CHAPTER ONE

INTRODUCTION

1.0 Background

Human immunodeficiency virus (HIV) isamong the greatest modern pandemics ever known.After the first cases were reported in 1981, it has grown to be among the main causes of mortality worldwide especially in Africa where it is currently ranked second among the top five killer diseasesafter lower respiratory tract infections (WHO, 2017).Since the start of the epidemic, it is estimated that the virus has infected about 76,100,000 (65,200,000 - 88,000,000) people while the number of carriers worldwide as at 2016 is estimated to be 36,700,000 (30,800,000 -42,900,000) (UNAIDS, 2017). According to UNAIDS report, about 1,800,000 (1,600,000 -2,100,000) people were newly infected while about 1,000,000 (830,000-1,200,000) people died from illnesses related to AIDS in 2016 (UNAIDS, 2017).

HIV is characterised by an extensive genetic diversity and groupedbased onthe relatedness of their nucleotidessequences. The extraordinary degree of genetic variability of the virus results from high nucleotide substitution rate occurring during replicationbecause of lack of reverse transcriptase (RT) proofreading capability and high viral replication rates. Wain-Hobson (1993) put the replication error rate at approximately one mutation foreach newly synthesized genome.Another contributing factor to the genetic variability of the virus is recombination whichoccurs at an estimated rate of 2.8 crossovers in every cycle of replication (Zhuang *et al.*, 2002). In addition, insertions and deletions have appreciable contribution to HIV-1 variations. The different strains of the virus sometimes are associated with certain geographic areas as well as shows preference for a particular transmission mode (Conroy *et al*., 2010). Globally, five HIV-1 strains are known to dominate: subtypeA, subtype B, and subtype C, recombinant of subtypes A and E known as CRF01_AE, and recombinantsubtypes A and G, CRF02_AG.

Africa, especially Central Africa is known to have the highest concentration of diverse strains of HIV-1 in the world. Although all strains are found in this region, subtypes A, C and CRF02_AG are the predominant ones with heterogeneous distribution (Tatem *et al*., 2012). Subtype C predominates in South as well as East Africa while CRF02_AG predominates in

West and West Central Africa (Montavon *et al*., 2000).There is dearth of information on the genetic heterogeneity of HIV in North Africa.However, the little available information suggests that subtype B may be the predominant subtype in that region (Annaz *et al*., 2011). Circulation of multiple subtypes in a given geographic area as obtained in Africa south of Sahara increases the chances of multiple strains of the virus infecting the hosts at the same time thereby increasing the chance of recombination.Consequent upon the constant evolution of the virus occasioned by mutation and recombination, new strains (other than already identified and documented ones) continues to emerge.

The clinical implications of the extensive variation of HIV strains can be quite enormous. The heterogeneity and rapid evolution rate poses enormous challenge to the diagnosisand treatmentof HIV and has hindered efforts to develop effective vaccine against the virus.For effective diagnosis, there is a need for universal assays that can detect all genetic forms of the virus. Majority of HIV immunoassays were originally designed using HIV-1 subtype B epitopes (Walter *et al*., 2000). Despite several modificationsaimed at improving the sensitivity and specificity of the assays, cases of false-negative results continued to arise especially for non-subtype B infected patients (Plantier *et al*., 2009a). False-negative results occur mainly as a result of low sensitivity in antigen detection emanating from inconsistencies witnessed among viral epitopes belonging to different genetic forms of the virus. Consequently, the different viral epitopes intended for detection in the assays may evade recognition by the monoclonal antibodies employed in the assay.Also, modifications or elimination occurring as natural polymorphisms within the immunodominant region (IDR) of *env* gp41 (major target of HIV-1 antibodies) as a result of genetic differences of HIV-1 could lead to decreased sensitivity or inability to detect antibody (Brennan *et al*., 2006). Occurrence of these innate polymorphisms in primer/probe sequences with the potential to decrease hybridization can also compromise reliable quantification of HIV-1 RNA or DNA in an infected person (Christopherson *et al*., 1997). This can have significant consequences on the ability to detect mother-to-child transmission of the virus early since thiscan be achieved almost esclusively by HIV-1 RNA or DNA detecting assays.

Ability of HIV to mutate and the diverse assortment of its antigen epitopes have also hindered efforts to develop effective vaccine.It is still not well understood how vaccines formulated against a particular subtype will protect against other subtypes.Many attempts to

develop a vaccine have focused on HIV-1 subtype B which predominates in European countries, US and Canada (Carr *et al*., 2010) and plays little role in HIV epidemics of Africa and Asia. The concern therefore is whether a subtype B-based vaccine will be effective against other subtypes. As efforts intensify in the area of development of effective test kits, production of effective antiretroviral drugs and vaccine development, it is imperative to put into consideration all the circulating strains of the virus.

In the area of treatment, occurrence of resistance to antiretroviral drugs has been linked to genetic subtypes.The differences in the susceptibility pattern to antitretroviral (ARV) drugs can be traced to variations in the composition of amino acids occurring among different HIV-1 clades.Treatments for HIV currently employs drugs such as nucleoside and non-nucleoside reverse transcriptase (RT) inhibitors (NRTIs and NNRTIs) in addition to protease inhibitors (PIs) which were produced using subtype B as a model in western countries. Resistance to some of these drugs however have been reported in some non-subtype B strains. For example, group O and HIV-2 isolates have shown significant natural resistance to NNRTIs (Smith *et al*., 2009) which can be attributed to resistance mutations occurring as natural polymorphisms.

In addition to the issue of sensitivity, development of resistance against certain drugs also contributes to failure of treatment with antiretroviral drugs.This results from acquisition of resistance-confering mutations in the viral *pol* gene encoding three major enzymes of the virus; protease (PR), reverse transcriptase (RT) and integrasethat provides target sites for antiretroviral drugs (Wainberg *et al*., 2011). There are indications that strains from certain subtypes and/or geographical regions are more likely to develop antiretroviral drug resistance against certain drugs than other variants (Wainberg, 2004). Codon usage and polymorphisms have also been shown to be subtype-specific and may affect the pathway to drug resistance (Ojesina *et al*., 2006).

Another class of antiretroviral drugs is the entry inhibitorssuch as Maraviroc (MVC) whichnegatively modulates the chemokine co-receptor-5 (CCR5) thereby preventing the binding of gp120.HIV-1 susceptibility to this class of drugs depends on the coreceptor usage (tropism).Studies have shown that HIV-1 generally uses CCR5 chemokine receptor early in infection(Dragic*et al*., 1996). In some individuals however, viruses that use other co-

receptors like CXCR4 are transmitted. The ability to use CXCR4 can also arise as a consequence of viral genetic changes over time. This change in tropism from CCR5-tropic to CXCR4-tropic is the principal mechanism of resistance developmentof HIV-1 to MVC (Moore and Kuritzkes, 2009). When mutations occur in the *env* that permits gp120 to bind an MVC-bound CCR5 receptor, it can also lead to MVC resistance. Such mutations are mostly situatedwithin the V3 loop of *env* gene (Iyidogan and Anderson, 2014). There are also single virus clones that have the abilityof using CCR5 as well as CXCR4 as coreceptors.Ability to use CXCR4 (either alone or with CCR5) is associated with poor prognosis in the patients (Koot*et al*., 1993; Richman and Bozzette, 1994).

1.1 JUSTIFICATION

In Nigeria, molecular studies have revealed co-circulation of multiple subtypes and CRFs of HIV-1 in different states and regionsof the country (Howard *et al*., 1994; Odaibo *et al*., 2001; Agwale *et al*., 2002; Odaibo *et al*., 2006; Ojesina *et al*., 2006; Sankale *et al*., 2007;Chaplin *et al*., 2011; Meloni *et al*., 2017). Majority of these studies however were done in the northern and western regions while there is limited information on thestrains circulating in eastern part of the country.Also, the few studies that investigated the drug resistant mutations (Ojesina *et al*., 2006; Odaibo *et al*., 2013) and coreceptor usage (Ajoge *et al*., 2011; Donbraye *et al*., 2015) of HIV-1 strains in the country were donein northern and western regions. With the growing evidence of increasing viral resistance to commonly used antiretroviral drugs and the possibility of use of CCR5-inhibitor like Maraviroc as an alternative in the country, it has become expedientto study the patterns of drug resistance mutations as well as coreceptor usage potential of HIV-1 strains circulating especially in the eastern part of the country. In addition, it is necessary to have information on all strains circulating in the country to inform policy in test algorithm andvaccine development.

1.2 AIM AND OBJECTIVES

1.2.1 AIM

The aim of this study was to characterize HIV-1 strains circulating in Southeastern parts of Nigeria.

1.2.2 SPECIFIC OBJECTIVES

The specific objectives of this studyare; to

1. Determine the HIV-1 strains circulating in Delta, Anambra and Imo states of Nigeria.
2. Identify the drug-resistance related mutations to protease inhibitors (PIs) and polymorphisms that exist in the circulating HIV-1 strains among the study population.
3. Detect and identify (if any) newly introduced HIV-1 strains not previously reported in the country.
4. Predict coreceptor usage of circulating HIV-1 strains in the study area by analysis of V3 amino acid sequenceof env protein

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND AND ORIGIN OF HIV

The history of HIV dates back to 1981 with the identification of acquired immunodeficiency syndrome (AIDS) among homosexuals in the US (Gottlieb *et al*., 1981) when majority of public health officials were still ignorant that they exist. In these patients were found diseases (syndromes) that were hitherto uncommon among young adults in the region. Commonest among these syndromes include Kaposi's sarcoma (KS), *Pneumocystis carinii* pneumonia (PCP) and *Mycobacterium avium* tuberculosis. Few years later, other cohorts were reported with similar syndromes in both US and Europe. Among them are haemophiliacs and injection drug users (IDUs) and recipients of blood transfusion. In early 1982, this new disease was described as "acquired immunodeficiency syndrome (AIDS)" by Center for Disease Control (CDC).Because AIDS encompasses a wide range of clinical diseases, the term "syndrome" was used. This definition was subsequently revised by the CDC to include more syndromes associated with advanced HIV disease (CDC, 1992).

A subcommittee of the International Committee for the Taxonomy of viruses (ICTV) proposed the designation"human immunodeficiency virus" (HIV) as the most appropriate name for AIDS virus.HIV-1 has been postulated to originate from transmisions of simian immunodeficiency virus (SIVcpzPtt) which infects chimpanzees in west-central Africa among different species. This resulted in group M and groups N and O (i.e pandemic and non-pandemic respectively) HIV-1 clades.It is yet to be identified if group O (outlier) viruses also originated from SIV.The most recently found group P has equally been postulated to originate from gorillas (Takehisa *et al*., 2009).

2.2 CLASSIFICATION

HIV is a member of genus *Lentivirus,* subfamily Orthoretrovirinaeand family Retroviridae. Members in this family synthesize proviral DNA from viral RNA template using the enzyme reverse transcriptase. The synthesized DNA is inserted into the cell chromosomeusing the enzyme integrase. Viral DNA replication occurs during the host cell's replication. Members of the genus *Lentivirus* are known as slow viruses because of the slow, progressive nature of the disease following prolonged sub-clinical infection (Table2.1).

