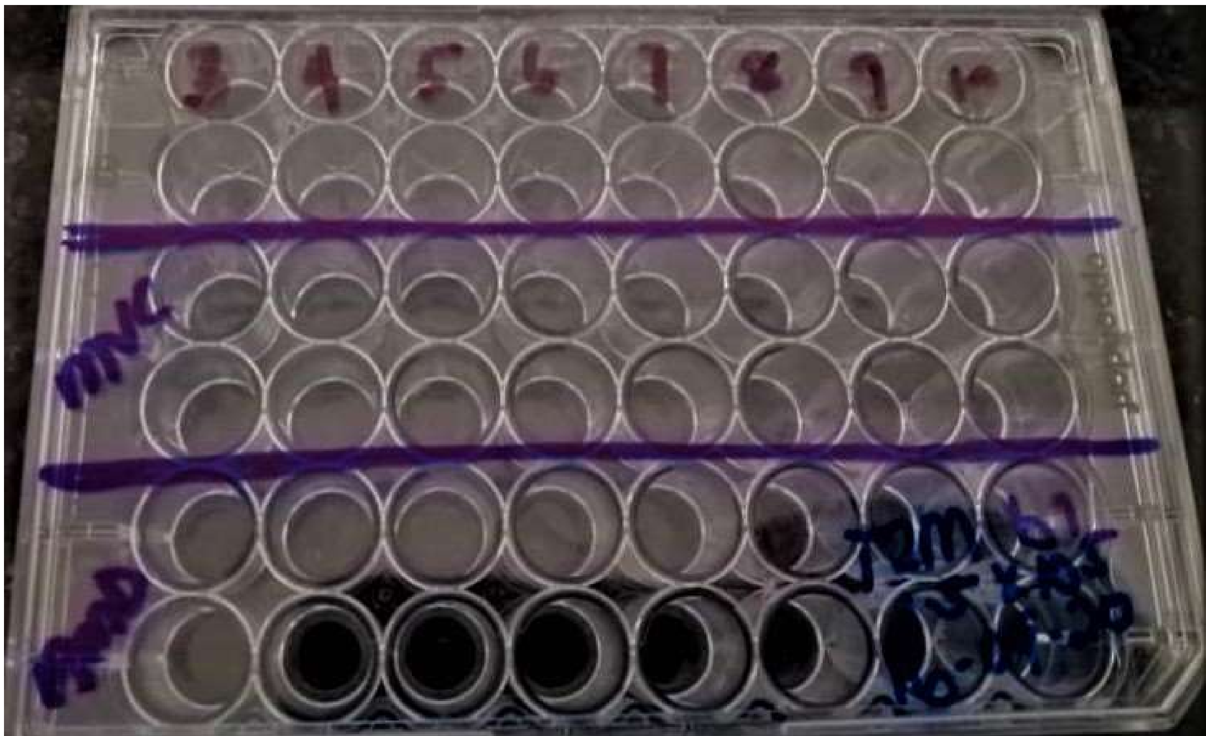

Figure 6: A 48-well cell culture plate demonstrating the experimental set up of infection.

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Each column represents the successive infection of cells without drugs, with maraviroc (MVC) and AMD-3100, each in duplicates.

After the 72-hour incubation, the infection response for each patient isolate and control set-up was determined by the measure of luminescence, in the luciferase assay, described in section 3.5.5 below.

3.5.5 Luciferase assay

Luciferase activity, measured as relative luminescence unit (RLU) was performed on the Tzm-bl infected cells as a quantitative determination of HIV-1 activity. TZM-bl cells possess luciferase and B-galactosidase reporter genes, which are activated by HIV-1 tat protein. The luciferase assay was performed with the Bright-Glo luciferase assay reagent, supplied by Promega, USA according to the manufacturer’s protocol. 50 µl of luciferin reagent was added to each well containing the lysed cells, including the uninfected cells. The plate was kept in a dark environment for 5 minutes, prior to acquiring luminescence on Promega plate count reader.

3.6 Statistical analyses

Data collected were entered in Ms-Excel version 2019 and analysed using GraphPad Prism Version 8 Statistical packages. Descriptive data were obtained for demographic data and clinical data for all participants. Drug resistant mutations were identified as described previously.

Column analyses and ANOVA were performed to determine the difference in infection response, measured as a function of luciferase in infected cells relative to uninfected cells. Differences in column means were further ascertained using the Sidak’s Multiple comparison tests and Brown-Forsythe tests. A p-value of < 0.05 was considered statistically significant.

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CHAPTER FOUR

4.0 RESULTS

4.1 Patient Demographics

Sixty-nine patients were enrolled at all the study sites into the study, following consent and willingness. Of the number, 19 of them were males while forty-seven of them were females, representing 27.53 % and 68.12 % respectively. Three participants (4.35 %) did not indicate their gender. The age of participants ranged from 8 years to 66 at the time of enrolment and the mean age was 38.

Eight participants representing 11.60 % of the study population did not indicate their ages. Four participants representing 5.80 % of the population were under 20 years old at the time of sampling. Twenty- four participants, representing 34.78 % of the population were between the ages of 21 and 40. Majority if the population were above 40 years of age, forming the highest 47. 83%

For all participants, the date of first diagnosis of infection was sought. Thirty-two participants (46.34 %) had been diagnosed in less than two years. 4 participants representing (5.79 %) of the population had been first diagnosed between two to five years and the last group which consisted 33 participants (47.83 %) had been diagnosed after five years following first diagnosis of infection. All the participants were treatment naïve at the time of sampling.

Some participants disclosed information on their likely exposure to infection. Five participants disclosed that they had homosexual orientation while thirty participants disclosed that they might have been infected from heterosexual contact. Twenty-two participants could not identify their likely exposure to infection and eight participants were unwilling to disclose how they might have acquired the infection. The demographics of the patients have been summarised in table 11 below.

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Table 11: Demographic characteristics of the participant

Variables	N (%)
Age (Range 8 – 66) mean = 38 years	
<20	4 (5.80)
21-40	24 (34.78)
>40	33 (47.83)
Unidentified	8 (11.60)
Total	**69 (100)**
Sex	
Male	19 (27.53)
Female	47 (68.12)
Unidentified	3(4.35)
Total	**69 (100)**
Date of diagnosis	
< 2years	32 (46.34)
2-5 years	4 (5.79)
>5 years	33 (47.83)
Total	**69 (100)**
Transmission	
Homosexual contact	5 (7.25)
Heterosexual contact	30 (43.48)
Other	4 (5.80)
Unidentified	22 (31.88)
Unwilling to disclose	8 (11.59)
Total	**69 (100)**

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4.2 Patients Clinical Characteristics

Immunological response for CD4 positive T lymphocyte counts and viral load were measured as clinical characteristics for all participants. We examined CD4 counts even though the WHO policy for ART initiation excludes CD4 counts. We obtained CD4 tests using methods described in 3.4.1 above, and categorised the responses into three main groups.

Twenty-two participants representing 31.88 % of the population, each had CD4 counts of less than 200 cells /mm3 respectively. Seventeen participants, representing 24.64 % of the study population had CD4 counts between 200 and 500 cells/mm^3. CD4 counts were undetermined for eight participants due to reagent scarcity at the time of sampling.

We determined the plasma levels of HIV-1 for all participants. The results were measured as copies of viral nucleic acid per millilitre of plasma.

Eight participants had viral load of less than 500 copies while five participants comprising a minority 7.25 % had viremia between 500 and 999 copies. Majority (24.64 %) of the participants had viral load between 1000 and 99000 HIV-1 copies per millilitre of plasma. 16 participants forming a second majority of 23.19 % had viremia greater than 100,000 copies. Samples for 4 participants showed no target definitions, indicating that the viral copies may be less than the threshold of 20 copies/ µl or unidentified HIV-1 RNA.

The summary of the clinical characteristics of the study participants is showed in Table 12 below.

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Table 12: Clinical characteristics (Immunological and Viral load) of participants.

Clinical variables	N (%)
Viral load copies/ µl	
< 500	8 (11.59)
500 – 999	5 (7.25)
1000 – 99,000	17 (24.64)
>100,000	16 (23.19)
TND	4 (5.80)
***	19
Total	**69 (100)**
CD4 Counts (cells/mm^3)	
< 200	22 (31.88)
200 – 499	17 (24.64)
> 500	22 (31.88)
Unidentified	8 (11.59)
Total	**69 (100)**

4.3 Amplification of HIV-1 protease and reverse transcriptase

HIV-1 protease was successfully amplified from 43 patient DNA. The amplifications had a band size of 463 base pair when resolved on 1.5 % agarose gel. A representative gel image is shown in figure 7 below.

