GENETIC VARIABILITY AND ANTIRETROVIRAL DRUG RESISTANCE AMONG DRUG-NAÏVE HIV TYPE-1 INFECTED PATIENTS IN KADUNA STATE, NIGERIA

BY

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BY
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DEPARTMENT OF MICROBIOLOGY
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NIGERIA

JUNE, 2023

DECLARATION

I declare that the work in this thesis entitled **"**Analysis of Viral Genetic Variability and Antiretroviral Drug Resistance among Drug-Naïve HIV Type-1 Infected Patients in Kaduna State, Nigeria" has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Mohammed Ibrahim TAHIR _____ _____

Name of Student Signature Date

CERTIFICATION

This thesis entitled **"**Analysis of Viral Genetic Variability and Antiretroviral Drug Resistance among Drug-Naïve HIV Type-1 Infected Patients in Kaduna State, Nigeria" by Mohammed Ibrahim Tahir meets the regulations governing the award of the degree of PhD in Microbiology of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Once again, I am forever grateful.

DEDICATION

I hereby dedicate this work to my mother, **Mallama Aliya Hussain**, May Allah (SWT) increase her in *Iman* and good health. Ameen.

ABSTRACT

An important feature of HIV pandemic is the global genetic diversity of the virus. Emerging HIV genetic variants results to adverse consequences related to pathogenesis, transmission, diagnosis, clinical management and vaccine production. Expansion of antiretroviral (ARV) types and regimens over the last few decades to reduce viral transmission, morbidity and mortality related to HIV disease has resulted in emergence of transmitted drug resistance which poses serious threat to the public health. The prevalence of transmitted drug resistance in resource-limited countries is about 5%, it is however projected to increase with ART expansion. This study was aimed at determining the viral genetic variability and antiretroviral drug resistance among drug-naïve HIV type-1 infected patients in Kaduna State, Nigeria. The study was a cross-sectional hospital based one that adopted the probability proportional to size (PPS) sampling method. Basic demographic data of each volunteer was captured using structured questionnaire and transcribed to electronic questionnaire using Epi Info® version 7.2.2.2.6 software. A total of fifty HIV infected drug-naïve adult participants were enrolled in this study from three voluntary counseling and testing (VCT) centers in Kaduna State, Nigeria. These participants were tested positive to HIV from April to October, 2018. The prevalence of HIV-1 was determined using Multispot™ HIV-1/HIV-2 rapid test kit. The CD4 cell counts and viral load were determined using FACS Flow cytometry and COBAS® TaqMan® HIV-1 Test respectively. Protease and reverse transcriptase regions of *pol* gene were sequenced using Sanger DNA sequencing reaction. Web-based resistance database was used to analyse the sequence reads. GraphPad Prism version 6.0 was used to analyse the collated data. Quantitative variables were presented as mean (±SD) and median (IQR), while Spearman correlation was used to compare the CD4 cell count and viral load. Level of significance of 95% was adopted to accept or reject null hypothesis at $p \leq 0.05$. Socio-demographic data of the study

participants were determined. All (100%) the HIV infected participants studied were found to be infected with HIV type 1. The study population was found to have relatively high median viral load 158,391 Copies/m with median CD4 cell count of 176 ceel/µL. However, few participants, despite being ART-naïve, had undetectable plasma viral load. Female had higher CD4 cells count values and lower plasma HIV-1 viral load value than male. The CD4 cells had strong reciprocal relationship with the plasma HIV-1 viral load. There was strong negative significant value of correlation coefficient between HIV-1 plasma viral load and CD4 cells count in the study population. The prevalence of transmitted drug resistance was found to be 19.5 % which is very high in the study area. The HIV-1 isolates were characterized into CRF02_AG, subtype G, CRF06_cpx and subtype C with frequency of 25(61%), 13(32%), 2(5%) and 1(2%) respectively. A protease inhibitor surveillance drug resistance mutation (SDRM) as M46MI, few other NRTI and NNRTI SDRM and other polymorphic mutations were detected among the drug-naïve HIV infected patients. The K103NSG mutation was highest with a frequency of 12.8 % (5/39). Genetic relatedness of the sequences from the isolates and reference sequences were presented in maximum likelihood phylogenetic tree. This study indicates that the baseline plasma viral load and CD4 cell count could affect prognosis, disease progression and transmission. The drug-naïve participants reported with undetectable plasma RNA could be ‘‘elite’’ controllers. This implies that, the patients may respond to the currently available highly active antiretroviral therapy (HAART) first line treatment.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Human immunodeficiency virus (HIV) is the aetiolologic agent of acquired immunodeficiency syndrome (AIDS) (Guatelli *et al*., 2002). The virus belongs to the *Lentivirus* genus in the family of *Retroviridae*. Two types of Human Immunodeficiency Virus (HIV) are known: the most common HIV-1, which is responsible for the worldwide AIDS epidemic, and the immunologically distinct HIV-2, which is much less common and less virulent but produces clinical symptoms similar to HIV-1 (Mercedes and Carlo, 2013). Both HIV-1 and HIV-2 are related to the non-human primate *lentivirus* simian immunodeficiency virus (SIV) (Guatelli *et al*., 2002). Infection with HIV type 1 is prevalent worldwide and is characterized by progressive deterioration of the immune system which can lead to death if not treated (Guatelli *et al*., 2002).

The HIV-1 is characterized by extensive and dynamic genetic diversity, generating variants which result to distinct molecular subtypes as well as recombinant forms; these forms demonstrate an uneven spatial global distribution. This diversity has role to play in understanding viral transmission, pathogenesis, and diagnosis and extremely influences approaches toward vaccine development (Buonaguro *et al*., 2007).

In 2021, 38.4 million people globally were reported living with HIV out of which 6.1million were from Western and central Africa (UNAIDS, 2022a). Nigeria, the most populous country in Africa, was ranked second in the world with the highest HIV-1 burden (Heipertz *et al*., 2016). It was estimated that 1.9 million people in Nigeria were living with HIV/AIDS, 74 000 new infections and 51 000 AIDS-related deaths in 2021 (UNAIDS 2022).

The virus is known for its highly recombinogenic nature, together with an extreme genetic variety, both attributable to an error-prone reverse transcriptase which gives rise to heterozygous virion. It exhibits considerable genetic diversity resulting from the high mutation rate of reverse transcriptase, high viral turnover, viral genomic recombination, and immune and therapeutic selection pressures (Akouamba, *et al*., 2005).

Sequence diversity of HIV-1 has resulted in identification of many subtypes of HIV-1 M group, as well as 70 circulating recombinant forms (CRFs) and a number of other unique recombinant forms of HIV-1 (Aik, 2008, Wenjun et al., 2017). As at May, 2023, over 100 CRFs have been identified, and more than 80 of them were published in the Los Alamos HIV database (Los Alamos National Laboratory, 2023). A remarkable degree of viral diversity within HIV-1 has resulted from mutational escape and adaptation in response to both immune activity and antiretroviral therapy (ART) (Santoro and Perno, 2013).

The strains of HIV-1 is subdivided into four genetic groups as M (major), O (outlying), N (new or non-M, non-O), and P, with varying regional distribution (Khairunisa *et al*., 2018). Group M is responsible for the pandemic and has been further divided into eleven subtypes and subsubtypes (Khairunisa *et al*., 2018), and recombination has however led to the circulation of mosaic HIV-1 strains as circulating recombinant forms (CRFs) and unique recombinant forms (URF) (Sagoe *et al.*, 2009). The eleven subtypes and sub-subtypes are A1, A2, B, C, D, F1, F2, G, H, K, and J (Wenjun *et al.,* 2017). Figure 1.1 shows evolutionary relationships among non-recombinant HIV-1 strains from the HIV-1/SIVcpz lineage. The phylogenetic tree shows the different HIV-1 groups, subtypes and sub-subtypes. Each of the internal branches defining a subtype or sub-subtype is supported by 100% of bootstraps. Subtype B is the predominantly localized in the Americas, Europe, and Australia, whereas there is a growing epidemic of non-B subtypes and CRFs in Africa

and Asia (Khairunisa *et al*., 2018). Within the group M, the average inter-subtype genetic variability is 15% for the *gag* gene and 25% for the *env* gene (Buonaguro *et al*., 2007).

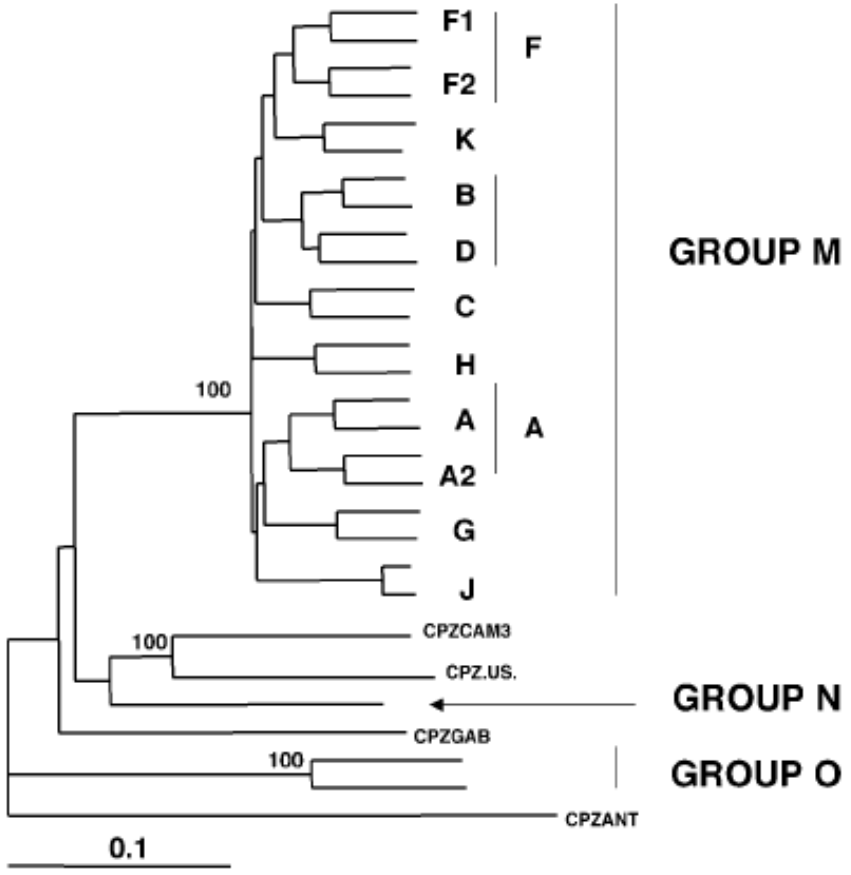

Figure 1.1: Evolutionary relationships among non-recombinant HIV-1 strains from the HIV-1/SIVcpz lineage, based on neighbor joining phylogenetic analysis of near full length genome sequences.
Source: (Peeters, 2000).

Recombination results from the simultaneous infection of a cell with two different proviruses, therefore permitting the encapsulation of one RNA transcript from each provirus into a heterozygous virion. After the subsequent infection of a new cell, the reverse transcriptase generates a newly synthesized retroviral DNA sequence that is recombinant between the two parental genomes (Renu and Chunfu, 2005).

Primary HIV-1 drug resistance (HIVDR) means that there is increased resistance of HIV-1 to antiretroviral drugs seen in individuals who have never received ART and likely have been infected with a drug resistant virus. In resource-limited settings where ART is being scaled up, the World Health Organization (WHO) recommends the surveillance of primary HIVDR (Sungkanuparph *et al.,* 2012). Primary HIVDR could be a major reason for treatment failure especially among individuals that initiated ART without baseline drug resistance testing.

Several studies have used the polymerase (*pol*), protease (*prot*), and reverse transcriptase (*RT*) genes for phylogeny. Also, the *pol* gene has been shown to be useful for subtyping in areas with multiple subtypes (Sagoe *et al.*, 2009).

1.2 Statement of Research Problem

Emerging HIV genetic variants results in adverse consequences related to pathogenesis, transmission, diagnosis, clinical management and vaccine development (Wenjun *et al.,* 2017). There has been expansion of ARV types and regimens over the last few decades which significantly reduced viral transmission, morbidity and mortality related to HIV disease. However, the emergence of transmitted drug resistance as a result of this expansion poses serious threat to the public health (Kotaki *et al*., 2015). The prevalence of transmitted drug resistance in resource-

limited countries is about 5%, and is projected to increase with ART expansion (Kotaki *et al*., 2015).

Antiretroviral drug resistance is the major obstacle for clinical management of ARV therapy as individuals infected with resistance HIV strain may not respond to the treatment. Current antiretroviral drugs comprise at least 16 drugs targeted against two enzymes, the reverse transcriptase (RT) and protease. The development of drug resistance to the nucleoside RT inhibitors (NRTIs), non-nucleoside RT inhibitors (NNRTIs) and protease inhibitors (PIs), is both a cause and a result of virologic treatment failure with incomplete virus suppression (Renu and Chunfu, 2005).

Most information of drug resistance is largely limited to developed countries with predominant HIV-1 subtype B infections. However, with introduction of ARV in other parts of the world, emerging data indicate that viral subtype may influence the effectiveness of antiretroviral treatment and that pre-existing mutations could reduce the effectiveness of ARV. In the absence of any drug exposure, RT and protease sequences from B and non-B HIV-1 are polymorphic among about 40 per cent of the first 240 RT amino acids and 30 per cent of the 99 protease amino acids. Some of these amino acid substitutions occur at high rates in non-subtype B viruses at positions associated with drug resistance in subtype B (Renu and Chunfu, 2005).

Resistance to antiretroviral drugs is an important issue in the clinical management of HIV-1-related disease, especially when there is an obviously high prevalence of HIV-1 antiretroviral resistance recently in treatment-naïve patients, even higher than those who have been infected longer. Despite being effective in decreasing the patient's plasma viral load to below detectable levels, HAART still fails to completely clear the virus as the viruses are maintained in long term reservoirs

such as memory T-cells. The HAART would never be able to reach 100% success rate as a result of sub- optimal drug concentrations, patient non-compliance and the emergence of resistant strain. The increasing use of HAART has greatly improved the rate of anti-HIV therapy as opposed to mono or dual-therapy. However, HAART failure has been shown with the increased number of the emerging resistant virus strains, capable of being transmitted to new hosts and amplified in the subsequent round of HAART (Aik, 2008).

The increased use of antiretroviral (ARV) drugs created the emergence of mutant strains resistant to treatment especially when there is no strict drug adherence. Thus, the World Health Organization (WHO) recommends epidemiological monitoring for newly infected patients with HIV (Kamangu *et al*, 2015). About 96% of new HIV-1 infection occurred in low- and middle – income countries (Miri *et al*., 2014).

Transmission and diverse distribution of HIV-1 subtype and its circulating recombinant forms (CRFs) in African sub-regions may be attributed to spatial accessibility, human migrations and movements through the available and efficient transportation system (Delatorre *et al*., 2014). The diversity poses a serious challenge for viral load determination, drug resistance testing, and HIV vaccine development (Akoamba *et al.*, 2005).

1.3 Justification of the Study

Studies related to HIV genetic variability were conducted in some parts of this country but there is paucity of data representing this proposed area of study (Raphael *et al*., 2012). Therefore, it is pertinent to understand the resistance pattern and diversity of HIV-1 and associated indicator markers such as CD4 cell count and plasma viral load in the study sites and to serve as representatives of the general population of drug-naïve HIV-1 infected adults in Kaduna State,

Nigeria. Data from this study will provide insights on possible viral pathogenesis and disease progression as they are linked to genetic variability. Information from genetic variability from this part of the world will assist in the quest for appropriate vaccine candidate and provide a glimpse on the circulating recombinant form (CRF) distribution in the study area.

Testing transmitted drug resistance among drug-naïve patients will reduce cost of therapy and avoid unnecessary adverse drug reactions and toxicity. The data generated from the work will help in the effort to reduce the emergence and transmission of HIV drug resistance and thereby achieving the global 90-90-90 targets for treatment: diagnosing 90% of all people with HIV infection; providing treatment to 90% of those diagnosed; and ensuring 90% of people on treatment achieve virological suppression by 2020 (WHO 2017).

Understanding regional and subtype-specific mutational patterns of HIV-1 transmitted drug resistance (TDR) will assist in selection of first-line antiretroviral (ARV) regimen. Workable diagnostic assay will be useful in middle and low-income regions where standard genotypic resistance testing is not affordable.

1.4 Aim of the Study

This study was aimed at determining the viral genetic variability and antiretroviral drug resistance among drug-naïve HIV type-1 infected patients in Kaduna State, Nigeria.

1.5 Objectives

The objectives of this study were to:

i. determine demographic and risk factors associated with HIV-1 infection;

ii. determine the relationship between CD4 T-cell counts and viral load among HIV-1 infected patients using FACS Flow cytometry and real time PCR respectively;

iii. detect Transmitted HIV Drug Resistance (TDR) among the drug-naïve patients using end point reverse transcriptase Polymerase Chain Reaction (RT-PCR), Sanger sequencing and web-based resistance database;

iv. characterize HIV isolates into sub-types, sub-subtypes and Circulating Recombinant Forms (CRF) using REGA HIV-1 Subtyping Tool and establish phylogeny among the HIV-1 isolates using MEGA 7.

v. detect type of mutations associated with the viral Protease and Reverse Transcriptase genes using specialized web-based resistance database;

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview of Human Immunodeficiency Virus

Human Immunodeficiency Virus (HIV) is the etiologic agent of acquired immunodeficiency syndrome (AIDS). AIDS emerged in 1981 in the human race as outbreak of *Pneumocystis carinii* pneumonia among homosexual men in the USA but was likely present in Africa many decades earlier (Girard *et al*., 2011). The disease is characterized by destruction of immune system through progressive depletion of CD4+ helper T cells which results in immunodeficiency syndrome. The syndrome eventually permits opportunistic infections of viral, bacterial, or parasitic origin to establish. Some form of cancers such as Kaposi sarcoma or non-Hodgkin B cell lymphomas may also emerge in AIDS-compromised individuals (Ren *et al*., 2016).

Majority of the HIV-1 strains analyzed till date have been subtyped. However, there are a few HIV-1 strains with genomes having regions represented from different subtypes (recombinants) seen in geographic areas where more than one type is circulating. This recombinant status is reinforced by the finding that irrespective of the regions of their genome analyzed they fail the criteria of a single designated subtype. These 'hybrid strains' are the products of recombination events taking place in the virus. If two different subtypes of HIV-1 infect a single cell, following replication the result can be a mosaic genome comprising regions from each of the two subtypes. This is due to the "template switching" ability of the reverse transcriptase enzyme. Two types of recombinant forms have been identified - circulating recombinant forms (CRF) and the unique recombinant forms (URF) (Kandathil *et al*., 2005). If the recombinant is identified in at least three epidemiologically unlinked individuals characterized by full-length genome sequencing, they are

designated CRFs. In addition to CRF, several HIV-1 strains with unique mosaic structures have been reported in epidemiologically linked persons. These forms known as unique recombinant forms (URF) have not shown any evidence of epidemic spread and are thought to arise due to secondary recombination of a CRF (Kandathil *et al*., 2005). Figure 2.1 shows mosaic genomic structure of some CRFs. Human immunodeficiency virus (HIV) is highly heterogeneous even within infected individuals, owing to rapid turnover rates, high viral load, and an error-prone reverse transcriptase enzyme, and recombination between viral genomes (Chin, 2017).

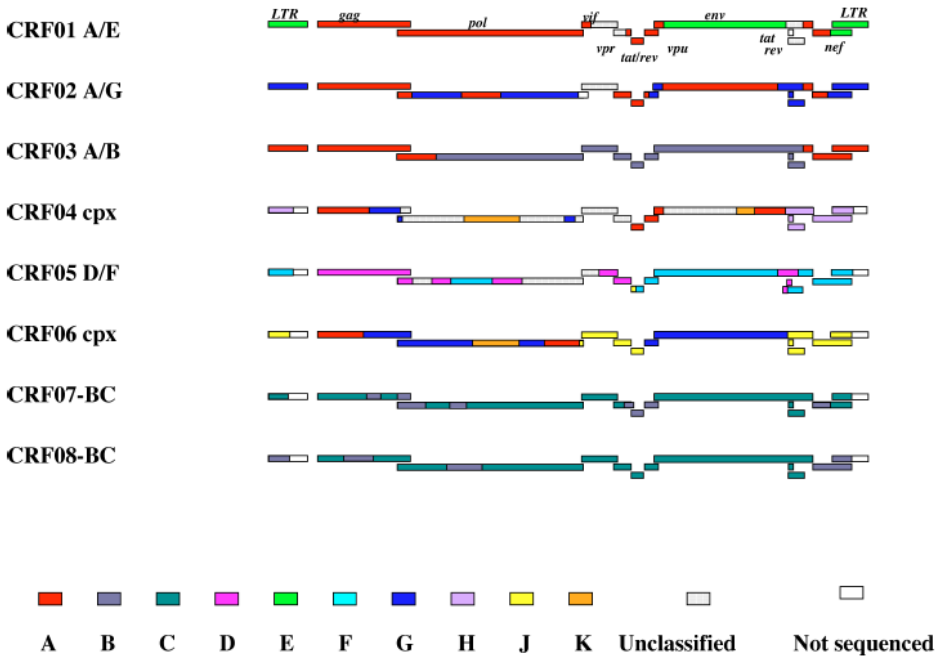

Figure 2.1 Schematic representation of the complex mosaic genomic structure. Structure from the actually published circulating recombinant forms (CRFs).
Source: (Peeters, 2000).

2.1.1 Basics of Human Immunodeficiency Virus Virology

Human Immunodeficiency Virus is a *lentivirus*, from the family *retroviridae* which possess RNA genome enclosed within a capsid and a lipid bi-layered envelope (Chinen *et al*., 2002). Other members of this family include the simian, the feline, and the bovine immunodeficiency viruses, the Visna virus of sheep, the caprine arthritis encephalitis virus and the equine infectious anemia virus (Girard *et al*., 2011). The HIV virions have spherical morphology of 100– 120 nm in diameter and consist of a lipid bilayer membrane that surrounds a dense nucleocapsid (Sierra *et al*. 2005).

The HIV genome consists of two identical positive-stranded RNA molecules of about 9.2 to 9.8 kilo base pairs each, which can be reverse-transcribed into double-stranded DNA. The HIV contains two long terminal repeats (LTR) at the end of its genome that include cis-regulatory sequences important for the expression of the provirus. Nine protein-coding genes (Table 2.1) lie between the 2 LTRs that includes three structural proteins (Gag, Pol, and Env), two regulatory proteins (Tat and Rev), and four accessory proteins (Vif, Vpr, Vpu, and Nef) (Ren *et al*., 2016) and the four accessory proteins are also called regulatory proteins.

The envelope is derived from host cell and contains 2 major viral glycoproteins, gp41 and gp120 which originated from enzymatic cleavage of the larger pre-protein gp160. They mediate viral entry and syncytium formation. The nucleoprotein has 3 structural proteins, p24, p16, and p9. The p24 form the capsid that houses the genomic RNA strands and the viral enzymes. The p16 is a matrix protein and anchored to the internal face of the envelope while p9 is a nucleocapsid but not covalently attached to the viral RNA (Chinen *et al*., 2002).

Table 2.1: HIV genes and gene products

Gene	Protein	Size	Function
Structural			
gag	Matrix (MA)	p17	Structural protein
	Capsid (CA)	p24	
	Nucleocapsid (NC)	p7	
	p6	p6	
pol	Protease (PR)	p12	Viral enzymes
	Reverse transcriptase	p66/p51	
	Integrase (IN)	p32	
env	Surface glycoprotein	gp120	Viral envelope glycoproteins
	Transmembrane (TM)	gp41	
Regulatory			
tat	Tat	p14	Transactivator of viral transcription
rev	Rev	p19	Transports unspliced mRNA to cytoplasm
vif	Vif	p24	Promotes virion infectivity
nef	nef	p27	Down-regulate class I MHC and CD4; enhances viral infectivity particles; facilitates T-cell activation
vpu[a]	Vpu	p16	Promotes release of viral particles; CD4 degradation
vpr	Vpr	p15	Arrest cell cycle in G_2; increase nuclear entry of the preintegration complex in non-dividing cells
vpx[a]	Vpx	p14	Facilitates viral replication and nuclear entry of the preintegration complex in macrophages

[a] *vpu* is found only in HIV-1; *vpx* is found only in HIV-2 and SIV. Adopted from: John *et al*., 2002

2.2 HIV Replication Strategies

Infection of host cells by the virions begins by an interaction between the extracellular domain of HIV-1 gp120 and cellular receptors (Sierra *et al*. 2005). The HIV enters lymphocytes and monocytes via cognate recognition of the viral glycoprotein gp120 with the cell surface CD4 molecule and a chemokine receptor. The receptors are either CXCR4 or CCR5 which have connection with cell tropism (Chinen *et al*., 2002). The CD4 is the major receptor for HIV-1 and HIV-2 and the seven transmembrane chemokine receptors CCR5 and CXCR4 are the main HIV-1 coreceptors *in vivo* (Sierra *et al*. 2005).

The cell tropism defines HIV strains into macrophage-tropic (M-tropic) and T-cell tropic (T-tropic). The M-tropic viruses also called R5 viruses uses CCR5 as coreceptor and mainly infect macrophages and poorly infect CD4 T cells. They are more easily transmitted sexually. The T-cell viruses on the other hand are otherwise referred to as X4 viruses and use the CXCR4. They are mostly expressed in CD4 T cells and induce the formation of syncytia in the infected cells. Mutation in the CCR5 gene can protect host cells from initiation of infection (Chinen *et al*., 2002). A primary HIV isolate has been reported to use the CD8 molecule to infect CD8 cells not requiring CD4 or chemokine receptor expression. Novel changes were observed in envelope sequences of the CD8-tropic viruses (Saha *et al*., 2001). After binding to coreceptor, viral and cellular membranes fuse and the viral core is released into the cytoplasm of the cell (Sierra *et al*. 2005).

Figure 2.2 elucidates replicative cycle of HIV. After viral entry, the virus RNA genome and viral protein are released by the capsid into the host cell cytoplasm. The viral RNA is transcribed into complementary DNA (cDNA) by viral reverse transcriptase and the RNA template thereafter degraded by the same enzyme.