TABLE 2.1: MEMBERS OF THE RETROVIRIDAE FAMILY

Subfamily:	Examples
ORTHORETROVIRINAE	
• Genus: *Alpharetrovirus*	Avian leukosis virus (ALV), Rous sarcoma virus (RSV), Avian carcinoma Mill Hill virus 2 (ACMHV-2), Avian myeloblastosis virus (AMV), Avian myelocytomatosis virus 29 (AMCV-29), Avian sarcoma virus CT10 (ASV-CT10), Fujinami sarcoma virus (FuSV), UR2 sarcoma virus (UR2SV), Y73 sarcoma virus (Y73SV)
• Genus: *Betaretrovirus*	Jaagsiekte sheep retrovirus (JSRV), Langur virus (LNGV), Mason-Pfizer Monkey virus (MPMV), Mouse mammary tumor virus (MMTV), squirrel monkey retrovirus (SMRV)
• Genus: *Gammaretrovirus*	Feline leukemia virus (FeLV), Gibbon ape leukemia virus (GALV), Guinea pig type-C oncovirus (GPCOV), Murine leukemia virus (MLV), Porcine type-C oncovirus (PCOV)
• Genus: *Deltaretrovirus*	Bovine leukemia virus (BLV), Human T-lymphotropic virus (HTLV)-1, -2, Simian T-lymphotropic virus (STLV)-1, -2, -3
• Genus: *Lentivirus*	Bovine immunodeficiency virus (BIV), Equine infectious anemia virus (EIAV), Feline immunodeficiency virus (FIV), Caprine arthritis encephalitis virus (CAEV), Visna/maedi virus (VISNA), Human immunodeficiency virus (HIV)-1, -2, Simian immunodeficiency virus (SIV)
• Genus: *Epsilonretrovirus*	Walleye dermal sarcoma virus (WDSV), Walleye epidermal hyperplasia virus 1 (WEHV-1), Walleye epidermal hyperplasia virus 2 (WEHV-2)
Subfamily:	
SPUMARETROVIRINAE	
• Genus: *Spumavirus*	Simian foamy virus (SFV), Human foamy virus (HFV), Bovine foamy virus (BFV), Equine foamy virus (EFV), Feline foamy virus (FFV)

SOURCE: Coffin*et al*. (1997)

Two types of HIV exists which differ genetically; HIV-1 and HIV-2. While HIV-1 predominates globally, HIV-2 is majorly found in Western part of Africa. In addition to type and class, HIV is also grouped into subtypes, CRFs and URFs.

There are 3 major groups of HIV-1. These include; group M (major), group O (outlier), and group N (new, non-M, and non-O). Group M with worldwide distribution was discovered first. This was followed by the discovery of group O (which is not as widely distributed as group M). On the other hand, identification of group N occurred in 1998 (Simon *et al*., 1998). More recently (in 2009) a new P (putative) groupwas reported from a Cameronian woman resident in France (Plantier *et al*.**,** 2009b). Group M accounts for majority of cases worldwide and is further classifiedinto nine major clades (A-D, F-H, J and K) and more than 80 CRFs presently.

Approximately 12% of global HIV-1 infections resulted from clade B while the remaining majority result from non-B clade (Hemelaar *et al*., 2006). Based on genetic variation of 15-20%, there are further distinct sequence clusters within some subtypes (variation between subtypes is usually 25-35%). In dually infected patients, inter-subtype recombinant genomes are also common. CRFs are described as inter-subtype recombinant viruses that have been continuously transmitted from one individual to another i.e infected three or more persons with no epidemiological relation.

2.3 HIV-1 GENETIC DIVERSITY AND DISTRIBUTION

HIV-1 strains are known for their tremendous level of genetic diversity. This resulted from its high mutation rate, reverse transcriptase enzyme lacking proof-reading ability and high rate of recombination that occurs during virus replication. Recombination occurs as a result of template switches that take place at the stage of reverse transcription because of the presence of two copies of single-stranded RNA in each retroviral particle. Other factors which result in the generation of genetically diverse strains of HIV-1 include insertions, deletions and duplications which are known to occur rapidly during the replication of this virus(Hu and Temin, 1990).There are differences in the global distribution of the different variants of the virus (Fig. 2.1). Africa which is suspected to be the source of the epidemic has been reported to have the highest genetic heterogeneity of HIV-1 with much of the diversity concentrated in Central Africa(Vidal *et al*., 2000).

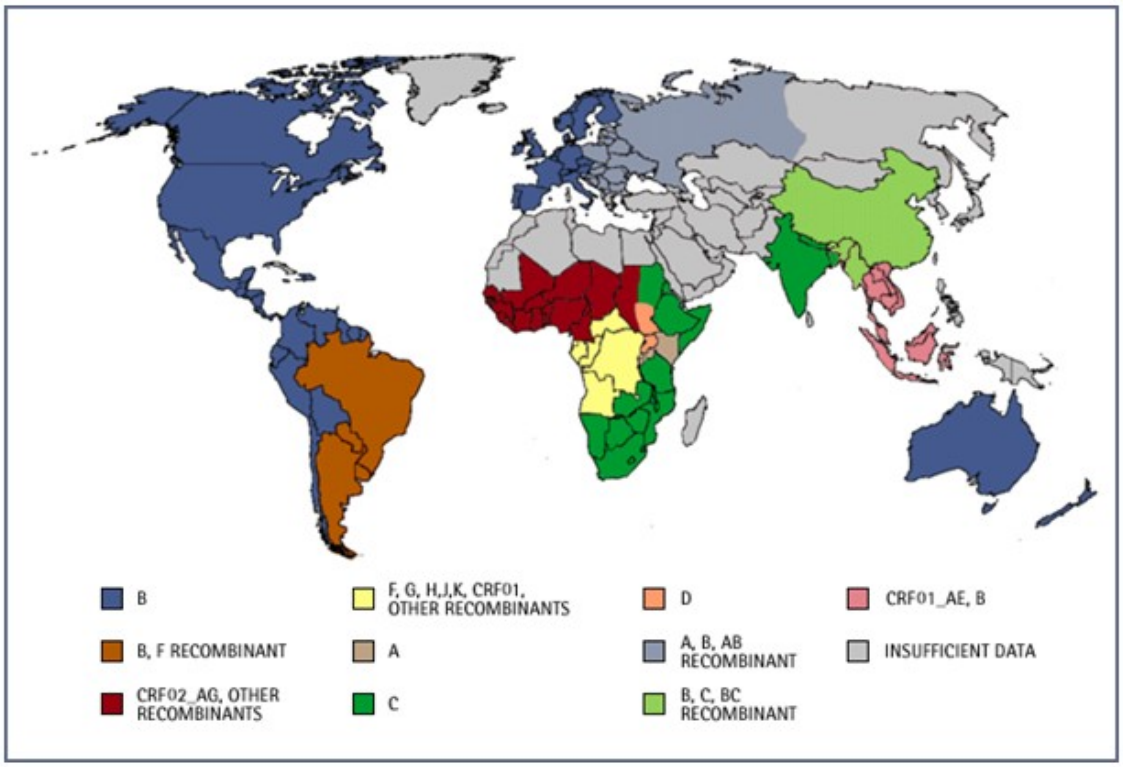


FIGURE 2.1: GLOBAL DISTRIBUTION AND GENETIC DIVERSITY OF HIV-1 SUBTYPES (CLADES) AND RECOMBINANTS.

SOURCE: McCutchan and Jackson, 2013

The different subtypes are heterogeneously distributed in the region. The predominant subtype in Southern and Eastern parts of Africa is C while most of the circulating viruses in

Western and West-Central Africa is CRF02-AG (Mc Cutchan, 2000). In developed countries like Western and Central Europe as well as the Americas, clade B predominates (Akouamba *et al*., 2005) while C is largely predominant in Southern Africa, India and Neighbouring countries (Neogi *et al*., 2009).Subtype D has been isolated exclusively from East as well as Central Africa and occasionally from South and West Africa (Conroy *et al*., 2010). Pure clade E has not been isolated instead; it has been reported as an A/E mosaic which has been reported from Thailand, China, Philippines, Central Africa and Nigeria (Janssens *et al*., 1997; Fayemiwo *et al*., 2014).Reports of subtype F have come from South America as well as Eastern Europe while G and A/G recombinants were reported from Western and Eastern Africa in addition to central Europe (Abecasis *et al*., 2011). Subtypes H and J were reported from Central Africa as well as Angola while clade K was reported from Congo DRC and Cameroun (Bartolo *et al*., 2005).

2.4 STRUCTURE OF HIV-1

Electron microscopy shows a core which is shaped like a cone and composed of p24 *gag* protein known as capsid. The capsid enclosed two similar RNA strands closely associated with the reverse transcriptaseand the nucleocapsid (NC) proteins (Figure 2.2). The diploid (2 × 9.2 kb) positive sense ssRNA HIV genome is composed of *gag,Pol* and *env* genes together with six additional assessory genes with regulatory functions. A myristylated p17 core (*gag*) protein surrounds the inner part of the viral membrane providing a matrix (MA) needed for the virion integrity (Gelderblom *et al*., 1989).The p17 is derived from the cleavage of 55 kDa *gag* gene. Phosphoprotein p24 which is a *gag* gene product is the most abundant of all viral proteins. The icosahedral nucleocapsid is constructed with this protein; the other two are p9 and p7closely associated with the viral genome.

On the viral surface aregp160, which has undergone cleavage inside the cell to give rise to two noncovalently linked components, gp120 and gp41.The V3 loop which is the receptor-binding ligand is used by the virus for attachment to the host cell receptor. It is located in the gp120 molecule and is the most important antigenic domains. Gp120 is highly glycosylated more than any other known viral protein, a property believed to be a protective device to impede access of neutralising antibodies.

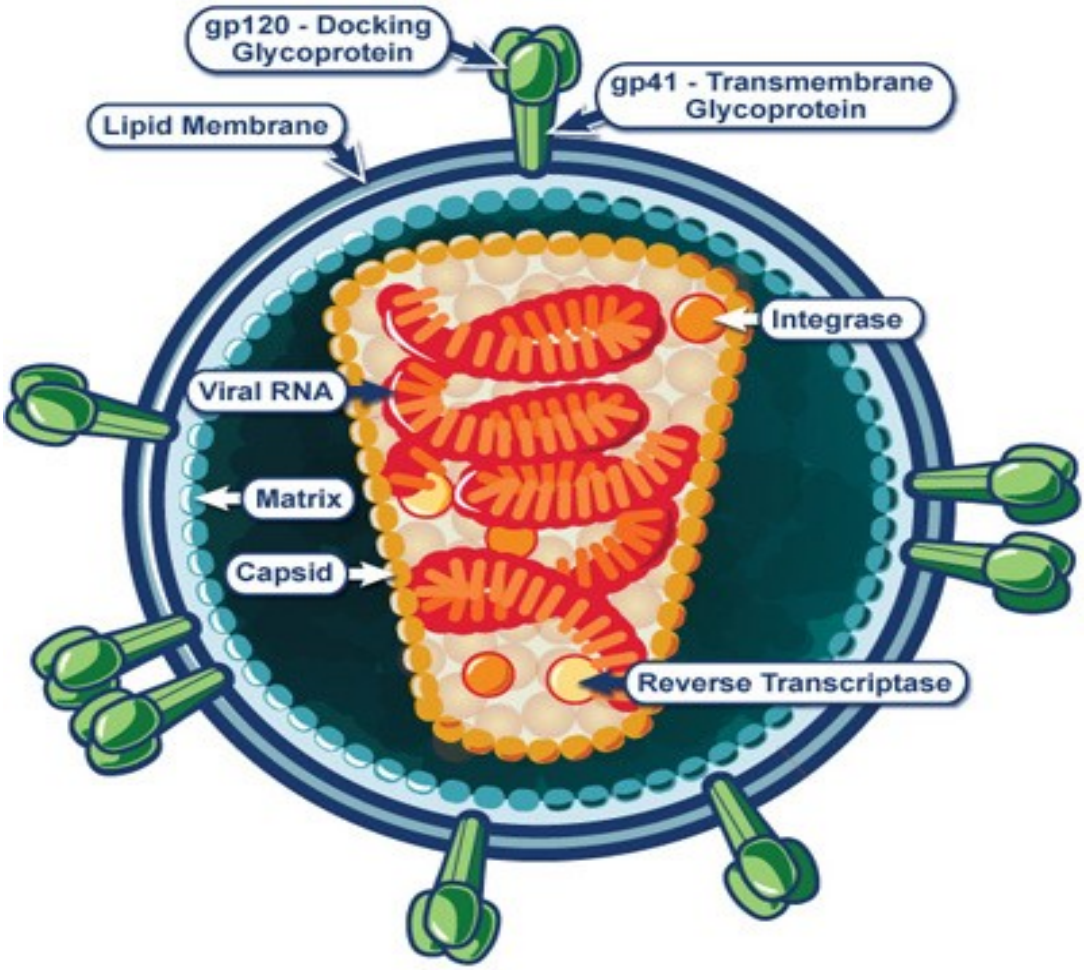


FIGURE 2.2: A SCHEMATIC DIAGRAM OF HUMAN IMMUNODEFICIENCY VIRUS

SOURCE: NIAID 2012

2.5 GENOME

There are nine open reading frames in the HIV genome (leading to nine primary translation products). As a result of cleavage of three of the products however, 15 proteins are made in all. The genome is composed of three major genes; *gag*, *pol* and *env* genes and six other accessory genes; *tat*, *rev*,*vif*, *vpr*, *vpu*, and *nef* (Fig. 2.3).