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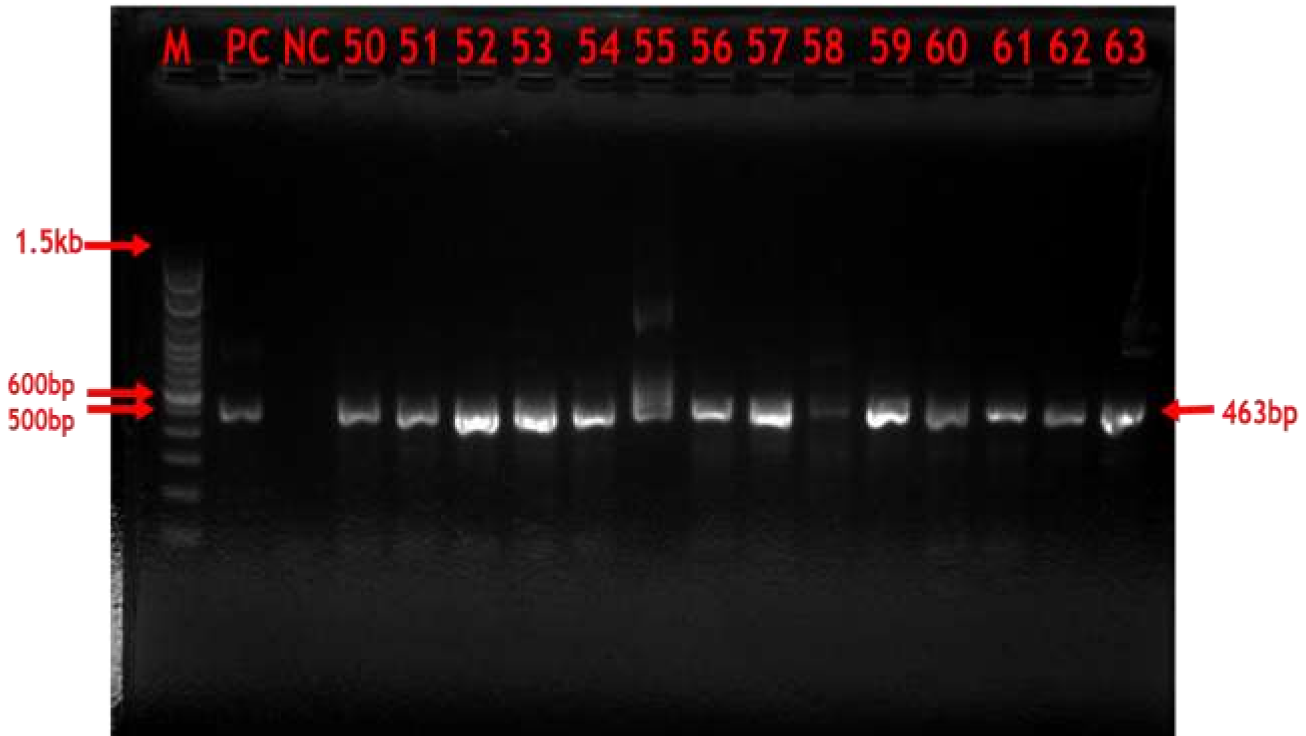

Figure 7: A representative gel image showing amplification of HIV-1 protease at 463 bp

M= Molecular marker, PC= Positive control, NC= Negative control, 50-63= selected sample ID

HIV-1 reverse transcriptase was successfully amplified from 37 patient samples. The remaining samples could not be amplified, which could be as a result of low viremia. The amplification showed PCR products of 887 bp when resolved on 1.5 % agarose gel. A representative of the gel electrophoresis is shown in figure 8 below.

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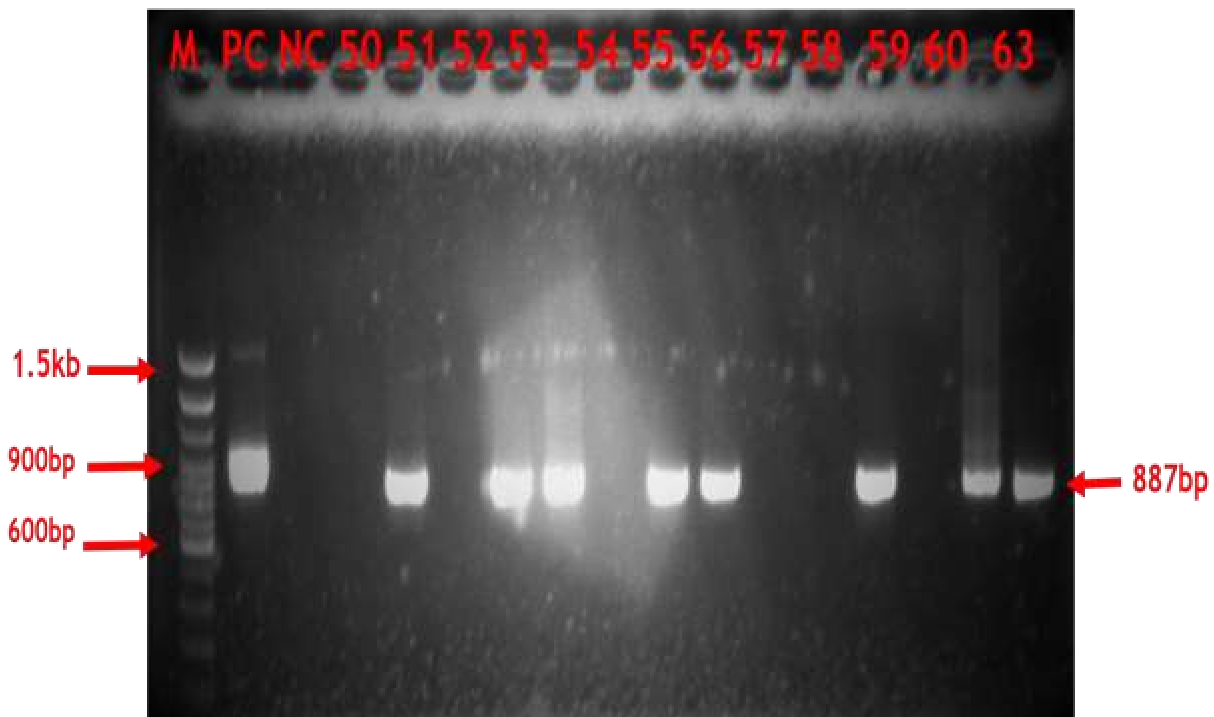

Figure 8: A representative gel image of the amplification of HIV-1 reverse transcriptase at 887 bp

M= Molecular marker, PC= Positive control, NC= Negative control, 50-63= selected sample ID

4.3 Circulating Sub-types of HIV-1

The circulating subtypes was determined following submission of edited sequences to the Stanford HIV database and COMET HIV sub-typing tool. Sixty-four percent of the circulating subtypes were CRF02_AG strains. Sub-types B and G followed 26% and 4% respectively. The minority represented populations were sub-types A, C and CRF06_cpx, accounting for 2% each, for samples used in this study. The summary of the distribution is presented in the pie chart below.