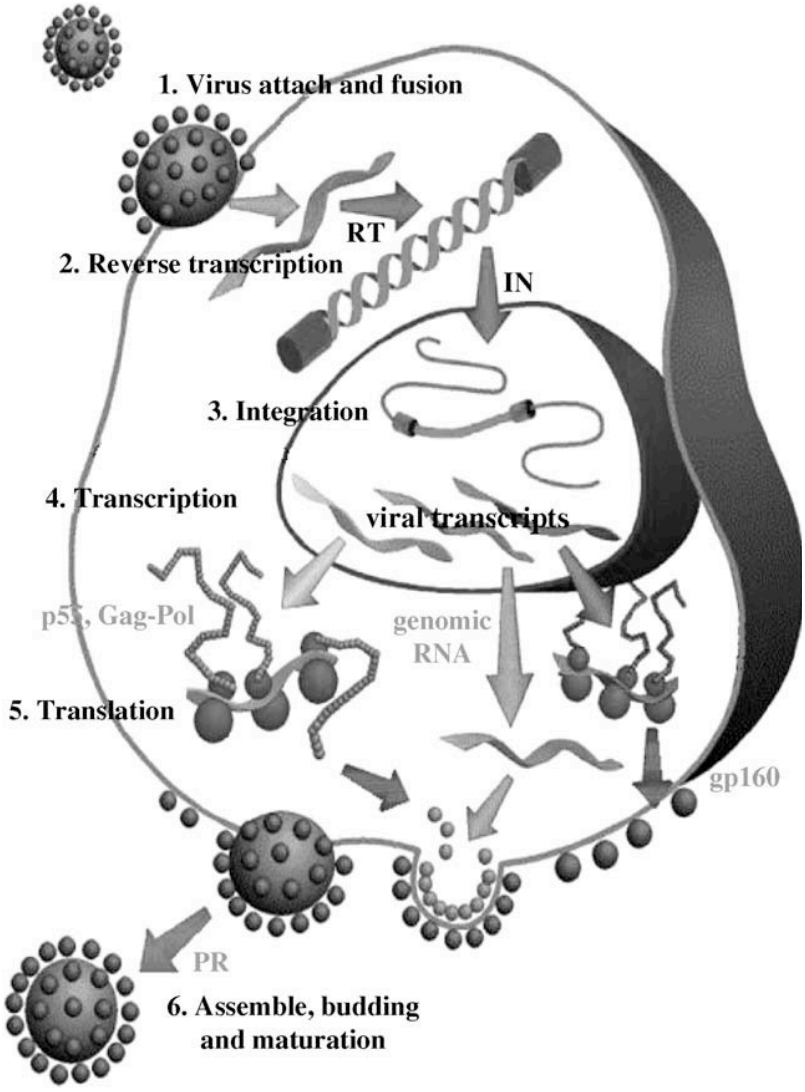

Figure 2.2: Replicative cycle of HIV.

The main steps in HIV replication are sequentially numbered from 1 to 6. (1) Virus binds to CD4 and the appropriate coreceptor resulting in fusion of the viral envelope and the cellular membrane and the release of viral nucleocapsid into the cytoplasm. (2) Following the uncoating viral RNA is reverse transcribed by the RT. (3) The resulting double-stranded proviral DNA migrates into the cell nucleus and is integrated into the cellular DNA by the IN. (4) The proviral DNA is transcribed by the cellular RNA polymerase II. (5) The mRNAs are translated by the cellular polysomes. (6) Viral proteins and genomic RNA are transported to the cellular membrane and assemble. Immature virions are released. Polypeptide precursors are processed by the viral PR to produce mature viral particles (Sierra *et al*. 2005)

After every 1500 to 4000 bases the reverse transcriptase incorporates an incorrect nucleotide which results to the rapid occurrence of mutations seen in HIV (Chinen *et al*., 2002). Presence of a cellular protein APOBEC3G influences the fidelity of the reverse transcription (Sierra *et al*. 2005).

The pre-integration complex enters the nuclear membrane through the nuclear pore directed by HIV-1 Vpr protein. In the nucleus, the viral DNA can be found in three forms as either linear, 1-LTR or 2-LTR circles. The LTR promoter is activated by cellular factors such as NFκB where Nef, Tat and Rev are produced in basal amounts from these DNA forms.

The linear dsDNA in the pre-integration complex is inserted into the host chromosome by the viral integrase (IN). The cellular RNA polymerase II transcribes the proviral DNA through binding of cellular factors to viral LTR and translation occur producing again basal amounts of Tat, Rev and Nef. When adequate amounts of Tat are produced, it controls further transcription of HIV-1 genes.

Multiply-spliced mRNAs are produced in the early stage of the replication cycle and from which the regulatory proteins Tat, Nef and Rev are expressed however, when sufficient amount of Rev is formed, non-spliced or singly-spliced species are produced and transported to the polysomes leading to the production of the other viral proteins and genomic RNA. The Rev binds to Rev responsive element (RRE) present in the singly-spliced and unspliced viral RNA molecules and facilitates their transport via the nucleus membrane, preventing them from splicing and allowing them to be translated in the cytoplasm.

The *env* gene is translated into the precursor protein gp160, which is glycosylated within the endoplasmic reticulum. The *gag-pol* gene is mainly translated to produce the Gag and Gag-Pol polyproteins. Gag polyprotein (p55) is a 55 kDa precursor that is proteolytically processed during

the maturation of the virus into six structural proteins which rearrange and produce the mature virion.

Ribosomal frame shift during translation result to the expression of Gag-Pol polyprotein that additionally includes the enzymatic proteins protease (PR), reverse transcriptase (RT), and Integrase (IN).

When translations are completed, the Env proteins move and insert into the plasma membrane. Gag and Gag-Pol polyproteins also move to the cellular membrane and start to assemble directed by the Gag polyprotein. Complex formed from viral enzymes, full-size genomic RNA, the cellular tRNAlys3 primer and cellular compounds associate buds through the plasma membrane producing an immature virion.

Decrease in number of the CD4 molecules present in the plasma membrane is important to avoid interaction with newly synthesized gp120 during viral assembly and budding. The CD4 molecules depletion is achieved by HIV-1 Nef, Env, and Vpu. The Nef in the early stages of infection accelerates endocytosis and subsequent degradation of CD4 molecules and MHC class I and II molecules on the cell surface. In later stages, the envelope precursor gp160 traps the newly-synthetized CD4 molecules within the endoplasmatic reticulum. The Vpu encourages the degradation of the CD4 molecules and releases the gp160 molecules allowing their maturation and trafficking. It also forms ion conducive pores believed to enhance virus release. Protease activation is triggered by budding which autocatalytically cleaves the Gag and Gag-Pol polyprotein releasing the structural proteins and enzymes (Sierra *et al*. 2005).

2.2.1.1 Latency

No productive latent infection can be resulted from HIV-1 infection of resting memory or naive CD4+ T cells, macrophages or mononuclear cells. Viral interruption to assume latency or to continue its life cycle is a molecular mechanism that is still not very clear (Sierra *et al*. 2005).

2.2.1.2 Long Terminal Repeats

The long terminal repeat (LTR) has origins in the unique 5' (U5) and 3' (U3) regions as well as the flanking R regions of the single-stranded RNA genome and it is approximately 640 bp. The reverse transcription process results in the placement of two identical LTRs, each comprised of a U3, R, and U5 region, at either end of the proviral DNA. The ends of the LTRs subsequently partake in integration of the provirus into the host genome. Once the provirus has been integrated, the LTR on the 5' end serves as the promoter for the entire retroviral genome, while the LTR at the 3' end provides for nascent viral RNA polyadenylation (Krebs *et al*., 2001).

It has been shown by functional mapping of the 5'LTR that the U3 and the R regions (U3R) contain a cluster of regulatory elements that are useful in the control of HIV-1 transcription and expression. Therefore, the number, location and binding patterns of the regulatory elements might be specific to some HIV-1 strains such as clade F2, CRF02_AG, CRF01_AE and CRF22_01A1 (Mbondji-wonje *et al*., 2018).

2.2.1.3 Gag Proteins

The HIV-1 Gag is described as a molecular machine that drives viral particle assembly and release. All the building blocks necessary for formation of a complete infectious virion are recruited by the Gag protein. These building components include both viral and cellular. The Gag is so efficient that it can form virus-like particles even when expressed in the absence of other viral proteins

(Göttlinger 2001). Gag-protease (Gag-Pro) or Gag-driven replication capacity correlates with markers of disease progression and significant inter-subtype differences in Gag-protease function have been reported. Patient-derived subtype C Gag-protease recombinant viruses replicate significantly slower than their subtype B counterparts (Kiguoya *et al*., 2017).

2.3 HIV Immunology

2.3.1 The Immune Response Processes

When HIV establishes infection, a robust host-mediated immune response is initiated however the response is usually unable to contain viral replication. In many cases a single transmitted/founder (T/F) virus is responsible for dissemination of infection. The virus reaches a peak viral load usually 10 – 15 days after infection before declining to a viral set point after about 30 days. Viral set point is a prognostic marker in the AIDS timeline.

The decline in peak viremia happens together with a rapid amplification in anti-viral CD8 T-cells, which have a downward selective pressure on the virus. However, despite this pressure HIV evades CD8 T-cell control of viremia by rapidly evolving susceptible T-cell target epitopes through mutations that affect epitope-HLA binding, mutations that interfere with intracellular epitope processing or mutations that prevent T cell receptor (TCR)-epitope-HLA recognition (Larijani *et al*., 2019).

2.3.2 Host Anti-HIV Immune Responses

The clinical course of HIV infection is determined by host immune response which consists of humoral and cellular components of innate and adaptive immunity. The innate system has an early response to pathogens while adaptive immune system responds relatively late.

2.3.2.1 Innate Immune Responses

Some important innate immune factors such as mannan-binding lectins (MBLs) and complement can readily inactivate virus while other circulating proteins can block HIV infection. Naturally occurring anti-Tat IgM and IgM anti-leukocyte antibodies inhibit effects of Tat and prevent HIV entry into host cells respectively. The HIV envelope gp120 and gp41 are neutralized by Anti-HIV antibodies and antibodies to cell surface proteins e.g. human leukocyte antigen (HLA) in some situations can mediate this activity.

Several monoclonal antibodies that recognize a consensus sequence of variety of HIV-1 and HIV-2 groups and clades have been derived from human sera that react with gp120or gp41. Antibodies that attach to virus-infected cells (via gp120 or gp41) can also be vital in direct killing of infected cells by antibody-directed cellular cytotoxicity (ADCC) (Levy, 2009).

Monocytes and HIV: Monocytes undergo changes in their frequency, phenotype and function during HIV infection. During the late-stage of HIV infection, CD16-expressing monocytes are first found to expand. This expansion of CD16+ monocytes was also reported during chronic phase of infection. The expansion observed in chronic infection and late-stage infection ranges from 30-50% as opposed to 5-10% in healthy persons. The expansion of CD16+ monocytes was reported principally in HIV-infected viremic persons but not individuals receiving antiretroviral therapy (Wacleche *et al*., 2018).

Dendritic Cells and HIV: Dendritic cells (DC) are important in both innate and adaptive immune processes in HIV infection. They are antigen presenting cells (APCs) for T and B cells and produce cytokines that control the immune response. They are found throughout the body however blood DCs consist of plasmacytoid dendritic cells (PDC) and myeloid dendritic cells (MDCs). Reduced

number of PDC is associated with the progression to AIDS and long-term survivors have higher number of this cell type than even healthy control (Levy 2009).

Vaginal and rectal mucosal tissues are the usual routes for primary HIV infection. The tissues have variety of dendritic cell (DC) subsets that can encounter the virus. The localization of DC plays an important role is spread of viral infection to CD4+ T cells. However, DCs were found to sequester virus for several days before efficiently disseminating the infection to CD4+ T-cells (Wacleche *et al*., 2018).

The HIV-1 co-receptors CCR5 and CXCR4 are highly expressed by DCs while CD4 are lowly expressed and as a result allowing attachment and entry via the viral glycoprotein gp120. Other molecules capable of binding to the gp120 and expressed by DC include myeloid dermal DC and MDDC express C-type lectin (CLR) receptor DC-SIGN as well as the mannose receptor (Wacleche *et al*., 2018).

Other Innate Immune Cells: Natural killer cells play a vital role in destroying HIV infected cells and are controlled by the production of cytokines such as IFNs and IL-12. Only cells that lack major histocompatibility class (MHC) I expression are destroyed by the NK cells. This is possible because of the interaction of NK-inhibiting receptors (KIRs) on NK cells with certain HLA moieties (Levy, 2009).

2.3.2.2 CD4 T-Lymphocytes

The CD4 T cell has important role in immune response of B cells and other T cells. The CD4 T cell is vital for efficient function of CD8 T cell immunity. Depending on the cytokines production, CD4 T cell are divided into TH1 and TH2 subsets. T-cell coproduction of IL-2 and IFN-gamma is useful for anti-HIV immunity. The interaction of CD4 cells with dendritic cells plays a key role in

determining their production of specific cytokines. Strong HIV specific CD4 cell responses alone and particularly in association with HIV-specific CD8 cells provide a good prognosis for the clinical course (Levy, 2009).

2.3.2.3 CD8 T-Lymphocytes

Non-Cytotoxic Activity: Like dendritic cells, CD8 cells can function in both the innate and adaptive immune system. The CD8 cell noncytotoxic antiviral response (CNAR), mediated by CD8 cell antiviral factor (CAF), blocks virus transcription without killing the infected cell. Activity of CNAR is highest in long-term survivors and when there is decrease in this activity, the virus replication resumes with progression to disease (Levy, 2009).

Cytotoxic Activity: The virus-infected cell is killed by the participation of classic CD8 in an adaptive immune activity. This response has been associated with long-term control of HIV infection. The activity can be detrimental if it lyses the autologous uninfected CD4 cells and APCs. These cells may remain HIV specific but not have the capacity for killing virus-infected cells because of the absence of perforin or other cytotoxic proteins (Levy 2009).

2.3.2.4 T-regulatory Cells

The CD4 T regulatory (T-reg) cells have received increased attention in HIV infection. These cells can be identified by several phenotypic markers, different functions, and various mechanisms of action. Importantly, they may be beneficial early in HIV infection by decreasing immune activation but detrimental later if they decrease the antiviral immune response. A bimodal response with T-reg cell activity, early but not late, can be observed in the SIV nonpathogenic primate models. The potential presence of CD8 T-reg cells in HIV infection should also be considered (Levy 2009).

2.3.3 Virus Resistance to Immune Responses

The HIV has a number of ways in which it resists immune activities. These include mutations of the viral peptide, defective presentation by HIV-infected cells, and incorrect expression of viral peptides by APCs. Viral escape mutants are more commonly encountered in acute infection in which the more dominant epitopes of viral proteins are first targeted (Levy 2009).

2.4 Global HIV Epidemiology

Human Immunodeficiency Virus is believed to be originated from multiple zoonotic transmissions of simian immunodeficiency virus (SIV) from nonhuman primates to humans in West and Central Africa (Chin, 2017). These simian immunodeficiency virus strains were harboured by more than 40 different nonhuman primate species and Independent zoonotic transmission events have resulted to emergence of many HIV lineages. The HIV type 1 (HIV-1) comprises of group M, N, O, and P while HIV type 2 (HIV-2) has group A–H. HIV-1 group M (Main) is responsible for the global HIV pandemic (Chin, 2017).

The HIV pandemic is a major global health challenge and as at 2017, about 37 million people were living with the disease. Even with the increase in scale up of antiretroviral treatment, in 2017 alone, about 940,000 people died from AIDS-related illnesses and 1·8 million people became newly infected with the virus (Hemelaar *et al*., 2018).

The important feature of HIV pandemic is the global genetic diversity of the virus. HIV-1 group M diversified into distinct subtypes, denoted by the letters A, B, C, D, F, G, H, J, and K. Recombinants between subtypes, denoted as circulating recombinant forms (CRFs) or unique recombinant forms (URFs) (Hemelaar *et al*., 2018). As of May, 2023, there were over 100 CRFs reported by Los Alamos HIV database (Los Alamos National Laboratory, 2023).

The current global distribution of HIV subtypes and CRFs can be linked to exportation of different viral strains from central Africa through spatial accessibilities which include human migration into new geographic regions where secondary epidemics were initiated (Daltore *et al*., 2014). The distribution of HIV-1 subtypes and recombinants varies and change over time across both globally and geographical regions. In a systematic review from 1990 to 2015 globally, subtype C accounted for 46.6% while subtype B had prevalence of 12.1%. subtype A was responsible for 10.3%, CRF02_AG had 7.7% while CRF01_AE, subtype G, and subtype D had 5.3%, 4.6% and 2.7% respectively. All CFRs accounted for 16.7% (Hemelaar, *et al*., 2018). .

Over the period covered by the review, West Africa was seen to be predominated majorly by CRF02_AG and subtype G while East Africa was dominated by subtype A, C and D. Central Africa had predominance of subtype A while southern Africa was predominated by subtype C. Some parts of Europe had up to 85% of subtype B while Southern Asia had about 80% of CRF01_AE (Hemelaar, *et al*., 2018). Subtype G emerged from Central Africa around late 1960s and spread to West and West Central Africa around 1975-1980. A study revealed that major subtype G clades in Nigeria were the GWA-I that most probably emerged in the country around the middle 1970s, and the GWA-II probably emerged in Togo or Ghana around the late 1970s (Daltore *et al*., 2014). Nazziwa *et al*. (2018) observed a large transmission cluster with 68% of Nigerian subtype G sequences and this is consistent with G cluster reported by Daltore *et al., (*2014). Three independent transmission clusters for CRF02_AG was originated from within Nigeria with merged estimates on the time of emergence from 1960-1980 (Nazziwa *et al*., 2018).

A systematic review and meta-analysis of HIV subtypes and CRFs from 2002-2015 in Nigeria reported pooled prevalence of Subtype G, CRF02_AG, CRF06_cpx, Subtype A and Subtype C as 38.27%, 37.81%, 6.6%, 14.05% and 2.80% respectively (Tahir *et al*., 2020).

2.4.1 HIV Molecular Epidemiology

The evolutionary indicators provided by the rapid and continuous changes of HIV can be exploited to infer associations between persons infected with genetically similar viruses. Molecular epidemiology is defined as the analysis of HIV sequence data (phylogenetics) integrated with socio-demographic, behavioral, and geographic information to help understand the epidemiology of a disease (Mehta *et al*., 2019). In the past, phylogenetic analyses of HIV sequences have been used to provide clues on the historical origins of HIV, and describe historical and current epidemics. These data, when generated and analyzed in real-time, can be used to improve the understanding of HIV transmission dynamics within a population, information that is critical to developing and implementing effective public health interventions (Mehta *et al*., 2019). Molecular methods alone or in combination with mathematical modeling can extrapolate about the transmission dynamics, critical epidemiological parameters (prevalence, incidence, effective number of infections, generation times, time between infection and diagnosis), or the spatiotemporal characteristics of epidemics (Paraskevis *et al*., 2016).

Molecular tools have been used to assess the impact of an intervention and outbreak investigation and are of great public health importance. Sometimes molecular sequence data may be more readily available than HIV surveillance data and can as a result allow for molecular analyses to be conducted more easily (Paraskevis *et al*., 2016).

Molecular epidemiology of HIV has many public health implications which include classification, dating, of origin of epidemics and studying transmission. It also assists in understanding global and regional pattern of epidemic spread. It tells the spatial characteristics of epidemic spread and assess the impact of intervention. Some critical epidemiological parameters such as prevalence,

incidence, generation times, time between infection and diagnosis can also be understood better by molecular epidemiology (Paraskevis *et al*., 2016).

The HIV molecular epidemiology can be used to describe factors associated with HIV transmission dynamics and demonstrates "hotspots" of transmission. It measures the impact of prevention interventions and also identifies and estimates the size of key populations for example People Living With HIV (PLWH) who are unaware, HIV uninfected and at increased risk of acquiring HIV infection. Again, HIV molecular epidemiology could be ued to identify the PLWH at highest risk of spreading HIV, directing the use of tailored, resource-intensive interventions to these persons. These interventions may include linkage to care, long-acting ART and directly observed therapy (Mehta *et al*., 2019).

2.4.2 HIV Transmission Model

Transmission of HIV from one person to another depends on the biologic properties of the virus, dose in the exposed body fluid and state or nature of the host susceptibility at both cellular and immunological levels. A free virus is involved in transmission of most viral infections; in the case of HIV, integration of the virus into the cellular chromosome establishes virus-infected cells as sources of transmission. They can transfer HIV to cells of both the immune system (e.g., T cells, macrophages, dendritic cells) as well as mucosal cells lining the vaginal and anal canals.

The transmission of HIV is associated with large amounts of virus in the genital fluids, which are often found with high plasma viral loads. Sexually transmitted diseases raise the level of infectious virus and infected cells in genital fluid and can enhance HIV transmission. Lack of circumcision has been reported to increase risk of infection for males and transmission has been reduced by circumcision of male adults (Levy, 2009).

Transmission chains of HIV-1 can be determined based on sequence similarity computed either directly from a sequence alignment or by extrapolating from phylogenetic tree. An HIV-1 transmission cluster can be described as a set of HIV-1 sequences that are aggregated in a nonrandom manner linked to their epidemiology. Evolutionary theory and sequence analysis have contributed significantly to understanding of HIV-1 epidemiology, for example by providing information about the time and geographical location of HIV-1 origins. Detailed analyses of viral sequences can give beneficial information about HIV-1 epidemics through identification of transmission linkages and by illustrating differences in transmission within and between populations (Hassan *et al*., 2017).

Multiple phylogenetic studies of HIV in sub-Saharan Africa have shown that communities "export" and "import" strains, which indicates that mobility-driven transmission frequently occurs. This transmission could result to source-sink dynamics which can be defined as situation where a community is sustaining a micro-epidemic in another community where transmission is too low to be self-sustaining (Okano *et al*., 2020).

In epidemiology, the Basic Reproduction Number (R0) is used to specify the sustainability threshold. The R0 is defined as the average number of secondary infections generated by one infected individual (over their lifetime) in a community where all individuals are susceptible. If R0 is greater than one, transmission is high enough to sustain an epidemic; if R0 is less than one, it is not (Okano *et al*., 2020). HIV-1 virions that breach the mucosa may have different fates. Empirical measurements of virus replication and diversification, together with a mathematical model of random virus evolution, allow for a precise molecular identification of transmitted/founder viruses that are responsible for productive clinical infection. R0 is the

reproductive ratio. R0>1 leads to productive clinical infection, whereas R0<1 results in an extinguished infection (Figure 2.3) (Shaw and Hunter, 2012).

The HIV-1 is transmitted by sexual contact across mucosal surfaces, by maternal-infant exposure, and by percutaneous inoculation. For reasons that are still incompletely understood, CCR5-tropic viruses (R5 viruses) are preferentially transmitted by all routes. Transmission is followed by an orderly appearance of viral and host markers of infection in the blood plasma. In the acute phase of infection, HIV-1 replicates exponentially and diversifies randomly, allowing for an unambiguous molecular identification of transmitted/founder virus genomes and a precise characterization of the population bottleneck to virus transmission. Sexual transmission of HIV-1 most often results in productive clinical infection arising from a single virus, highlighting the extreme bottleneck and inherent inefficiency in virus transmission.

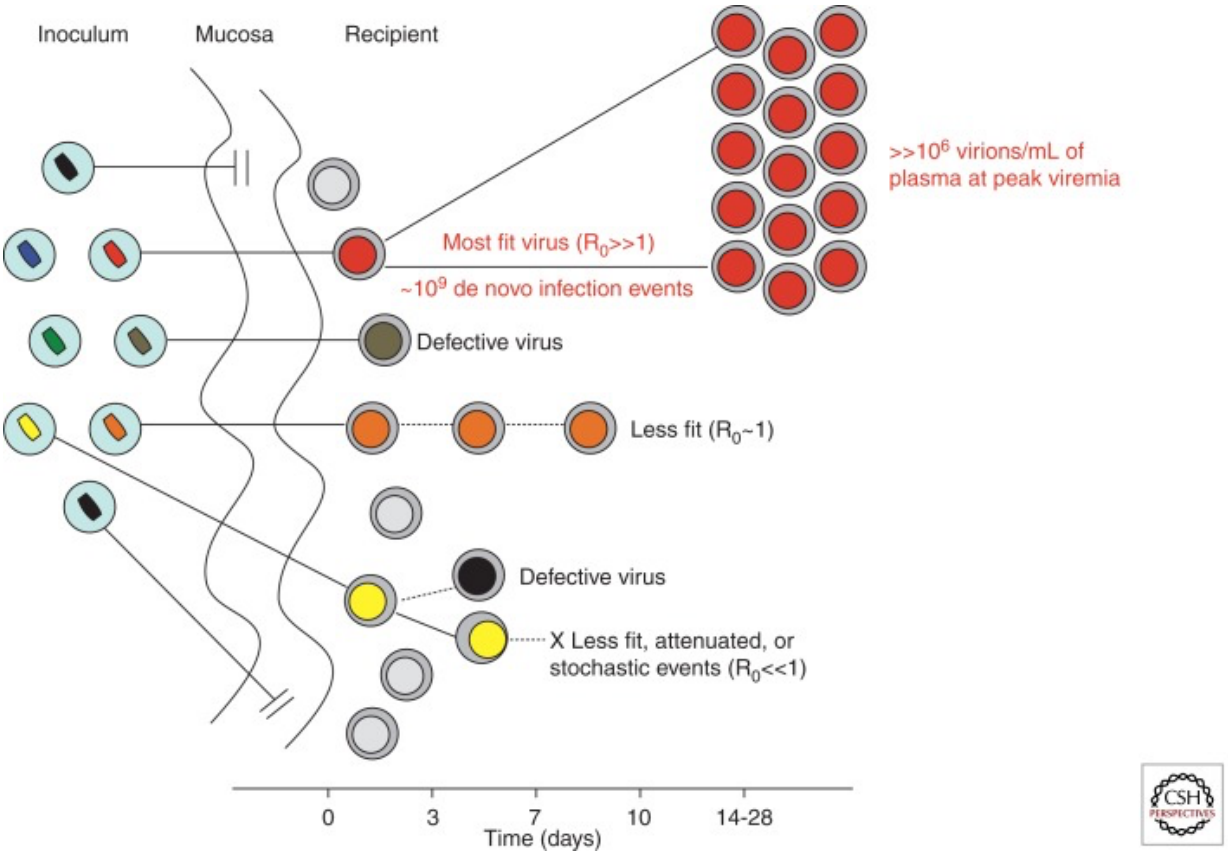

Figure 2.3: HIV-1 transmission model.

Source: Shaw and Hunter, 2012.

It remains to be determined if HIV-1 transmission is largely a stochastic process whereby any reasonably fit R5 virus can be transmitted or if there are features of transmitted/founder viruses that facilitate their transmission in a biologically meaningful way. Human tissue explant models of HIV-1 infection and animal models of SIV/SHIV/HIV-1 transmission, coupled with new challenge virus strains that more closely reflect transmitted/founder viruses, have the potential to elucidate fundamental mechanisms in HIV-1 transmission relevant to vaccine design and other prevention strategies (Shaw and Hunter, 2012).