2.5.1 Gag (group specific antigens**)** is the genomic region which encodes the capsid proteins. It is derived from the cleavage by viral protease during viral maturation of the precursor p55 myristylated polyprotein. Other products of the cleavage are p17, p24, p7 and p6 proteins. Gag associates with plasma membrane (site of virus assembly).Because of its role in viral assembly, this 55-kDa *gag* precursor is known as assembling.

2.5.2 Pol (polymerase) encodes protease, reverse transcriptase and integrase enzymes which are synthesized as a precursor polyprotein known as *gag-pol*. During maturation, pol polypeptide is cleaved out of the gag by the viral protease and goes ahead to digest it thereby separating the protease (p10), RT (p50), RNase H (p15) and integrase (p31) activities.

2.5.3 Env (envelope) is the viral glycoproteins synthesized as a precursor (gp160). Processing of this glycoprotein yields envelope surface gp120 and gp41 which traverses membrane. The ligand for the CD4 receptor and chemokine co-receptors for HIV-1 is found on gp120.

2.5.4 Tat (transactivator of transcription) Tat is needed for the initiation of transcription in HIV-1 (Bagashev and Sawaya, 2013). It lso promotes elongation phase of HIV-1 transcription hence, inability to express tat leads to generation of short transcripts.

2.5.5 Rev (regulator of expression of virion proteins) is also necessary for HIV expression.It is localized in the nucleolus and is19-kD in size. It induces the transition from the early to the late phase within the nuclei and nucleoli of infected cells (Malim *et al*., 1989).Proviruses without *rev* functions are deficient in expressing viral late genes but are able to transcribe. Consequently, they do not produce virions.

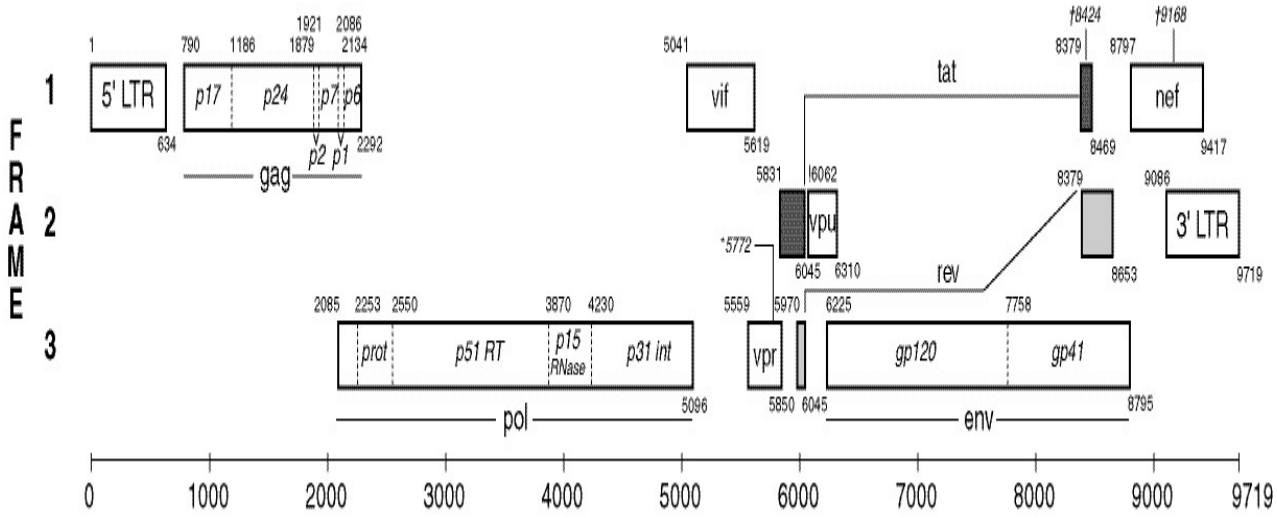


FIGURE 2.3: STRUCTURE OF HIV-1 GENOME

Rectangles = open reading frames, Small numbers at the top left of each rectangle = gene start (i.e position of A in the ATG start codon). Number in the lower right =last position of the stop codon

SOURCE: Korber *et al*. (1998)

2.5.6 Vif (Viral infectivity factor) is found in almost all lentiviruses.By affecting assembly/maturation during virus production, *vif* promotes infectivity.Viral particles produced without *vif*are defective while there is no much effect on the cell-to-cell transmission of virus.

2.5.7 Vpr (viral protein R) is believed to aid pathogenesis of HIV-1 infection *in vivo*.It has been proposed that Vpr functions by targeting the importation of preintegration complexes (PIC) into the nucleus, arresting cell growth (Hrimech *et al*., 1999), and transactivation of cellular genes.

2.5.8 Vpu (viral protein U) is a 16-kd integral membrane proteinknown to degrade CD4 in the endoplasmic reticulum and facilitating viral budding(Dube *et al*., 2010).Vpu is restricted to HIV-1 and some SIVs.

2.5.9 Nef (negative regulatory factor)is a 27-kd myristylated protein synthesized by an open reading framesituated at the 3' end of the primate lentiviruses and is multifunctional in nature. Other forms exist including the nonmyristylated variants. It is found in all primate lentiviruses(Das and Jameel, 2005).Some of the functions include; enhanced spread of the virus and rapid progression of disease *in vivo*(daSilva *et al*., 2009).

2.6EVOLUTION

Extensive viral diversity is as a result of infidelity of the reverse transcriptase, the dimeric nature of the RNA(Temin, 1993),the rapid virus turnover(Ho *et al*., 1995), and the huge population size.The resulting mutations and recombination leads to emergence ofspecies that differs genetically in each infected individual, termed 'quasispecies'. Further evolution of the virus results from selection pressures applied by factors such as the immune system or antiviral agents(Thomson*et al*., 2002).

2.7REPLICATION AND PATHOGENESIS

2.7.1 REPLICATION:

The replication of HIV is grouped into stages as shown in figure 2.4:

2.7.1.1 Binding and Fusion: Infection with the virus commences following the attachment of viral glycoprotein gp120 to the cell's CD4 receptor(Wyatt *et al*., 1998). This is followed by attachment to either of the other two co-receptors and subsequent entry into the host cell cytoplasm. Some strains (R5 variants) bind to CCR5co-receptors while some (X4 variants) binds to CXCR4co-receptors. However, some strains (R5X4 variants or dual tropic) use both co-reptors for entry.

The affinity of the binding between gp120 and CD4 molecule is known to be greater than that for CD4 molecules natural ligand, the class II MHC complex (Dalgleish *et al*., 1984). Since this binding is required for HIV infection, virtually any cell expressing the CD4 surface molecules may be a target for infection. Following CD4 attachment, conformational change occurs in the gp120 which exposes a co-receptor binding domain. A fusion peptide of trans-membrane glycoprotein gp41 is exposed after structural rearrangements following interaction with the co-receptor. This is followed by fusing together of viral envelope with cell membrane and subsequent liberation of viral genome into the cytoplasm.

2.7.1.2 Reverse Transcription: Once inside the cytoplasm, the HIV-1 RNA genome undergoes reverse transcription using the enzyme reverse transcriptase into a linear, dsDNA molecule. Due to its RNase H activity, the reverse transcriptasealso degrades the RNA of the RNA/DNA hybrid resulting from first round of polymerization. The newly synthesized DNA moves to the nucleus and incorporates into the host DNA.

2.7.1.3 Integration: This step is facilitated by the action of the viral integrase. During processing, two to three nucleotides that follow highly conserved CA bases near the 3′ end of each DNA strand are removed by the viral integrase prior to integration. This processed viral DNA is then inserted into cellular DNA in a sequence-independent manner facilitated by an action of viral integrase protein as a provirus(Lewis *et al*., 1992). The integratedHIV DNA is called provirus.Transcription by cellular RNA polymerase II cannot occur in resting T cells but only in replicating cells.