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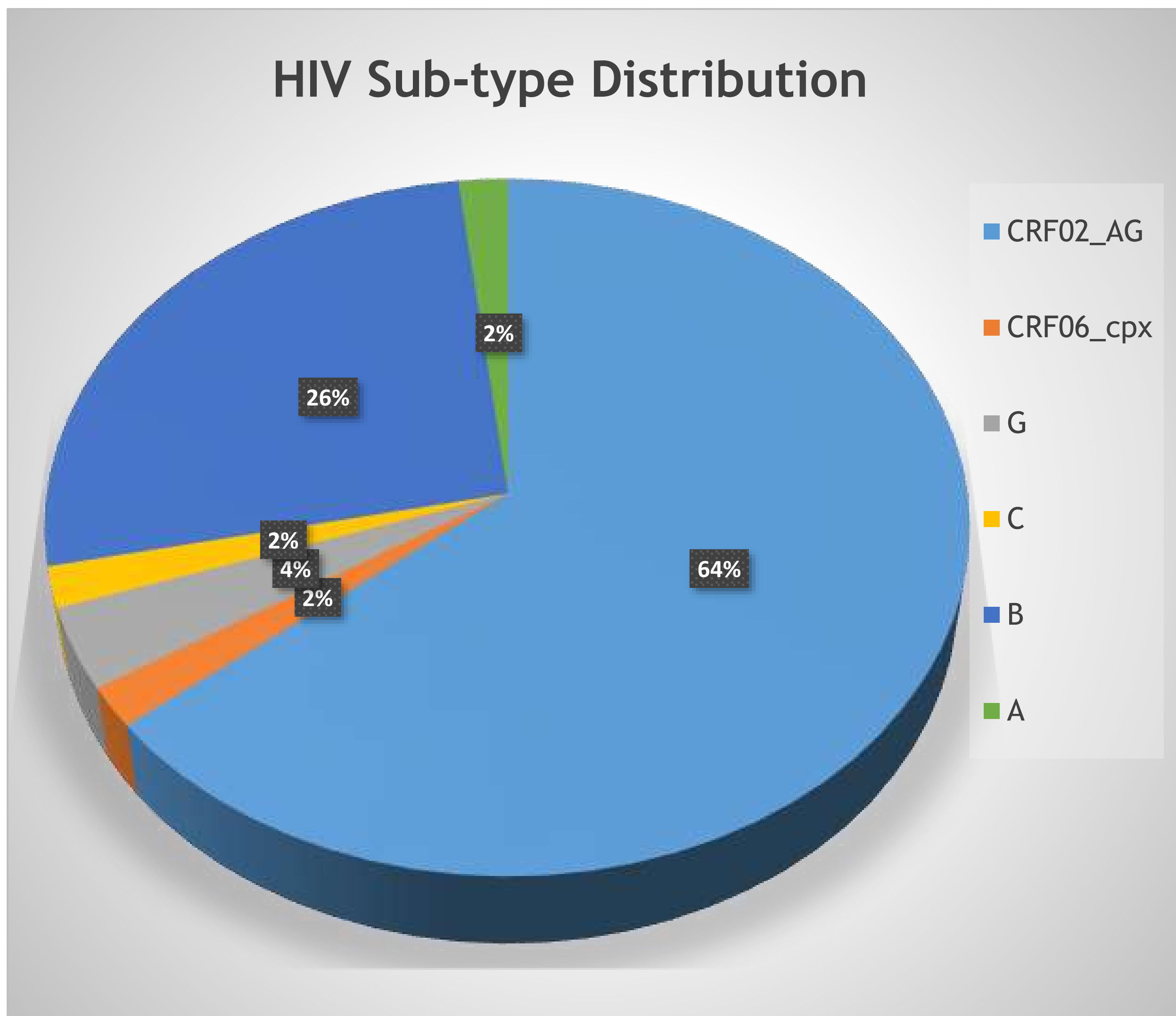

Figure 9: A pie chart showing the distribution of circulating HIV-1 subtypes

4.4. Identified HIV-1 Drug Resistance

43 sequences and 37 sequences were successfully obtained for protease and reverse transcriptase amplicons respectively. After extensive sequence editing, several HIV-1 drug resistance mutations were found. However, the reports and discussions will be limited to seven drug resistant mutations that are on the WHO drug resistance surveillance list. There were no NRTI mutations observed in this study. All mutations observed were either NNRTIs and PIs and have been presented in table 13 below.

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Table 13: Table showing HIV-1 DR mutations observed in the study.

NRTI	NNRTI Mutations (N)		PI Mutations (N)	
	K103N (1)	*NVP/EFV*	V32L (1)	*(ATV/LPV/DRV) *r*
	V179I (1)	*ETR/RPV*	V11L (1)	*DRV*r*
	A98G (1)	*DOR/EFV/ETR/NVP/RPV*	L10LF (1)	*(ATV/LPV/DRV) *r*
	E138A (2)	*ETR/RPV*		

NVP- Nevirapine, ETR- Etravirine, RPV- Rilpivirine, ATV- Atazanavir, LPV- Lopinavir, DRV- Darunavir, r- Ritonavir. The numbers in parenthesis indicate the number of events.

The four NNRTI drug mutations observed in this study are K103N, V179I, A98G and E138A. K103N is a major NNRTI drug resistance. It is a non-polymorphic mutation that causes high-level reductions in susceptibility to nevirapine and efavirenz. K103N was found in one individual, who carried a CRF02_AG subtype. E138A, also a non-polymorphic mutation was observed in two different individuals. Both individuals carried CRF02_AG sub-types and this mutation reduces the drug susceptibility of etravirine in many individuals.

V179I is a minor NNRTI mutation that is selected in patients receiving etravirine and rilpivirine. It was found in one individual in this study, who carried a sub-type A strain of the virus.

The PI drug mutations that were found are V32L, V11I and L10F. V32L is a non-polymorphic PI-selected mutation which is associated with reduced susceptibility to PI drugs. V11I and L10F are non-polymorphic accessory mutations, which reduce susceptibility to ritonavir-

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boosted darunavir, when present with other mutations. They however do not confer any resistance on their own. The three PI mutations observed occurred in three different individuals.

An important PI drug mutation K20I was observed in 34 participants. K20I is a very common occurring mutation that reduces susceptibility to selected ARVs in sub-type B. However, it is a consensus sequence in CRF02_AG sub-type and therefore does not confer any drug resistance. None of the sub-types B observed in this study possessed the K20I drug mutation.

Nearly all the sub-types found in circulation in this study was represented in the incidence of drug resistance, with CRF06_cpx being the only exception. The summary of the occurrence of the drug resistance mutations and the sub-types is presented in the figure 10 below.

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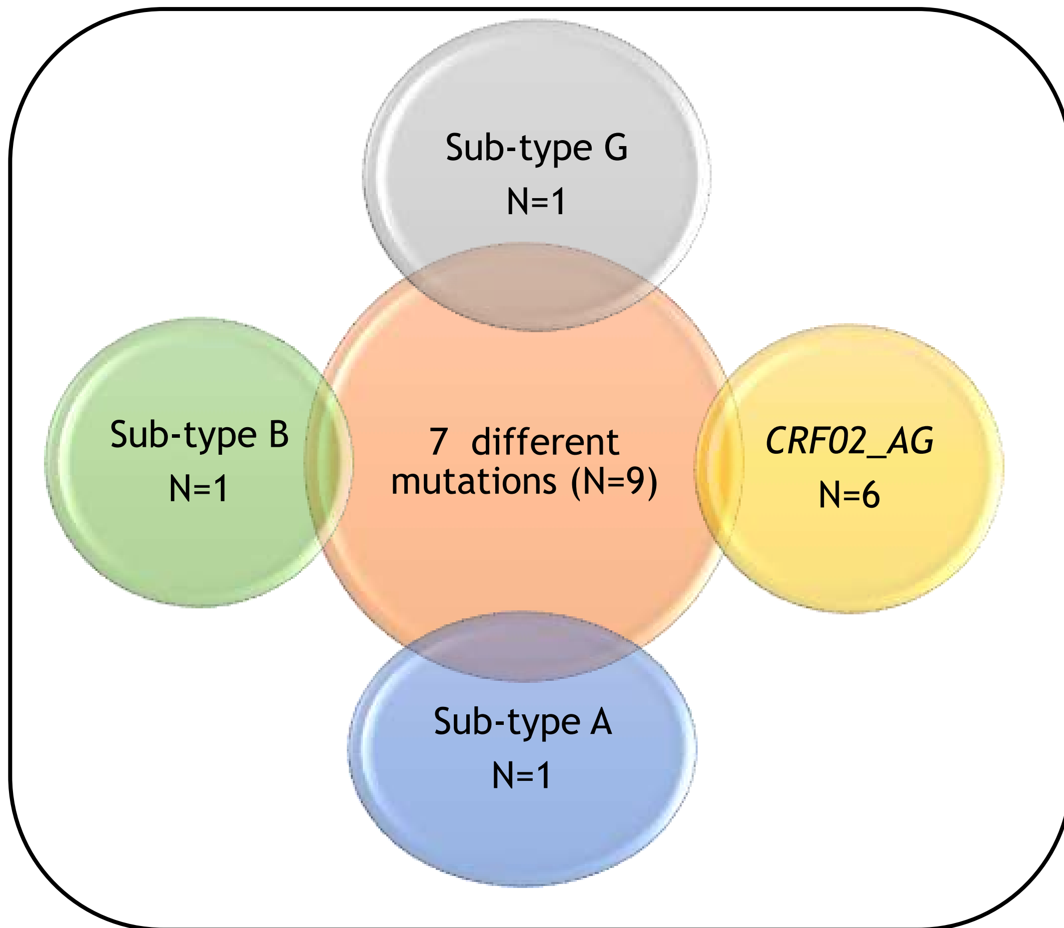

Figure 10 shows the contributions of the circulating subtypes to the observed drug mutations

N is the number of mutations.