2.5 Prevention and Treatment of HIV Infection

Despite progress in development and delivery of efficacious HIV prevention interventions, more than one million people are newly infected with HIV every year. UNAIDS have called for a reinvigoration of HIV prevention methods and suggest that 25% of global HIV spending should be allocated to prevention activities (Krishnaratne *et al*., 2016). Transmission of HIV can be prevented through use of barrier protection during sex (e.g., latex condoms), safer drug injection techniques, and adherence to universal precautions in the healthcare setting (DeHaan *et al*., 2021). Condoms reduce the probability of HIV infection per sex act as much as 95% and reduce HIV incidence in sero-discordant couples by 90 – 95% when used consistently (Foss *et al.,* 2004). Antiretroviral drugs reduce risk of mother-to-child transmission of HIV and are also efficient for post-exposure prophylaxis for parenteral and sexual exposures. Sexual transmission may be lower in couples in which one partner is infected with HIV and the other is not and the infected partner is on antiretroviral therapy (ART) (Anglemyer *et al*., 2013).

Topical microbicide formulations are applied vaginally or rectally as a strategy for HIV prevention (Cutler and Justman, 2008). Microbicides are chemical agents that prevent infection by HIV, other enveloped viruses and many sexually transmitted pathogens. Some microbicides double as contraceptives while others are not (Weber *et al*., 2005). More than 90% of pediatric infection are resulted from 'HIV mother-to-child transmission' (MTCT), during pregnancy, labour, delivery or later by breastfeeding. The MTCT has been reduced significantly in the last few years due to prophylactic perinatal antiretroviral therapy of pregnant women living with HIV (Moretton *et al*., 2017).

Nucleotide sequences from HIV infected persons are increasingly used to detect transmission cluster which will become even more important in efforts to prevent HIV transmission and reduce incidence (Oster *et al*., 2018).

2.5.1 Antiretroviral Therapy

The first antiretroviral agent approved for use is Zidovudine in 1987 and in the mid-1990s, three HIV protease inhibitors (PI) were also approved. Approval of more antiretrovirals continued and by 2014, 28 antiretroviral drugs belonging to six different mechanistic classes were approved for use in the United States (Pau and George, 2014). The main aims of antiretroviral therapy are to achieve and maintain suppression of plasma viremia and improve overall immune response. It also prolongs survival and reduces HIV-associated morbidity. Risk of Transmission of HIV to uninfected individuals is also reduced with antiretrovirals (Pau and George, 2014).

2.5.1.1 Rationale for Combination Antiretroviral Therapy

Combination of different agents targeting different steps within the HIV life cycle gives either a synergistic or additive antiviral. Four different combinations are commonly prescribed to

treatment-naïve patients (Pau and George, 2014). The combinations include a backbone of 2-NRTIs, plus one of these classes of drugs: a PI (usually boosted with ritonavir), a non-nucleoside reverse transcriptase inhibitor (NNRTI), an integrase strand transfer inhibitor (INSTI), or the C-C chemokine receptor type 5 (CCR5) antagonist maraviroc (Pau and George, 2014). Transmission of drug-resistant HIV may occur, therefore a genotypic resistance testing is generally recommended before initiation of therapy in order to avoid prescription of suboptimal therapy. In the face of the efficacy of these antiretroviral regimens, some patients may experience treatment failure with HIV drug resistance. Therapy may be more complex in these patients and require the use of multiple drug classes, based on genotypic or phenotypic drug susceptibility testing (Pau and George, 2014).

2.5.1.2 Nucleoside Reverse Transcriptase Inhibitors

The Nucleoside Reverse Transcriptase Inhibitors (NRTIs) are the critical component of most combination regimens. They exhibit a mechanism of action by phosphorylation intracellularly to their active diphosphate or triphosphate metabolites, which then inhibit the enzymatic activity of the HIV reverse transcriptase by incorporating into the nucleotide analogue, causing DNA chain termination or by competing with the natural substrate of the virus (Pau and George, 2014). Today, combination antiretroviral regimen therapy comprises 2-NRTI as a backbone and a third or fourth drug, typically an NNRTI, a boosted-PI, an INSTI, or a CCR5 antagonist (Pau and George, 2014). Examples of older NRTIs include zidovudine, stavudine, didanosine and zalcitabine, which have serious toxicities. However, newer NRTIs such as abacavir, tenofovir, lamivudine and emtricitabine have relatively less toxicities (Pau and George, 2014). The most commonly used NRTIs are tenofovir and abacavir, and the duo are used in combination with emtricitabine or lamivudine. (Pau and George, 2014).

Resistance to NRTIs is developed through discrimination when reverse transcriptase is able to differentiate an NRTI from its analogue (e.g., M184V or K65R point mutations), or phosphorolytic removal of NRTIs after incorporation into viral DNA (e.g., T215Y point mutation), also known as thymidine analogue mutations (Dionne, 2019).

2.5.1.3 Nonnucleoside Reverse Transcriptase Inhibitors

Unlike NRTIs, Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs) do not involve phosphorylation in their mechanism of activity. They are however noncompetitive inhibitors of reverse transcriptase which lead to a conformational change and, consequently, decreases the action of this enzyme (Pau and George, 2014). Examples of NNRTIs include Nevirapine, Delavirdine, Efavirenz, Etravirin and Rlipivirine (Dionne, 2019). The second-generation NNRTIs were developed to retain activity in the presence of certain high-level resistance mutations to the first-generation NNRTIs (i.e., K103N) (Dionne, 2019).

2.5.1.4 Protease Inhibitors

The Protease Inhibitors (PIs) exhibit their antiretroviral activity by binding to HIV proteases which lead to blockage of the proteolytic activities of the enzyme. Once the enzyme activities are blocked mature and infectious virions cannot be formed. Examples of PIs include ritonavir, saquinavir, lopinavir, fosamprenavir, tipranavir, atazanavir, darunavir, indinavir and nelfinavir (Pau and George, 2014).

2.5.2 Prophylaxis

Prophylaxis for HIV is divided into post-exposure prophylaxis (PEP) and pre-exposure prophylaxis (PrEP). The PEP is the administration of antiretroviral therapy to an HIV negative person who may have been exposed to HIV. This exposure can be occupational or non-

occupational, with non-occupational exposure being related to sexual or intravenous drug exposures. PrEP is the use of antiretroviral drugs in a high risk, HIV negative individuals, to prevent future HIV infections (Territo and Justiz, 2021).

2.5.2.1 Pre-Exposure Prophylaxis

Evidences have supported the efficacy of Pre-Exposure Prophylaxis in decreasing the incidence of HIV infection among high-risk population when combined with other behavioral preventive measures (Tetteh *et al*., 2017). The recommended drugs for oral PrEP are TDF or a combination of TDF/FTC which are potent and have satisfactory resistance profile with limited adverse effects (Tetteh *et al*., 2017). However there a number of concerns with implementation of PrEP which include high costs, safety screening, toxicity due to continuous use, adverse drug reactions, poor adherence, likely to abuse the drug and possible decreased condom use as an additional protective method (Tetteh *et al*., 2017).

2.5.2.2 Post-Exposure Prophylaxis (PEP)

HIV Infection from an exposure can be prevented by rapid administration of antiretroviral medication as PEP. The first dose of PEP should be administered within 2 hours of an exposure (ideal) and no later than 72 hours after an exposure (DeHaan *et al*., 2021). These exposures can either come from occupation contact with blood or another blood-containing fluids or non-occupational contact by sexual or injection-drug use (Rey, 2011). It is assumed to reduce the risk of HIV transmission by at least 80%, though its efficacy has not been completely determined. Considering the relative inefficiency of HIV transmission, and the basis that the use of PEP is often a one-time event, there are no human randomized control studies to justify the practice (Mayer *et al*., 2017). However, there are multiple animal studies that demonstrate that post-exposure dosing of antiretrovirals can protect against HIV acquisition (Mayer *et al*., 2017). There are many

guidelines to help the clinicians in their decision to offer PEP and to improve its cost-effectiveness (Rey, 2011; Mayer *et al*., 2017). A study found that the fixed dose combination of Elvitegravir/Cobicistat /TDF/FTC, a.k.a. "the QUAD pill" was well tolerated with high completion rates. It is a single pill regimen taken once daily (Mayer *et al*., 2017).

2.5.3 HIV Vaccine Development

Even with progress in highly active antiretroviral therapy, pre-exposure prophylaxis, microbicides, and other preventive approaches, a vaccine to prevent HIV-1 infection is still greatly needed to curtail the epidemic (Heger *et al*., 2018). Genetic diversity of HIV-1 remains a major obstacle to the development of vaccine because of need to have vaccine that will protect against divergent HIV subtypes and recombinant. The design, testing, and implementation of HIV vaccines need latest and accurate knowledge of the distribution of HIV subtypes and recombinants in different parts of the world. This is because vaccine immunogen sequences need to match, as closely as possible, the viral sequences circulating in the target population (Hemelaar *et al*., 2018).

Hundreds of vaccine candidates were clinically tested since 1987 however only six HIV vaccine efficacy trials have been completed (Gao *et al*., 2018). Three factors have majorly affected prompt HIV vaccine development which includes the genetic variability nature of the virus, the absence of important information on the immunological protection mechanism against the virus and the lack of good animal models (Sisay *et al*., 2018). Nevertheless great events were recorded in the timeline of HIV vaccine development such as RV144 clinical trial and the recent identification of broadly neutralizing HIV-1 antibodies. Out of six HIV-1 vaccine trials, only RV144 showed efficacy through induction of functional antibodies to V1V2 region of HIV envelope (Hsu and O'Connell, 2017). The RV144 has demonstrated an efficacy of 31.2% and thereby revived hope

and can be served as a proof that an effective HIV vaccine is possible (Heger *et al*., 2018; Sisay *et al*., 2018).

There are many vaccine candidates that are undergoing clinical and preclinical trials. For instance, the two-viral vector-based vaccine trials, Imbokodo and HVTN 702, that resulted from years of scientific testing and clinical development and are currently representing the best efforts in HIV vaccine development (Gao *et al*., 2018). In due course, an effective HIV vaccine is expected to elicit a diverse antibody response that includes broadly neutralizing antibodies (bNAbs) and non-neutralizing antibodies with Fc receptor-mediated antiviral effector functions (Pollara *et al*., 2017).

2.6 HIV-1 Pathogenesis

Destruction of CD4+T lymphocytes and subsequent loss of immune competence is considered to be the hallmarks of AIDS. Two hypotheses have been proposed in attempt to identify mechanisms by which HIV-1 causes disease. A hypothesis proposed that HIV deplete CD4+T lymphocytes by directly infecting and killing them. Another hypothesis was based on the observation that HIV-1 infection indirectly impairs cell function, perhaps because of an aberrant reaction to the infection by the host's immune response (Stevenson, 2003).

The CD4 was first demonstrated as a receptor for HIV and after an interval of about a decade the chemokine receptors CCR5 and CXR4 were identified as coreceptors for HIV-1. This distribution of the receptors allows infection in addition to CD4+T cells of macrophages and dendritic cells. The virus has adapted to these cells and therefore, may exist in various replication states and tissue compartments and survive even in the presence of active host immune responses and antiretroviral treatment regimens (Stevenson, 2003). During acute or early infection, the R5 viruses are first

observed in the blood and then more cytopathic X4 viruses emerges. Some studies have reported that the R5 virus often evolves into a dual-tropic R5/X4 virus and then to an X4 virus (Levy, 2009).

2.6.1 Factors Influencing HIV Pathogenesis

2.6.1.1 Depletion of CD4 Cells

The major reason for opportunistic infections and cancers associated with HIV infection is the destruction of CD4 T cells. Factors that influence CD4 cell death and HIV replication include certain cytokines (e.g., TNF-α) and HIV proteins (e.g., Tat, Nef, Vpr, Vpu). The CD4 cell can also be lost by the conventional disruption in metabolic processes and cell membrane integrity by HIV causing necrosis. The CD4 cell apoptosis may result from direct virus infection or indirect effect of immune activation. Autophagy and CD8 cytotoxic T-cell activity against normal CD4 cell have also been reported to cause CD4 Cell death (Levy 2009).

2.6.1.2 Immune Activation

Production of pro-inflammatory cytokines such as TNF- α as hyperimmune response can result to increased HIV production and loss of CD4 and CD8 T cells through apoptosis (Levy, 2009). Human Immunodeficiency Virus infection is hardly cleared by the immune system and unlike other chronic viral infections, HIV causes intensive non-antigen-specific T-cell activation. Typically, T cells proliferating in response to infection will give rise to a small proportion of long-lived resting memory cells, preserving T-cell homeostasis. However, the widespread activation of T cells in HIV infection may continually drain the naïve and resting memory CD4+ T-cell pools, causing failure of T-cell homeostasis and eventual CD4+ T-cell depletion (Hunt 2007).

2.6.1.3 Co-factors in HIV Pathogenesis

Coinfection of HIV with other microbial infections such as herpesvirus or GB virus C can enhance or delay HIV pathogenesis respectively. Some polymorphisms in the human histocompatibility locus can affect the level of anti-HIV response by cellular component of immune complex. These human histocompatibility antigen polymorphisms can result to fast or slow progressors and High-risk exposed HIV-seronegative individuals (Levy 2009). Relative expression of chemokine receptors (e.g., CCR5), chemokines, and other cytokines have been reported to influence disease progression. In the same vein, homozygosity for variant MBL2 alleles is associated with an increased risk of HIV infection and progression to AIDS (Levy 2009).

2.6.1.4 Intrinsic Intracellular Factors

During HIV reverse transcription, APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G) (and 3F) a cytosine deaminase, alters single stranded DNA synthesis and thereby, causing production of an inactive DNA which is destroyed by the host cells. The human immunodeficiency virus type 1 depends on Vif (viral infectivity factor) to overwhelmed the antiviral function of the APOBEC3G. Higher expression of APOBEC3G and 3F in a cell is associated with lower viral replication during early infection. These proteins could also be responsible for the resistance or latent infection of resting CD4 cells (Levy 2009).

2.7 Overview of HIV-1 Laboratory Diagnoses

Accurate and prompt HIV diagnosis of HIV infection increase survival rates of the infected individuals and reduce the spread of the virus (Kabir *et al*., 2020). Treatment of patients with ART is being monitored to evaluate effectiveness and emergence of possible resistance through

measurement of CD4 cells, viral RNA load and mutations that could cause drug resistance (Kabir *et al*., 2020).

The genetic variability of HIV-1 may pose significant problems for the specificity and/or sensitivity of serological and molecular diagnostic tests, which may represent a serious risk factor for the spreading of unidentified infections. Fourth-generation HIV immunoassays are designed to detect both the HIV p24 antigen and antibody in a single test in order to reduce the seroconversion window. The sensitivity of these assays for the p24 antigen and seroconversion, however, can vary significantly. Nevertheless, a comparative study reported in 2007 has shown that some of these immunoassays have levels of specificity and sensitivity comparable to those of second- and third-generation assays designed to detect either antigens or antibodies and can identify most of the known group M subtypes, the most diffused CRFs (CRF01_AE and CRF02_AG), and group O viruses (Buonaguro *et al*., 2007).

2.7.1 HIV Screening Using Rapid Diagnostic Test (RDT) Kits

In 1985, the first HIV antibody was developed to screen blood products rather than diagnosis of AIDS. Serologic tests for HIV have been characterized into generations based on the principle of the tests. Every succeeding generation led to a shorter false-negative window period between infection and detection (Branson, 2019).

First-generation assays detected IgG antibody with test-negative window of 6 to 12 weeks (Branson 2019). The second-generation assays have recombinant antigens while third-generation assays included IgM detection, reducing the test-negative window to approximately 3 weeks post-infection. Fourth- and fifth-generation HIV assays included p24 antigen detection to the screening assay, reducing the test-negative window to 11 to 14 days (Alexander 2016; Branson 2019).

The HIV testing is a vital intervention in both diagnoses and management of HIV infection. The laboratory diagnosis includes series of tests with an algorithm for resolving discordant test results to maximize overall sensitivity and specificity (Branson 2019).

2.7.2 The HIV Plasma RNA Quantification

The HIV RNA quantification (also called viral load) has potential benefits as it assist to check treatment failure and challenges with drug adherence by patients. It is also essential in early infant diagnosis *and* applications include quality assessment, resistance surveillance, and vaccine efficacy studies (Calmy *et al*., 2007). New devices for point-of-care HIV viral load are available to reduce turnaround time and hence improve HIV management (Drain *et al*., 2019). A viral load of less than 200 copies/ml designates successful of viral suppression. However, when the viral load rises above 1000 copies/ml after six-months from date of ART initiation, it shows the emergence of drug-resistance and may lead to virological ART failures (Kabir *et al*., 2020).

2.7.3 Drug Resistance Testing

Effort to reduce the emergence and transmission of HIV drug resistance (HIVDR) is a vital feature of the global response to antimicrobial resistance (AMR). Prevention, monitoring and response to HIVDR is key to building and sustaining gains in HIV treatment scale-up, and achieving the global 90-90-90 targets for treatment: diagnosing 90% of all people with HIV infection; providing treatment to 90% of those diagnosed; and ensuring 90% of people on treatment achieve virological suppression, by 2020 (WHO 2017). Drug resistance testing shows which drugs not to use and saves costs associated with switching drugs too early or using drugs that are no longer effective. Toxicity of inactive drugs can also be avoided and it may prevent further development of resistance and improved short term response in several prospective studies (WHO 2017).

The HIV-1 drug resistance testing is conducted in vitro phenotypically or genotypically. Phenotypic in vitro susceptibility assays measure ARV susceptibility in cell culture. The susceptibility is reported as the antiretroviral concentration that inhibits HIV-1 multiplication by 50% (IC_{50}). The IC_{50} is compared to that of a drug-susceptible reference strain, and reported as a ratio, and termed as fold change, of the IC_{50} of the patient's virus relative to the reference control. Phenotypic resistance testing is usually reserved for drug development, drug resistance research, or complex clinical cases because it is not cost effective and lengthy turnaround time (Clutter *et al*., 2016).

Standard genotypic resistance testing (SGRT) comprises the use of dideoxyterminator Sanger sequencing of HIV *protease*, *reverse transcriptase* (RT), and/or *integrase* to detect recognized clinically significant DRMs. The sanger sequencing is carried out on PCR products amplified from cDNA that has been reverse-transcribed from plasma RNA (Clutter *et al*., 2016). The French National Agency for AIDS Research (ANRS AC11) Resistance Group (2015) has recommended resistance study in several populations. The HIV drug-resistance testing in plasma is recommended in persons with HIV infection at entry into care regardless of whether antiretroviral therapy (ART) will be initiated immediately or deferred. Genotypic testing in plasma is recommended as the preferred resistance testing to guide therapy in antiretroviral (ARV)-naive patients. Standard genotypic drug-resistance testing in plasma in ARV-naive persons involves testing for mutations in the reverse transcriptase (RT), protease (PR) and integrase (IN) genes (ANRS 2015).

HIV drug-resistance testing in plasma should be performed to assist in the selection of active drugs when changing ARV regimens in persons with virologic failure (confirmed plasma HIV RNA >50 copies/mL). In persons with plasma HIV RNA >50 but <200 copies/mL, testing may be unsuccessful in plasma and testing using proviral DNA (cell associated HIV DNA) could be

considered. In any case of virological failure, plasma drug level measurements of ARVs should be performed (ANRS 2015).

Drug-resistance testing in the setting of virologic failure should be performed while the person is taking prescribed ARV drugs or, if not possible, within 4 weeks after discontinuing therapy. If greater than 4 weeks has lapsed since the ARVs were discontinued, resistance testing may still provide useful information to guide therapy, recognizing that previously selected resistance mutations can be missed (ANRS 2015).

Drug-resistance testing in the setting of virologic success in patients with previous episodes of virologic failures, not documented with resistance testing in plasma, should be performed if a therapeutic change is needed for other reasons (side effects, treatment simplifications, etc.) using proviral DNA sequencing (cell associated HIV DNA), recognizing that previously selected resistance mutations can be missed (ANRS 2015).

In any case of HIV RNA >50 copies/mL in CSF of a patient treated by ARV, genotypic resistance testing should be performed on the CSF. ARV drug level measurements should be also performed in CSF (ANRS 2015).

2.8 Molecular Basis of HIV Drug Resistance

High rate of HIV-1 reverse transcriptase processing error, genetic recombination and the accumulation of proviral variants during the course of infection result to HIV-1 genetic variability. The selection of drug-resistant variants is influenced by the level at which viral replication continues during incompletely suppressive therapy, the ease of acquiring particular drug resistance mutation (DRM), and the effect of DRMs on drug susceptibility and virus replication. Virtually all clinically significant DRMs arise only as a result of selective drug pressure and are otherwise

nonpolymorphic. Only single DRM may be sufficient to cause reduced susceptibility to some ARVs while other may require multiple DRMs (Clutter *et al*., 2016). Drug resistance mutation can be characterized as primary DRMs which directly reduce drug susceptibility and accessory DRMs which enhance fitness of variants containing primary DRMs or that contribute further reductions in susceptibility (Clutter *et al*., 2016).

There is essentially no cross-resistance between drug classes. Viruses that are highly resistant to drugs in one ARV class are completely susceptible to ARVs from unused classes. In contrast, significant cross-resistance within a drug class is common because most DRMs reduce susceptibility to multiple ARVs of the same class. The first-line ART regimens are adequately potent to completely block HIV-1 replication and have a genetic barrier to resistance good enough to sustain virological suppression. Therefore, most cases of virological failure and drug resistance arise from poor drug adherence which exposes the virus to the incompletely suppressive ARV levels capable of exerting drug selective pressure (Clutter *et al*., 2016).

2.8.1 Nucleoside Reverse Transcriptase Inhibitors Resistance Mutations

There are two genetic mechanisms of NRTI resistance: (i) discriminatory mutations that assist RT to distinguish between dideoxy-NRTI chain terminators and the dNTPs of the cell, and consequently inhibiting NRTIs from being incorporated into viral DNA; and (ii) primer unblocking mutations that enable the phosphorylytic excision of an NRTI-triphosphate from viral DNA. These unblocking mutations are also called thymidine analogue mutations (TAMs) because they are selected by the thymidine analogues AZT and stavudine (d4T)

The commonest NRTI-resistance mutations are M184V/I which are selected by and cause high-level (>200-fold) reduced susceptibility to the cytosine analog NRTIs, 3TC and FTC. M184V/I

are also selected by, and causes low-level resistance to abacavir (ABC). During virological failure on a regimen containing either 3TC or FTC, M184I often emerges before M184V. However, in most patients M184V will outcompete M184I within several weeks. Despite the high-levels of 3TC and FTC phenotypic resistance associated with M184V/I, NRTIs are continued to be used to treat viruses with M184V/I because maintenance of these DRMs reduces virological fitness and increases susceptibility to AZT and TDF.

The next most common discriminatory mutations include K65R, K70E/G/Q, L74V/I, Y115F and the Q151M complex of mutations. K65R is selected primarily by TDF and to a lesser extent by ABC and d4T. K65R increases susceptibility to AZT. Even though K65R reduces TDF susceptibility in vitro by only two-fold, it is, after M184V, the most common DRM in patients with VF on a TDF-containing regimen. L74V/I and Y115F are selected primarily by ABC. However, L74I and Y115F are also often selected by TDF, particularly in LMICs where resistance tests are often performed after prolonged VF. K70E/G/Q are also selected by TDF and ABC-containing regimens and are associated with minimal reductions on in vitro susceptibility to these NRTIs.

The Q151M is an uncommon DRM that usually occurs only in heavily treated patients with prolonged virologic failure. It usually occurs in combination with several accessory DRMs (A62V, V75I, F77L and F116Y) and is associated high-level resistance to AZT and ABC and intermediate resistance to TDF, 3TC, and FTC. The TAMs include M41L, D67N, K70R, L210W, T215F/Y and K219Q/E. Although they are selected only by AZT and d4T, they confer lower levels of cross-resistance to TDF and ABC.

The TAMs occur in two distinct but overlapping patterns denoted as type I and type II. The type I TAMs, which include M41L, L210W and T215Y, cause higher levels of phenotypic and clinical cross resistance to TDF and ABC than the type II TAMs (D67N, K70R, T215F and K219Q/E). The presence of all three type I TAMs markedly worsens clinical response to TDF- and ABC-containing regimens. The TAMs and discriminatory mutations other than M184V/I occur through two separate opposing pathways. Viruses already containing multiple TAMs are more likely to develop additional TAMs than K65R when treated with TDF or ABC. Some viruses with multiple TAMs will also develop a double-amino acid insertion at RT position 69 referred to as T69S_SS (indicating a wild type threonine replaced by three serines) or simply T69insertion. In combination with multiple TAMs this DRM causes high level resistance to AZT, ABC, and TDF, and intermediate resistance to 3TC and FTC (Clutter *et al*., 2016).

2.8.2 Non-Nucleoside Reverse Transcriptase Inhibitors Resistance Mutations

Because NNRTIs have relative low barrier to resistance, to develop high-level resistance, one DRM in the case of nevirapine (NVP), one to two DRMs in the case of efavirenz (EFV), and two DRMs in the case of etravirine (ETR), are required. Rilpivirine (RPV) has an analogous structure to ETR; however, its genetic barrier to clinically significant resistance is lower than ETR because it is administered at a much lower.

There is a high level of cross-resistance within the NNRTI class as a result of two mechanisms: (i) most NNRTI-resistance mutations reduce susceptibility to two or more NNRTIs; and (ii) the low genetic barrier to NNRTI resistance allows for the emergence of multiple independent NNRTI-resistant lineages, even if not all are detectible by (Clutter *et al*., 2016).

The most common NNRTI mutations are L100I, K101E/P, K103N/S, V106A/M, Y181C/I/V, Y188C/H/L, G190A/S/E, and M230L. Each causes an intermediate or a high-level phenotypic resistance to NVP. All with the exception of Y181C/I/V cause intermediate- or high-level phenotypic resistance to EFV. With the exception of K103N/S, V106A/M, Y188C/H, and G190A/S, they cause phenotypic ETR and RPV resistance. In addition, the previously unrecognized mutations E138K/G/Q/A are among the most common mutations to emerge in patients receiving RPV (Clutter *et al*., 2016).

2.8.3 Protease Inhibitors Resistance Mutations

The commonly used Protease Inhibitors (PIs) are boosted lopinavir (LPV/r), boosted atazanavir (ATV/r), and DRV/r however; ATV/r and DRV/r are the two most commonly recommended PIs in high income countries and LPV/r is widely used in second-line regimens in LMICs. LPV/r and DRV/r have high genetic barriers to resistance because multiple DRMs are required before antiviral activity is compromised. LPV/r and DRV/r have also been shown to be effective at maintaining virological suppression in ARV-experienced patients undergoing regimen simplification. Several lines of evidence suggest that ATV/r has a lower genetic barrier to resistance than LPV/r and DRV/r: (i) fewer mutations are needed for reductions in in vitro susceptibility; (ii) smaller reductions in in vitro susceptibility are required to increase rates of clinical virological failure; (iii) ATV/r monotherapy has also been less effective than LPV/r and DRV/r for regimen simplification. PI DRMs develop much less frequently in patients receiving an initial LPV/r, ATV/r, or DRV/r-containing regimen than do NRTI and NNRTI DRMs in patients receiving NRTI/NNRTI containing regimens (Clutter *et al*., 2016).