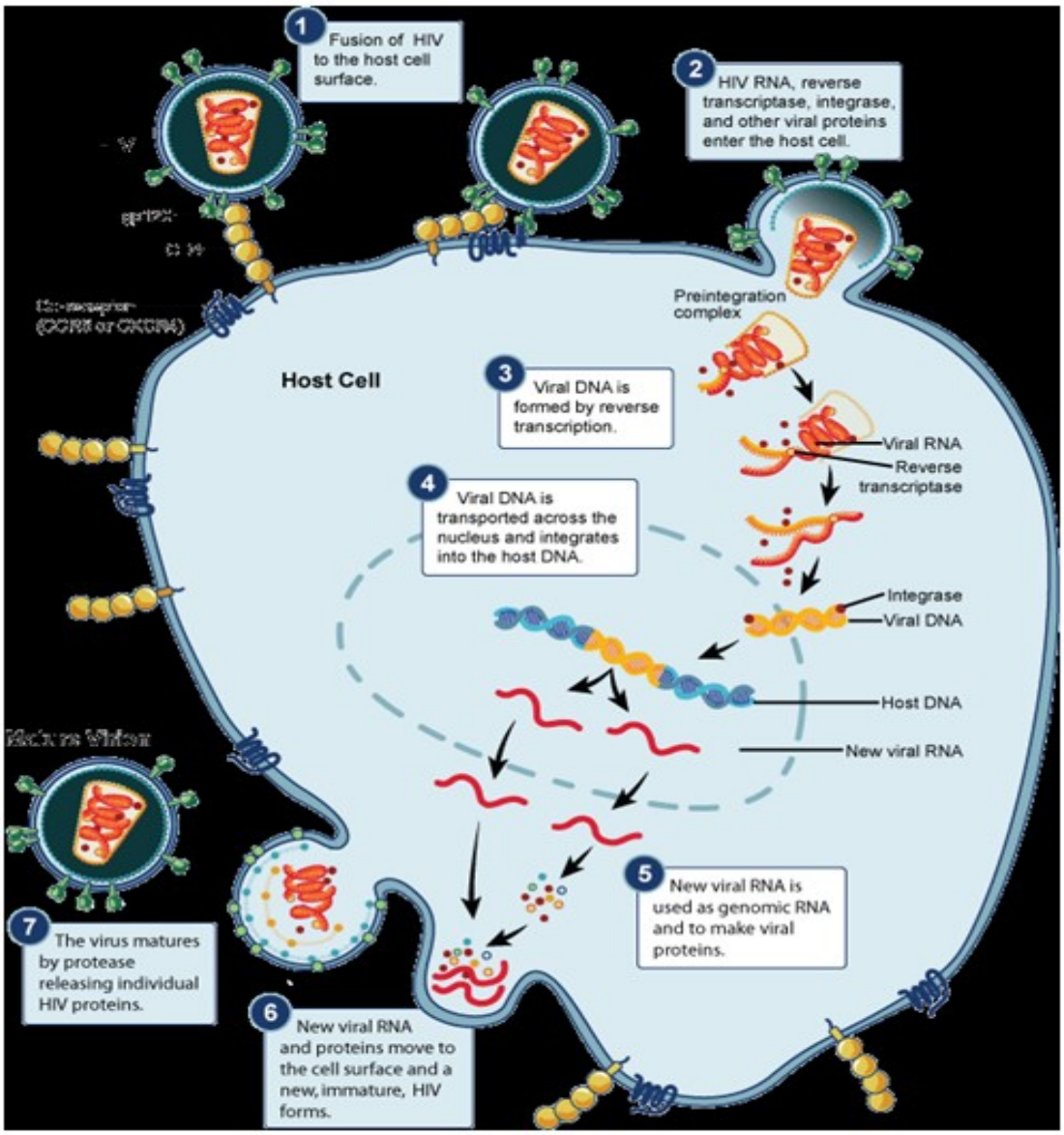


FIGURE 2.4: REPLICATION CYCLE OF HIV-1

SOURCE: NIAID, 2012

2.7.1.4Transcription and translation: On activation of the host cell, the provirus with the help of host cell RNA polymerase transcribes copies of mRNAs and other genomic materials. Translation of the mRNAs yields viral polyproteins including structural (*gag*) and non-structural (*gag-pol*). Association of these polyproteins with other viral proteins and RNA forms immature virion.

2.7.1.5Assembly: At production, HIV-1 virions are usually immature and become fully mature as the particles bud from infected cell membrane(Cravin and Parent, 1996).At this maturation stage,*gag-pol* and *gag* polyproteins are cleaved with the help of proteolytic enzymes.This proteolytic cleavage initiated at the plasma membrane using enzyme protease is a necessary step which improves progeny virion release(Kaplan *et al*., 1994).The cleavage products of *gag* include; matrix, capsid, nucleocapsid and p6. In addition, the cleavage yields p2 and p1 which are spacer peptides.Cleavage of *gag-pol*yields MA and CA. In addition, its C-terminal end yields p2, NC, transframe protein (TF), PR, RT, and IN upon cleavage. These processed protein products together with copies of HIV's RNA genetic material are then assembled into new virus particles.

2.7.1.6Budding: The newly assembled virus is then released by budding. During budding, part of the host cell's outer membrane is taken up by the new virus to form its outer envelope. Theviral envelope is studded with HIV glycoproteins which are made up of protein and sugar combination.These glycoproteins are used as viral ligand for attachment to the host cell's CD4 and co-receptors.The newly budded HIV virions then proceed to infect other susceptible cells.

2.7.2 PATHOGENESIS:

Factors that determine the pathogenesis of HIV infection include; life cycle of the virus, host cell environment and viral load. The rate and severity of disease progression is also determined by age and genetic differences among infected individuals, the virulence of the infecting viral strain, and presence of other pathogens.Infection by HIV-1 can theoretically be divided into the following stages: primary, chronic asymptomatic and late stage HIV-1 infection.

2.7.2.1 Primary HIV infection:

Depletion of the cellular immune system is the hallmark of HIV-1 infection(Vergis and Mellors, 2000).Person to person transmission of HIV occurs through genital secretions, notably semen or via blood. Infected cells at the site of viral entry migrates to their regional lymph nodes(Mascola *et al*., 2000) and gets sequestered and replicates appreciably within 2–6 weeks and released into circulation resulting in high plasma viraemia. This is referred to as primary HIV infection (PHI).

PHI is characterized by high level of virus in the plasma.Symptoms of 'seroconversion illnesses may accompany PHI which comprises of rash, lymphadenopathy, arthropy and fever. However, in some individuals this period is clinically asymptomatic. As a result of primary immune response, there is spontaneous decline in the viraemia after 2–4 weeks.Despite the suppression of the viraemia level following seroconversion, complete elimination is never achieved.

The viraemic peak also corresponds to temporary decline of peripheral blood $CD4^+$ T-cells.Though this condition is generally modest and transient, it may sometimes result to $CD4^+$ T-cells decline to $<200 \times 10^9/l$ and eventually to overt reversible clinical immunosuppression (Weber, 2001). Following reduction in the viraemic peak,there is rebound in the level of $CD4^+$cell towards but slightly lower than baseline levels.During this phase also, $CD8^+$ T cells capable of destroying HIV-1-infected cells and secrete IFN-γ emerge. Their emergence occurs as plasmaviraemia peaks and decreasesthereafter.This can be used to monitor progress of disease.

Despite production of high levels of antibody, their neutralizing responses against HIV are weak. As a result of this, viruses capable of resisting the neutralizing activities of the antibodies emerge(Wei *et al*., 2003).With these setbacks for antibody response, HIV-1-specific $CD8^+$ T cells are largely responsible for controlling HIV-1 infection duringacute phase. The major clearance of HIV virions seen in the patient soon after infection is attributed to a cytotoxic T ($CD8^+$ T) cell and not antibody response.

2.7.2.2 Chronic asymptomatic HIV-1 infection:

PHI is followed by a period of long asymptomatic infection. There is relative balance between rate of replication and immunological response with little or no clinical manifestations of infection.There is rebound of CD4+cell level to near baseline but never pre-infection levels.

2.7.2.3 Late stage HIV-1 infection:

Evidence of increase in CD4 decline begins to emerge in late stage HIV disease, when the CD4 count is less than 200 x $10^9/mm^3$. Increased CD4 cell loss and failure of production of proportionate amount to match with the loss leads to a slow, sustained decline in CD4 cells which is usually observed in this phase of the infection. Decline in the $CD4^+$ cell counts to below 200-400 per µl paves the way for opportunistic infections with various microorganisms.This eventually overwhelms immune system and results in AIDS.

2.7.3 THE CDC CLASSIFICATION AND STAGING SYSTEM FOR HIV INFECTION

Three stages have been identified for a typical HIV infection by the Centers for Disease Control and Prevention (CDC).This system (as revised in 1993) categorizesHIV disease severity on the basis of level of CD4 cell andpresence of specific HIV-related conditions. These stages are Categories A, B, and C (Fig 2.5).

2.7.3.1 Category A, the first stage: Detection of infection at this stage is difficult without blood test.Mononucleosis-like illness manifests in about half of esposed individuals within about three weeks of infection. Some individuals in this category on the other hand are asymptomatic. Viraemia usually peaks soon after exposure with rapid decline in the CD4 cell population. CD4 cells numbers recovers with the appearance of antibodies to HIV and drops again gradually over the years as the infection progresses.

2.7.3.2 Category B: this stage is characterized by symptomatic conditions with commencement of indications of immune failure. There is emergence of persistent infections such as yeast infections, diarrhoea, shingles and certain cancerous conditions of the cervix.

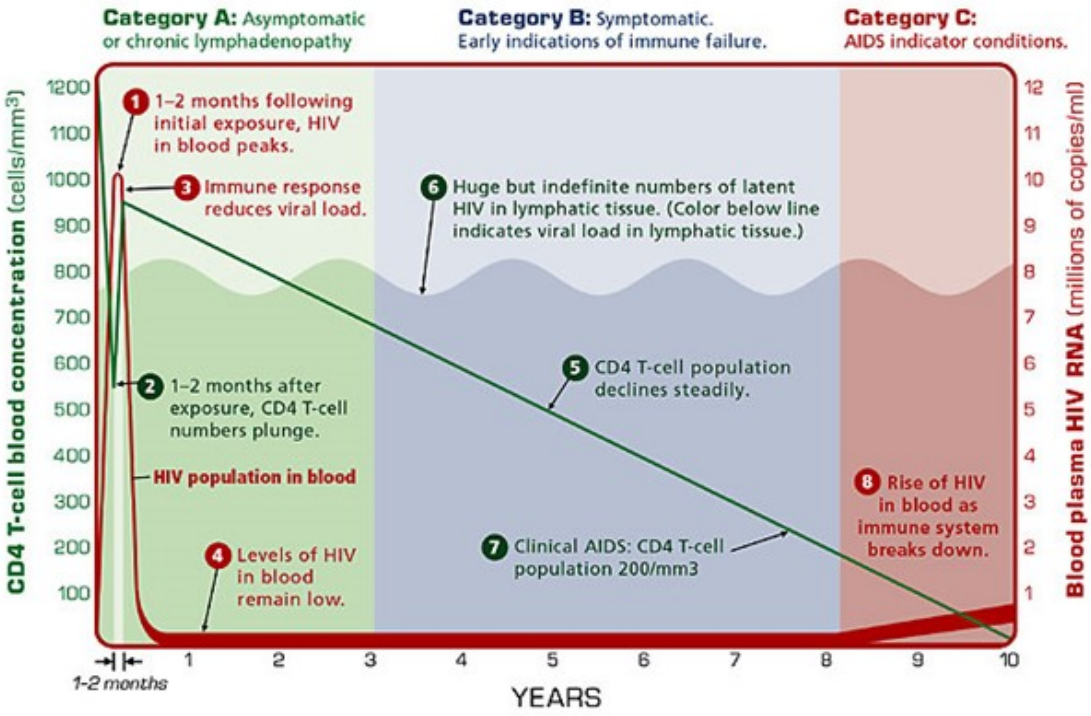


FIGURE 2.5: TYPICAL PROGRESSION OF HIV INFECTION AND AIDS

SOURCE: Piot, (1998)

In some patients who lack criteria for AIDS, certain neurological diseases such as myelopathy and peripheral neuropathy caused by HIV are seen. In about 25% of patients, subacute encephalitis termed AIDS-dementia complex is manifested.

2.7.3.3 Category C: this stage is synonymous with AIDS. AIDS is defined by either diagnosis of one of the AIDS-defing conditions, or by measurement of CD4 levels <200 cells/mm^3 (from the normal level of 800-1200 cells/mm^3). The opportunistic infections associated with AIDS appear at this stage. AIDS inevitably leads to death in the course of less than a year in untreated cases (Asjo, 2002).

2.7.4 WHO CLINICAL STAGING AND DISEASE CLASSIFICATION SYSTEM

This system was developed by WHO for resourse-constrained settings. Under it, diagnosis, evaluation and management of patients is based purely on clinical findings and CD4 cells enumeration is not needed. In many resource-limited countries where access to CD4 testing is not guaranteed, this staging system serves to guide decision on appropriate time to commence antiretroviral treatment for patients. The stages are 1 through 4as shown in Table 2.2.