4.5 HIV co-receptor phenotypes

4.5.1 Infection response of NL4-3 HIV and patient isolates

In determining the co-receptor phenotypes, NL4-3 HIV, which is an HIV-X4 laboratory strain was used as control virus. The infectivity of the virus is shown in figure 10 subsequently, showing an increased response in luciferase response relative to uninfected cells. However, HIV p-125 which is an HIV-CCR5 laboratory strain did not show any promise after several attempts and optimizations. It was determined that, only the stock of the harvested virus

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achieved effective infection of Tzm-bl cells. Dilutions of the stock virus showed little or no luciferase activity.

In determining the infection of Tzm-bl cells with patient isolates, optimizations were done using virus in PBMCs and plasma from the same patient. Patients isolates in PBMCs and plasma showed different infection responses, with plasma isolates showing a higher luciferase activity, compared to uninfected cells. Plasma samples diluted did not show any positive response when excessively diluted with media. There was significant difference in infection using the laboratory strains produced and patient plasma virus, relative to the uninfected cells. The statistical response is shown in tables 14 and 15 below. The summary of the infection response is shown in figure 11 below.

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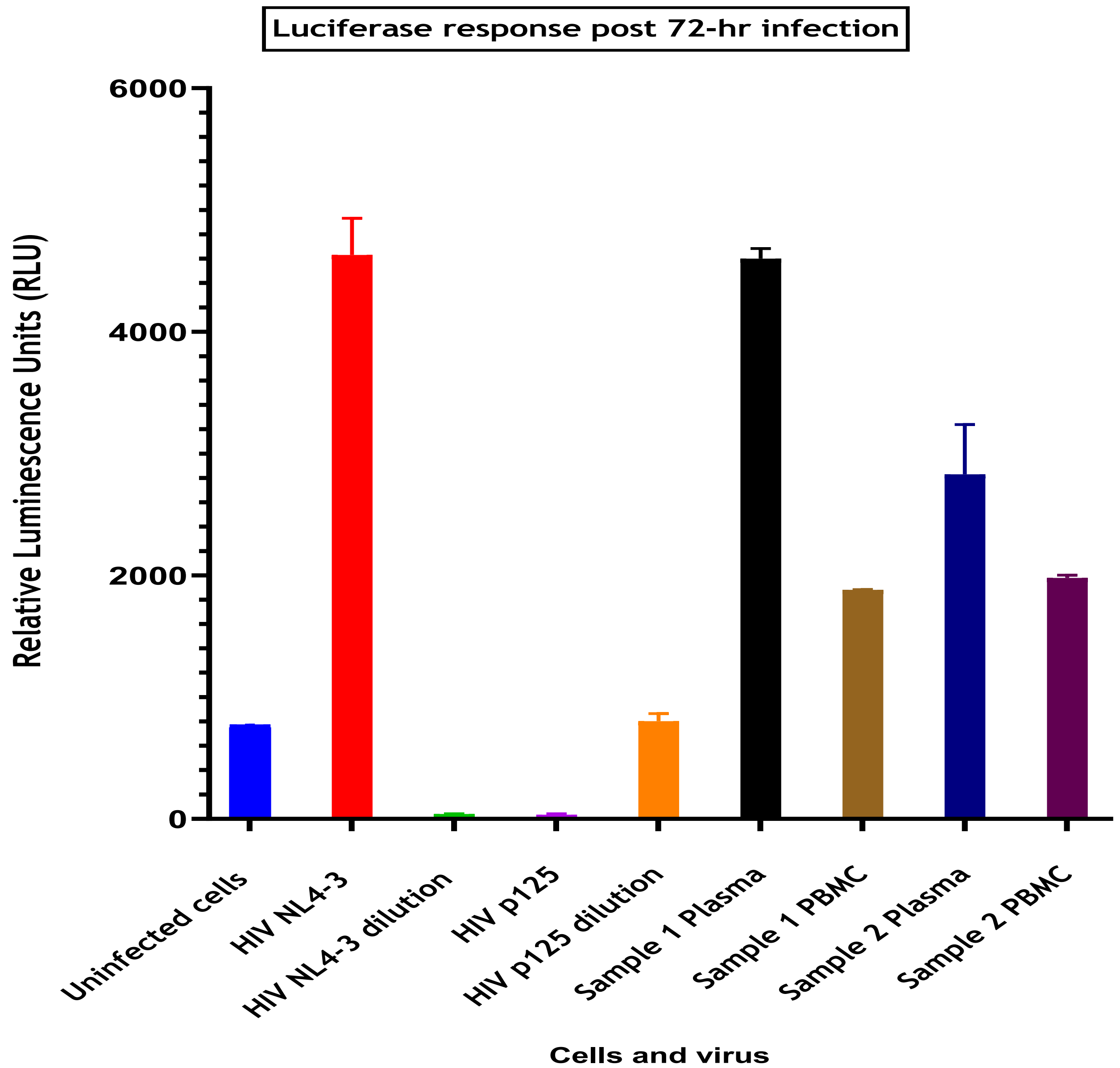

Figure 11: A bar graph showing infection response with isolates from patient plasma and PBMCs, relatively compared to a lab-produced CXCR4 HIV.

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Table 14: ANOVA table showing difference in means of infection using laboratory produced virus from patient isolates

ANOVA					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	50012616	8	6251577	F (8, 9) = 210.4	P<0.0001
Residual (within columns)	267387	9	29710		
Total	50280003	17			

SS is sum of Squares, DF is degree of freedom, MS is Mean of SS and P is the statistical value at alpha

Table 15: Post-hoc test showing the extent of individual mean infection responses between the groups

Brown-Forsythe post-hoc test	
F (DFn, DFd)	3.669e+029 (8, 9)
P value	< 0.0001
P value summary	****

4.5.2 Inhibitory concentration of HIV-1 co-receptor antagonists

AMD-3100 was initially tested and the inhibitory concentration was determined in an infection experiment. Due to the unsuccessful production of HIV p-125 a HIV-1 CCR5-tropic virus, maraviroc, a commercial HIV-1 CCR5 antagonist was not used in the experiment. The minimum inhibitory concentration was only determined for AMD-3100 against HIV NL4-3.

One, two, five and ten micromolar concentrations of AMD-3100 was used in optimizing the inhibition assay. It was observed that 10 µM effectively inhibited HIV NL4-3 replication in

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Tzm-bl cells. There was no difference when the concentration of drug was reduced to 5 µM and further down to 2 µM, suggesting a minimum inhibitory concentration of 2 µM against patient isolates and NL4-3. The results are shown in figure 12 below. Also, the comparison of mean, of the different drug concentrations following statistical analyses are shown in tables 16 and 17 subsequently.