The DRMs with the greatest impact on susceptibility are in the substrate cleft including V32I, G48V/M, I50V/L, V82A/T/L/F/S/C/M, and I84V/A/C. In addition, several mutations in the

enzyme flap including M46I/L and I54V/M/L/T/S/A, and in the enzyme core L33F, L76V, and N88S also markedly reduce susceptibility. Most PI-resistant viruses also require one or more accessory protease mutations and one or more compensatory gag cleavage-site mutations.

The requirement for multiple accessory mutations outside of protease practically contributes to the high genetic barrier to resistance of boosted PIs. The ATV-selected DRM, I50L, is outstanding for causing high-level resistance to just one PI. However, each of the remaining major mutations reduces susceptibility to two or more PIs. Due to the large number of PI DRMs and the fact that different DRMs at the same position can have markedly different effects on PI susceptibility, PI cross resistance is complex and that is the case for DRMs at positions 50, 54 and 82. Due to their high genetic barriers to resistance, DRV/r and LPV/r are often used in patients failing therapy, even those who are PI-experienced (Clutter *et al*., 2016).

2.8.4 Integrase Strand Transfer Inhibitor Resistance Mutations

Raltegravir (RAL) was the first FDA-approved Integrase Strand Transfer Inhibitor (INSTI) and has many and available associated information. RAL resistance occurs often by overlapping mutational pathways with DRMs signature as N155H±E92Q; Q148H/R/K±G140S/A; and Y143C/R. The presence of two of these signature DRMs is usually associated with >150-fold reduced RAL susceptibility. Although each of these DRMs alone is associated with much lower levels of phenotypic resistance, they are likely a indication of high-level resistance. Elvitegravir (EVG) shares two mutational pathways with RAL: N155H±E92Q and Q148H/R/K±G140SA. E92Q alone is particularly common in patients receiving EVG and is often the most common EVG-resistance DRM in clinical trials. There are also two non-polymorphic DRMs – T66I/K and S147G – that occur more commonly with EVG and cause lower-levels of cross-resistance to RAL.

Considering the extensive overlap between the genetic mechanisms of resistance to RAL and EVG, there are no scenarios in which patients with VF while receiving one of these INSTIs should be switched to the other INSTI (Clutter *et al*., 2016).

2.8.5 HIV-1 Tropism

Maraviroc allosterically inhibits gp120 Env of CCR5 (R5)-tropic HIV-1 strains from binding to the seven-transmembrane G protein-coupled R5 receptor. Whereas HIV-1 gp120 binds to the N-terminus and second extracellular loop region of R5, maraviroc binds to a pocket formed by the transmembrane helices. In patients receiving R5 inhibitors, the most common mechanism of VF is the expansion of pre-existing CXCR4 (X4) tropic viruses that are intrinsically resistant to R5 inhibitors. The HIV-1 can also develop maraviroc resistance mutations that allow HIV-1 gp120 to bind to an inhibitor-bound R5 receptor. However, reports of such resistance have been documented primarily in vitro and in only a small number of clinical viral isolates. More than 80% of patients are initially infected with exclusively R5 tropic viruses. X4 tropic viruses usually emerge during later stages of infection, and about 50% of patients chronically infected with HIV-1 harbor X4 tropic viruses. X4-tropic viruses are often present in low proportions relative to R5-tropic viruses when they emerge. A tropism assay should be performed to confirm that a patient is not infected with X4- tropic variants before using maraviroc. Tropism can be detected phenotypically or genotypically. The phenotypic profile test assesses the tropism of complete env genes amplified from patient samples using reporter cell lines expressing R5 or X4. Given a sufficiently high plasma virus level, it has the sensitivity to detect X4 variants at populations less than 1%. Genotypic testing for tropism involves sequencing the envelope V3 loop and using algorithms to predict tropism from the sequence. Positively charged residues at positions 11 and 25 of the V3 loop of gp120 and several less common combinations of mutations primarily but not exclusively

within the V3 loop are associated with X4 tropism. SGRT has a specificity of ~85% and sensitivity of 50–70% for detecting the presence of X4 tropic variants. The low sensitivity of SGRT arises from the fact that X4 tropic variants are often present at levels below the 20% sensitivity of SGRT and because some of the determinants of tropism lie outside of the V3 loop. However, sequencing multiple independent aliquots of viral cDNA or using NGS deep sequencing technology can increase the sensitivity of genotypic tropism testing. Several clinical laboratories in high-income countries offer one or more genotypic approaches for detecting minor X4 tropic viruses.

2.9 Effect of Subtypes on HIV-1 Drug Resistance

All mutations that cause resistance in subtype B viruses do same in non-B subtypes. However, several DRMs preferentially occur in certain HIV-1 subtypes. The majority of these subtype-specific differences in DRM distribution are attributed to differences in codon usage. The NNRTI DRM V106M occurs more often in subtype C viruses from patients treated with NVP or EFV because V106M requires a single base-pair change in subtype C viruses – GTG (V) => ATG (M) – but a two base-pair change in all other subtypes – GTA (V) => ATG (M).

CRF01_AE viruses favorably develop the NRTI DRM V75M, subtype G viruses favorably develop the PI DRM V82M, and subtype A viruses favorably develop the NNRTI DRMa G190S by a similar mechanism. In subtype B viruses, integrase G140 is encoded primarily by GGT or GGC whereas for nearly all other subtypes, G140 is encoded primarily by GGG or GGA. Therefore, a single G to A change at the first position of the G140 codon results in a serine in subtype B viruses, but not in other subtypes. G140S is an important compensatory mutation for Q148 mutations and this may explain why Q148 mutations occur significantly more commonly among subtype B patients compared to non-B patients with RAL VF.

Several lines of evidence suggest that K65R is more likely to emerge in subtype C viruses than viruses belonging to other subtypes. First, biochemical studies have shown that the unique subtype C sequence in the region of K65R, specifically five consecutive adenosines preceding the adenosine at the second position in the K65 codon, facilitates mutation during reverse transcription). Second, K65R emerges more rapidly during in vitro passage of subtype C viruses than during passage of subtype B viruses in the presence of tenofovir. Third, three retrospective studies have suggested that in patients who develop VF on a TDF-containing regimen are at a somewhat higher risk of developing K65R if they are infected with a subtype C virus. Despite the reported tendency to acquire the K65R DRM, a recent study showed that when demographic and clinical factors are controlled for among patients receiving at TDF-containing regimen, infection with a subtype C virus does not increase the risk of developing VF.

2.10 Transmitted Drug Resistance

Transmitted Drug Resistance (TDR) is a type of HIVDR that occurs when primary HIV infection is caused by a DRM bearing virus. The prevalence of TDR by SGRT is about 12% to 24% in the U.S., and 10% in Europe. Though with limited data, it has been suggested that TDR prevalence is below 5% in most of the LMICs in sub-Saharan Africa and South / Southeast Asia, intermediate TDR rates of 5 to 10% have been reported from Latin America, Eastern Europe, and high-income Asian. Rising temporal trends in TDR continue to be reported from many regions, mainly connected to increases in NNRTI. Since DRMs reduce viral fitness, many transmitted drug-resistant viruses steadily revert to wild type over a period of several years.

In the absence of selective drug pressure, PI and NNRTI DRMs gradually revert to wild type after an average of approximately 3 years. Within the NRTI class, the rate of reversion is more variable: M184V usually fades within one year; T215FY also fades rapidly; but T215 revertants persist for

more than ten years. The most common transmitted NNRTI-associated DRMs include K103N, Y181C, G190A, and K101E which combined accounted for >80% of NNRTI-associated TDR. The most common NRTI-associated DRMs include M184V, the TAMs, the revertants, T69D, and F77L. Not surprisingly, M184V occurs more commonly in recently infected patients compared with chronically infected patients. The most common PI SDRMs were L90M, M46I/L, I85V, I54V, N88D, I84V, D30N, G73S, and they occurred less frequently. Although transmitted INSTI-associated resistance has been practically absent in ongoing surveillance efforts, there have been at least four case reports of INSTI-associated TDR (Clutter *et al*., 2016).

The TDR arises from two distinct sources: (i) from patients failing therapy who have developed ADR, or (ii) from an ART-naïve person with TDR, a phenomenon termed "onward transmission". With a greater number of intervening transmission events in the case of onward transmission, the prevalence of DRMs with high fitness costs, such as M184V, is likely to decrease. Onward transmission is therefore responsible for established drug-resistant lineages that typically contain fit drug-resistant strains.

In high-income countries, SGRT is recommended at the time of a patient's initial diagnosis. If therapy is delayed, SGRT may be repeated at the time of ART initiation if the patient is considered to have been at risk for superinfection. In patients with transmitted NRTI resistance, the selection of first line therapy should be based on the extent of NRTI resistance. Both LPV/r and DRV/r-containing regimens have been shown to be highly effective for treating patients with multiple NRTI and NNRTI-associated DRMs following the failure of a first-line NRTI/NNRTI-containing regimen. Therefore, they are each also likely to be highly effective at treating ARV-naïve patients harboring multiple NRTI-resistance mutations. However, first-line ATV/r, RAL, or EVG-containing regimens should be used only for treating patients with minimal transmitted NRTI

resistance. The use of DTG-containing regimens in this scenario may be useful but needs further study. Due to historically lower levels of TDR in LMICs and cost-effectiveness considerations, individual-level baseline SGRT is not recommended by the WHO in LMICs. However population-level HIVDR surveillance programs are strongly recommended and they have played a key role in tracking TDR trends and in shaping first-line ARV regimen recommendations (Clutter *et al*., 2016).

In LMICs, ART programs are facing important challenges such as ARV stock outs and suboptimal retention in care. Patients experiencing HIV treatment interruptions are more likely to carry DRMs. When these patients re-engage into care, they most frequently re-initiate treatment with the same first-line regimen with which they were previously treated, despite the increased risk of resistance relative to the truly ART-naive population. Therefore, to assess the predicted activity of first-line ART, the WHO recommends monitoring pre-treatment resistance (PDR) not only among individuals initiating ART for the first time but also among individuals re-initiating first-line ART. Primary drug resistance (PDR) is indeed the result of either TDR or acquired drug resistance (ADR), the later due to prior ARV treatment among re-starters, prior exposure to PMTCT among women, or prior ARV prophylaxis. The PDR surveys will be critical given the more recently observed signals of high level HIVDR in several LMIC settings, including Angola (16%), Cuba (22%), Mexico (12%), Papua New Guinea (16%), Botswana (10%) and South Africa (14%). In the absence of SGRT-guided therapy, national PDR surveillance among individuals starting ART is critical to predict the activity of available first-line ART and inform the composition of these regimens. Where PDR levels remain low, patients are anticipated to do well on the WHO-recommended first-line regimen of EFV + TDF/3TC or FTC. Where PDR levels are high, alternative first-line regimens may become necessary for all starters or for a subset of those

reporting prior ARV drug exposure (that is, people who defaulted from ART and restart, women exposed to PMTCT and perhaps those infected despite ARV drug prophylaxis [i.e. post-exposure prophylaxis and PrEP]). Although these surveys are strongly recommended by WHO and are essential to address data gaps in LMIC, their implementation has been slow due to lack of awareness of HIVDR as a public health threat, limited resources, and competing national and donor priorities (Clutter *et al*., 2016).

2.11 Acquired Drug Resistance

More than 70-80% of patients with virologic failure develop ADR. M184V/I is usually selected as the initial NRTI DRM for cytidine analog (3TC or FTC) containing regimens. Among patients receiving an NNRTI-containing regimen, one or more NNRTI DRMs are also typically seen early in VF. Other NRTI DRMs usually occur after M184V/I and the NNRTI. INSTI DRMs may develop early in treatment, but PI DRMs are rare and are generally a late occurrence.

Resistance is detected more often in patients with infrequent viral load testing because VF is usually detected later, after DRMs have had time to accumulate. Through this mechanism of prolonged VF, infrequent viral load testing may jeopardize the efficacy of second-line options, particularly NNRTI- and INSTI-based regimens. Patterns of ADR at VF vary according to regimen and patient adherence levels. Indeed, the lack of any ADR in the setting of treatment failure usually indicates non-adherence. Because all recommended first-line regimens contain a cytidine analog, M184V is the most commonly acquired DRM in both high-income countries and LMICs. The later NRTI DRMs to develop depend on the second NRTI in the regimen. Although both d4T and AZT are thymidine analogs, they differ somewhat in the mutations they select. AZT selects almost exclusively TAMs whereas d4T selects primarily TAMs but may also select discriminatory mutations including K65R. TDF selects primarily for K65R and less commonly for K70E/G/Q

and Y115F. Abacavir selects primarily L74V/I, Y115F, and less commonly K65R (Clutter *et al*., 2016).

The NNRTI DRMs are selected early and often by NNRTI-based regimens, occurring in the vast majority of patients with VF. In both high-income countries and LMICs, K103N followed by Y181C, G190A/S, Y188L, and V106M are the most commonly observed acquired NNRTI DRMs. After failing a first-line NNRTI-based regimen containing EFV or NVP, cross-resistance to the second generation NNRTIs RPV and ETR is common and has been associated with VF. In previously PI-naïve patients, PI DRMs often occur in approximately 10% or fewer patients failing PI-based therapy. As noted previously, a high proportion of patients with VF on an initial PI based regimen but without PI DRMs will achieve virological suppression with improved adherence alone. In patients with VF on a first-line RAL- or EVG-based regimen, INSTI resistance is not uncommon. In contrast, treatment-emergent INSTI resistance is rarely observed in patients failing first-line DTG-based regimens. In high-income countries, SGRT is recommended in patients with VF when the viral load is >1000 copies/mL, and should be considered when the viral load is >50 copies/mL.

In patients with VF, the ability to detect ADR is much higher, if SGRT is performed while a patient is on the failing regimen or within four weeks of discontinuation. Genotypic testing can indicate whether VF is due primarily to non-adherence – i.e., if no DRMs are present – and can also be used to help design second-line therapy when ADR is the cause of VF. Patients who develop VF without DRMs can often be treated successfully with adherence counseling alone. In contrast, patients with VF who have developed DRMs on a first-line regimen usually require a change in ARVs. In high-income countries where SGRT is routinely performed in the setting of VF, a second-line regimen should include at least two, preferably three, fully active agents. As a general

principle, adding a single fully active ARV to a failing regimen is not recommended due to the risk of selecting for resistance to the new ARV. A fully active agent is defined as an ARV which is expected to have activity based on the patient's treatment history and SGRT results. Managing patients with ADR who are failing second-line regimens is more challenging, and often requires expert guidance. With complex patterns of resistance, phenotypic testing may be useful to determine the activity of various ARVs. The number of available fully active ARVs diminishes with each successive treatment failure (Clutter *et al*., 2016).

Therefore, a salvage regimen in patients who have experienced multiple VFs and who have multiclass resistant viruses is likely to be complicated and to require multiple ARVs that may have only partial residual activity. Late salvage therapy ARVs can be placed into the following categories: (i) ARVs belonging to a previously unused drug class. Enfuvirtide is a highly active treatment option for late salvage therapy. However, it is not a popular treatment choice among clinicians and patients because of its requirement for parenteral intramuscular administration. Although maraviroc is well tolerated, it is often not an option because patients with multiple VFs tend to have more advanced immunosuppression and an increased likelihood of harboring X4-tropic viruses; (ii) ARVs belonging to a previously used drug class that retain significant antiviral activity including the PIs DRV/r and tipranavir/r, the INSTI dolutegravir, and in some instances the NNRTI ETR; (iii) NRTI combinations, which often retain in vivo virological activity even in the presence of reduced in vitro NRTI susceptibility; (iv) ARVs associated with previous VF episodes and treatment-emergent drug resistance that may have regained their activity as a result of viral reversion to wild type; and (v) investigational ARVs . In most LMICs, a diagnosis of ADR is made empirically and the selection of second-line therapy is guided by WHO recommendations. A patient is presumed to have ADR when two viral loads performed 3 months apart are both above

1,000 copies/mL, and a switch to second-line therapy is indicated (World Health Organization, 2015b). The preferred second-line options include a boosted PI, namely ATV/r or LPV/r, and two NRTIs. Subsequent options include DRV/r plus two NRTIs or LPV/r plus RAL. These alternatives are equally efficacious, but due to higher costs they are not currently preferred options in second-line regimens. (Clutter *et al*., 2016).

Another consideration is that, as previously noted, PI-based regimens rarely select for PI DRMs, but failure of a RAL-containing regimen often selects for INSTI DRMs that may jeopardize INSTI activity in subsequent regimens. Second-line NRTI selection is directed by the first-line NRTIs used: if VF developed on TDF/FTC or 3TC, then AZT/3TC is recommended; if VF developed on AZT/3TC, then TDF/FTC or 3TC is recommended (World Health Organization, 2015a). In some LMICs with limited access to SGRT, resistance testing is recommended at VF of second-line therapy (Republic of South Africa Department of Health, 2014) and is managed according to the resistance detected and regimens available (Clutter *et al*., 2016).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study area was Kaduna State, Nigeria which is in the centre of the northern Nigeria and more than 1000km from the Atlantic Ocean. It is located on latitude of 11° 12'' N and longitude of 07°37'' E. The state has its capital at Kaduna city. According to the National Population Commission, the 2006 census puts the population of the State at 6,066,562 (NPC, 2006). It is characterized by a tropical climate with two seasons; a wet season of approximately 210 days (May to October), with a mean annual rainfall of 1092.8mm, and the dry season which spans between November and April. The monthly mean temperature shows a maximum of 39.7°C in June and 13.8°C in December (Ukegbu, 2005). The State has a cosmopolitan nature as it is still the administrative hub for all northern States. It has many institutions of learning that provide opportunities to people across all walks of life. It has 23 local government areas and three senatorial zones as Kaduna North, Kaduna Central and Kaduna South. A hospital was selected from each of the three senatorial zones for this study.

3.2 Study Design

The study was cross-sectional hospital-based one that employed probability proportional to size (PPS) sampling method as recommended by World Health Organization (WHO 2014).

3.3 Study Population

The study population consisted of HIV infected adult individuals of both sexes and who were antiretroviral naïve and attending Barau Dikko Teaching Hospital, Kaduna and enrolled at point of diagnosis, Sir Patrick Ibrahim Yakowa Memorial Hospital, Kafanchan or Hajiya Gambo

Sawaba General Hospital, Zaria. Each selected hospital represented a socio-political zone of Kaduna State.

3.4 Inclusion and Exclusion Criteria

The HIV-positive persons who were enrolled at Antiretroviral Clinic, who consented and were not placed on any antiretroviral therapy were included in the research. Persons who did not satisfy the criteria were excluded from this study.

3.5 Sample Size Determination

The sample size for this study was determined using the revised formula of Lwanga and Lameshow (1991) at 95% confidence level and a reported 3.0% pre-treatment HIV-1 drug resistance rate in a cohort study among patients eligible for commencement of antiretroviral therapy in Abuja, Nigeria by Diallo *et al*. (2015).

$N = \frac{z^2pq}{d^2}$ where,

N= Sample size

Z= Statistics for a level of 95% confidence interval= 1.96

P= pre-treatment HIV-1 drug resistance rate = 3.0 % (Diallo *et al*., 2015).

d = level of significance (allowable error) = 5%

q= 1-p

thus,

$$N = \frac{(1.96^2) \times 0.03 \times (1 - 0.03)}{(0.05^2)}$$

$N = 44$

The calculated sample size was 44, but it was increased to 50 to reduce sampling error. Probability proportional to size (PPS) sampling as recommended by WHO (2014) was adopted to allocate

sample size across the selected hospitals based on number of ART initiators by hospital during a six-month period. Table 3.1 shows the distribution of the sample size across the study area.

3.6 Ethical Approval and Informed Consent

The study was conducted in accordance with the Declaration of Helsinki, and letter of ethical approval was obtained prior to commencement of study from the Health Research Ethics Committees of Ministry of Health, Kaduna State and Barau Dikko Teaching Hospital, Kaduna with reference number MOH/ADM/744/VOL.1/471 (Appendix Ia) and 18-0001(Appendix Ib) respectively. Individual informed consent form (Appendix II) was filled by each volunteer prior to sample collection. Volunteers gave written consent after comprehending what the research was all about.

3.7 Data Collection by Use of Questionnaire

Basic demographic data such as age, sex and marital status of each volunteer was captured using structured questionnaire (Appendix III) and transcribed to electronic questionnaire using EpiInfo® version 7 software.

Table 3.1: Distribution of sample size across the three selected hospitals in Kaduna State, Nigeria

Name of Hospital	HIV patients enrolled in the last 6 months	Calculated sample size	Number of actual samples collected
BDTH	222	24.50	25
SPIYMH	133	14.68	14
HGSGH	98	10.82	11
Total	453	50.00	50

Keys: BDTH = Barau Dikko Teaching Hospital, Kaduna, SPIYMH = Sir Patrick Ibrahim Yakowa Memorial Hospital, Kafanchan, HGSGH = Hajiya Gambo Sawaba General Hospital, Zaria

3.8 Sample Collection, Transportation and Storage

Samples were collected from April, 2018 to October, 2018. Every participant was assigned a unique and confidential identifier code for questionnaire and samples. Nine milliliters (9ml) of blood was collected from each participant in an Ethylenediaminetetraacetic acid (EDTA) anticoagulated vacutainer tube by a trained phlebotomist. One milliliter (1ml) of the well mixed whole blood was transferred to a 2ml-container for CD4 cells count. The remaining 8ml of blood was centrifuged at 1,500g for 10 minutes and the plasma was transferred using clean Pasteur pipette into two cryovials for HIV typing, RT-PCR and plasma viral load estimation. The CD4 cells count analysis was conducted in the respective hospitals where the samples were collected, while plasma samples were transported in cold chain to DNA Labs, Kaduna and stored at -20°C for further molecular analysis. Samples for HIV viral load from the three study areas were transported in cold chain to ART laboratory, Ahmadu Bello University Teaching Hospital, Shika, Zaria for HIV-1 plasma viral load determination.

3.9 Detection of HIV Types Using Rapid Diagnostic Test (RDT) Kit

Plasma samples of the study participants that were previously tested positive by the National testing algorithm kits (Alere Determine™ HIV-1/2, USA; Uni-Gold™, Ireland; HIV-1/2 STAT-PAK®, USA) were tested using MULTISURE® HIV Rapid Test (MP Biomedicals, Germany).

Principle: A rapid diagnostic kit operates on the principle of antigen-antibody interaction. The strip contains a membrane with a test line coated with capture antibodies specific to the target antigen. When a sample is applied to the strip, the target antigen, if present, binds to the capture antibodies on the test line, forming an antigen-antibody complex. The strip also has a control line coated with different antibodies, which serves as an internal control. Labeled antibodies or

particles conjugated to a visual indicator bind to the antigen-antibody complex, resulting in a visible signal at the test line.

MULTISURE® HIV Rapid Test (MP Biomedicals, Germany) was used to detect HIV type in participants' plasma. The kit and samples were brought to room temperature prior to commencement of the assay. The test device was labeled with the sample number and 25µl plasma was added into the kit's square well (Figure 3.1a). When the sample front reached the blue indicator line, 3 drops of Chase Buffer were added into the kit's oval well. The 'HIV Pull Tab' was pulled out until resistance was felt. One drop of Chase Buffer was added into the square well and result was read after 20 minutes. Results were interpreted as presented in Figure 3.1b.

3.10 CD4 Cell Enumeration

CD4 cell enumeration was carried out using BD FACSCount™ (BD Biosciences, USA) flow cytometry system. Appropriate protocol disk was inserted into the drive and power was turned on. The fluid reservoir was filled and waste reservoir emptied. All necessary daily maintenance activities were observed. Participants' whole blood sample and control were placed on the blood mixer and allowed to mix for 10 minutes.

Principle: Flow cytometry is a technique used to estimate CD cell counts by labeling cells with fluorescently labeled antibodies specific to their surface markers. A cell suspension is prepared, and antibodies are added, binding to their respective markers. The suspension is then analyzed in a flow cytometer, which detects light scattering and fluorescence emissions from the labeled cells. Data analysis allows for the identification and quantification of specific cell populations based on their surface marker expression, such as CD4+ T cells or CD8+ T cells. The cell counts are estimated by determining the number of events within specific gates or populations.

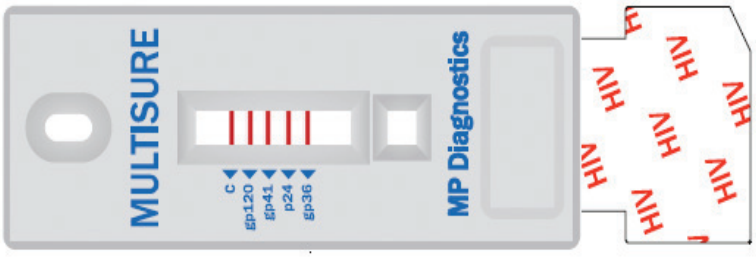

Figure 3.1a: Multisure Test Device RDT kit used for HIV typing.

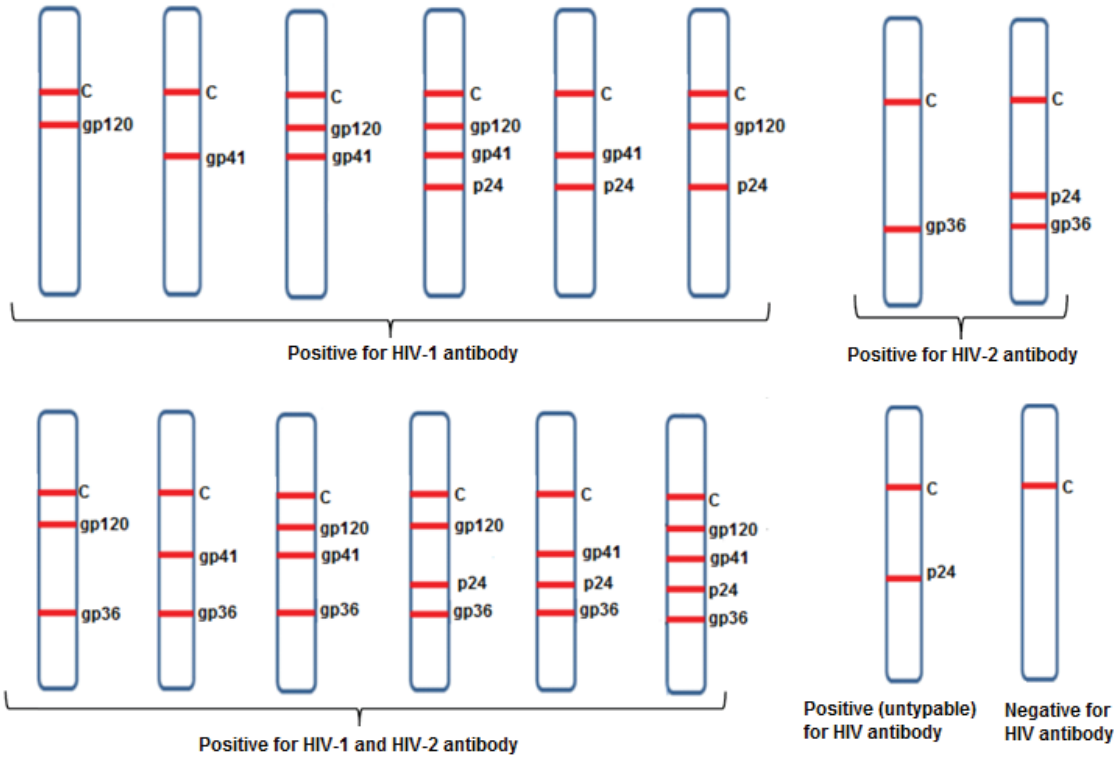

Figure 3.1b: Interpretation of the test results for HIV typing

3.10.1 Checking Controls

Pairs of the control beads (Low, Medium and High) were vortexed for 5 seconds upside down and 5 seconds upright. The reagent tubes were opened with the coring station and 50µl of normal blood was reverse pipetted into each of the tubes. The tubes were recapped with new caps and vortexed upright for 5 seconds. The recapped tubes were incubated in the dark at room temperature for 60 minutes. The tubes were uncapped and 50µl of fixative solution was reverse pipetted into each tube. The tubes were recapped and vortexed upright for 5 seconds. A pair of zero/low and medium/high control beads were removed from the control kit and placed on the control workstation. The reagent tubes were uncapped and discarded accordingly. The first control beads pair were vortexed for 5 seconds upside down and 5 seconds upright and reverse pipetted 50µl of Zero control beads (yellow top) into the CD_4 reagent tube labelled zero (Green top) and low control beads (red top) into the CD_8 reagent tube labelled low (Clear top). The second control beads pair were vortexed for 5 seconds upside down and 5 seconds upright and reverse pipetted also 50µl of Medium control beads (blue top) into the CD_4 reagent tube labelled medium (Green top) and high control beads (purple top) into the CD_8 reagent tube labelled high (Clear top).