2.8 OPPORTUNISTIC INFECTIONS ASSOCIATED WITH AIDS

Exposure to certain organisms including bacteria, fungi, parasites and even viruses in immunocompetent individuals do not necessarily lead to clinical manifestations. However, exposure of immunocompromized individuals such as AIDS patients to similar organisms leads to serious health implications from opportunistic infections (OIs).These infections are reffered to as “opportunistic” because of their ability to thrive in the environment of suppressed immune system.OIs in AIDS patients occur following the droping of $CD4^+$ cells to <200cells/mm^3 and areusually a major contributor of mortalityamong these patients.CDC has identified more than 20 OIs regarded as AIDS-defining conditions. Irrespective of CD4 count, infection with HIV and one or more of these OIs are considered diagnostic of AIDS.

2.8.1 FUNGAL OPPORTUNISTIC INFECTIONS

2.8.1.1 Candidiasis (Oral and Esophageal)

Candidiasis of the oropharynx otherwise known as thrush is the commonest intraoral lesion among HIV-infected persons.

TABLE 2.2: WHO CLINICAL STAGING OF HIV AND AIDS FOR ADULTS AND ADOLESCENTS

Clinical staging	Case definition
Primary HIV infection	Asymptomatic, acute retroviral syndrome
Clinical stage 1	Asymptomatic, persistent generalized lymphadenopathy
Clinical stage 2	Moderate unexplained weight loss, Recurrent respiratory infections, Herpes zoster, Angular cheilitis, Recurrent oral ulceration, Papular pruritic eruptions, Seborrheic dermatitis, Fungal nail infections
Clinical stage 3	Unexplained severe weight loss, Unexplained chronic diarrhea for >1 month, Unexplained persistent fever for >1 month, Persistent oral candidiasis (thrush), Oral hairy leukoplakia, Pulmonary tuberculosis, Severe presumed bacterial infections, Acute necrotizing ulcerative stomatitis, gingivitis, or periodontitis, Unexplained anemia, Neutropenia, Chronic thrombocytopenia
Clinical stage 4	HIV wasting syndrome, *Pneumocystis* pneumonia, Recurrent severe bacterial pneumonia, Chronic herpes simplex infection, Esophageal candidiasis, Extrapulmonary tuberculosis, Kaposi sarcoma, Cytomegalovirus infection, Central nervous system toxoplasmosis, HIV encephalopathy, Cryptococcosis,Disseminated nontuberculosis mycobacteria infection, Progressive multifocal leukoencephalopathy, Candida of the trachea, bronchi, or lungs, Chronic cryptosporidiosis (with diarrhea), Chronic isosporiasis, Disseminated mycosis, Recurrent nontyphoidal *Salmonella* bacteremia, Lymphoma (cerebral or B-cell non-Hodgkin), Invasive cervical carcinoma, Atypical disseminated leishmaniasis, Symptomatic HIV-associated nephropathy, Symptomatic HIV-associated cardiomyopathy, Reactivation of American trypanosomiasis

Source:Adapted from WHO, 2007

It was among the initial manifestations of HIV-induced immundeficiencies to be recognized (Gottlieb *et al*., 1981). Presence of oral thrush in an adult with no other known causes of immunosuppression is an indication of HIV infection.

2.8.1.2 Pneumocystis Infections:

Pneumocystis jirovec is commonly found in the lungs of many individuals. This fungus is normally kept under control by the immune system but can become pathogenic under a weakened immunity.It causes opportunistic disease (especially pneumocystis pneumonia-PCP) in people with severe immunodeficiency. PCP was once the major cause of mortality inAIDSpatients but most cases are easily treated these days.

2.8.1.3 Cryptococcal Disease:

Cryptococcosis is a fungal disease caused by *Cryptococcus neoformans* and affects the central nervous system (CNS) and can also be systemic.Cryptococcal infections are usually without symptoms, self-limiting and confined to the lungs in immunocompetent patients but could be life-threatening in AIDS patients.

2.8.2 BACTERIAL OPPORTUNISTIC INFECTIONS

2.8.2.1 *Mycobacterium avium* Complex (MAC):

Thisconsists of several related ubiquitous species of *Mycobacterium* (*Mycobacterium avium*, *M intracellulare*, and other species of *Mycobacterium* that have not been classified) that are rarely disease-causing in individuals with intact immunity. In advanced AIDS patients, MAC is one of the commonest causes of opportunistic infections(Chaisson *et al*., 1992).Severe immunosuppression increases the risk of disseminated MAC (DMAC).

2.8.2.2 Other bacterial infections

Other bacteria known to cause opportunistic infections in AIDS patients include *Staphylococcus aureus*(causes bacterial skininfections),*Haemophilus influenza*(causes invasive *Haemophilus influenzae*),*Pseudomonas aeruginosa*(causes pneumonia in HIV-infected hospitalized patients), and Salmonella (causes Salmonellosis and bacteremia).

2.8.3 VIRAL OPPORTUNISTIC INFECTIONS

2.8.3.1 Cytomegalovirus Disease:

Cytomegalovirus (CMV) infection is usually asymptomatic in immunocompetent individuals but contributes significantly to morbidity and mortality in AIDS conditions(Hoover *et al*., 1993).The infection is kept latent by immunological activities in immunocompetent individuals.Following loss of immune functions especially cellular arm of immunity in AIDS patients, there is reactivation and replication of the virus.

2.8.3.2 Herpes Simplex, Mucocutaneous:

HSV-1 and 2 infections are common among HIV-1 infected individuals. In infected persons, there is frequent oral and genital shedding of the viruses with increased shedding among those co-infected with HIV than among HSV-infected/HIV-1 uninfected individuals (Kim *et al*., 2006).

2.8.3.3. Kaposi sarcoma:

This is a common cancer among AIDS patients (Eltom *et al*., 2002). It is caused by human herpes virus 8.Transmission occurs through deep kissing, unprotected sex, and sharing needles. Vertical (mother to child) transmission also occurs. AIDS patients have greatly increased risk of developing KS.

2.8.3.4 Herpes Zoster/Shingles:

Varicella-zoster virusinfection results in chicken pox in the infected person. After the primary infection, the virus enters a latent phase in cranial nerve and dosal root ganglia. Reactivation of the infection leads to shingles or herpes zoster which is a mucosal infection that occurs along a dermatome.Zoster occurs more often in HIV patients probably as a sign of immunodeficiency but is not regarded as AIDS-defining illness(Wright and Johnson, 1997).

2.8.4 PROTOZOAN OPPORTUNISTIC INFECTIONS

2.8.4.1 Toxoplasmosis:

This is caused by an intracellular protozoan, *Toxoplasma gondii*which infects mostly the central nervous system of immunosuppressed patients leading to severe neurologic disease. Human infection usually occurs through the consumption of improperly cooked meat that

contains the cysts or vegetables containing the oocysts. Infection can also occur when exposed to oocysts-infested cat faeces.

2.8.4.2 Cryptosporidiosis:

This is caused by *Cryptosporidium parvum*which usually infects small intestinal mucosa resulting in watery diarrhea in immunocompromised persons (Janoff *et al*., 1990). The infection is highly contagious and spreads by fecal-oral route.It is usually a self-limiting disease in immunocompetent individuals but in patients with AIDS, it can lead to severe chronic diarrhea, electrolyte imbalance, malabsorption and weight loss.

2.8.4.3 Isosporiasis

This is a disease caused by the protozoan *Isospora belli*. It is spread by consumption of oocysts-infested food. It is also contracted by drinking water contaminated with feces of infected humans(Lindsay *et al*., 1997). It can also be spread through oral-anal sex.*I belli* causes aweakning diarrhea with wasting and improper absorption in patients with advanced HIV disease.

2.9EPIDEMIOLOGY

2.9.1 PREVALENCE

By the end of year 2016, about 36,700,000 [30,800,000 –42,900,000] were estimated of living with HIV worldwide (UNAIDS, 2017). Out of this number, 1,800,000 (1,600,000 - 2,100,000) represents total new infections, 1,700,000 (1,400,000 -1,900,000) represents new infections in people who are 15 years and above while 160,000 (100,000-220,000) represents total new infections among people who are 14 years and below. An estimated 1,000,000 (830,000-1,200,000) people died of AIDS-related illnesses in the same year. Heaviest burden of the epidemic is in Africa south of the Sahara with varying degrees of burden seen in other countries and regions of the world. Out of the total 36,700,000 people infected at the end of 2016, 19,400,000 (17,800,000 -21,100,000) are from Eastern and Southern Africa while 6,100,000 million (4,900,000 -7,600,000) people are from Western and Central Africa. These together accounted for about 69.5% of the total number of people living with HIV at the end of 2016 (Table 2.3).

TABLE 2.3: HIV REGIONAL DATA –2016

Region	People living with HIV (total) 2016	New HIV infections 2016			AIDS-related deaths (total) 2016	Total number accessing antiretroviral therapy 2016	Total number accessing antiretroviral therapy June 2017
		Total	Aged 15+	Aged 0-14			
Eastern and southern Africa	19.4 million [17.8 million–21.1 million]	790 000 [710 000–870 000]	710 000 [630 000–790 000]	77 000 [52 000– 110 000	420 000 [350 000–510 000]	11.7 million [10.3 million–12.1 million]	12.5 million [11.0 million–13.0 million]
Asia and the Pacific	5.1 million [3.9 million–7.2 million]	270 000 [190 000–370 000]	250 000 [180 000–380 000]	15 000 [7700– 26 000]	170 000 [130 000–220 000]	2.4 million [2.1 million–2.5 million]	2.5 million [2.2 million–2.6 million]
Western and central Africa	6.1 million [4.9 million–7.6 million]	370 000 [270 000–490 000]	310 000 [220 000–410 000]	60 000 [35 000– 89 000]	310 000 [220 000–400 000]	2.1 million [1.9 million–2.2 million]	2.3 million [2.0 million–2.4 million]
Latin America	1.8 million [1.4 million–2.1 million]	97 000 [79 000–120 000]	96 000 [78 000–120 000]	1800 [1300–2400]	36 000 [28 000–45 000]	1.0 million [896 000–1 059 000]	1.1 million [937 000– 1.1 million]
The Caribbean	310 000 [280 000–350 000]	18 000 [15 000–22 000]	17 000 [14 000–21 000]	<1000 [<1000–1000]	9400 [7300–12 000]	162 000 [143 000–169 000]	170 000 [150 000–177 000]
Middle East and North Africa	230 000 [160 000–380 000]	18 000 [11 000–39 000]	17 000 [10 000–36 000]	1400 [<1000–3300]	11 000 [7700– 19 000]	54 400 [47 800–56 500	58 400 [51 400–60 700
Eastern Europe and central Asia	1.6 million [1.4 million–1.7 million]	190 000 [160 000–220 000]	190 000 [160 000–220 000]	-*	40 000 [32 000–49 000]	434 000 [382 000–452 000]	474 000 [417 000– 493 000]
Western and central Europe and North America	2.1 million [2 million–2.3 million]	73 000 [68 000–78 000]	72 000 [67 000–78 000]	-*	18 000 [15 000–20 000]	1.7 million [1.5 million–1.7 million]	1.7 million [1.5 million–1.8 million]

*Estimates were unavailable at the time of publication

SOURCE: UNAIDS (2017)

In Nigeria, records show that there has been a slight decrease in prevalence rate of the infection. A look at the sentinel studies conducted in the country so far shows that the epidemic is on the decrease after the initial peak of 5.8% in 2001 (Figure 2.6). Despite this seeming success, Nigeria is still with the second heaviest burden of HIV in Africa with an increasing number of persons harbouring the virus. Latest statistics on the prevalence shows that 3.5 million people are infected placing Nigeria 3rd on the list of countries with heaviest burden of HIV/AIDS after India and South Africa.