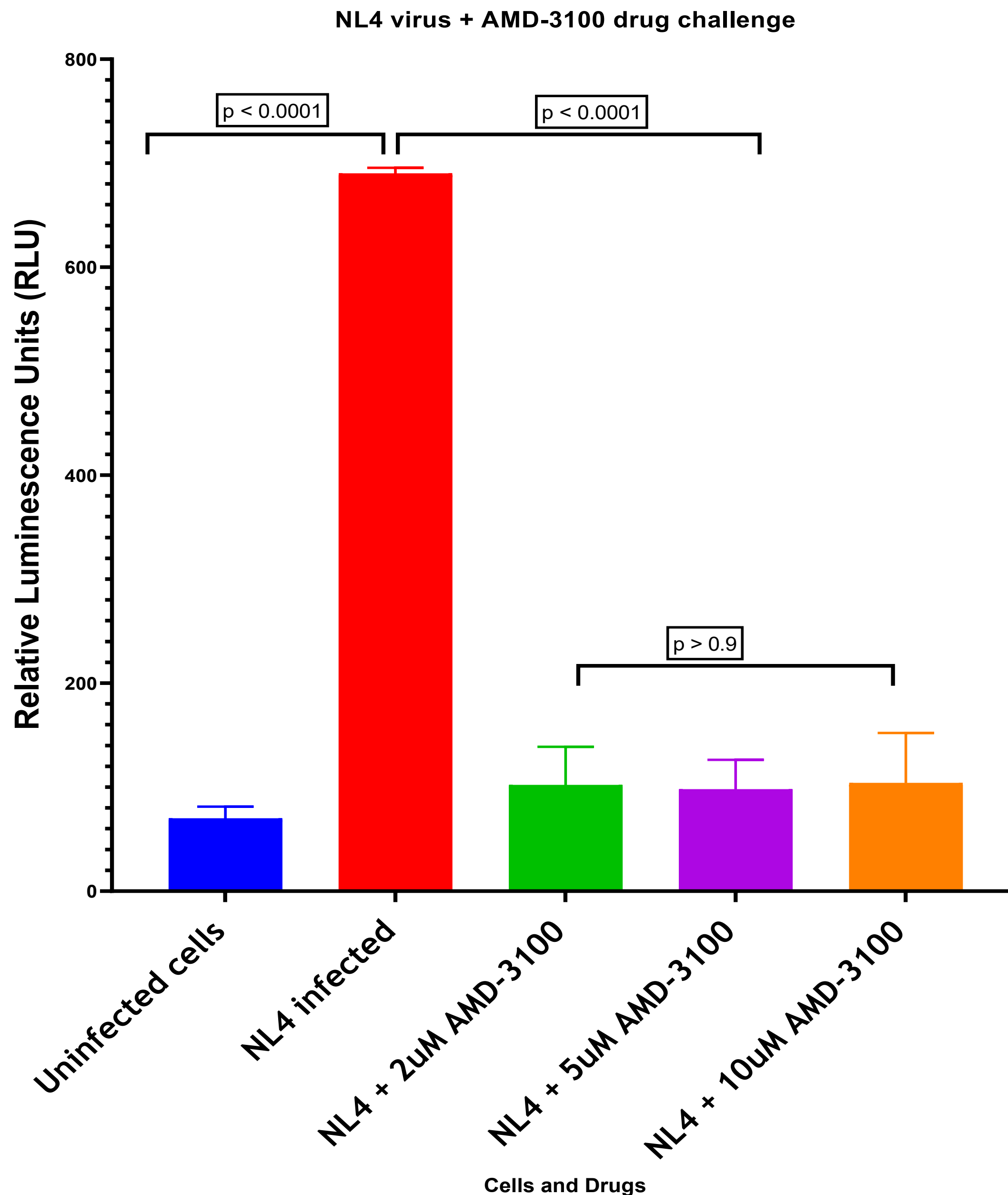

Figure 12: A bar graph showing the effective drug concentrations that sufficiently reduces infection with lab-produced CXCR4-HIV

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Table 16: ANOVA showing the difference in the infection responses in drug concentration assay

ANOVA					
	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	570854	4	142714	F (4, 5) = 154.3	P<0.0001
Residual (within columns)	4624	5	924.8		
Total	575479	9			

SS is Sum of Squares, DF is Degree of Freedom, MS is Mean Squares, P value is the statistical value of the data at alpha of 0.05

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Table 17: Multiple comparism of individual mean responses of three drug concentrations in infection inhibition

Sidak's multiple comparisons test				
	Mean Diff.	**95.00% CI of diff.**	**Significance**	**P Value**
Uninfected cells vs. NL4 infected	-620.0	-760.8 to -479.2	****	<0.0001
Uninfected cells vs. NL4 + 2uM AMD-3100	-32.00	-172.8 to 108.8	ns	0.9765
Uninfected cells vs. NL4 + 5uM AMD-3100	-28.00	-168.8 to 112.8	ns	0.9898
Uninfected cells vs. NL4 + 10uM AMD-3100	-34.00	-174.8 to 106.8	ns	0.9665
NL4 infected vs. NL4 + 5uM AMD-3100	592.0	451.2 to 732.8	****	<0.0001
NL4 infected vs. NL4 + 2uM AMD-3100	588.0	447.2 to 728.8	****	<0.0001
NL4 infected vs. NL4 + 10uM AMD-3100	586.0	445.2 to 726.8	****	<0.0001
NL4 + 2uM AMD-3100 vs. NL4 + 5uM AMD-3100	4.000	-136.8 to 144.8	ns	>0.9999
NL4 + 2uM AMD-3100 vs. NL4 + 10uM AMD-3100	-2.000	-142.8 to 138.8	ns	>0.9999

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CHAPTER FIVE

5.0 DISCUSSION OF RESULTS

5.1 Demographic responses of participants.

Generally, HIV-1 infection is reported in more females than males (Boerma *et al.*, 2016; Deeks *et al.*, 2015; Hamers *et al.*, 2011; Nichols *et al.*, 2018), an observation which has been reported in nearly all HIV-1 endemic populations including previous reports from Ghana. In our report, more females were recorded than males. The large number of females in this study had nothing to do with consenting, participation and admittance at HIV care clinics. Generally, more females reported to the HIV care clinics than males. It is therefore consistent with previous findings that the female HIV population in Ghana is greater than the male population in Ghana. While many reasons may explain this phenomenon, the most frequently attributed reason is the predisposition of women genitalia to acquiring sexually transmitted diseases, more often than males do (Deeks *et al.*, 2015; Melo *et al.*, 2019).

Participants in this study were mainly adults, representing ages above 18 years. Majority of the individuals aged between 20 years and 55 years with a mean age of 38 years, which is indicative of their maximum exposure to the risk of transmission of HIV, including but not limited to sexual contact. Thirty participants openly provided information that they got infected through sexual contact and it is consistent with many reports that suggest that HIV is mainly transmitted through sexual contact, accounting for more than 90% of all new infections (Melo *et al.*, 2019).

This study also solicited for information regarding the date of known diagnosis of HIV infection. The information was necessary in order to observe the contribution of the period of infection to drug mutational patterns and phenotypic co-receptor types. All the participants who carried drug mutations have had infection between two years and 10 years. None of the individuals who had less than two years of known diagnosis of HIV carried any of the seven

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relevant mutations found in this study. Several reasons account for the evolution of drug resistance and chiefly among them is random viral replication leading to the evolution of new clones as has been discussed in earlier paragraphs of this report. It is therefore consistent with many reports that drug resistance is low in ART-naïve populations.

5.2 Clinical characteristics of participants

HIV-1 primarily infects $CD4^+$ T lymphocytes, which double as markers of immune activation. Untreated HIV infection depletes $CD4^+$ T lymphocytes, leaving the immune system compromised and susceptible to many opportunistic infections (Deeks *et al.*, 2015; Fromentin *et al.*, 2016). Generally, as $CD4^+$ T cell population decreases, viremia increases and vice versa but the report is different in sub-HIV populations known as elite controllers and Long Term Non Progressors (LTNPs). Patients in this study had fairly considerable $CD4^+$ T cell counts, with an average of 409 cells/mm^3 of whole blood. This observation is noteworthy because majority of the patients have had infection for more than two years without treatment, by which time a significant reduction in $CD4^+$ T population is expected as seen in classical HIV-1 pathogenesis. Also, all of the individuals that were enrolled showed no symptoms of disease, demonstrating little or no progression to disease.

Also, we considered the viremia of all patients. There was a weak correlation (Spearman's r = 0.4) between viremia and $CD4^+$ T populations. While viremia was considerably high (mean = 137,694 copies of virus per ul of plasma), $CD4^+$ T was fairly good. Many factors could explain the reason for this observation. Firstly, patients sampled in this study demonstrate to be LTNPs and elite controllers. This inference is much appreciated when individual $CD4^+$ T counts are matched with viral load.

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Also, disease progression has been strongly linked with HIV-1 subtypes. HIV-1 subtype B has been shown to be more virulent and accounts for the majority of the most severe cases of morbidity (Beloukas *et al.*, 2016). The majority of the participants in this study carried non-B clades of HIV-1. Even though it was observed that the HIV-1 B clade population is gradually increasing in the Ghanaian HIV population, the dominance of the non-B clades could be the reason for the delayed progression to disease of participants that were enrolled in this study.

While the delayed progression to disease of participants holds a good outlook for the HIV situation in Ghana, it is important to continue to monitor the situation, especially in the growing evidence of the sub-type B HIV-1 in Ghana.

5.3 Circulating phenotypes of HIV-1 and Transmitted Drug Resistant Mutations

This report found CRF02_AG as the predominant circulating subtype of HIV-1, followed by sub-type B with minority populations of sub-types A, C, G and CRF06_cpx. Even though majority of the HIV population live in sub-Saharan Africa, sub-type B is not the dominant clade in the sub-Saharan region (Deeks *et al.*, 2015; Ndembi *et al.*, 2008). HIV-1 circulating sub-types reports from other African countries including Ghana show that newly infected individuals in Ghana carry clades other than B (Barth *et al.*, 2010; Beloukas *et al.*, 2016; Deletsu *et al.*, 2020).