The lot code and normal control identity were entered into the machine. The first reagent pair (CD_4-Zero and CD_8-Low) were vortexed upright for 5 seconds. The CD4 –Zero tube was uncapped and placed the reagent pair in the sample holder so that the CD4 –Zero tube was in the run position and **RUN** was pressed. This was repeated for all the controls. Controls have passed the check before proceeding to test the patients' samples.

3.10.2 Sample Analysis

The reagent tubes were labeled with patients' laboratory numbers. The tubes were vortexed upside down for 5 seconds, then upright for 5 seconds and opened with Coring Station. Fifty microliter

of blood was reverse pipetted into each of the reagent tube. The pipette tips were changed between each tube. The reagent tube was capped and vortexed upright for 5 seconds. the reagent tube was placed in the FACSCount workstation, the cover closed to protect reagent from light, and incubated for 60 minutes at room temperature in the dark. After the incubation step was completed, the tubes were uncapped and reverse pipetted 50µl of fixative solution into each reagent tube while changing tips between tubes. The reagent tube was recapped with new caps and vortexed upright for 5 seconds. All the tubes were run on the FACSCount instrument within the day of preparation.

3.11 Plasma HIV-1 Viral Load Estimation

Plasma HIV-1 RNA was quantified using COBAS® TaqMan® HIV-1 Test, v2.0 (Roche Molecular Systems, Inc., USA) following the ART Laboratory standard operating procedure. The machine has a detection limit of 20 copies/mL with linear range of 20–100,000,000 copies/mL.

Principle: The principle of HIV viral load measurement using real-time polymerase chain reaction (PCR) technique involves quantifying the amount of HIV genetic material (RNA) present in a patient's blood sample. Real-time PCR is a highly sensitive and specific molecular technique that allows for the accurate and precise detection and quantification of viral RNA. The process begins with the extraction of RNA from the patient's blood sample. The extracted RNA is then reverse transcribed into complementary DNA (cDNA) using an enzyme called reverse transcriptase. This step converts the viral RNA into DNA, which can be amplified and detected using PCR. In the real-time PCR technique, specific primers and a fluorescent probe that binds to a region of the HIV genome are added to the cDNA mixture. The primers are designed to target a conserved region of the HIV genome, ensuring accurate and reliable detection of different HIV strains. The fluorescent probe emits fluorescence only when it binds to the amplified DNA during the PCR process. The PCR reaction is performed in a specialized instrument called a real-time PCR machine, which

monitors the amplification of DNA in real-time. As the PCR cycles progress, the fluorescence emitted by the probe is detected and measured by the instrument. The amount of fluorescence produced is directly proportional to the amount of viral RNA present in the patient's sample.

The machine generates a fluorescence curve, and the cycle threshold (Ct) value is determined, which represents the cycle number at which the fluorescence signal reaches a significant threshold above the background noise. The Ct value is inversely proportional to the initial amount of viral RNA in the sample. By comparing the Ct value to a standard curve generated using known concentrations of HIV RNA, the viral load in the patient's sample can be quantified.

3.11.1 Maintenance and Priming

The COBAS® TaqMan® Instrument was primed using AMPLILINK software and all maintenance schedules were observed.

3.11.2 Loading of Reagent Cassettes

The HIV-1 CS1 (HIV-1 Magnetic Glass Particles Reagent Cassette) was placed onto a reagent rack. The HIV-1 CS2 (HIV-1 Lysis Reagent Cassette), HIV-1 CS3 (HIV-1 Multi-Reagent Cassette) and HIV-1 CS4 (HIV-1 Test-Specific Reagent Cassette) were placed onto a separate reagent rack. The reagent rack containing HIV-1 CS1 was loaded onto rack position A of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer and the AMPLILINK Software, Version 3.1.x Series.

The reagent rack containing HIV-1 CS2, HIV-1 CS3 and HIV-1 CS4 were loaded onto rack position B, C, D or E of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer and the AMPLILINK Software, Version 3.1.x Series.

3.11.3 Loading of Disposables

A configuration was chosen and the appropriate number of reagent cassette racks, sample racks with Input S-tubes, SPU racks, K-tip racks, K-tube racks and K-carriers on K-carrier racks were loaded onto the respective rack positions of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer and the AMPLILINK Software, Version 3.1.x Series.

The Sample Processing Units (SPUs) were placed in the SPU rack(s) and the rack(s) loaded onto rack position **J, K** or **L** of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer and the AMPLILINK Software, Version 3.1.x Series. Full K-tube rack(s) were loaded onto rack position **M, N, O** or **P** of the COBAS® AmpliPrep Instrument as described in the Manual. Full K-tip rack(s) were loaded onto rack position **M, N, O** or **P** of the COBAS® AmpliPrep Instrument also as described in the Manual.

3.11.4 Ordering and Loading of Specimens

A barcode label clip was attached to each sample rack position where a specimen (S-tube) was to be placed. One of the specific barcode label clips for the controls [CTM (–) C, HIV-1 L(+)C and HIV-1 H(+)C] was attached to each sample rack position where the controls (S-tube) are to be placed. The barcode label clips for controls had the same control lot number as the lot number on the control vials in the kit. One Input S-tube was placed into each position containing a barcode label clip. Using the AMPLILINK software, Specimen order was created for each specimen and

control in the Orders window Sample folder. The Test file name in the Orders window was HIMAPU96 or HIMAPU48. Appropriate test file was selected and completed by saving.

Specimen and control orders were assigned to sample rack positions in the Orders window Sample Rack folder. The Sample Rack Order report was printed to use as a worksheet. Specimen and control racks were prepared in the designated area for specimen and control addition as follows: [CTM (–) C, HIV-1 L(+)C and HIV-1 H(+)C] for 3 to 5 seconds.

One thousand microlitre (1,000µL) of each specimen and control [CTM (–) C, HIV-1 L(+)C and HIV-1 H(+)C] was transferred to the appropriate barcode labeled Input S-tube using a micropipette or with an aerosol barrier or positive displacement RNase-free tip. The barcode label clips for controls had the same control lot number as the lot number on the control vials in the kit. Correct control was assigned to the position with the appropriate control barcode clip. The upper part of the S-tubes was avoided from contamination with specimens or controls. The sample rack(s) filled with Input S-tubes were loaded onto rack positions F, G or H of the COBAS® AmpliPrep Instrument.

3.11.5 Start of COBAS® AmpliPrep Instrument Run

The COBAS® AmpliPrep Instrument was started using the AMPLILINK software as described in the Manual.

3.11.6 End of COBAS® AmpliPrep Instrument Run and Transfer to COBAS® TaqMan® Analyzer

Flags or error messages were checked for in the system screen and processed specimens and controls were removed from the Instrument sample racks. All generated waste were removed and discarded accordingly.

3.11.7 Amplification and Detection

The COBAS® TaqMan® Analyzer run was started within 120 minutes following completion of specimen and control preparation.

3.11.8 Sample Run

Appropriate steps were performed to transfer the K-tubes to the COBAS® TaqMan® Analyzer. The instrument was started automatically after insertion of sample rack(s). At the completion of the run, result reports were printed. Flags or error messages in the result report were checked. Specimens with flags and comments were interpreted as described in the *Results* section. After acceptance, data were stored in the archive.

3.11.9 Interpretation of Results

The COBAS® TaqMan® Analyzer automatically determined the HIV-1 RNA concentration for the specimens and controls. The HIV-1 RNA concentration was expressed in copies copies/mL. For statistical purposes, samples with a plasma viral load < 20 copies/mL (Target Not Detected) were assigned the highest possible value of 20 copies/mL. The assigned value does not have any significant impact on the clinical outcome of the patients.

3.12 Sequencing of Protease and Reverse Transcriptase Regions in the *pol* Gene for detection of transmitted drug resistance, characterization of HIV and mutation studies

Regions that code for the production of protease and reverse transcriptase enzymes in the *pol* gene were amplified by PCR and sequenced using DNA Sanger sequencing method.

3.12.1 Viral RNA Extraction

AccuPrep® Viral RNA extraction kit (Bioneer Corporation, South Korea) was used for RNA extraction from plasma samples. Ten microlitre (10 µL) of proteinase K was added to a 1.5 mL tube and 200 µL of plasma added. Three hundred microlitre (300 µL) of VP Buffer was added into the tube and mixed by vortexing for 10 seconds. The tube was incubated for 10 min at 56°C. Three hundred microlitre (300 µL) of isopropanol was added, briefly vortexed and centrifuged down the liquid clinging to the walls and lid of the tube. The mixture was transferred to a binding column tube fitted in a collection tube and centrifuged at 11,000 rpm for 1 minute. The flow-through was discarded and collection tube fitted back. Five hundred microlitre (500 µL) of VW1 buffer was added to the column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and collection tube fitted back. Six hundred microlitre (600 µL) of RWA2 buffer was added into the column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and collection tube fitted back. The column was centrifuged more at 13,000 rpm for 1 minute to remove ethanol completely. The binding column was transferred to a 1.5mL tube for elution and 50 µL of ER buffer was added and allowed to stand for 1 minute to permeate the column. The RNA was eluted by centrifuging at 13,000 rpm for 1 minute. The eluted RNA was stored at -20°C until use.

3.12.2 Amplification of Partial Protease Region

Principle: PCR involves three main steps: denaturation, annealing, and extension. In the denaturation step, the DNA template containing the target sequence is heated to a high temperature, typically around 95°C. This causes the DNA double helix to separate, resulting in two single strands. During the annealing step, the temperature is lowered to allow specific DNA primers to bind to complementary sequences flanking the target region. These primers are short DNA

sequences that are designed to be complementary to the specific DNA regions of interest. The primers act as starting points for DNA synthesis.

In the extension step, the temperature is raised to an optimal range for the DNA polymerase enzyme to synthesize new DNA strands. The enzyme uses the primers as a template to add nucleotides and extend the DNA strands. This process generates two copies of the target DNA sequence, one for each of the original DNA strands. Each PCR cycle doubles the amount of DNA, resulting in an exponential increase in the target DNA region. This amplification process typically involves multiple cycles, typically ranging from 25 to 35 cycles, to achieve a significant amount of the desired DNA sequence.

The amplified DNA can then be analyzed using various techniques, such as gel electrophoresis, sequencing, or further downstream applications. PCR has revolutionized molecular biology and has numerous applications, including genetic research, diagnostics, forensics, and infectious disease detection.

The primers used for the amplification of protease and reverse transcriptase regions are presented in Tables 2.2. Each primer was reconstituted and diluted to 10nM concentration with molecular grade water. Thirteen microliter (13µl) of molecular grade water was added to RT PCR Premix (Bioneer Corporation, South Korea). One microliter (1µl) of forward primer (DRPRO5) and 1 µl of reverse primer (DRPRO2L) were added. Five microliter (5 µl) of RNA template was also added. The mixture was placed in PTI 100^{TM} thermocycler (MJ Research, Inc., USA) and the cycling condition was set as below in a one-step RT PCR reaction:

i. 42°C for 60 minutes (reverse transcription)
ii. 94°C for 5 minutes
iii. 94° C for 30 seconds (denaturation)

iv. 52° C for 40 seconds (annealing)

v. 72° C for 1 minutes (extension)

vi. Steps iii–v repeated 35 times

vii. 72°C for 5 minutes (final extension)

For the second round of the nested PCR, Hot-start PCR Premix (Bioneer Corporation, South Korea) was reconstituted with 15 µl molecular grade water, and 1 µl of forward primer (DRPRO1M) and 1 µl of reverse primer (DRPRO6) and 3 µl of first run PCR product were added. The mixture was placed in the thermocycler and the temperature set as below:

I. 94° C for 5 minutes (initial denaturation)

II. 94° C for 30 seconds (denaturation)

III. 55°C for 40 seconds (annealing)

IV. 72°C for 1 minutes (extension)

V. Cycle II – IV 35 times

VI. 72°C for 5 minutes (final extension)

Table 3.2: Primer sequences used for the amplification of HIV-1 protease and reverse transcriptase genes

Gene	Primer	Nucleotide sequence	Amplicon size (bp)	Reference
Protease	DRPR05	5'-AGACAGGYTAATTTTTTAGGGA-3'		
	DRPR02L	5'-TATGGATTTTCAGGCCCAATTTTTGA-3'		
	DRPR01M	5'-AGAGCCAACAGCCCCACCAG-3'	460	
	DRPR06	5'-ACTTTTGGGCCATCCATTCC-3'		Khairunisa *et al*., 2018, ANRS 2015
Reverse transcriptase	RT1L	5'-ATGATAGGGGGAATTGGAGGTTT-3'		
	MJ4*	5'-CTGTTAGTGCTTTGGTTCCTCT-3'		
	DRRT4L	5'-TACTTCTGTTAGTGCTTTGGTTCC-3'	887	
	RT7L	5'-GACCTACACCTGTCAACATAATTGG-3'		
	DRRT6L	5'-TAATCCCTGCATAAATCTGACTTGC-3'		

*MJ4 is the only primer used from ANRS

3.12.3 Amplification of Partial Reverse Transcriptase region

The primers for amplification of *protease* and *reverse transcriptase* gene are presented in Table 3.2. Each primer was reconstituted and diluted to 10nM concentration with molecular grade water Thirteen microliter (13µl) of molecular grade water was added to RT PCR Premix (Bioneer Corporation, South Korea). One microliter (1µl) of forward primer (RT1L) and 1 µl of reverse primer (DRRT4L/MJ4) were added. Five microliter (5 µl) of RNA template was also added. The mixture was placed in PTI 100^{TM} thermocycler (MJ Research, Inc., USA) and the cycling condition was set as below:

i. 42˚C for 60 minutes (reverse transcription)
ii. 94˚C for 5 minutes (initial denaturation)
iii. 94˚C for 30 seconds (denaturation)
iv. 52˚C for 40 seconds (annealing)
v. 72˚C for 1 minutes (extension)
vi. Steps iii – v repeated 35 times
vii. 72˚C for 5 minutes (final extension)

For the second round of the nested PCR, Hotstart PCR Premix (Bioneer Corporation, South Korea) was reconstituted with 15 µl molecular grade water, and 1 µl of forward primer (RT7L) and 1 µl of reverse primer (DRRT6L) and 3 µl of first run PCR product were added. The mixture will be placed in thermocycler and the temperature set as below:

i. 94˚C for 5 minutes
ii. 94˚C for 30 seconds (denaturation)
iii. 52˚C for 40 seconds (annealing)
iv. 72˚C for 1 minutes (extension)

v. Steps ii – v repeated 35 times

vi. 72°C for 5 minutes (final extension)

3.13 Gel Electrophoresis

Principle: Agarose gel electrophoresis is a technique used to separate and analyze DNA or RNA molecules based on their size. It involves the use of an agarose gel matrix, which is created by dissolving agarose powder in a buffer solution and allowing it to solidify in a casting tray with wells. The DNA or RNA samples, along with a loading buffer, are loaded into the wells, and an electric field is applied across the gel. The negatively charged molecules migrate through the gel matrix, with smaller fragments moving faster and farther than larger ones due to size-dependent sieving. After electrophoresis, the gel is stained and visualized using DNA-specific dyes, and the separated fragments can be analyzed based on their migration patterns, providing information about their size. By exploiting the principle of size-dependent sieving, the technique allows visualization and analysis of nucleic acids.

Agarose powder weighing 1.5g of was dissolved in 100ml of 1X Tris-acetate EDTA buffer. The solution was heated in a microwave until the agarose completely dissolved and was allowed to cool in a water bath set at 50°C. Five microliter (5µl) of ethidium bromide was added to the cooled gel and it was poured into the prepared casting tray with comb. It was allowed to cool for 30 minutes at room temperature and comb was removed. The gel was placed in electrophoresis chamber and covered with 1X Tris-acetate EDTA buffer. The PCR product and 100 bp molecular marker were loaded onto the immersed gel and electrophoresed at 120 Volt for 45 min. Gel documentation system was used to visualize DNA bands. The amplicon size was determined by extrapolating from the molecular marker bands to determine the result. Amplicon of 460 base pairs was expected for protease gene and 887 base pairs for Reverse Transcriptase gene.

3.14 DNA sequencing

The amplicon was cleaned and sequenced using DRPRO6 for protease and DRRT6L for reverse transcriptase. Sanger DNA sequencing method was employed using ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA).

3.14.1 DNA Clean-up for Sequencing

Gel extraction kit (Bioland scientific, USA) was used to purify the DNA from gel for sequencing reaction. The amplicon was excised from the agarose gel and weighed in 1.5mL microtube. Three gel volume of Buffer GB was added and incubated at 60°C for 8minutes while agitating the tube every 2 minutes till the gel melted completely. One gel volume of isopropanol was added and mixed. Seven hundred and fifty microliter (750µL) of the mixture was transferred into a DNA mini column with collection tube and centrifuged at 11,000 rpm for 1 min at room temperature. The flow-through was discarded and the column was placed back to the collection tube. This step was repeated with more mixture. Seven hundred and fifty microliter (750µL) of DNA Wash Buffer was added to the column and centrifuged at 11,000 rpm for 1 min at room temperature and the flow-through was discarded. The empty column was centrifuged at 11,000 rpm for 2 min to remove the residual ethanol. The column was placed in a clean 1.5 ml microcentrifuge tube and 20µL elution buffer was added to the column. It was incubated at room temperature for 1 min. centrifuged at 11,000 rpm for 1 min to elute the DNA.

3.14.2 Sanger Sequencing Reaction

Principle: The Sanger sequencing method, also known as chain-termination sequencing, is a classic DNA sequencing technique that revolutionized the field of genomics. The principle of

Sanger sequencing is based on the incorporation of chain-terminating nucleotides during DNA replication.

The process begins with the DNA template to be sequenced, which is denatured to obtain single-stranded DNA. Then, a DNA primer that is complementary to the target region is added. DNA polymerase, the enzyme responsible for DNA replication, is also included in the reaction. The DNA synthesis reaction contains a mixture of regular nucleotides (dATP, dTTP, dCTP, and dGTP) and chain-terminating dideoxynucleotides (ddATP, ddTTP, ddCTP, and ddGTP). These chain-terminating nucleotides lack the 3'-OH group necessary for DNA strand elongation.

During DNA replication, when a chain-terminating nucleotide is incorporated into the growing DNA strand, it effectively terminates further extension. This results in a series of DNA fragments of varying lengths, each terminating at a specific position corresponding to the incorporated chain-terminating nucleotide. The DNA fragments generated are then separated using gel electrophoresis. The fragments migrate through the gel based on their size, with smaller fragments traveling faster than larger ones. The gel is typically composed of a polyacrylamide matrix for high-resolution separation.

After electrophoresis, the DNA fragments are visualized using fluorescence or radioactivity detection methods. The sequence of the DNA template is determined by analyzing the patterns of DNA fragments on the gel. The position at which a specific chain-terminating nucleotide is incorporated indicates the corresponding base in the DNA sequence. This process is repeated for each of the four chain-terminating nucleotides, resulting in a series of sequencing reactions. The results are then analyzed and interpreted to reconstruct the original DNA sequence of the template.

Sanger sequencing reaction was conducted using ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA).

3.15 Bioinformatics

The results of DNA sequencing generated as chromatographs were edited where necessary using FinchTV software. Consensus sequences were submitted to the NCBI database. The sequence reads were compared against the non-redundant nucleotide sequence collection at Center for Biotechnology Information (NCBI) Genbank using the web interface of NCBI-BLAST. HIV-1 subtyping was statistically done by maximum likelihood (ML) phylogenetic reconstruction in MEGA X; a bootstrap of 1000 replicates was used to generate the consensus evolutionary tree and assess the strength of cluster from each node. Phylogenetic inferences were performed by the neighbor-joining (NJ) method. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-86574.03) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 74 nucleotide sequences. Codon positions included were $1^{st} + 2^{nd} + 3^{rd}$ + Noncoding. There were a total of 9912 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

REGA HIV-1 Subtyping Tool - Version 3.0 was used to subtype the sequence and thereby the HIV isolate. Drug resistance and specific mutations were detected using specialised resistance web-based database developed by GENAFOR (www.geno2pheno.org) and Stanford University HIV Drug Resistance Database (Rhee *et al*., 2003). Multiple sequence alignment was performed using Mega 7 and TCoffee to identify regions of similarity, difference, structure and function using reference hxb2 sequence. Each sequence was aligned with available sequences in NCBI database

using BLAST® and some hits were retrieved and used for multiple sequence alignment alongside our original sequences.

3.16 Data analysis

Epi Info version 7.2 was used to collate data from questionnaires and results of laboratory analyses. GraphPad Prism version 6.0 was used to analyse the collated data. Quantitative variables were presented as mean (±SD) or median (IQR), while Spearman correlation was used to compare the CD4 cells count and viral load. Level of significance of 95% was adopted to accept or reject null hypothesis at $p \leq 0.05$. Pattern of mutations was correlated with HIV-1 subtypes.

CHAPTER FOUR

4.0 RESULTS

4.1 Socio-demographic characteristics of drug-naive HIV positive research participants in Kaduna State, Nigeria

A total of 50 HIV infected drug-naïve participants were enrolled for this study with a median age of 35.0 (IQR: 25-39.25) years with age range of 20 to 66 years. Of the 50 patients recruited 32 (64%) were female. A total of 21 (42%) of the participants represented the age group of 31 - 40 while age group of greater than 50 years constituted the lowest number with only 1 (2%) participant. Participants with secondary level of education had the highest frequency of 23 (46%) while only 3 (6%) had no form of education. Self-employed participants were the highest recruited (33: 66%) and only 2 (4%) were non-governmental employees. Out of the 50 participants 22 (44%) were either married men or women while 19 (38%) were not married. Eight (16%) and 1 (2%) of the participants were either widow/widower and divorced respectively. These socio-demographic characteristics are depicted in Table 4.1.

4.2 Attitude and Practice Associated with Risk of HIV infection transmission among study participants

Table 4.2 described the risk factors associated with HIV infection among the study participants. Forty-five participants had responded to sexual orientation whereby 42/45 (93.33%) were heterosexual and 3/45 (6.67%) were homosexual. The study participants were exposed to HIV transmission risk factors that included blood transfusion, dental procedure and surgery, with frequency of 6/50 (12%), 13/50 (26%) and 8/50 (16%), respectively. Other risk factors associated with HIV transmission among the participants were local practices of surgery, commercial pedicures, contact with blood or blood product, and sharing of hair clippers with frequencies of 14/50 (28%), 22/50 (44%), 18/50 (36%) and 25/50 (50%) respectively.

Table 4.1: Sociodemographic characteristics of HIV-1 infected drug-naive patients in the study area.

Variable	Frequency	% (95% Confidence Interval)
Gender		
Male	18	32 (22.92– 50.81)
Female	32	64 (49.19– 77.08)
Age Group (years)		
20 – 30	20	40 (26.00 – 54.00)
31 – 40	21	42 (28.00 - 56.00)
41 – 50	8	16 (6.00 - 26.00)
>50	1	2.0 (0.00 – 6.00)
Level of Education		
Primary	6	12(4.53-24.31)
Secondary	23	46 (31.81-60.68)
Tertiary	14	28 (16.23-42.49)
Informal	4	8 (2.22- 19.23)
None	3	6 (1.25- 16.55)
Occupation		
Civil Servant	6	12(4.53-24.31)
NGO-employed	2	4(0.49 - 13.71)
Self-employed	33	66 (51.23- 78.79)
Student	9	18 (8.58- 31.44)
Marital status		
Divorced	1	2 (0.05-10.65)
Married	22	44 (33.66-62.58)
Single	16	32 (19.52-46.70)
Widow/Widower	9	18 (8.58-31.44)
Sexual orientation (n=45)		
Heterosexual	42	93.33(81.73 - 98.60)
Homosexual	3	6.67 (1.40 - 18.27)

Median: 35.0 (IQR: 25 – 39.25) years

Individuals with skin scarification such as tattoo and tribal marks constituted 23/50 (46%) while female participants that ever had child delivery at home were 15/22 (68.18%). Participants that were in polygamous marriage, those that had multiple sexual partners and had partner living with HIV were 3/34 (11.76%), 16/47 (34.04%) and 14/47 (29.79%) respectively.

4.3 Prevalence of HIV type 1 among HIV infected patients in Kaduna State

Fifty participants' samples reacted to both HIV-1 gp120 and HIV-1 gp41 antibodies and concurrently out of these 50 samples 48 reacted to p24 antibodies. None reacted to HIV-2 gp36 antibodies (Table 4.3). Therefore, all the 50 participants studied were found to be infected with HIV type-1.