According to the 2014statistics, highest concentration of the infection is found in the North Central geopolitical zone of the country with prevalence of 5.8% followed by South-East and South-South geopolitical zones with prevalence of 4.9% each while the least prevalence of 1.9% is found in North West geopolitical zone (FMoH, 2015) (Figure 2.7).

The report also shows highest rate of the infection in North-Central state of Benue with a prevalence of 15.4% followed by Akwa Ibom state in the South South zone with a prevalence of 10.8%. The least prevalence was observed in Borno state in North East zone and Zamfara state in North West zone with prevalence of 1.1% and 0.9% respectively as shown in Figure 2.8.

2.9.2 HIV TRANSMISSION

HIV is transmitted principally through three modes: sexual intercourse, exchange of blood, and perinatal transmission.

2.9.2.1 Sexual transmission

Sexual intercourse remains one of the commonest means of HIV transmission. Homosexual practices among males remains a principal means of spread of the epidemic especially in the Western developed parts of the world.Similarly, heterosexual practices are the major mode of transmission in developing countries especially in Africa (Padian *et al*., 1997). Transmission occurs mainly by penile-vaginal and penile-anal intercourse and in some cases by fellatio. During penile-vaginal intercourse, both the male and female partners stand the risk of acquiring the virus but the risk is higher for the female (receptive) partner. In anal intercourse between homosexual men, the receptive partner stands greater risk of being infected by the virus(Moss *et al*., 1987).

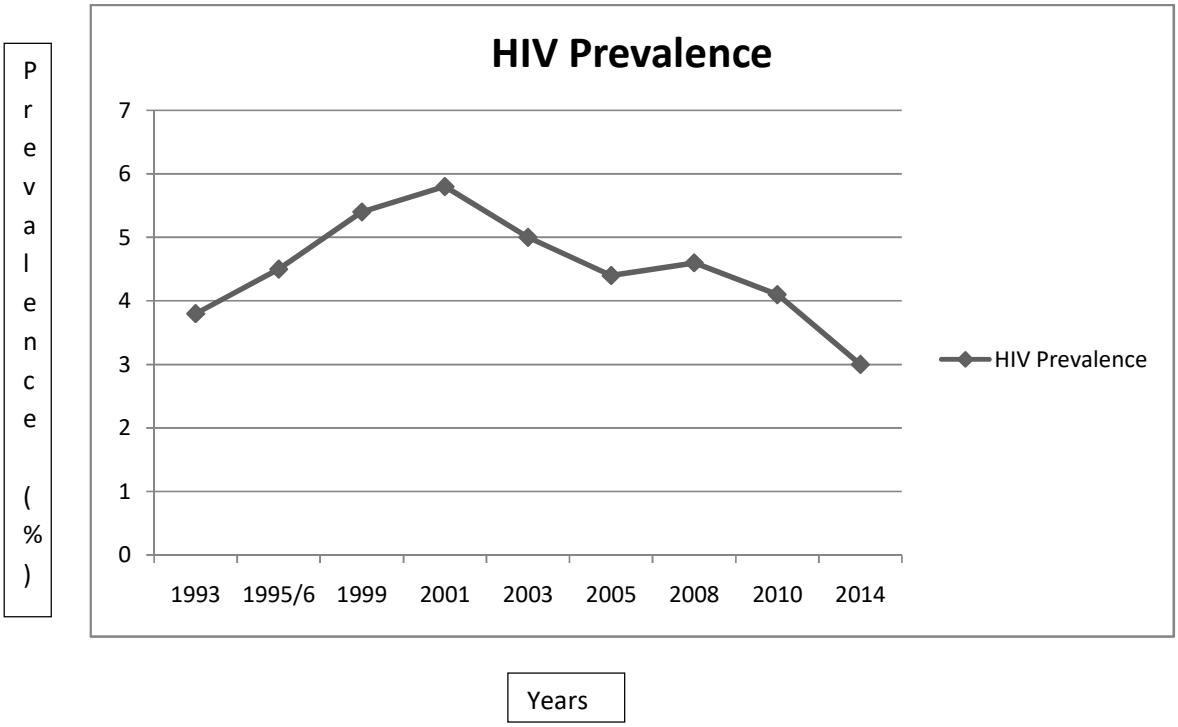


FIGURE 2.6: TRENDS IN HIV SEROPREVALENCE AMONG PREGNANT WOMEN IN NIGERIA, 1991-2014

SOURCE: FMoH(2015)

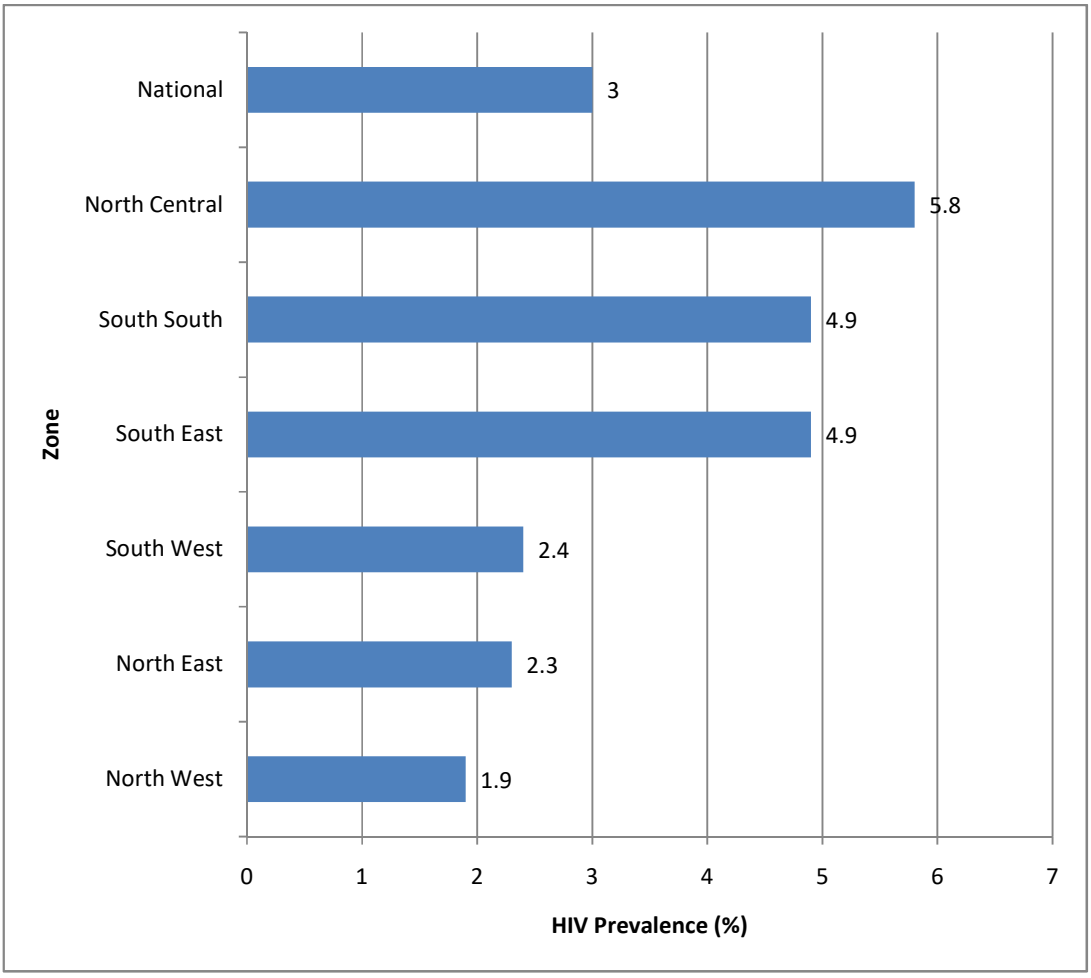


FIGURE 2.7: HIV PREVALENCE AMONG PREGNANT WOMEN ATTENDING SENTINEL ANTE-NATAL CLINICS BY NIGERIAN GEOGRAPHICAL ZONES IN 2014

SOURCE: FMoH, 2015

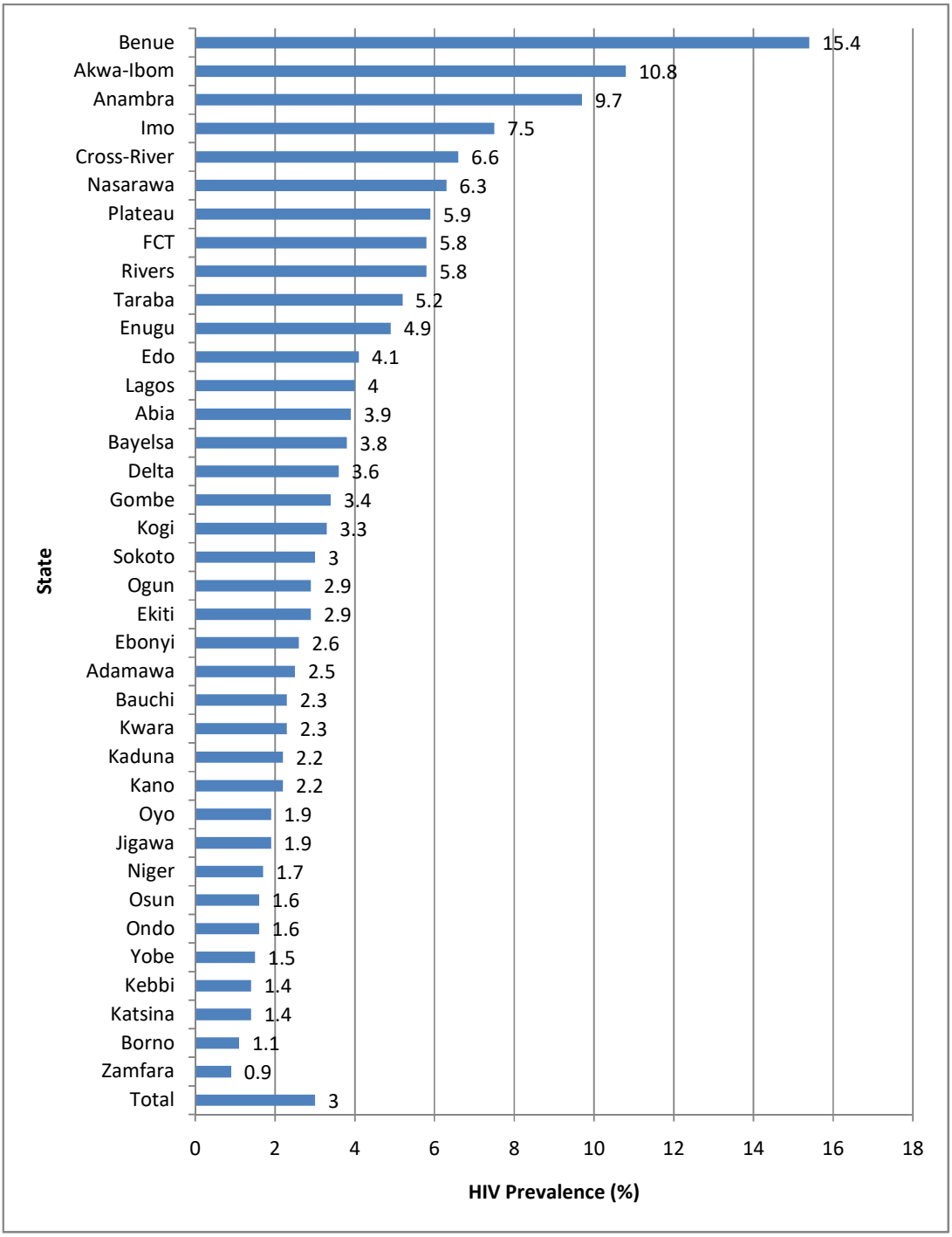


FIGURE 2.8: HIV SERO-PREVALENCE AMONG PREGNANT WOMEN IN SENTINEL ANTENATAL CLINICS BY STATES IN NIGERIA IN 2014

Source: FMoH (2015)

Anal heterosexual intercourse also poses a risk for acquisition of the virus for the female partner(Padian *et al*., 1987). It has also been reported that the act of fellatio can transmit HIV (Dillon *et al*., 2000). It is believed that female partner in the act of fellatio stand the same risk of transmission as the receptive oral partner in male homosexual couples.Theoretically, transmission from cunnilingus is possible although this is yet to be reported and documented.