HIV sub-type B has accounted for majority of newly diagnosed patients in Europe, scoring as high as 70% -80 % of newly diagnosed cases in Europe and in the U.S (Beloukas *et al.*, 2016; Dana S. Clutter *et al.*, 2016). The sub-type B clade is known to be responsible for the Western epidemic, as well as many parts of Asia and has been used to establish most drug resistance analyses (Tzou *et al.*, 2020; Ueda *et al.*, 2019). Even though drug resistance profiles between B and non-B sub-types may be different, the established reports of drug resistance in B sub-

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types are applicable to non-B subtypes due to resistance-related positions that are common to many of the HIV-1 strains (Barth *et al.*, 2010; Tzou *et al.*, 2020). Studies have shown that the non-B and CRF clades have mainly been associated with immigration, heterosexual transmission and in males (Beloukas *et al.*, 2016).

Drug resistance has been reported in nearly all HIV-1 endemic countries. Drug resistance reports vary by country due to measures that may be in place to reduce such incidences. Drug resistant mutations are described based on their effect on the susceptibility or response to the drugs. They occur at very different rates in different populations and are largely affected by many factors including circulating sub-types, adherence to drugs and random replication-driven mutations (D. S. Clutter *et al.*, 2017; De Luca *et al.*, 2015).

Transmitted drug resistance is HIV-DR that occurs when primary HIV infection is caused by a drug resistant mutant-bearing virus and arises from patients who develop acquired resistance from HIV replication and or from an ART-naïve with TDR (D. S. Clutter *et al.*, 2017).

ART initiation in many resource-limited countries is empirical, usually without genotypic tests. The situation is not very different in Ghana as individuals initiate ART without genotypic tests (Barth *et al.*, 2010). Drug resistant mutations are described based on their effect on the susceptibility or response to the drugs. They occur at very different rates in different populations and are largely affected by many factors including circulating sub-types, adherence to drugs and random replication-driven mutations (D. S. Clutter *et al.*, 2017; De Luca *et al.*, 2015).

Only a couple of studies have looked at pre-treatment drug resistance in Ghana (Bonney *et al.*, 2013; Alexander Martin-Odoom *et al.*, 2017). One of such is the HIV-DR threshold survey. The study enrolled participants actively in 2009, and found only two major drug resistance mutations, M184V and Y181C at a prevalence of 5 %. Even though they found minor reverse

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transcriptase mutations including A98G, K103R, K101Q and E138Q, the authors reported that transmitted drug resistance is very low in Ghana according to the WHO guidelines for characterising transmitted drug resistance. However due to the evolving nature of HIV, they recommended that the study be repeated every two years (Bonney *et al.*, 2013; Alexander Martin-Odoom *et al.*, 2017).

Despite the high incidence of major mutations including M184V an NRTI mutation and K103N, an NNRTI in ART-experienced individuals, they are hardly transmitted prior to initiation of ART. K103 mutation was observed in one individual in this study, which is low and a good promise for the use of NNRTI in managing HIV-1 infection in Ghana. The poor transmission of M184V mutants among ART-naïve individuals could be due to reasons including reduced transmission efficiency and reduced viral fitness (Barth *et al.*, 2010; De Luca *et al.*, 2015).

A98G and E138A are NNRTI mutation which confers very little resistance to the NNRTI ARVs. It is not frequently reported in drug resistance studies, even in ART-experienced HIV individuals (Barth *et al.*, 2010; Tzou *et al.*, 2020).

Reports have shown that HIV-1 sub-types affect drug mutation patterns. Majority of drug resistant mutations occur in certain HIV-1 subtypes and these subtype-specific differences are attributed to differences in codon calls (Dana S. Clutter *et al.*, 2016; De Luca *et al.*, 2015).

Waters et al previously reported that the transmission of CCR5-tropic HIV is mainly through heterosexual contact, with X4-tropic viruses less transmitted. They established that an increase in X4- isolates in ART-naïve individuals advances clinical progression to disease (Waters *et al.*, 2008). Also, INSTIs and entry inhibitors have become a better alternative in anti-retroviral therapy in resource-rich countries, with the decision now being replicated in developing

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countries. They have better barriers to drug resistance in lower-income countries including Ghana where little or no resistance have been reported yet (Tzou *et al.*, 2020).

No major PI associated mutations were found in any individual, which means that isolates potentially have full susceptibility to protease inhibitor ARVs and this is usual of ART-naïve HIV-1 isolates (Ajoge *et al.*, 2012; Bonney *et al.*, 2013; Hamers *et al.*, 2011; Kelentse *et al.*, 2020). A study done in Nigeria which is one of the high-burdened HIV countries in West-Africa and targeted at ART-naïve pregnant women recorded no protease inhibitor associated mutations (Imade *et al.*, 2014). It is therefore obvious that drug mutations in the protease region are rarely transmitted, showing promise because protease inhibitors remain a very crucial component of second-line therapy of ART and any growing threats of drug resistance could potentially derail the gains made.

The mutations that were observed in the protease region in this were V32L, V11L and L10LF, all accessory mutations. These mutations only reduce susceptibility when present with other mutations. Very few studies have typically discussed these mutations (Budambula *et al.*, 2015), due to their scarce incidence. It is recommended that the general observation of their low transmission is worth considering in future research.

The growing population of HIV-1 sub-type B in Ghana leaves much to be desired because sub-type B has accounted for many of the HIV-DR cases in the Europe and America. It will be important to understand the host and pathogen genomics of the B clade in Ghana and the sub-Region as a whole, to protect the current gains of antiretroviral therapy.

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Limitations

The current study had limitations which are very typical of every scientific research. The samples used in this study were collected between 2018 and 2019, which suggests that the response may be different in subsequent years. In order to keep an updated HIV-DR profile of the HIV-1 population in Ghana, it is important to conduct continuous surveillance of DR in the HIV population in Ghana.

Also, the sample size used in this study is very limited and enrolled at 3 major hospitals in Ghana. Even though the hospitals serve as referral hospitals for many people living with HIV, there are other satellite antiretroviral clinics in Ghana. Enrolling more participants at the smaller clinics that provide antiretroviral therapy sites would have given a national outlook of the HIV-DR situation in Ghana.

Another limitation of this study was the difficulty in transforming competent *E. coli* with R5-specific HIV DNA. R5-specific HIV was obtained from a donor lab at the Microbiology and Immunology Department of the Washington University in St. Louis. After several attempts, while optimizing at full considerations, the transformation was unsuccessful. The difficulty in transforming the cells may have been due to degraded nucleic material of the HIV clones that were used.

Another limitation in determining the phenotypic co-receptor was the absence of CD4/CXCR4 and CD4/CCR5 -specific secondary cell lines. Cell lines that stably express the CD4 protein and either of the chemokines would have best facilitated the infection assays. However, the cells were not received timely as a result of border closures due to the COVID-19 pandemic.

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CONCLUSION

This study provides useful findings on the circulating sub-types and the transmitted drug resistance report of HIV-1, in the Ghanaian population. It was observed that CRF02_AG remains the predominant sub-type of HIV-1 in Ghana, with sub-type B following, in increased numbers when compared to reports from previous studies. Sub-types A, C and G were also found in this study.

Seven important clinical mutations, that are on the WHO's Drug Resistance surveillance were found to be present in 9 individuals. These are K103N, E138A, V179I and A98G which are NNRTIs. The remaining are V32L, V11L and L10LF which are accessory protease inhibitor mutations. K103N, which is the most threatening of the seven mutations occurred just once, suggesting a very low prevalence of transmitted drug resistance in Ghana.

This study had short comings in extensively determining tropism in the ART-naïve populations due to time and resource constraints, as a result of the COVID-19 pandemic. Even though lab strains of HIV- specific X4 and R5 were produced and the minimum drug inhibitions determined, determination of patient isolates co-receptor phenotypes was not entirely successful. The preliminary results did not show any obvious pattern of the known HIV co-receptor phenotypes using the established drug inhibition assay. With the limited data obtained, it is inconclusive to make valid claims on the selected patient isolates co-receptor phenotypes.

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RECOMMENDATIONS

Generally, the study is concise in its objectives as well as methods. While great effort and skill was employed to answer all the research questions, a few genuine concerns need to be addressed.