Table 4.2: Some risk Factors Associated with HIV-1 infected drug-naive patients in the Study Area

Risk factor	Frequency (n=50)	% (95% Confidence Interval)
Tattoo and Tribal marks	23	46 (31.81-60.68)
Partner(s) living with HIV(*n=47*)*	14	29.79 (17.34-44.89)
Sharing hair clipper	25	50 (35.53-64.47)
Child delivery at home (*n=22*)*	15	68.18% (45.13-86.14)
Surgery	8	16 (7.17-29.11)
Local surgery	14	28 (16.23- 42.49)
Blood transfusion	6	12 (4.53-24.31)
Dental procedure	13	26 (14.63-40.34)
Contact with blood or blood product	18	38 (24.65-52.83)
Commercial pedicure	22	44 (29.99-58.75)
Multiple sexual partners(*n=47*)*	16	34.04 (20.86 - 49.31)
Polygamy (*n=34*)*	4	11.76 (3.30- 27.45)

**Not applicable to all respondents*
Univariate analysis showing the frequency and percentages with 95% CI.

Table 4.3: Confirmation of HIV-1 among HIV infected drug-naive patients in the study area

Antigens reacted to	Number positive	Percentage
HIV-1 gp120	50	100
HIV-1 gp41	50	100
HIV-1 and HIV-2 p24	48	96
HIV-2 gp36	0	0

4.4 CD4 cell enumeration among drug-naïve HIV infected participants in Kaduna state, Nigeria.

The median and mean CD4 cells among the participants were 176.0 cell/µl (IQR: 74.75- 402.8) and 250.8 cell/µl (SD: 225.2) with the minimum CD4 cell count of 8 cell/µl and the maximum of 900 cell/µl. Female had median and mean CD4 cell count of 191.50 (IQR: 58.50-480.50) and 265.75 (SD: 237.68) cell/µl whereas male had median and mean CD4 cell count of 155 (IQR: 83-286) and 224.28 (SD: 204.96) cell/µl respectively (Figure 4.1). This apparent difference between the two means was not statistically significant (p=0.7850). The distribution of CD4 cell count by age groups are presented in Table 4.4. There was no significant different in CD4 cells count between the age groups (p= 0.9371) (Table 4.4). Several participants, 17 (34%) had a CD4 cell count that falls between 51-200 cell/µl. Nine (18%) had CD4 cell count >50 cell/µl while 7 (14) had CD4 cell count > 500 cell/µl (Table 4.5).

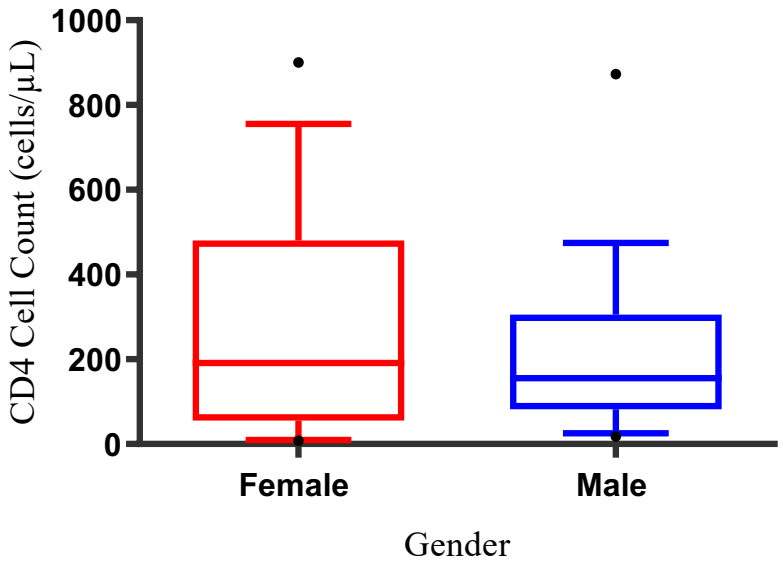

Figure 4.1. Box and whisker plot depicting CD4 cell count by gender in the study area

Table 4.4: Distribution of CD4 cell count by age groups of drug-naïve HIV infected participants in Kaduna State.

Age group (Years)	Frequency (%)	Median (IQR) CD4 cell count (cell/µl)
20 – 30	20 (40)	200.00 (159.84-368.26)
31 – 40	21 (42)	159.00 (151.19-390.00)
41 – 50	8 (16)	149.00 (75.02- 227.48)
>50	1 (2)	363.00

p= 0.9371

Table 4.5: Distribution of CD4 cell count of drug-naïve HIV infected participants in Kaduna State

CD4 Categories (cell/µl)	Frequency (%)	Median (IQR)
≤50	9 (18)	18(16 – 33)
51- 200	17 (34)	111(83 – 182)
201- 350	8 (16)	261(226.5 – 278.5)
351- 500	9 (18)	426 (379 – 496)
>500	7 (14)	588 (522 – 900)

4.5 HIV-1 plasma viral load among drug-naïve HIV-1 infected participants in Kaduna State, Nigeria

Of the 50 drug-naïve HIV-1 infected participants, 47(94%) had detectable plasma levels of HIV-1 RNA. The median (IQR) HIV-1 plasma viral load among the participants was 158,391 (44,730 - 548,281) copies/ml. Female had median (IQR) HIV-1 plasma viral load of 134619 (29,324 - 700,757), while their male counterparts had median (IQR) of 222,221 (44,730 – 452,113) (Figure 4.2). There was no significant difference between female and male viral load ($p>0.999$). There was no significant difference in HIV-1 plasma viral load across the age groups ($p= 0.5203$) (Table 4.6). Five (10%) participants had plasma viral load of <1000 copies/ml and 30 (60%) of the participants had plasma viral load of >100,000 copies/ml (Table 4.7). Table 4.8 shows summary of CD4 cell count and plasma viral load among the participants.

4.6 Correlation between CD4 cell count and HIV-1 RNA plasma viral load

There was a significant moderately strong, negative correlation between HIV-1 plasma viral load and CD4 cell count (Spearman correlation coefficient = -0.5007, P = 0.0002) (Figure 4.3). Table 4.8 presents the summary of cell count and HIV-1 RNA plasma viral load.

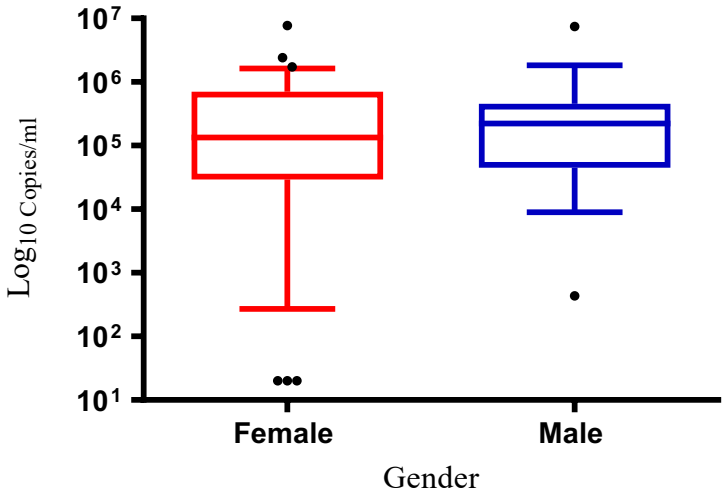

Figure 4.2. Distribution of Viral Load in relation to gender of the study population

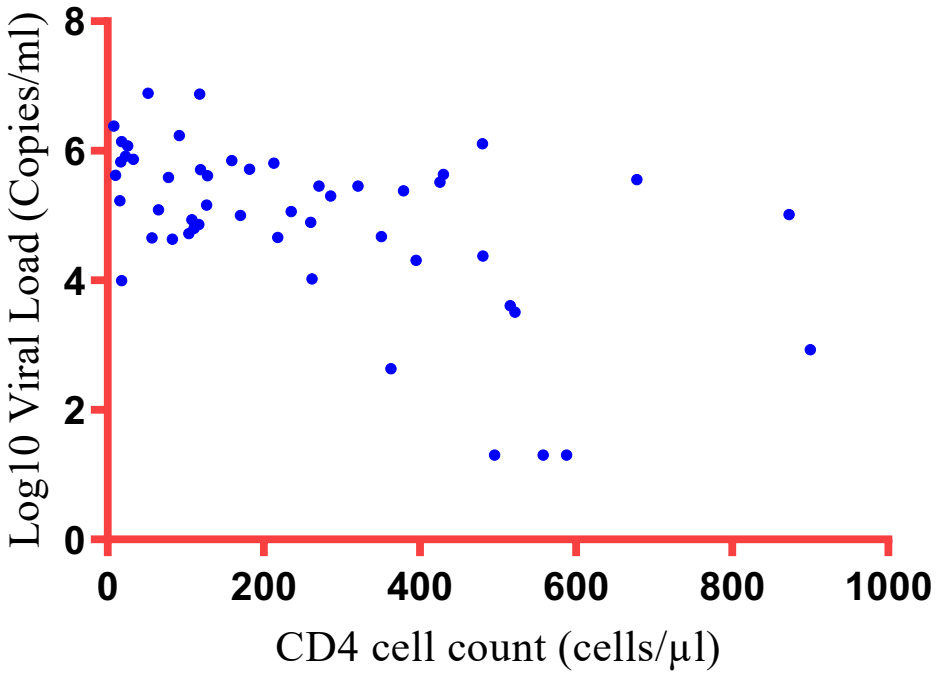

Figure 4.3 : Correlation of CD4 cell count and HIV-1 plasma Viral Load

Table 4.6: HIV-1 plasma viral load in relation to age groups of drug-naïve HIV infected participants in Kaduna State

Age group (Years)	Frequency (%)	Median (IQR) Viral Load (copies/ml)
20 – 30	20 (40)	101,187 (-147,108 – 1,458,948)
31 – 40	21 (42)	362,120 (109854.36 – 1598451.92)
41 – 50	8 (16)	90,152 (3184.29 – 375256.71)
>50	1 (2)	435

Table 4.7: Distribution of plasma viral load of drug-naïve HIV infected participants in Kaduna State

Viral load Categories (Copies/ml)	Frequency (%)	Median (IQR)
≤1000	5 (10)	20 (20 – 435)
1001- 10,000	3(6)	4,105 (3,216 – 9,926)
10,001- 100,000	12(24)	46,922 (33,645 – 68,283.5)
>100,000	30(60)	425,250 (243,436 – 827,276)

Table 4.8: Summary of CD4 cell count and plasma viral load among drug-naïve HIV-1 infected participants in Kaduna state, Nigeria

Statistics	CD4 cell count (cell/µl)	Viral load (copies/ml)	Viral load (log_{10})
Mean	250.8	651,396	4.946
Median	176.0	158,391	5.199
SD	225.2	1.517×10^6	1.278
25^{th} percentile	74.75	44,730	4.651
75^{th} percentile	402.8	548,281	5.737
Minimum	8.000	20.00	1.301
Maximum	900.0	7.727×10^6	6.888

4.7 PCR and Sequencing data

Out of the 47 (94%) samples with detectable plasma viral load that were subjected to reverse transcription polymerase chain reaction (RT-PCR) to amplify both protease and reverse transcriptase regions of the HIV-1 *pol* gene, 3 could not be amplified due relatively low viral load of ≤3,216 copies/ml. Most of the ANRS primers however, failed to amplify the regions of interest. Gel photo of the amplicon sizes of 460bp and 887bp for protease and reverse transcriptase regions is presented in Plate I.

Protease and reverse transcriptase regions were not amplified in 2 and 5 samples with appreciable plasma viral load (≥4,105 copies/ml) respectively. Protease and reverse transcriptase regions were successfully sequenced from 42 (89.4%) and 39 (83.0%) samples respectively (Table 4.9).

4.8 Genetic diversity of HIV-1 infected in drug-naïve patients

A total of 41 samples from the drug-naïve HIV infected patients were characterized into CRF02_AG, subtype G, CRF06_cpx and subtype C with frequency of 25(61%), 13(32%), 2(5%) and 1(2%) respectively (Figure 4.4) using REGA HIV-1 Subtyping Tool – Version 3. 0.

4.9 Phylogenetic Analyses of the Protease and Reverse Transcriptase Sequences of the Pol Region Using MEGA-X

All sequences from this study were submitted to NCBI database for Nucleotide sequence accession numbers. Figure 4.5 and 4.6 show rooted tree of different genetic subtypes and CRFs in the protease and reverse transcriptase region respectively using references sequences and 24 retrieved Nigerian sequences from Los Alamos HIV Sequence database. Maximum likelihood tree was constructed using MEGA-X with 100 bootstrap value. The tree was rooted against Simian Immunodeficiency Virus pol sequence (JN091694). The nodes of the reference sequences were indicated with red diamond-shaped label, the retrieved sequences were indicated using green

Table 4.9: Summary of Sequencing of regions of HIV-1 *Pol* Gene among HIV-1 Infected Drug-Naïve Participants in Kaduna State

Event	Protease region	Reverse Transcriptase region	Total
Successful sequencing reaction	42	39	81
Failed RT-PCR (Considerable viral load)	2	5	7
Failed RT-PCR (LLV)*	3	3	6
TND (<20 copies/mL)	3	3	6
Total	50	50	100

TND = Target not detected. *LLV: low level viraemia defined by viral load of <10,000 copies/mL, RT-PCR= Reverse transcription polymerase chain reaction

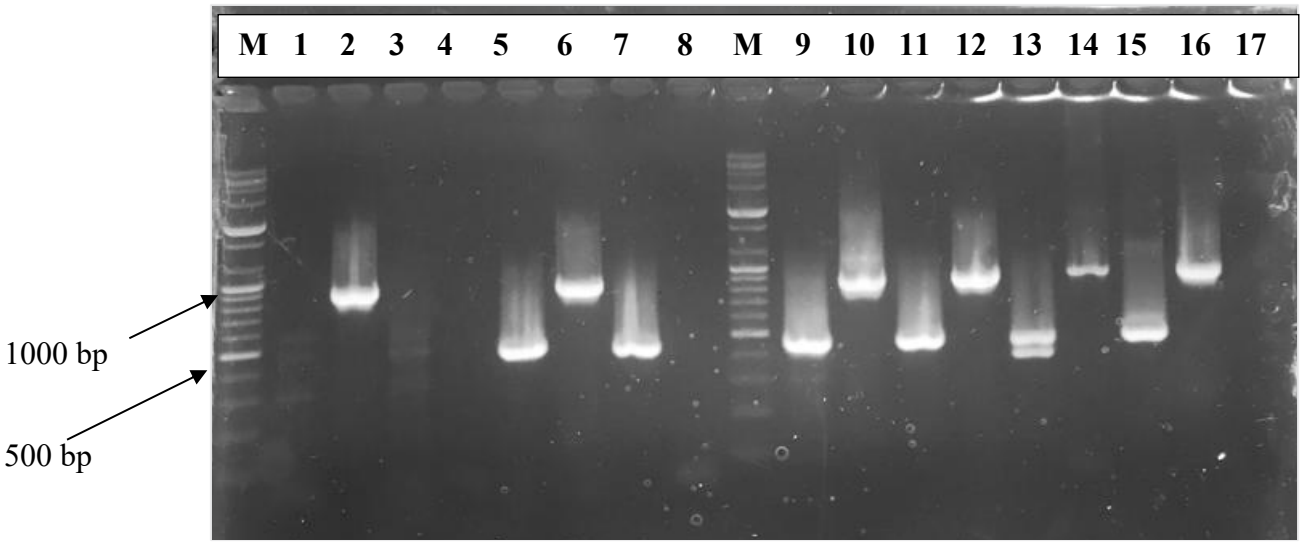

Plate I: Agarose Gel Photo of Electrophoresed RT-PCR Product of Region of Protease and Reverse Transcriptase of Some Samples.
M: molecular marker of 100bp

Lane 1, 5, 7, 9, 11, 13, 15 = 460bp amplicon of protease region.
Lane 2, 6, 10, 12, 14 16 = 887bp amplicon of reverse transcriptase region,
Lane 17 = Negative control.

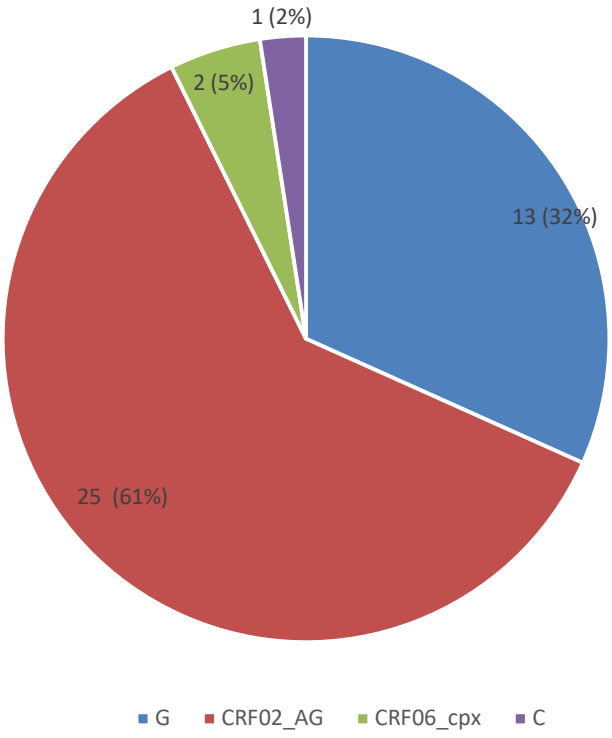

Figure 4.4: Genetic Diversity of The *Pol* Gene Among Drug-Naïve Patients in Kaduna State

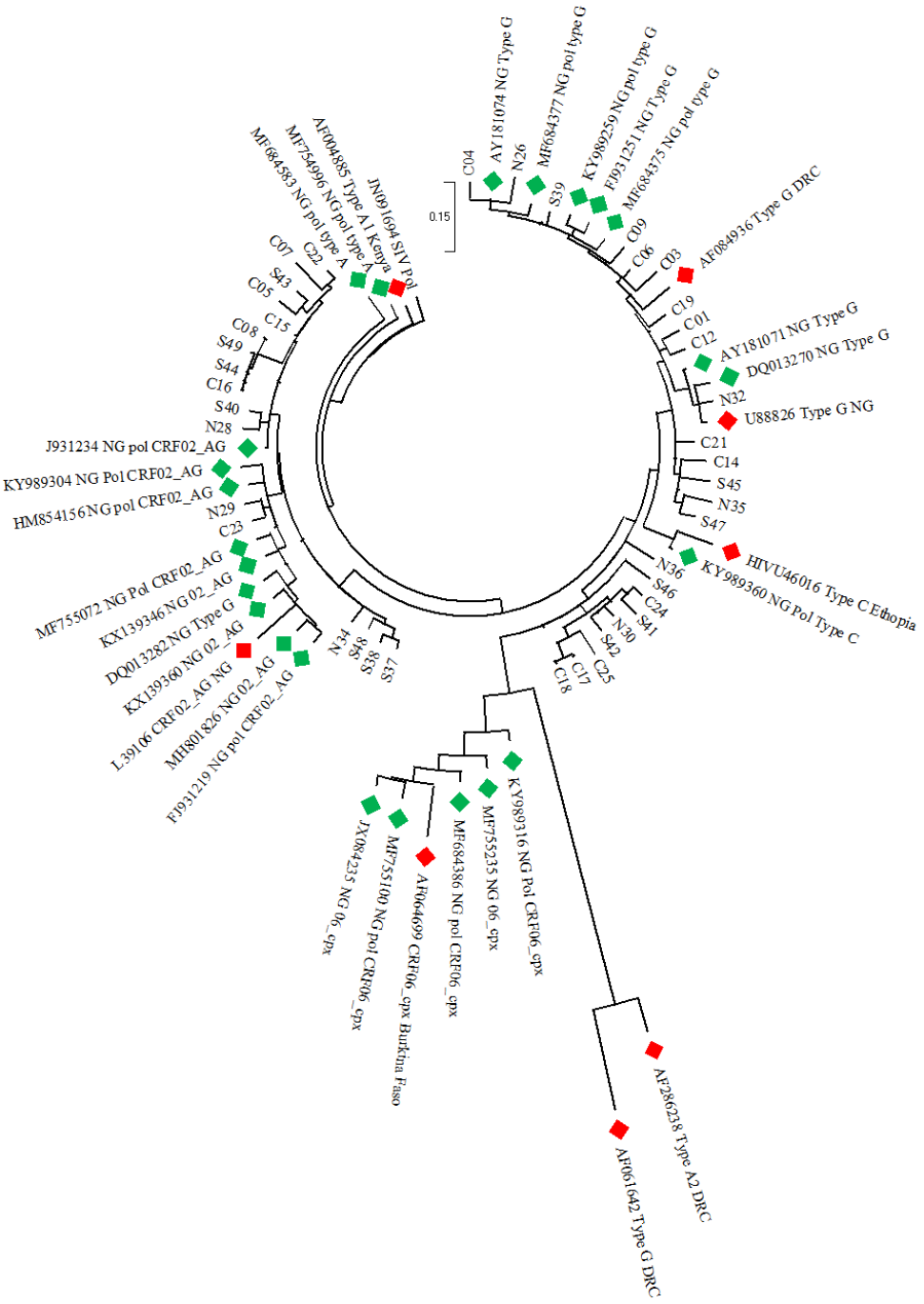

Figure 4.5: Rooted Maximum Likelihood Phylogenetic Tree of Isolates from Antiretroviral Treatment-Naïve Patients by Protease Gene of the Pol Region of HIV-1. The tree was constructed using MEGA-X

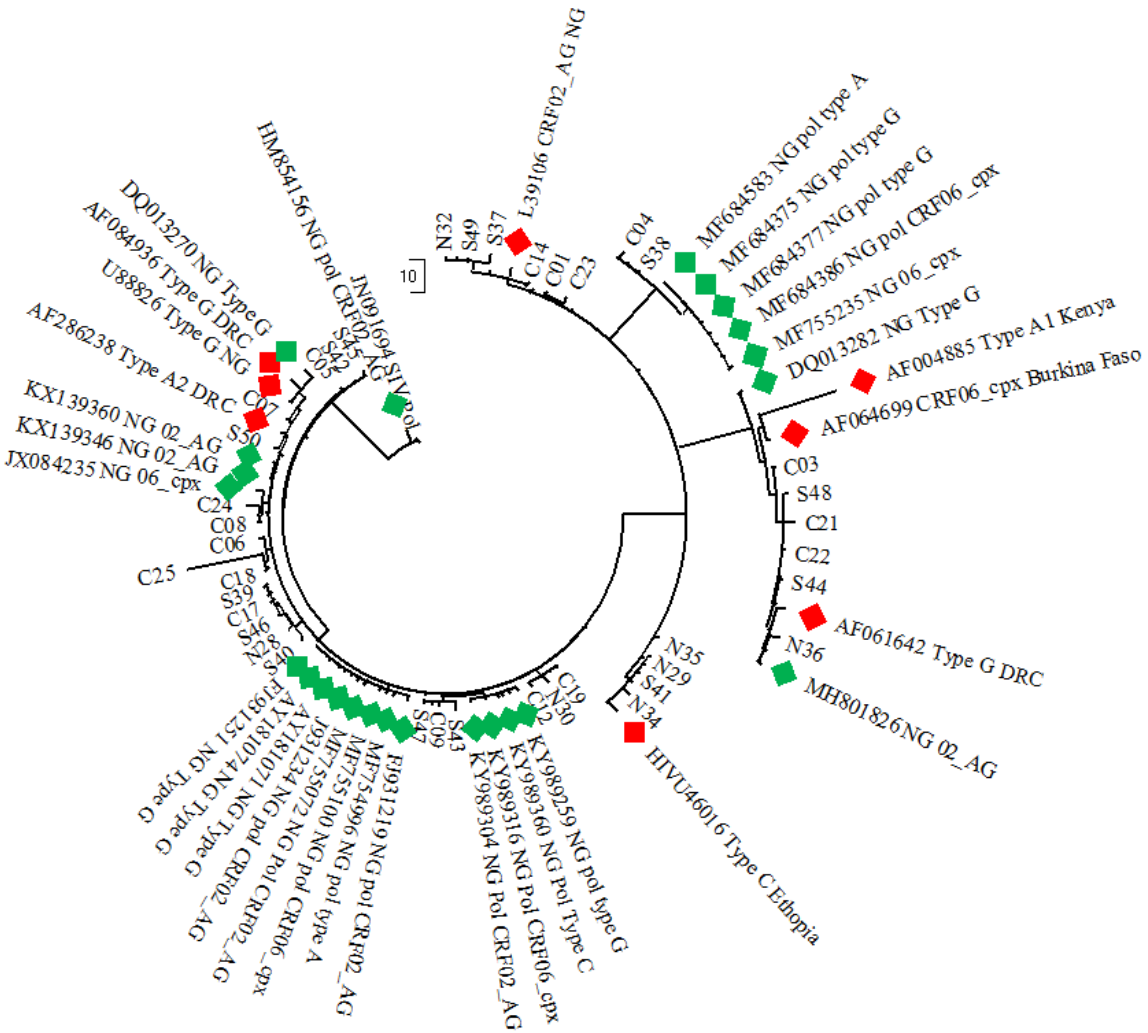

Figure 4.6: Rooted Maximum Likelihood Phylogenetic Tree of Isolates from Antiretroviral Treatment-Naïve Patients by Reverse Transcriptase Gene of the Pol Region Of HIV-1. The tree was constructed using MEGA-X

diamond-shaped label while the study sequences were left without labels. These sequences were aligned using ClustalW and an extract from the alignment was presented as Plate II in the Appendix III.

The phylogenetic tree illustrates different clusters among the isolates. By the protease region, subtypes AG and G clustered around their respective reference sequences and Nigerian sequences retrieved from Los Alamos HIV sequence database. The C14 and N45 sequences identified as CRF06_cpx clustered together with common ancestral node which in turn share common subtree with subtype G sequences. The S44 sequence and the only subtype C obtained in this study shared the same subtree with all the subtype G, CRF02_AG and CRF06_cpx except subtype A. This pattern of cluster seen in protease region is not demonstrated in phylogenetic tree of reverse transcriptase region.

4.10 Association between genotypes and mean CD4 cell count and HIV plasma viral load

Table 4.10 shows the isolated subtypes and CRFs and their respective CD4 cell count and HIV plasma viral load. The average HIV load was highest among patients with G subtype and lowest in the only patient with subtype C. The observed difference was however not statistically significant (p=0.9024). The average CD4 cell count was highest among patients with CRF02_AG and lowest in the only patient with subtype C, the difference was not statistically significant (p=0.9154).

Table 4.10: Mean CD4 Cell Count and HIV Viral Load in Relation to Subtypes and CRF

Subtype/CRF	Frequency of subtype/CRF	Mean CD4 ± SD (Cell/µl)	Mean viral load ± SD (Copies/ml)
G	13 (30.95)	203.9 ± 37.34	919,219 ± 575,830
CRF02_AG	25 (61.90)	211.3 ± 45.76	659,530 ± 304,137
CRF06_cpx	2(4.76)	104.0 ± 78.00	854,259 ± 337,306
C	1(2.38)	78	387438
p-value		0.9154	0.9024

p-values determined by One-way ANOVA

4.11 Prevalence of HIV-1 Transmitted Drug Resistance Among Drug-Naïve Infected Patients

Overall prevalence of HIV-1 transmitted drug resistance (TDR) among the study participants was 19.5% (8/41). The prevalence of TDR to Protease Inhibitors (PIs), Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) was 2.4% (1/41), 7.7% (3/39) and 17.9% (7/39), respectively. Patients with subtype G had the highest TDR (4/41, 9.8%) while patients with CRF02_AG had the lowest TDR (3/41, 7.3%). The observed difference in prevalence of TDR among the subtypes and CRFs was not statistically significant (χ^2=3.373, df=3, p=0.3376) (Table 4.11).