2.9.2.2Blood, blood products, tissue transplantation and artificial insemination

HIV can also be acquired through infected blood transfusion as well as transfusion of infected blood products into an uninfected person(Donegan *et al*., 1994).However, as a result of mandatory screening, the risk of transmission from these sources has been reduced significantly in many parts of the world. Organ and tissue transplantation especially organs that contain blood or highly vascular tissues such as liver, pancreas, heart, kidney, skin andbone are other efficient means of transmission of the virus. Artificial insemination; both cervical and intrauterine can also lead toacquisition of HIV.

2.9.2.3 Vertical/perinatal

Transmission of HIV also occurs perinatally or vertically from infected mother to her offspring. This can occur during pregnancy (*in utero*), labor and delivery (intrapartum) or breastfeeding (postpartum). Infection *in utero* occurs when the foetus comes in contact with mother's blood as a result of bleeding in the placenta or swallows infected amniotic fluid.

Seroconversion during pregnancy, high viral load(Dunn *et al*., 1992), poor feeding, presence of other STIs and poor adherence to antiretroviral drug therapy are all maternal factors which increase the chance of mother to child transmission.The risk of *intrapartum*HIV transmission is increased by factors such as delivery through the vagina, vaginal tissue rupture, contact with mother's blood and vaginal secretions and chorioamnitis. Chorioamnitis in pregnancy leads toproliferation of leucocytes which are the targets of the virus. The baby's chance of acquiring the virus is also increased by high marternal viral load(Dunn *et al*., 1992).

Breastfeeding is the most important risk factor of transmission postnatally. Cracked nipples, mastitis (breast inflammation), prolonged breast-feeding, seroconversion after delivery, elevated viral load, and low $CD4^+$ cell count are risk factors of transmission during breastfeeding. Mixed feeding also increases the probability of transmission to the baby.This

is because it is believed that the introduction of new foods triggers the baby's immune response by attracting leucocytes to the gastrointestinal tract (GIT) thereby providing enough target cells for HIV.

2.9.2.4OTHER MEANS OF TRANSMISSION

2.9.2.4.1 Transmission in health care settings

Transmission from health worker to patient and vice versa, as well as patient to patient can occur through the use of instruments or equipments in the health-care settings.Transmission through needle-stick injuries and injuries from other sharp objects are common in hospitals and other clinical services(Cardo *et al*., 1997).

2.10LABORATORY DIAGNOSIS OF HIV

2.10.1 SEROLOGICAL METHOD: Serological assays are mainly used in the diagnosis of HIV infection despite the several alternative methods provided by modern molecular biology.Serological diagnosis is based on the detection of various markers. While the direct detection identifies whole viral particles, viral nucleic acids or circulating antigens, indirect serologic detection identifies specific antibodies to HIV in biologic samples.While some serological assays are used for screening, others are used for confirmation of the results of the screening assays(Schochetmann *et al*., 1989). There is need for high sensitivity for screening assays in order to capture all potentially infected individuals (low false negative rate), while confirmatory tests would be highly specificto distinguish true infections from non-specific reactions (low false-positive rate)(Constantine *et al*., 1994).

2.10.1.1 SCREENING ASSAYS: Screening assays for HIV detection can be divided into rapid tests and ELISA-based assays.

A. RAPID TESTS: These are qualitative immunoassays designed as a point-of-care assay to assist in HIV diagnosis.The rapidity is such that results are available in less than 30 minutes. The test can be as accurate as ELISA method if carried out by experienced personnel.It can be employed in different rooms such as emergency,labour andautopsy rooms as well as doctors' offices, point-of-care testing, funeral homes, small blood banksand situations where immediate treatment is recommended for exposures and large-scale or

community-wide HIV testing programmes (CDC, 1998). The assays are simple to perform, robust and easy to interpret. There may not be need for additionalreagents to carry out the assay. The kits can be transported easily without encountering damages inflicted by inadequate temperature storage and are widely used in developing countries where facilities may be suboptimal such as unstable electricity(Ketema *et al*., 2001).

B. ELISA-BASED ASSAYS: Detection of HIV antibody can be achieved by enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA). Because ELISA assays are simple to perform, comparativelyaffordable, standard, highly sensitive and reproducible with high turnaround time,they are useful in many settings(Max *et al*., 2002).There are different varieties of ELISA assays with all working with the same principle. ELISA assays involves using enzyme conjugates to bind specific HIV antibody. Addition of substrates/chromogens produces colour change as a result of reaction catalysed by the bound enzyme conjugate.

Indirect ELISA is the most widely used. In this assay, antibody to HIV in the sample reacts with HIV antigen coated on the walls of 96-well microtiter plate.This is followed by a period of incubation (usually for 30 minutes) at 37^0C or 40^0C. Thereafter, the plate is washed to remove unbound antibody and other components of the serum.A conjugate (an antihuman immunoglobulin linked to an enzyme) is then added which attaches to specific antibody already complexed with the antigens on the walls of the microtiter plate (solid phase). Again, another round of washing is carried out to remove excess and unreacted reagents. Addition of an appropriate substrate to the enzyme then results in colour development. The intensity (measured as optical density-OD using spectrophotometer) of the colour is a measure of the concentration of the specific HIV antibody.

The indirect ELISA method belong to first generation tests which used purified antigens derived from disrupted whole HIV virions. Developed in 1985, the assays proved to be extremely sensitive with less than 0.1% false negatives but less specific (1% false positives).The assays were able to capture HIV antibodies after about an average of 40 days of infection(Fanales-Belasio *et al*., 2010). In a bid to improve on the specificity and sensitivity of the assays, 2^{nd} generation EIA was developed in 1987 also on indirect format

but HIV recombinant antigens and peptides replacing full viral lysate. These tests decreased the window period (Weber, 2006)to about 33-35 days post-infection.

In order to further improve on the sensitivity and specificity of the assays, 3rd generation EIA was developed in 1994 based on a new format. The solid phase is coated with recombinant HIV-1 and HIV-2 proteins and/or peptides. This reacts with the patient's antiserum upon addition of the sample. On addition of similar immobilized HIV antigen conjugated to an enzyme (alkaline phosphatase or horseradish peroxidase), the HIV antigen reacts with already binded antibody from the serum thereby sandwishing it between two antigen molecules. Addition of the substrate causes colour development which is proportional to the antibody concentration.This arrangement is known as sandwich format and is the basis of third generation EIAs(Max *et al*., 2002). Sensitivity and specificity of the assays were improved and the period of antibody detection was further decreased to about 22 days post-infection using this generation of tests(Weber, 2006).

This was subsequently followed by the development of fourth generation ELISA assay.In this assay, HIV antigen (p24) and antibody are simultaneously detected.p24 antigen is the first serologic marker to be detected in an infected individual (Allain and Launa, 1986). Emergence of antibody to p24 is temporarily associated with declining p24 Ag levels and with immune complexes of these reactants. P24 Ag disappears with the appearance of its corresponding antibodies. It remains undetectable for years and reappears and rises coincidentally with the development of clinical manifestation of AIDS.Fourth generation ELISA is an improvement over third generation ELISA (Fig 2.9).

2.10.1.2 CONFIRMATORY ASSAYS

Because of the highly sensitive nature of screening assays, there is the need for specificity which can achieved in assays like western blot (WB), indirect immunofluorescence assay (IFA), radioimmunoprecipitation assay (RIPA), or line immunoassay (LIA) to eliminate cases of false positive results in HIV infection diagnosis. For this purpose, WB and LIA are used to confirm results of screening assays.

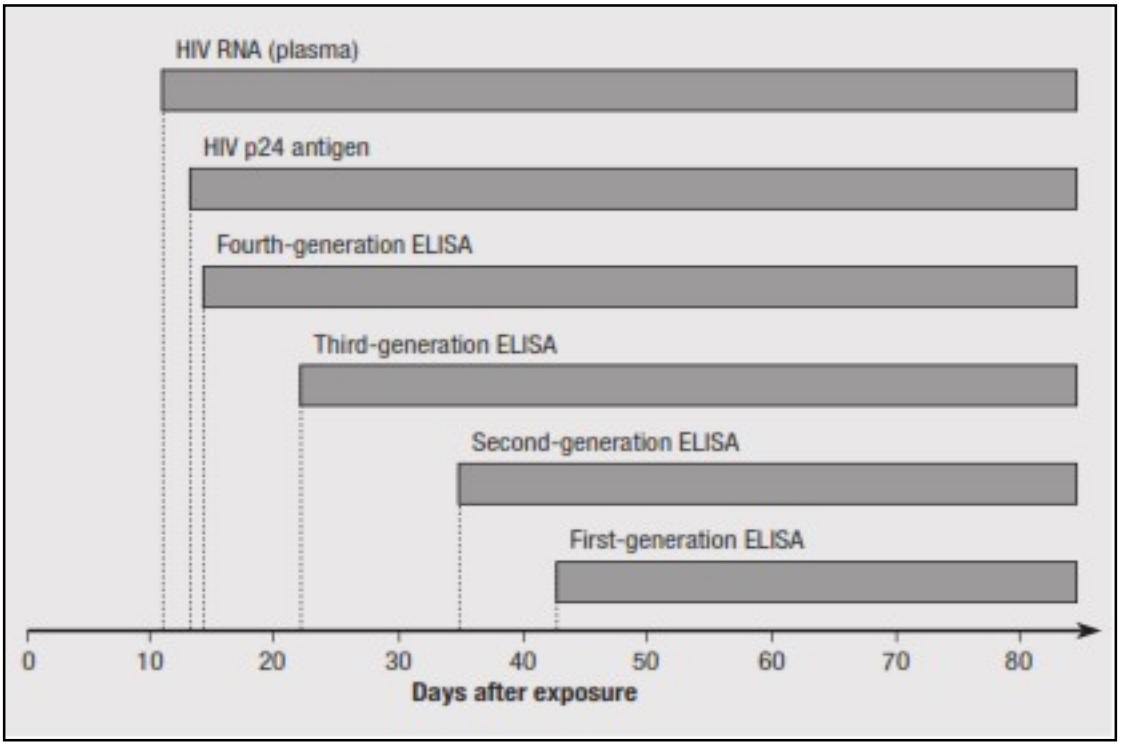


FIGURE 2.9: TIME OF DETECTION OF SPECIFIC MARKERS OF HIVINFECTIONS, ACCORDING TO STANDARDIZED, COMMERCIALLY AVAILABLE KITS. ONCE DETECTED, ASSAY FOR THE MARKERS REMAIN POSITIVE FOR THE REST OF THE LIFETIME. TIME 0 INDICATES TIME OF HIV INFECTION WHILE HORIZONTAL BARS AND ARROW INDICATE REST OF THE LIFE OF THE INFECTED PERSON.