It is important to note that samples used in this research were collected from consenting participants between 2019 and 2019, suggesting that observations may be a little different given that enrolment continues. It is therefore recommended that the study continues for a larger sample participant number to validate the current data and also to serve as a surveillance model for circulating sub-types and transmitted drug resistance of HIV-1 in Ghana.

There is the need to understand the growing adaptation of the HIV-1 B clade in Ghana, using host and pathogen genomics studies. This is important because reported cases of high drug resistance have the sub-type B predominant in their populations.

Finally, the phenotypes of circulating HIV-1 co-receptors need to be studied at length in both ART-naïve and ART-experienced populations in order to have good knowledge on how maraviroc and other new entry inhibitors will fare in the Ghanaian population.

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APPENDICES

Appendix 1: Ethical Approval

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH

Established 1979 A Constituent of the College of Health Sciences
University of Ghana

Phone: +233-302-916438 (Direct)

+233-289-522574

Fax: +233-302-502182/513202

My Reference: DF 22

Dr. Osbourne Quaye
University of Ghana Department of Biochemistry, Celll and Molecular Biology
P.O.Box LG 54
Legon

RE: Our Study # 030/16-17

At: NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH-IRB

Dear Dr. Osbourne Quaye:

Meeting Date: 11/1/2017

At: NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH-IRB

Protocol Title:

Effect of immune checkpoint molecules in progression, persistence and latency establishment of HIV-1 in ART-naïve or exposed patients

This is to advise you that the above referenced Study has been presented to the Institutional Review Board, and the following action taken subject to the conditions and explanation
provided below.

Internal #: 1814
Expiration Date: 10/31/2018
On Agenda For: Renewal
Reason 1: Progress Report **Reason 2:**
Description:
IRB ACTION: Renewed
Condition 1:
Action
Explanation:

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Yours Sincerely,

NMIMR-IRB
IRB Administrator

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Appendix 2: Consent form and Questionnaire adapted for participant enrolment.

NMIMR-IRB CONSENT FORM TEMPLATE

Title: **Effect of immune checkpoint molecules in progression, persistence and latency establishment of HIV-1 in ART-naïve or exposed patients**

Principal Investigators: Dr. Edward K. Maina & Dr. Osbourne Quaye

Address: West African Center for Cell Biology of Infectious Diseases (WACCBIP), P. O. Box LG 581 Legon, Accra

Co-Investigator: Mr. Joshua Kuleape

General Information about Research

Researchers' statement

We would like to tell you about a study being conducted by a Research Team from the West African Center for Cell Biology of Infectious Diseases (WACCBIP) and Noguchi Memorial Institute for Medical Research (NMIMR). We would like to tell you about the study to find out if you would be willing to participate. The purpose of this consent form is to give you the information you will need to help you decide whether to be in the study or not. Please read the form carefully. You may ask questions about the purpose of the research, what we would ask you to do, the possible risks and benefits, your rights as a volunteer, and anything else about the research or this form that is not clear. When we have answered all your questions, and if you qualify based on the screening criteria, you can decide if you want to be in the study or not. This process is called 'informed consent'. We will give you a copy of this form for your records.

Purpose of the Research

This is a research study. We are conducting a study to learn more about the how the human body fights the virus that causes HIV infection and what strategy the virus uses to avoid destruction by the body's immune system. We are asking HIV infected individuals and uninfected controls to enroll and participate in this study. If you agree to participate as a study participant, we will take you for counseling before we ask you for approximately 4 teaspoons (20 ml) of your blood for HIV-1 confirmation and subsequent use of the sample in the proposed study. The aim of the study is to better understand HIV-1 infection in order to improve HIV control and possibly find new treatment for the infection.

Procedure

This is what will happen if you decide to have your blood taken for the purpose of the study. First, you will meet with a counselor. The counselor will give you more information about the risks and benefits of your participation in the study. They will explain the importance of HIV infection. Approximately 20 ml of blood will be taken from a vein with a sterile needle for HIV-1 status confirmation and other study procedures. Your blood will be transported to the laboratory at the West African Center for Cell Biology of Infectious Diseases for the purpose of the proposed study. Your samples may be stored and used in future HIV studies. Specimens in the bank will be stored for five (5) years, until research funding is exhausted or the specimen is no longer usable. Health information about you will be retrieved from the hospital/clinic main health records if you choose to be part of this research study. We will be able to inform your doctor about your results after the test.

Possible Risks and Discomforts

The risks of drawing blood may include temporary discomfort from the needle stick, bruising, bleeding, and, rarely, infection. There also may be other side effects or discomforts that we cannot predict at this time. Trained medical personnel will perform the blood collection procedures for laboratory tests and will make every effort to minimize any discomfort. Using well-trained and experienced medical personnel will lessen these risks.

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Possible Benefits
You may not benefit directly from taking part in this research study. However, the results of this research may guide the future treatment of HIV infected people. Health policy makers may also gain further understanding of HIV-1 infection that might lead to formulation of policies and programs to benefit HIV infected individuals

Confidentiality
Your personal information will be held in the strictest confidence, and no identifying information of any kind will be released to any other person or agency without your specific permission in writing. Your samples collected for research purposes will be labeled with a code number and will be taken to the WACCBIP and NMIMR for processing. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

Compensation
You will be compensated (GHC 50.00) for participating in the study to cater for transportation and other expenses incurred for participating in the study.

Voluntary Participation and Right to Leave the Research
Please understand that taking part in this research is entirely voluntary and that you may refuse to take part or withdraw at any point.

Contacts for Additional Information
If you have any questions about this study you may contact Dr. Edward Maina on 0265154115, if you are still not satisfied and need further explanation you can contact Dr. Osbourne Quaye on 0207915923 or Mr. Joshua Kuleape on 0540820905.

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

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VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title **"Effect of immune checkpoint molecules in progression, persistence and latency establishment of HIV-1 in ART-naïve or exposed patients"** has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

______________________ ______________________________

Date Name and signature or mark of volunteer

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

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

______________________ ______________________________

Date Name and signature of witness

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

______________________ ______________________________

Date Name Signature of Person Who Obtained Consent

3

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Data Abstraction Form

Effect of immune checkpoint molecules in progression, persistence and latency establishment of HIV-1 in ART-naïve or exposed patients

Dr. Osbourne Quaye & Dr. Edward K. Maina
West African Center for Cell Biology of Infectious Pathogens,
University of Ghana, Legon

- Study Site____
- Sample and patient ID__________________
- Date of Collection_______________

1. Demographic Information

Diagnostic status; HIV infection _______________ AIDS _______________

Age; _______________

Date of known +HIV status; _____/_____/_________

Age at Diagnosis_______________

Gender; Male _______________ Female_______________

Level of Education; _______________ Employment status; _______________

Income; _______________

2. Risk Behavior

A) Men Sex with Men

B) Injecting Drug Use

C) Hemophilia or coagulation disorder

D) Heterosexual contact

E) Mother with HIV infection (prenatal transmission)

F) Receipt of blood transfusion, blood component or tissue

G) Other risk not reported or risk unidentified

3. Laboratory Data

Most recent CD4 Count _________cells//ul ______% Date______/_____/______

Previous CD4 count

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CD4 count ________________cells//ul ______% Date _____/_____/_____

CD4 count ________________cells//ul ______% Date _____/_____/_____

CD4 count ________________cells//ul ______% Date _____/_____/_____

CD4 count ________________cells//ul ______% Date _____/_____/_____

Lowest CD4 _________________ Date _____/_____/_____

4. **HIV Detection Tests (Quantitative viral load)**

Most recent VL; Copies/mL: _________Log: __________Collection Date: __/__/__

Previous VL;

Test 1: HIV-1 RNA/DNA NAAT (Quantitative viral load)

Result: Detectable _________________Undetectable __________

Copies/mL: _____________Log: ______________ Collection Date: __ __ /__ __ /__ __

Test 2: HIV-1 RNA/DNA NAAT (Quantitative viral load)

Result: Detectable_________________ Undetectable __________

Copies/mL: _____________ Log: ______________ Collection Date: __ __ /__ __ /__ __

Test 3: HIV-1 RNA/DNA NAAT (Quantitative viral load)

Result: Detectable_________________ Undetectable __________

Copies/mL: ____________ Log: ______________ Collection Date: __ __ /__ __ /__ __

Lowest VL ______________ Date _____/_____/_________

5. **ART History**

Date started ART_____/______/______

CD4 count at start of ART; CD4 count ______________cells//ul ______%

Viral Load at start of ART; Copies/mL: _____________Log: ___________

ARV regimen currently on____________________ Duration_________________

1st Line ____________________________ Duration_____________

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Specify ARVs_______________________ Duration_______________