Patients with TDR had relatively lower CD4 cell count mean ± SD of 162.9 ± 86.49 cell/µl than patients without TDR with CD4 cell count mean ± SD of 267.6±34.02 cell/µl. However, this difference observed was not statistically significant (p=0.2319). Similarly, lower HIV plasma viral load mean ± SD of 367,046 ± 142,615 copies/ml was obtained among patients with TDR while patients without TDR had HIV plasma viral load mean ± SD of 705,558 ± 253,710 copies/ml. This difference was however not statistically significant (p= 0.2510).

Table 4.11: Occurrence of at Least One Surveillance Drug Resistance Mutation among the HIV Subtype and CRFs

Type/Subtype	Frequency (%) of subtype/CRF	Number of patients with ≥ 1 SDRM
G	13 (31.71)	4 (9.8%)
CRF02_AG	25 (60.98)	3 (7.3%)
CRF06_cpx	2(4.88)	1 (2.4%)
C	1(2.44)	0 (0%)
Total	**41**	**8 (19.5%)**

SDRM = Surveillance drug resistance mutation, χ^2=3.373, df=3, p=0.3376

4.12 Resistance-Associated Mutations and Polymorphisms

A total of 11 patients had at least one mutation associated with drug resistance out of which 8 had at least one surveillance drug resistance mutation (SDRM). One patient with research number C07 infected with CRF02_AG strain had 1(2.4%) protease major mutation which is M46MI and no protease accessory mutation was detected in any of the patients. A patient with CRF02_AG circulating form had 2 Thymidine-associated mutations (TAM) of NRTI class as L210LW and T215Y. Out of the 2 patients identified with CRF06_cpx, 1 had T215Y TAM. Gene mutation at position 184 was highest among NRTI resistance mutations with frequency of 7.7 % (3/39). In NNRTI resistance mutations, K103NSG was highest with a frequency of 12.8 % (5/39) while K101E, A98G and G190A had frequency of 7.7% (3/39) each. Table 4.12 shows the frequencies of occurrence of various resistance mutations identified among the drug-naïve patients.

Table 4.12: Frequencies of Drug Resistance Polymorphisms Among Dug-Naïve Patients

	Number of patients infected with resistant subtypes			
Gene and associated Mutation	G	CRF02_AG	CRF06_cpx	Total
Protease				
Major mutations				
M46MI	-	1	-	1
Accessory mutation				
Nil				
Reverse transcriptase				
NRTI mutations				
Thymidine-associated mutations				
L210LW	-	1	-	1
T215Y	-	1	1	2
Other NRTI mutations				
A62AV	-	1	-	1
Y115F	-	1	-	1
F116Y	-	1	-	1
Q151M	-	1	-	1
M184VI	-	2	1	3
K70KQ	-	2	-	2
NNRTI mutations				
K101E	1	2	-	3
V106I	2	-	-	2
E138AG	1	1	-	2
K103NSG	1	3	1	5
A98G	1	2	-	3
Y181IC	-	2	-	2
V108VI	-	1	-	1
G190A	-	2	1	3

Key: NRTI = Nucleoside Reverse Transcriptase Inhibitor, NNRTI = Non-Nucleoside Reverse Transcriptase Inhibitor

4.13 Predicted Drug Resistance Profile of Drug-Naïve Patients Infected With HIV-1

Human Immunodeficiency Virus Drug resistance mutation profiles of patients are presented in Table 4.13. Resistance to protease inhibitors drug class was detected in only 1 patient with potential low-level resistance to both ritonavir boosted Atazanavir and lopinavir. Resistance to drugs in NRTI class showed 3 patients had high-level resistance to emtricitabine and/or lamivudine while abacavir, zidovudine and tenofovir each recorded high-level resistance in 1 patient. Two patients had intermediate resistance to abacavir and/or zidovudine respectively. One and two patients had low-level resistance to abacavir and tenofovir respectively. Potential low-level resistance was detected in emtricitabine and lamivudine with frequency of 1 each (Figure 4.7).

In resistance to NNRTI class, 6 patients had high-level resistance (HLR) to efavirenz and/or nevirapine and 4 patients had HLR to rilpivirine. High-level resistance was equally recorded in doravirine and etravirine with frequency of 1 patient each. Two patients had intermediate resistance (IR) to etravirine and/or nevirapine and 3 patients had IR to doravirine, efavirenz and/or rilpivirine. One patient had low-level resistance to etravirine. Three patients recorded potential low-level resistance to etravirine while the same level of resistance is recorded in nevirapine and rilpivirine with a frequency of 1 patient.

Table 4.13: NRTI and NNRTI Drug Resistance Mutations Profile of Drug-Naïve Patients

Patient's Identifier	Type/Subtype	NRTI Mutation	NNRTI Mutation	SDRMs	Resistance drugs
C01	G	-	K101E, V106I, E138A	K101E	ABC[1], AZT[1] FTC[1], 3TC[1] TDF[1], DOR[4], EFV[3], ETR[4] NVP[4], RPV[5]
C04	G	-	K103N	K103N	ABC[1], AZT[1] FTC[1], 3TC[1,] TDF[1], DOR[1], EFV[5], ETR[1] NVP[5], RPV[1]
C05	CRF02_AG	M184V, L210LW, T215Y	A98G, K101E, G190A	K101E, M184V, G190A, L210W, T215Y	ABC[4], AZT[4] FTC[5], 3TC[5] TDF[3] DOR[4], EFV[5] ETR[3] NVP[5], RPV[5]
C08	CRF02_AG	-	A98AG, K101KE, E138G, Y181I	K101E, Y181I	ABC[1], AZT[1] FTC[1], 3TC[1] TDF[1], DOR[4], EFV[5], ETR[5] NVP[5], RPV[5]
C14	CRF06_cpx	M184V, T215F	K103S, G190A	K103S, M184V, G190A, T215F	ABC[3], AZT[4] FTC[5], 3TC[5] TDF[1], DOR[1], EFV[5], ETR[2] NVP[5], RPV[3]
C19	G	-	A98G	-	ABC[1], AZT[1] FTC[1], 3TC[1] TDF[1], DOR[3], EFV[3], ETR[2] NVP[4], RPV[3]
N29	CRF02_AG	-	K103N	K103N	ABC[1], AZT[1] FTC[1], 3TC[1] TDF[1], DOR[1], EFV[5], ETR[1] NVP[5], RPV[1]
N32	G	-	V106I	-	ABC[1], AZT[1] FTC[1], 3TC[1] TDF[1], DOR[3], EFV[1], ETR[2] NVP[2], RPV[2]
N36	CRF02_AG	-	K103Q	-	ABC[1], AZT[1] FTC[1], 3TC[1] TDF[1], DOR[1], EFV[1], ETR[1] NVP[1], RPV[1]
S40	CRF02_AG	A62AV, K70Q, Y115F, F116Y, Q151M, M184I	K103N, V108VI, Y181C	K103N, Y115F, F116Y, Q151M, Y181C, M184I, G190A	ABC[5], AZT[5] FTC[5], 3TC[5] TDF[5], DOR[5], EFV[5], ETR[4] NVP[5], RPV[5]
S43	CRF02_AG	K70KQ	-	-	ABC[3], AZT[1] FTC[2], 3TC[2] TDF[3], DOR[1], EFV[1], ETR[1] NVP[1], RPV[1]

Key: 1 = susceptible; 2 = Potential low-level resistance (mutation net drug score of 10-14); 3 = Low-level resistance (mutation net drug score of 15-30); 4 = Intermediate resistance (mutation net drug score of 31-59); 5 = High-level resistance (mutation net drug score of ≥60). ABC- Abacavir; AZT- zidovudine; FTC- emtricitabine; 3TC- lamivudine; TDF- tenofovir; DOR- doravirine; EFV- efavirenz; ETR- etravirine; NVP- nevirapine; DRV/r- ritonavir boosted darunavir; ATV/r - ritonavir boosted Atazanavir; RPV- ritonavir boosted rilpivirine; LPV/r- ritonavir boosted lopinavir

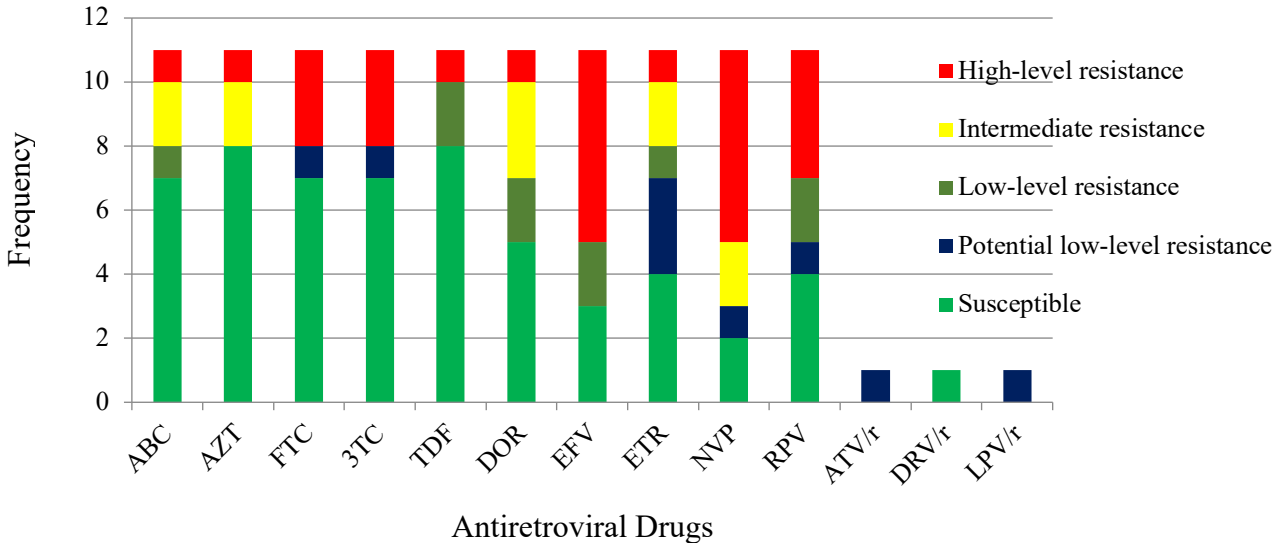

Figure 4.7: Predicted Antiretroviral Drug Resistance of Patients with Drug Resistance Mutations.

These drug predictions were based on the Stanford HIVdb v7.0. ABC- Abacavir; AZT- zidovudine; FTC- emtricitabine; 3TC- lamivudine; TDF- tenofovir; DOR- doravirine; EFV- efavirenz; ETR- etravirine; NVP- nevirapine; DRV/r- ritonavir boosted darunavir; ATV/r - ritonavir boosted Atazanavir; RPV- ritonavir boosted rilpivirine; LPV/r- ritonavir boosted lopinavir

CHAPTER FIVE

5.0 DISCUSSION

No previous study on HIV-1 diversity and transmitted drug resistance has been reported from the study area to the best of our knowledge. Therefore, it is pertinent to understand the resistance pattern and diversity of HIV-1 and associated characteristic markers such as CD4 cell count and the plasma viral load in the study sample as representative of the general population of drug-naïve HIV-infected adults in Kaduna State, Nigeria.

5.1 Socio-demographic and risk factors associated with the study participants

The median age of the participants in this study was 35 years with a range of 20 and 66 years. Takuva *et al*. (2012) have reported a similar median age of 36 years while Govender *et al.* (2014) reported a median age of 34 years among adults in testing centers in South Africa. Other study has recorded relatively lower median age of 32 years (Lawn *et al*., 2006).

More females were reported in this study than male and this could be attributed to the fact that female were found to be more infected than their male counterpart in the study area as reported by the Nigeria HIV/AIDS Indicator and Impact Survey (NAIIS) (2019) where female and male of age range 15 to 64 years recorded prevalence of 0.7% and 0.4% respectively in Northwestern Nigeria. The reason could be that more female patients presented themselves to the hospital during the survey. Female tend to be more often tested for HIV than male in the course of antenatal and other gynecological services rendered to them and thereby could be the reason for this ratio. Akinsegun *et al*. (2012) and Attah (2017) have also reported female to have higher frequency among HIV infected individuals in the study area.

The highest frequency of participants recorded among the 31-40 years age group in this study could be connected to the high-risk behaviors associated with the age category such as high sexual

activities. This age category is part of the active labour force and sexually active. Similar age range of 31-40 years was reported in Nigeria to have a high prevalence of 33.48% among HIV infected participants (Nwozor and Nwankwo 2013). All the participants in this study were exposed to at least one or more risk factors associated with HIV transmission. Therefore, this attribute to the possible risk that predisposed them to the HIV transmission.

5.2 Prevalence of HIV-1 among the HIV infected participants

All the 50 participants were found to be infected with HIV type-1. This could be as a result of remarkable decrease of HIV-2 prevalence since the 1990s and simultaneous increase in HIV-1 prevalence (Olaleye *et al*., 1993; Zeh *et al*., 2005; Omobolaji *et al*., 2011). This decrease in HIV-2 prevalence could be seen in study reported by Olaleye *et al*. (1993) where 3854 sera samples were collected between 1985-1990 in Ibadan Nigeria and recorded HIV-2 prevalence of 1.3%. Nearly a decade later, Zeh *et al*. (2005) reported a decreased prevalence of 0.3% of HIV-2 among 420 blood samples collected from 35 States of Nigeria in 1999. Decline of HIV-2 prevalence could be attributed to low infectivity when compared to HIV-1 (Campbell-Yesufu and Gandhi, 2011).

The antibody against p24 antigen was not detected in two out of the 50 samples assayed. The p24 antigen is the first viral protein to appear after acute infection and as the infection progresses; antibody to p24 is produced with parallel loss in p24 antigen. However, as infection progress 25-50% adult patients lose detectable anti-p24 and antigenaemia recur. Although anti-p24 antibodies are not neutralizing, their continued presence in infected patients is correlated with a delayed progression to AIDS (Stickler *et al*., 1992).

5.3 CD4 cells enumeration among drug-naïve HIV-1 infected participants

The median CD4 cell count for the drug-naïve HIV-1 infected participants in this study was 176.0 cells/µl. This low median CD4 cell count is expected as the study participants were drug-naïve. Decreased CD4 cell count has been reported to increase risk of clinical events and developing resistance (Phillips *et al*., 2001). Opportunistic infections may set in among the participants due to this low median CD4 cell count. In 2013, Anude *et al.* in Nigeria reported comparable median baseline CD4 cell count of 142 cells/ µl. Lawn *et al.* (2006) have reported a relatively lower baseline median CD4 cell count of 97cells/ µl. Govender *et al.* (2014) reported in Soweto of South Africa a median CD4 cell count of 364 cells/µl. This finding demonstrated that female had higher median CD4 cell count than male; a result in agreement with several reports (Akinbami *et al*., 2012; Maskew *et al*., 2013; Govender *et al*., 2014; Attah, 2017) although our comparison had no statistical significant difference. The gender variation of the CD4 cell count has been linked to hormonal difference such as estradiol (Rodriguez-Garcia *et al*., 2013). Twenty-six (52%) of the participants had CD4 cell count < 200 cells/ µl and ART initiation at lower CD4 cell count has been associated with early mortality primarily from Immune Reconstitution Inflammatory Syndrome (IRIS) (Mohas *et al*., 2013). Akinsegun *et al*. (2012) reported similar result of 51.9% among ART-naïve HIV patients.

5.4 HIV-1 plasma viral load among drug-naïve HIV-1 infected participants

A total of 47 (94%) study participants had detectable levels of plasma HIV-1. The high percentage is not unconnected to the study population category as drug-naïve HIV infected individuals mostly have detectable plasma HIV level (Odaibo *et al.*, 2013). Previous studies have reported relatively lower proportion of study participants with detectable levels of plasma HIV-1 of 90.5% (Odaibo *et al.*, 2013) in Nigeria and 91.5% (Haokip and Heigrujam, 2018) in India. The participants with

undetected plasma RNA could be HIV or ‘‘elite’’ controllers which are defined as drug-naïve HIV-infected individuals having undetectable viremia by standard assays (<50 copies RNA/mL) (Deeks and Walker, 2007; Hatano *at al.,* 2009). These patients with undetectable viral load could have been affected by social desirability bias and concealed their real ART status. The success rate for drug resistance testing decreases with low-level viremia as PCR fails to amplify the region of interest for sequencing reaction (Gonzalez-Serna *et al*., 2014; Douglas, 2014). Plasma with undetectable HIV RNA copies could pose challenge of unsuccessful nucleic acid amplification of target region. Undetectable viremia or plasma HIV-1 RNA levels below 1500 copies per milliliter were not associated with heterosexual HIV transmission, whereas the risk of transmission increased substantially with increasing viral loads (Quinn *et al*., 2000).

We reported in this study a median viral load of 158,391 copies/ml. Odaibo *et al*. (2013) has reported similar higher mean of 450,370 copies/ml in Nigeria. Three different South African and a Botswana studies reported median plasma viral load of less than 100,000 copies/ml among drug-naïve patients (Lawn *et al*., 2006; Govender *et al*., 2014; Martinson *et al*., 2014; Farahani *et al.,* 2016). This notable difference could be linked to the various assays used for the plasma RNA quantification. High baseline plasma viral load can affect prognosis, disease progression and transmission (Quinn *et al*., 2000; Farahani *et al*., 2016).

This study reported female to have lower median HIV-1 plasma viral load than their male counterparts. This reciprocates the CD4 cell count where female had higher value than male. This data suggest that women may be a low-risk group for HIV. This could be because of differences in the genital anatomy between male and female. Majority of the patients (84%) had viral load >10,000 copies/ml and 60% had viral load >100,000 copies/ml. In a study, slow rate of achieving viral suppression during treatment has been connected with patients with viral load of >100,000

copies/ml (Philips *et al*., 2001). Another study by Farahani *et al*. (2016) reported that the hazard of developing composite outcome for the individuals with baseline HIV-1 RNA >10,000 copies/ml to be 2.3 times higher than that for those with baseline HIV-1 RNA <10,000 copies/ml. Higher viral load in patients was found to deplete CD4 cells much faster than in those with relatively lower viral load and this is because there are more viruses to infect the CD4 cells and deplete them(Farahani *et al*., 2016). In similar drug-naïve studies by Rangarajan *et al.* (2016) and Farahani *et al*. (2016) 60% and 53% of the study participants had viral load of >10,000 copies/ml respectively. Additionally, Rangarajan *et al.* (2016) reported 16% patients that had viral load >100,000 copies/ml.

5.5 Correlation between CD4 cell counts and plasma HIV viral load

There was a significant moderately strong, negative Spearman correlation between HIV-1 plasma viral load and CD4 cell count in this study. This reciprocal association could be for the reason that plasma viral load strongly predicts the rate of decrease in CD4 cell count and progression to AIDS although, correct prognosis of HIV infected individual is better known when both CD4 cell and plasma viral load are used (Mellors *at al.,* 1997). Rangarajan *et al*. (2016) and Govender *et al*. (2014) reported similar relationship however, Farahani *et al*. (2016) reported negative but weak correlation. Method, sensitivity and specificity of assays may cause this variation.

5.6 HIV genetic diversity and transmitted drug resistance

Protease and reverse transcriptase regions were amplified and sequenced from 89.4% and 83.0% of samples with detectable plasma viral load respectively. The level and difference in the amplification rate may possibly be due to low level viremia or variations in the targeted regions of interest. This could also be the reason the ANRS primer failed to amplify the targeted region. When there are too much variations between the annealing sites at the target region and the

designed primer sets, primer annealing may fail and hence there will not be amplification. Similar study conducted in Kinshasa, Democratic Republic of Congo reported amplification rate for protease and reverse transcriptase region of 84.9% and 94.8% respectively (Kamangu *et al*., 2015).

The evolution and spread of HIV has resulted to several distributions of subtypes, CRFs and URFs which change over time particularly in various regions and generally in global scale. This makes the pandemic complex and dynamic. Therefore, it is essential to continuously monitor the diversity and spread of HIV-1 regionally and worldwide as the pandemic develops. All the strains identified in this study were non-subtype B and this is consistent since subtype B is not predominant in Nigeria. Subtype B is reported to be predominant in North America, Western Europe, and Australia (Kantor *et al*., 2005). Genetic diversities reported in this study could have many implications as Fayemiwo *et al.* (2014) reported HIV-1 genetic diversity has consequences in antiretroviral therapies, development of diagnostic test and vaccine. HIV-1 diversity influences antiretroviral therapies as subtypes differ from one another by 10-12% of their nucleotides and 5%–6% of their amino acids in protease and reverse transcriptase which in turn can impact the spectrum of mutations that occur during selective drug pressure (Kantor *et al*., 2005).

Roughly, 60% of the isolated strains were circulating recombinant forms which indicates preponderance of the CRFs in Kaduna State Nigeria. The CRF02_AG was found to be the predominant strain in the study area. This was not unexpected as the CRF was earlier shown to have emerged among several African countries and became epidemic in the African continent, mostly in West and West Central Africa, where it accounts for 50 to 70% of the circulating strains (Requejo, 2006). Infection with CRF02_AG has been reported to facilitate transmission because it is characterized by high viral loads (Delgado *et al*., 2008) and has also been shown to have high

in vitro replication capacity than its parental subtype A and G. Therefore, this capacity may contribute to its dominant spread (Konings *et al*., 2006; Njai *et al.,* 2006).

Diversity in subtype distribution was observed before year 2000 as subtypes A (61%) and G (31%) were dominant in the southern and northern Nigeria, respectively, and CRF02-AG was not detected (Peeters *et al*., 2000). Afterward, several studies reported that subtype A have significantly reduced with minor reduction of subtype G and a remarkable increase of CRF02_AG (Diallo *et al*., 2015).

Some studies reported CRF02_AG to be predominant in Nigeria (Charurat *et al*., 2012; Anejo-Okopi *et al*., 2013; Rawizza *et al*., 2013; Crawford *et al*., 2014; Heipertz Jr 2016) while other studies reported subtype G to be predominant (Etiebet *et al*., 2013; Fayemiwo *et al*., 2014; Imade *et al*., 2014; Negedu-Momoh *et al*., 2014; Diallo *et al*., 2015). However, a recent systematic review and scoping analysis of reported data from 2002 to 2017 in Nigeria showed Subtype G as the most predominant HIV-1 strain in Nigeria (Tahir *et al*., 2019).

Clustering of the isolates observed in the phylogenetic trees in this study in separate subtree can explain the evolutionary dynamism of HIV-1 virus. Since these isolates were isolated at different time and from different places with the reference sequences, viruses from these two distinct sources of the same subtype or CRF may not have 100% same genetic configuration. Therefore, spatial and temporal factors must have affected their genetic variability. Phylogenetic tree of reverse transcriptase gene did not give the same pattern with protease gene and this can be attributed to the different mutation and recombination rates associated with each of the gene. Reverse transcriptase gene was reported to have low recombination event rate (Ren *et al*., 2016).

In this this study an overall transmitted drug resistance prevalence of 19.5% was obtained. Global scaling up of ART continue to have unintended consequence of transmitted drug resistance especially in resource-limited settings. Inadequate, poor accessibility and adherence to drugs could contribute to this. It is established that HIV transmitted drug resistance will emerge in the region where ART has been widely available for years (Bennett *et al*., 2008). Therefore, HIV transmitted drug resistance in Nigeria is not unexpected as the ART has been scaled up for the past 2 decades. Transmitted drug resistance is defined as presence of at least 1 surveillance drug resistance mutations (SDRMs) (Bennett *et al*., 2008). Transmitted drug resistance has therapy implication as it limits therapeutic options and increases the likelihood of therapy failure. The SDRM list has 93 mutations including 34 NRTI-resistance mutations at 15 RT positions, 19 NNRTI-resistance mutations at 10 RT positions, and 40 PI-resistance mutations at 18 protease positions (Bennett *et al*., 2009).

The high TDR in this study infers that many HIV-infected persons receiving antiretroviral therapy with possible drug adherence problem continue to participate in risk behavior. This is high level transmitted HIV drug resistance (> 15%) as classified by WHO (WHO, 2012) and to the best of our knowledge is the highest reported in Nigeria. This high-level TDR prevalence can be attributed to the remarkable scale-up of provision of ARVs by the international donors to Low- and middle-income countries including Nigeria (Sungkanuparph *et al*., 2012). The Nigeria government, with funding from the U.S. President's Emergency Plan for AIDS Relief (PEPFAR), and other international and local donors, has intensely scaled up ART intervention over the years. By the year 2006 about 90,000 patients were initiated on ART and by 2009 the number increased to 300,000. At the end of 2012 about half a million patients were initiated on ART (Dalhatu *et al*.,

2016). This number increased by 2015 to 853,992 patients covering 28.1% of population living with HIV (Adeyinka *et al*., 2018).

Diallo *et al*. (2015) and Imade *et al*. (2014) reported < 5% TDR prevalence in 2015 and 2014 respectively while Anejo-Okopi *et al*. (2013) and Ojesina *et al*. (2006) reported moderate level of TDR of 8% and high-level TDR of 17% respectively. A study in Germany has reported a similar high prevalence of 17.2% (Machnowska *et al*., 2019). Onywera *et al*. (2017) and Reynolds *et al.* (2016) in Kenya and Uganda have reported TDR prevalence of 9.2% and 4% respectively. A meta-analysis consisting of 95 studies from 32 sub-Saharan African countries had reported pooled TDR prevalence of 2.8% (Rhee *et al*., 2015).

There was only one sequence with SDRM detected against protease inhibitors and at least SDRMs in three sequences were detected against NRTI while NNRTI had the seven sequences with at least one SDRMs associated with them. This observed difference in mutation frequency could be because only efavirenz or nevirapine as NNRTI is used with many choices from NRTI to formulate various highly active antiretroviral therapy (HAART) regimens available in Nigeria. Therefore, people living with HIV on HAART have more contact with NNRTI than other classes of antiretrovirals.

Lower CD4 cell count and HIV plasma viral load mean was obtained among patients with TDR with no statistically significant difference. This could be as a result of low sample size of the TDR patients. in In PIs, NRTIs and NNRTIs-associated mutations and polymorphisms were determined in the study. The one sample with M46MI mutation in the protease region was associated with potential low-level resistance to both Atazanavir and lopinavir boosted with ritonavir. Diallo *et al*. (2015) has reported 1 M46L mutation in Abuja, Nigeria.

Two TAMs were reported in a single sample with CRF02_AG strain. This individual might have been infected by HIV infected persons failing zidovudine or stavudine-based therapy. M184VI and K103NSG had highest frequencies in this study and are commonly found in patients failing NNRTI-based regimens, such as tenofovir/lamivudine/efavirenz, tenofovir/emtricitabine/efavirenz, zidovudine/lamivudine/nevirapine, tenofovir/lamivudine (or emtricitabine)/nevirapine and abacavir/lamivudine/efavirenz, which are commonly used as first-line ART in Nigeria (FMOH, 2016).