2nd Line _______________________ Duration_______________

Specify ARVs_______________________ Duration_______________

Switch from 1st to 2nd Line ARV regimen date_____/_____/_____

Treatment history:

ARV_______________________ Dates (from- to) _______________

ARV_______________________ Dates (from- to) _______________

ARV combination

- o HAART
- o SALVAGE
- o UNKNOWN
- o OTHER (mono or dual)

History of herbal medicine while on ART? Yes_______ No_______

How long_______________________________________

History of herbal Medicine without ARV? Yes_______ No_______

How long_______________________________________

Resistance

Resistance Test Done; Yes_______No_______ Date_____/_____/_____

Resistance Test Type; ○ Genotype ○ Phenotype ○ Virtual Phenotype

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Stage of disease:
1. Asymptomatic
2. Symptomatic
3. AIDS

WHO Clinical stage

WHO Stage I   WHO Stage II   WHO Stage III   WHO Stage IV

Malaria

Positive   Negative

Antimalarial drugs

1.
2.
3.
4

6. AIDS Indicator Diseases

AIDS Indicator Diseases	Initial Dx Date	Presumptive	Definitive
1. ________________	_____/_____/_____	○	○
2. ________________	_____/_____/_____	○	○
3. ________________	_____/_____/_____	○	○
4. ________________	_____/_____/_____	○	○
5. ________________	_____/_____/_____	○	○

7. Co-infections

Hepatitis B

Yes ________________ No ________________ Unknown ________________

Acute________________________ Chronic________________________

Diagnosis date_____/_____/_____

NMIMR INSTITUTIONAL REVIEW BOARD
VALID UNTIL
0 4 SEP 2019
APPROVED DOCUMENT

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Hepatitis C

Yes ____________________No ________________Unknown ________________

Acute___________________________Chronic__________________________

Diagnosis date______/______/______

HPV

Yes ____________________No ________________Unknown ________________

Acute___________________________Chronic__________________________

Diagnosis date______/______/______

Sexually transmitted disease

Yes _________________No ________________Unknown ________________

If yes, Specify___________________________

Acute___________________________Chronic___________________________

Diagnosis date______/______/______

TB

Date of last tuberculin skin test:

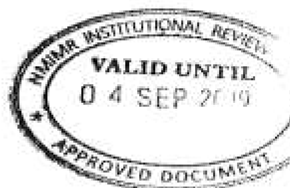
Positive: ____________Negative ____________Not done________________

Diagnosis date______/______/______

Others

Specify___________________________

Diagnosis date______/______/______



8

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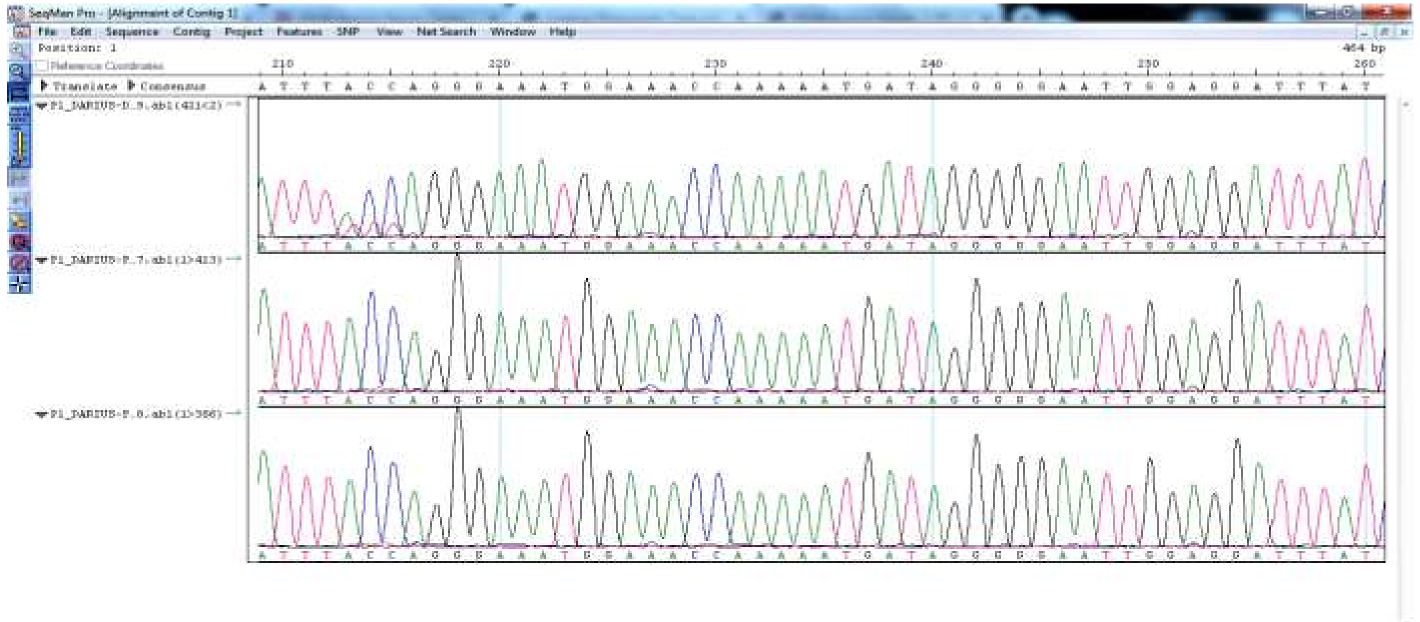
Appendix 3: A photo representation of sequence editing in Seqman pro.



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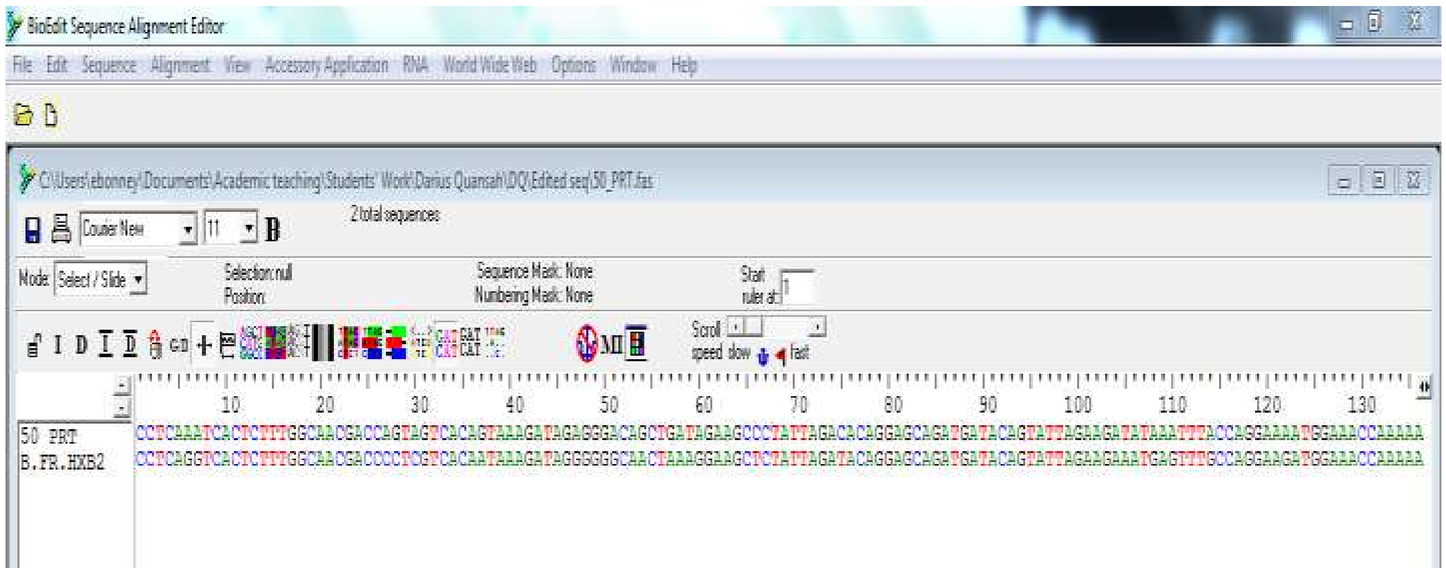
Appendix 4: A photo representation of a patient isolate sequence aligned to HXB2 HIV



Appendix 5: A photo representation of a HIV-DR report of a participant in the Stanford HIV-DB