In this study, the highest frequency of high-level resistance to efavirenz and nevirapine which are NNRTI mostly used in ART regimens in Nigeria and therefore, the people living with HIV on ART are frequently more exposed to them than any other ARV. However, 4 patients had high level resistance to rilpivirine which is drug that has not been used in Nigerian ART guidelines or regimens. Three patients had intermediate resistance to doravirine which has not been used as a part of combination of HAART in Nigeria. This could be as a result of multiple mutations that may predispose resistance to more drugs not been exposed in a certain population.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION, RECOMMENDATIONS AND LIMITATIONS

6.1 Summary

This study was cross-sectional and hospital-based where 50 adult ART-naïve HIV infected persons were recruited by probability proportional to size (PPS) sampling method from three selected hospitals in Kaduna State, Nigeria. Samples were collected for CD4 cell count, viral load and HIV pol region sequencing from the eligible participants and assayed using flow cytometry, real time PCR techniques and reverse transcriptase PCR. The RT PCR amplicons were sequenced and the reads subjected to bioinformatics. Data were collected using Epi-info® 7 and analysed using GraphPad Prims 6 statistical software package.

Socio-demographic data associated with the study participants were determined. The study sample was predominantly female with frequency of 64%. Age group 31 – 40 years constituted the highest frequency of 42%.

All the HIV infected participants studied were found to be infected with HIV type 1. The study population was found to have relatively high median viral load of 158,391 (IQR: 44,730- 548,281) copies/ml. However, few participants, despite been ART-naïve yet had undetectable plasma viral load. The median CD4 cell count was found to be 176.0 cell/µl (IQR: 74.75- 402.8). Female had higher CD4 cells count and lower plasma HIV-1 viral load than male. CD4 cells had strong reciprocal relationship with the plasma HIV-1 viral load. There was strong negative significant correlation between HIV-1 plasma viral load and CD4 cells count in the study population.

The prevalence of transmitted drug resistance was found to be 19.5 % which is very high in the study area. The HIV-1 isolates were characterized into CRF02_AG, subtype G, CRF06_cpx and subtype C with frequency of 25(61%), 13(32%), 2(5%) and 1(2%) respectively.

Protease inhibitors, NRTI and NNRTI surveillance drug resistance mutations and other polymorphic mutations were detected among the drug-naïve HIV infected patients. The K103NSG mutation was highest with a frequency of 12.8 % (5/39). Genetic relatedness of the sequences from the isolates and reference sequences were presented in maximum likelihood phylogenetic tree.

6.2 Conclusion

Socio-demographic data associated with the study participants were determined. All (100%) the HIV infected participants studied were found to be infected with HIV type 1. The study population was found to have relatively high viral load (158,391 Copies/mL). However, few participants (3), despite been ART-naïve yet had undetectable plasma viral load. Female had higher CD4 cells count and lower plasma HIV-1 viral load than male. CD4 cells had strong reciprocal relationship with the plasma HIV-1 viral load. There was strong negative significant correlation between HIV-1 plasma viral load and CD4 cells count in the study population.

The prevalence of transmitted drug resistance was found to be 19.5 % which is very high in the study area. The HIV-1 isolates were characterized into CRF02_AG, subtype G, CRF06_cpx and subtype C with frequency of 25(61%), 13(32%), 2(5%) and 1(2%) respectively. Protease inhibitors, NRTI and NNRTI surveillance drug resistance mutations and other polymorphic mutations were detected among the drug-naïve HIV infected patients. The K103NSG mutation was highest with a frequency of 12.8 % (5/39). Genetic relatedness of the sequences from the isolates and reference sequences were presented in maximum likelihood phylogenetic tree.

6.3 Recommendations

i. Further study is recommended on the HIV-1 infected drug-naïve individuals with undetectable viral load to ascertain viral set points and other host and viral immunological indices.

ii. Pretreatment resistance testing should be the standard of care for recently infected individuals. This will help in selecting appropriate first-line treatment regimen and arresting further spread of drug resistant HIV strains

iii. There is need to design of consensus primer sets that could amplify all HIV non-B subtypes that are predominant in this part of the world.

iv. It is necessary to standardize an affordable in-house genotyping system as conducted in this work for middle- and low-income countries. Commercially available kits such as ViroSeq™ HIV-1 and TRUGENE® HIV-1 genotyping systems cost up to $120 and $150 per test respectively

v. To understand better the concept of HIV drug resistance, efforts must be extended to proteomics to understand how protein-protein interactions affect drug resistance.

6.4 Limitation

i. Social desirability bias might have affected this study as patients could present themselves as drug-naïve while they must have been enrolled in another center and loss to follow-up or relocated.

ii. Some samples with high viral load yet have load failed to amplify for both protease and reverse transcriptase genes. These samples might have had target nucleotides sequence with variability at the annealing site of the primer sets used in this work.

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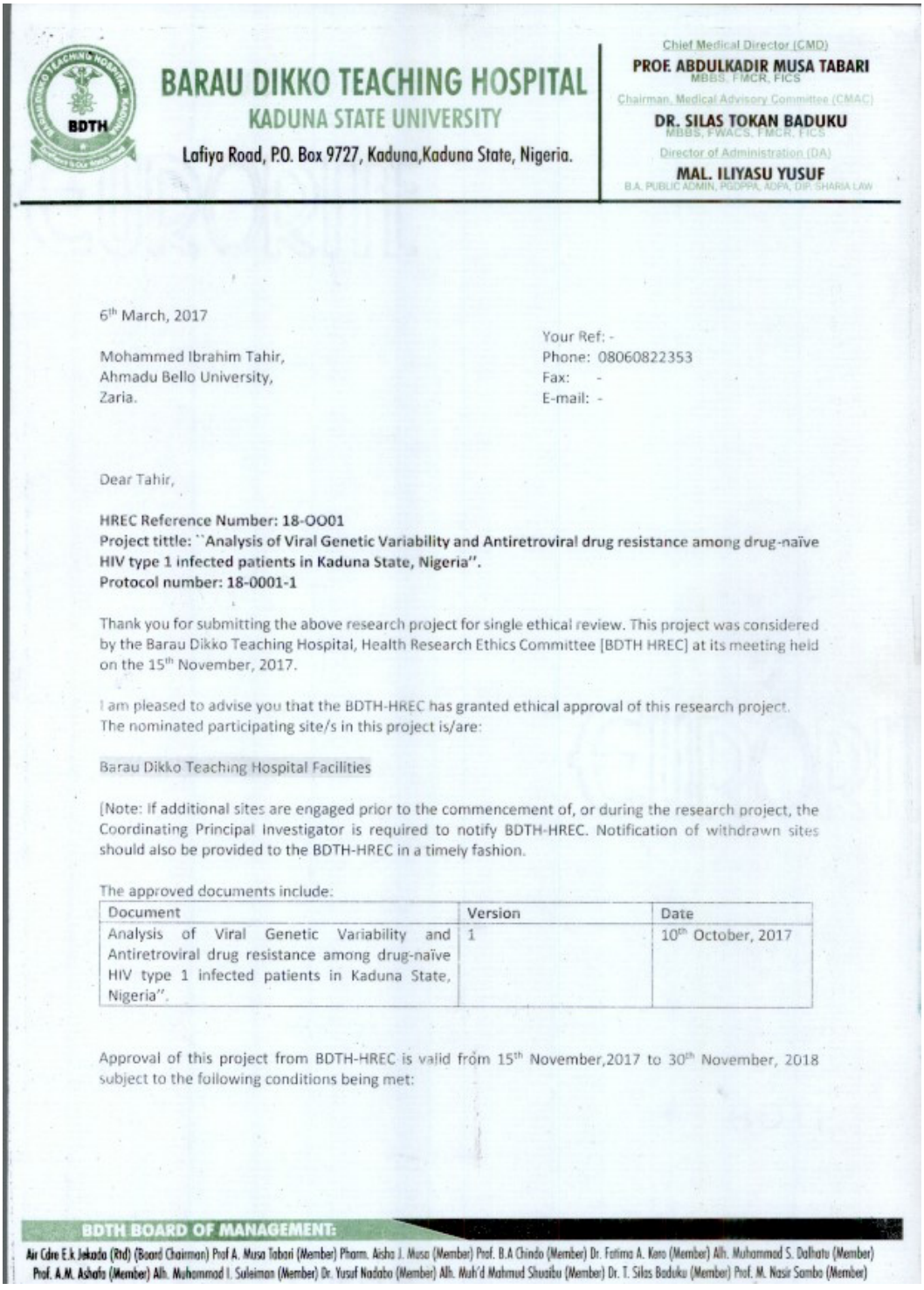
APPENDICES
APPENDIX Ia
Ethical clearance I



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MBBS, FWACS, FMCR, FICS
Director of Administration (DA)
MAL. ILIYASU YUSUF
B.A. PUBLIC ADMIN, PGDPPA, ADPA, DIP. SHARIA LAW

6th March, 2017

Mohammed Ibrahim Tahir,
Ahmadu Bello University,
Zaria.

Your Ref: -
Phone: 08060822353
Fax: -
E-mail: -

Dear Tahir,

HREC Reference Number: 18-OO01
Project tittle: ``Analysis of Viral Genetic Variability and Antiretroviral drug resistance among drug-naïve HIV type 1 infected patients in Kaduna State, Nigeria''.
Protocol number: 18-0001-1

Thank you for submitting the above research project for single ethical review. This project was considered by the Barau Dikko Teaching Hospital, Health Research Ethics Committee [BDTH HREC] at its meeting held on the 15th November, 2017.

I am pleased to advise you that the BDTH-HREC has granted ethical approval of this research project. The nominated participating site/s in this project is/are:

Barau Dikko Teaching Hospital Facilities

[Note: If additional sites are engaged prior to the commencement of, or during the research project, the Coordinating Principal Investigator is required to notify BDTH-HREC. Notification of withdrawn sites should also be provided to the BDTH-HREC in a timely fashion.

The approved documents include:

Document	Version	Date
Analysis of Viral Genetic Variability and Antiretroviral drug resistance among drug-naïve HIV type 1 infected patients in Kaduna State, Nigeria''.	1	10th October, 2017

Approval of this project from BDTH-HREC is valid from 15th November,2017 to 30th November, 2018 subject to the following conditions being met:

BDTH BOARD OF MANAGEMENT:
Air Cdre E.k. Jakada (Rtd) (Board Chairman) Prof A. Musa Tabari (Member) Pharm. Aisha J. Musa (Member) Prof. B.A Chindo (Member) Dr. Fatima A. Karo (Member) Alh. Muhammad S. Dalhatu (Member) Prof. A.M. Ashafa (Member) Alh. Muhammad I. Suleiman (Member) Dr. Yusuf Nadaba (Member) Alh. Muh'd Mahmud Shuaibu (Member) Dr. T. Silas Baduku (Member) Prof. M. Nasir Samba (Member)

- The Coordinating Principal Investigator will immediately report anything that might warrant review of ethical approval of the project.
- The Coordinating Principal Investigator will notify the BDTH-HREC of any event that requires a modification to the protocol or other project documents and submit any required amendments in accordance with the instructions provided by the HREC.
- The Coordinating Principal Investigator will submit any necessary reports related to the safety of research participants in accordance with BDTH-HREC policy and procedures.
- The Coordinating Principal Investigator will report the BDTH-HREC and notify the HREC when the project is completed at all sites.
- The Coordinating Principal Investigator will notify the BDTH-HREC if the project is discontinued at a participating site before the expected completion date, with reasons provided.
- The Coordinating Principal Investigator will notify the BDTH-HREC of any plan to extend the duration of the project past the approval period listed above and will submit any associated required documentation.
- The Coordinating Investigator will notify the BDTH-HREC of his or her inability to continue as Coordinating Principal Investigator including the name of and contact information for a replacement.

This letter constitutes ethical approval only. This project cannot proceed at any site until separate research governance authorization has been obtained from the CEO or delegate of the institution under whose auspices the research will be conducted at that site.

Should you have any queries about the BDTH-HREC's consideration of your project please contact Admin officer, Barau Dikko Teaching Hospital, Lafia Road Kaduna.
The BDTH HREC wishes you every success in your research.

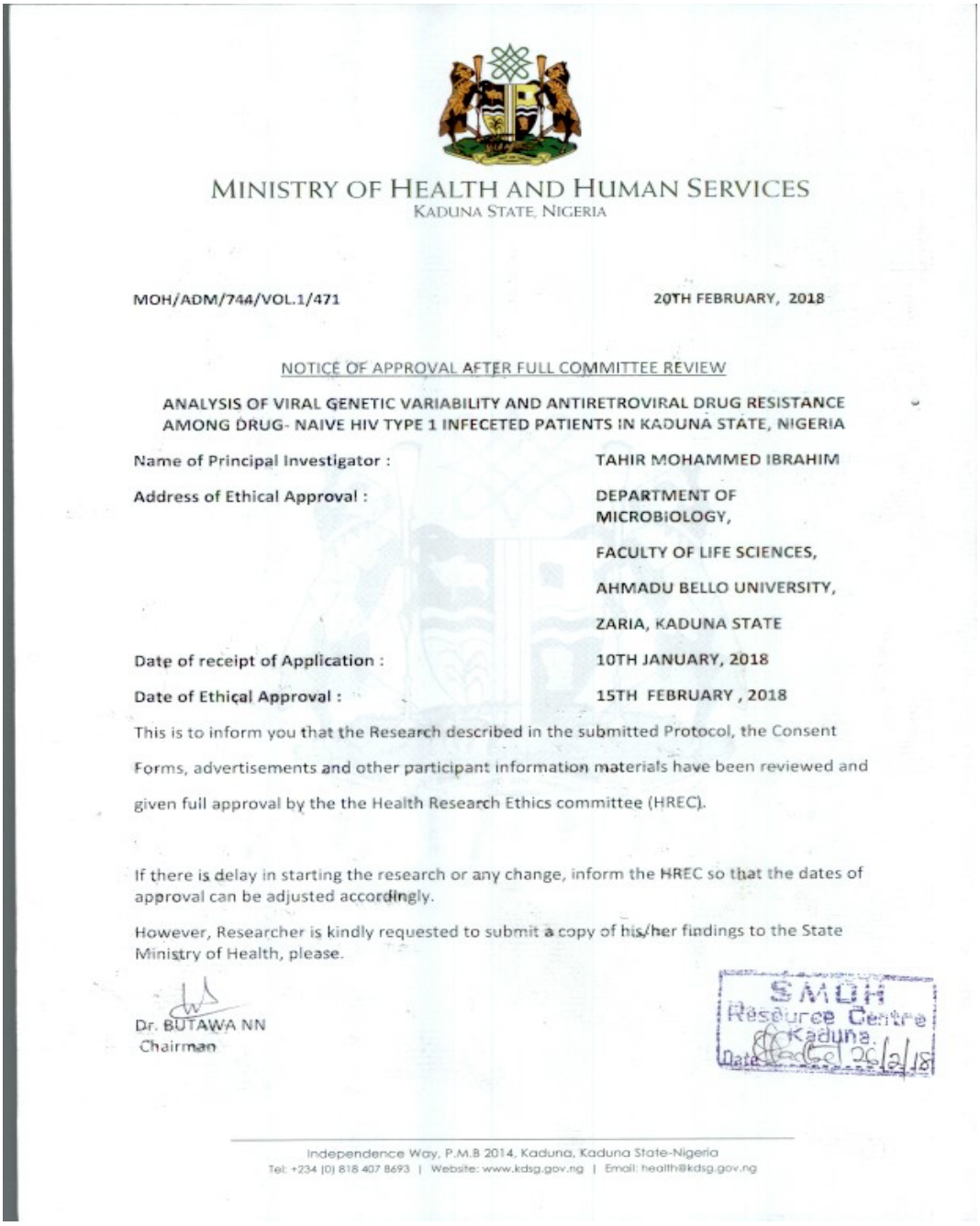
Yours faithfully,

06/03/2018

Dr. J M Banda
Secretary
BDTH HREC This HREC is constituted and operates in accordance with the National Health Research Ethics Committee (NHREC)

APPENDIX Ib

Ethical Clearance II



MINISTRY OF HEALTH AND HUMAN SERVICES
KADUNA STATE, NIGERIA

MOH/ADM/744/VOL.1/471 20TH FEBRUARY, 2018

NOTICE OF APPROVAL AFTER FULL COMMITTEE REVIEW

ANALYSIS OF VIRAL GENETIC VARIABILITY AND ANTIRETROVIRAL DRUG RESISTANCE AMONG DRUG- NAIVE HIV TYPE 1 INFECETED PATIENTS IN KADUNA STATE, NIGERIA

Name of Principal Investigator :	TAHIR MOHAMMED IBRAHIM
Address of Ethical Approval :	DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA, KADUNA STATE
Date of receipt of Application :	10TH JANUARY, 2018
Date of Ethical Approval :	15TH FEBRUARY , 2018

This is to inform you that the Research described in the submitted Protocol, the Consent Forms, advertisements and other participant information materials have been reviewed and given full approval by the the Health Research Ethics committee (HREC).

If there is delay in starting the research or any change, inform the HREC so that the dates of approval can be adjusted accordingly.

However, Researcher is kindly requested to submit a copy of his/her findings to the State Ministry of Health, please.

Dr. BUTAWA NN
Chairman

SMOH Resource Centre Kaduna. Date Received 26/2/18

Independence Way, P.M.B 2014, Kaduna, Kaduna State-Nigeria
Tel: +234 (0) 818 407 8693 | Website: www.kdsg.gov.ng | Email: health@kdsg.gov.ng

Appendix II: Informed consent form

DEPARTMENT OF MICROBIOLOGY

AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

INDVIDUAL INFORMED CONSENT FORM

This Informed Consent Form is for HIV infected individuals attending Barau Dikko Teaching Hospital, Kaduna. We are inviting you to participate in this research work titled **"Analysis of Viral Genetic Variability and Antiretroviral Drug Resistance among Drug-Naïve HIV Type-1 Infected Patients attending selected Hospitals in Kaduna State, Nigeria."**

Principal Investigator: Tahir, Mohammed Ibrahim
Co-investigators: Prof M. Aminu-Mukhtar
Dr A. B. Suleiman
Dr S. A. Opaluwa

Name of Proposal: Analysis of Viral Genetic Variability and Antiretroviral Drug Resistance among Drug-Naïve HIV Type-1 Infected Patients attending selected Hospitals in Kaduna State, Nigeria

This Informed Consent Form has two parts:

- Information Sheet (to share information about the research with you)
- Certificate of Consent (for signatures if you agree to take part)

You will be given a copy of the full Informed Consent Form

PART I: INFORMATION SHEET

Introduction

I am Tahir Mohammed Ibrahim, a Ph.D. student of the Department of Microbiology, Ahmadu Bello University, Zaria carrying out a research work. We are inviting you to participate in this research work titled **"Analysis of Viral Genetic Variability and Antiretroviral Drug Resistance among Drug-Naïve HIV Type-1 Infected Patients attending selected Hospitals in Kaduna State, Nigeria".**

I am going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.
There may be some words that you do not understand. Please, ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask me or your doctor.

Purpose of the research

The purpose of this research is to determine the Molecular Diversity and the prevalence of mutations associated with resistance to antiretrovirals (ARVs) in treatment-naïve and experienced patients attending selected hospital in Kaduna State, Nigeria.

Participant selection

We are inviting drug naïve HIV infected individuals in the aforementioned hospital to participate in this research.

- *Do you know why we are asking you to take part in this study? YES.....NO……..*
- *.Do you know what the study is about? YES..... NO. .....*

Voluntary Participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. Whether you choose to participate or not, all the services you receive in this hospital will continue and nothing will change. If you choose not to participate in this research project, you will be offered the service that is routinely offered in this hospital. You may change your mind later and stop participating even if you agreed earlier.

- *If you decide not to take part in this research study, do you know what your options are? YES…….. NO……..*
- *Do you know that you do not have to take part in this research study, if you do not wish to? YES…… NO ……..*
- *Do you have any questions? YES…… NO. ……*

Procedures and Protocol

When you have agreed to participate in this research, we will take blood from your arm aseptically by venipuncture, and dispense into sterile labeled EDTA anticoagulant container. The samples will be taken to the laboratory for analysis. At the end of the research any leftover blood will be destroyed.

Description of the Process

In the hospital small amount of blood will be taken from you as described above. This blood will be tested for the presence of HIV-1 antibodies. If the antibodies of the virus are detected, the blood will be used to detect drug resistance using Polymerase Chain Reaction (PCR) and DNA sequencing techniques.

We will also ask you a few questions about your general health and other measures so as to investigate the epidemiologic risk factors associated with the presence of the virus.

Duration

The study is designed to have contact with the volunteers only once from which their samples will be collected and processed over the period of the research.

Risks

There is a risk that you may share some personal or confidential information by chance, or that you may feel uncomfortable talking about some of the topics. However, we do not wish for this to happen. You do not have to answer any question or take part in the research if you feel the question(s) are too personal or if talking about them makes you uncomfortable.

Benefits

If you participate in this research, you will have the following benefits:

- ✓ You will have the opportunity of being tested for HIV resistance gene
- ✓ You will have a better knowledge and understanding of HIV infection
- ✓ There may not be any benefit to the society at this stage of the research, but future generations are likely to benefit.
- ✓ Apart from these few benefits listed above, your participation is likely to help us find answer to the research questions.

Reimbursements
You will not be provided with any special incentive or travel allowance for you to take part in the research. But we advise that your attendance in the hospital will have to be on your normal weekdays. However, you will have the opportunity of doing free HIV resistance study.

- *Can you tell me if you have understood correctly the benefits that you will have if you take part in the study?.....*
- *Do you know that the study will not pay for any costs?.....*
- *Do you know the kind of benefit you can get by participating in this research?.....*
- *Do you have any other questions?.....*

Confidentiality
With this research, it is possible that if others in the community are aware that you are participating, they may ask you questions. We will not be sharing the identity of those participating in the research.
The information that we collect from this research project will be kept confidential. Information about you that will be collected during the research will be put away and no-one but the researchers will be able to see it. Any information about you will have a number on it instead of your name. Only the principal investigator will know what your number is and we will lock that information up with a lock and key. It will not be shared with or given to anyone except the principal investigator.

- *Did you understand the procedures that we will be using to make sure that any information that we as researchers collect about you will remain confidential?.....*
- *Do you have any questions about them?.....*

Sharing the Results
The knowledge that we get from doing this research will be shared with you before it is made widely available to the public. Confidential information will not be shared. There will be small meetings and these will be announced. After these meetings, we will publish the results in order that other interested people may learn from our research. The publication will not capture any information linked to you.

Right to Refuse or Withdraw
You do not have to take part in this research if you do not wish to do so, refusing to participate will not affect the services rendered to you in this hospital in any way. You will still have all the benefits that you would otherwise have in this hospital. You may stop participating in the research at any time that you wish without losing any of your rights as a patient. Services given to you in this hospital will not be affected in any way.

Who to Contact
If you have any questions you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact the following:

Tahir Mohammed Ibrahim: 08060822353.

This proposal has been reviewed and approved by Scientific Ethical Committee (SEC) of the Ministry of Health and Human Services, which is a committee whose task is to make sure that research participants are protected from harm.

- *Do you know that you do not have to take part in this study if you do not wish to? ……*
- *Do you know that you can ask me questions later, if you wish to? ……..*
- *Do you know that I have given the contact details of the person who can give you more information about the*

study?.....

- *You can ask me any more questions about any part of the research study, if you wish to. Do you have any questions?.....*

PART II: CERTIFICATE OF CONSENT

I have read the foregoing information, or it has been read and translated to me in language that I understand. I have also talked it over with the doctor to my satisfaction. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I understand that my participation is voluntary. I know enough about the purpose, methods, risks and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I have received a copy of this consent form and additional sheet to keep for myself. I therefore consent voluntarily to participate as a participant in this research.

Name of Participant: .....

Signature of Participant: .....

Date: .....
Day/month/year

Statement by Witness
I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness: .....

Signature of witness: ……...

Date …….
Day/month/year

Statement by the Researcher/Person explaining Consent
I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. ***Blood sample will be taken***

I confirm that sufficient information, including about risks and benefits, to make an informed decision have been fully explained to the participant. The participant was given an opportunity to ask questions about the study, and all the questions asked by participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

Name of Researcher/Person explaining the consent: Tahir, Mohammed Ibrahim.

Signature of Researcher /Person explaining the consent: _____

Date _____ Day/month/year

APPENDIX III

Plate II: An extract from outcome of multiple sequence alignment of a portion of pol region using ClustalW

APPENDIX IV
QUESTIONNAIRE

Analysis of Viral Genetic Variability and Antiretroviral Drug Resistance among Drug-Naïve HIV Type-1 Infected Patients attending selected Hospitals in Kaduna State, Nigeria

QUESTIONNAIRE

Name (Optional): _____ Hospital Name: _____

Patient's Care No.:_____ Participant's Unique Serial code: _____

Date: _____

1. Age [ ]
2. Sex: M [ ] F [ ]
3. Address: _____
4. Phone No._____ Alternative phone No._____
5. Level of Education: Primary [ ] Secondary [ ] Tertiary [ ] Informal only [ ] None [ ]
6. Occupation: Civil servant [ ] Self-employed [ ] NGO-employed [ ] Retired [ ] Student [ ] others (specify) _____
7. Marital Status: Married [ ] Single [ ] divorced [ ] widow/ widower [ ]
8. If married, what is the nature of the marriage? Monogamy [ ]Polygamy []
9. Ever had sex? Y[ ] N[ ]
10. Number of Sexual Partners: 1 [ ] ≥ 2[ ]
11. Sexual orientation: Heterosexual [ ] homosexual [ ]
12. Have ever travelled out of the country? Yes [ ] No [ ]
    If yes where_____

13. Have you ever had:

i.	scarification?	Yes [ ] No [ ]
ii.	do you a partner(s) living with HIV?	Yes [ ] No [ ]
iii.	parent(s) living with HIV?	Yes [ ] No [ ]
iv.	sharing hair clipper?	Yes [ ] No [ ]
v.	needle-stick injury?	Yes [ ] No [ ]
vi.	child delivery at home?	Yes [ ] No [ ] NA [ ]
vii.	surgery?	Yes [ ] No [ ]
viii.	local surgery (e.g. belu, cupping)?	Yes [ ] No [ ]
ix.	blood transfusion?	Yes [ ] No [ ]
x.	dental procedure?	Yes [ ] No [ ]
xi.	exposure to blood, body fluid or tissues?	Yes [ ] No [ ]
xii.	commercial nail cutting?	Yes [ ] No [ ]
xiii.	do you have hepatitis infection?	Yes [ ] No [ ]
	If yes, were you treated?	Yes [ ] No [ ] If
	yes, name of drug_____	