SOURCE: Butto *et al*. (2010)

A. WESTERN BLOT (WB)

This is employed as a confirmatory assay for screening assay-reactive samples. In this method, HIV denatured proteins are blotted on strips of a nitrocellulose membrane and incubated with the patient's serum. Antibodies against various viral proteins, if present attaches to their complimentary proteins. An anti-human immunoglobulin labelled with an enzyme and a matching substrate reveal the occurrence of the reaction between the antigen and antibody as a colorimetric reaction which appears as "bands" on the strip.

Detectable antigens by Western Blot for HIV-1 can be grouped into three: the env glycoproteins (gp41, gp120, and gp160), the Gag or nuclear proteins (p17, p24/25, p55) and the Pol or endonuclease-polymerase proteins (p34, p40, p52, p68). According to CDC guideline, positive result is recorded when the sample is reactive to at least 2 of the following antigens: p24, gp41, gp120/160 while negative result is recorded by absence of all the bands.When the sample is reactive to one or more antigens but fall short of the requirements for positivity, it is regarded as indeterminate result. Most available WBs includes HIV-2 proteins (synthetic peptides) so that HIV-2 infections can also be captured.

B. INDIRECT IMMUNOFLUORESCENCE ASSAY (IFA)

This technique involves artificially infecting cell (lymphocytes) with HIV and fixing the cell to microscope slide. This is followed by the addition of serum. If the antibody is present, it reacts with the cell-associated HIV. After washing, antiimmunoglobulin antibodies labelled with fluorescence material is added and viewed under fluorescent microscope. Indeterminate results from WB can sometimes be resolved using this technique. The technique however employs the use of costly microscope and requires subjective interpretation that can only be provided by well-trained personnels.

C. LINE IMMUNOASSAY (LIA)

LIA makes use of recombinant or synthetic peptide antigens applied on a nitrocellulose strip as against bloting by electrophoresis as in the case of Western blot. By employing artificial antigens, contaminants arising from cell culture which can interfere and cause false reactions are avoided.This technique is an alternative to the classic WB and IFA confirmatory assays.Figure 2.10 illustrates progression of HIV-1 markers in acute infection.

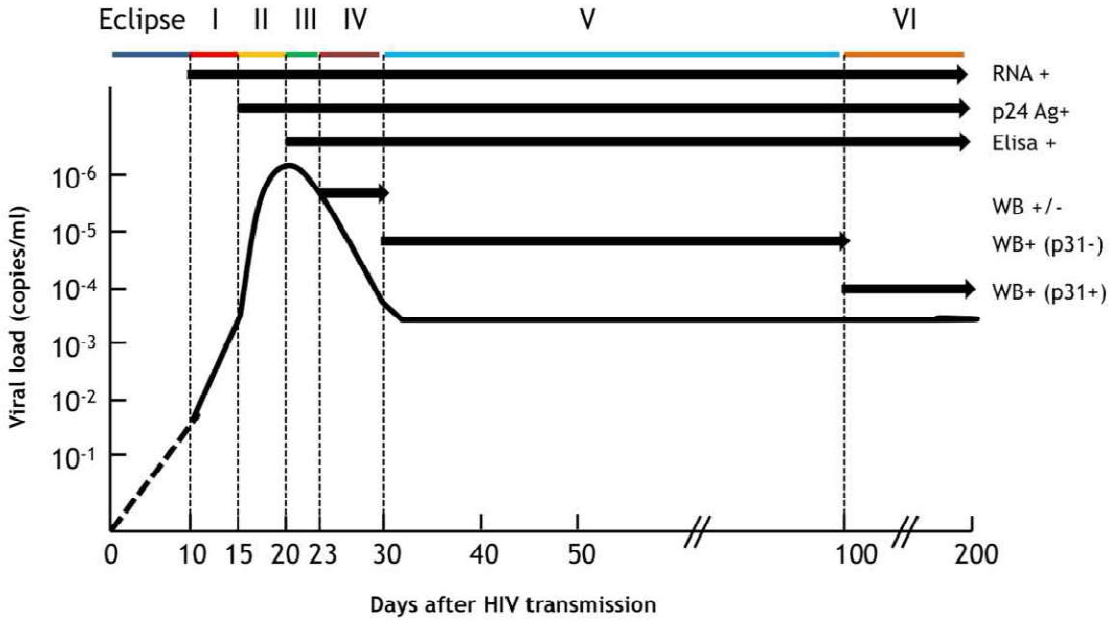


FIGURE 2.10: PROGRESSION OF HIV-1 MARKERS IN ACUTE INFECTION.

Stage Interpretation

I = Definitive HIV RNA viraemia

II = p24 antigenemia

III = HIV EIA antibody reactive

IV = I, Western blot indeterminate

V = Western blot positive without p31 pol band

VI = P, Western blot positive with p31 pol band

Source: Adapted from (Fiebig *et al*., 2003)

2.10.2 MOLECULAR BASED ASSAYS

Different types of diagnostic assays which are able to quantitatively detect HIV-1 RNA in plasma have been developed. These nucleic acid-based assays have been quite useful in evaluating the progress of disease and efficacy of ART, determining primary infection, diagnosis in infants born to infected mothers and recipients of HIV vaccine. It has also been used for the diagnosis of HIV infections prior to seroconversion. Table 2.4 shows the various types of viral load assays recommended by WHO for the diagnosis of HIV infection.

2.10.3 DIAGNOSIS IN THE NEWBORN

Since the invention of the first tests, it has been difficult to make a definitive laboratory diagnosis of HIV infection in the neonate. This is as a result of the presence of marternal antibodywhich may persist for up to 1 year after birth before seroreversion. The immune system of the newborn acquires the ability to produce antibody (seroconvert) several months later. Early detection of HIV in perinatal infection is possible using highly sensitive and specific commercially available virologic assays.

The timing of transmission of HIV from mother to child (in utero at the time of birth or breastfeeding) is a critical consideration in the appropriate diagnosis of infants. Early infection and estimation of time of infection can be done using several algorithms including PCR for HIV DNA and RNA detection as well as culture method. These assays can define and rule out infection in infants (<18months).PCR is a highly sensitive assay that is capable of detecting 1 DNA molecule in a clinical specimen. The reaction involves the use of thermal cycler (a machine which regulates temperatures needed for the amplification of the DNA). DNA template contains the sequence of interest while DNA polymerase is the enzyme that catalyses the reaction. Primers defines/primes the target sequence while nucleotides (dNTPs or deoxynucleotide triphosphates) serves as “building blocks” for new DNA strands to be synthesized. They include; dTTP, dCTP, dATP and dGTP. In the reaction, buffer solution Tris-HCL is also needed to provide a conducive chemical environment for optimum activity and stability of the DNA polymerase. Divalent cations which could be Magnesium or Manganese ions act as cofactor of the enzyme. Taq DNA polymerase requires free magnesium that binds to template DNA, primers and dNTPs. Also, monovalent cation, potassium ions facilitates primer annealing while gelatine or bovine serum helps in stabilization of the enzyme.

TABLE 2.4: SOME VIRAL LOAD ASSAYS RECOMMENDED BY WHO

Assays	Technology	Probe target	Linear range (RNA copies/ml)	HIV-1 clade recognition
Abbot Real Time HIV-1	RT-PCR	*pol*-INT	40-10,000,000	Group M (subtypes A-H), several CRFs and Groups O and N
Amplicor HIV-1 Monitor Test v1.5	RT-PCR	*gag*-p24	Standard protocol: 400 to >750,000; Ultra-sensitive: 50 - >100,000	HIV-1 Group M (subtypes A-H)
Cobas Amplicor HIV-1 Monitor Test, v1.5	RT-PCR	*Gag*	Standard protocol: 400 to >750,000; Ultrasensitive: 50 - >100,000	HIV-1 Group M (subtypes A-H)
Cobas AmpliPrep/Cobas TaqMan HIV-1 Test, v2.0	RT-PCR	*gag*-p41 and 5´LTR	20-10,000,000	Group M, several CRFs, and Group O
Versant HIV-1 RNA 1.0 Assay (kPCR)	RT-PCR	*gag*-p24	37-11,000,000	HIV-1 Groups M and O
Versant HIV-1 RNA 3.0 Assay	bDNA	*pol*-INT	50-500,000	HIV-1 Group M
NucliSens EasyQ HIV-1 v2.0	NASBA	*gag*-p24	10- 10,000,000	HIV-1 Group M (subtypes A-J), CRF01_AE, and CRF02_AG

RT-PCR= reverse transcriptase polymerase chain reaction, bDNA= branched-chain DNA, NASBA= isothermal nucleic acid sequence-based amplification

Source: Adapted from WHO (2010)

The use of serologic methods including HIV ELISA, immunofluorescent assays and western blot are appropriate for diagnosis of infants more than 18 months when trans-placental antibody has disappeared in HIV exposed infants. Presently, however, it is still difficult to achieve conclusive diagnosis in the infants <6 months of age.Detection and quantitation of several virological and immunological markers is still the basis for the diagnosis in acute infection.

2.10.4VIRAL ISOLATION

HIV culture is carried out by cocultivating clinical specimenwith mononuclear cells from a HIV-1 positive donor which have been activated with phytohemagglutinin (Hollinger *et al*., 1992).This method can be used qualitatively and quantitatively if adequate amounts of patient specimen are used and yields highly efficient result (Hollinger *et al*., 1992).After incubating for two to four weeks, the supernatant is tested for the presence of viral RNA or p24 antigen which indicates the presence of the virus.

2.11PROGNOSTIC TESTS

There is a need for follow-up after the initial diagnosis of HIV infection. The patient is usually monitored at regular intervals to ascertain the extent of disease progression and determine the appropriate time for commencement of therapy and to assess response to therapy for those already receiving ART. A couple of assays are available to monitor patients with HIV-1 after commencement of therapy.

2.11.1 Peripheral Blood CD4+ T Lymphocyte Counts

CD4 count is not considered a diagnostic assay for HIV, rather a procedure of enumerating the cells to determine their level in the blood.The procedure is utilized for monitoring the functionality of immune system in HIV-positive individuals. By monitoring the CD4 count, it can be estimated with some level of accuracy the period of developing AIDS-related complications. Decreasing CD4 T-cell level is an indication of a progressing HIV infection.The counts are best for evaluating immediate risk of development ofOIs or neoplasia. On the other hand, long-term clinical outcome can better be predicted using plasma HIV-1 RNA levels. Thus, the recommended approach for prognostic assessment isenumeration of CD4+ lymphocytes in combination with determination of plasma viral load (Valentine *et al*., 1998; Holland *et al*., 2000).

2.11.2 Plasma HIV RNA Levels or Viral Loads

There are commercial assays available to detect plasma HIV-1 RNA. Theseassays are highly sensitive and also suitable for monitoring HIVinfected individuals regardless of disease stage(Ho *et al*., 1989). Plasma RNA levels show a wide dynamic range that correlates significantly with the clinical stages of the disease and therefore can be useful forpredicting the time to AIDS and death. Additionally, plasma viral load declines rapidly as a result administration of effective antiviral therapy (O'Brien *et al*., 1997).Consequently, viral load is now routinely measured as part of management for HIV patients.

2.12TREATMENT OF HIV AND AIDS

HIV is controlled by special group of chemotherapeutic agents known as antiretroviral drugs.For now HIV drugs do not cure the infection but the goal is to improve on the quality of patient's lives and decrease mortality by reducing viral load, improving CD4 count, controlling co-infections etc.Antiretroviral drugs act by targeting specific processes that are unique to the virus without killing the host cell. Different identified stages in the replication cycle of HIV that can possibly be targeted for chemotherapy include; Receptor binding, Viral envelope and host cell membrane fusion, Uncoating of the nucleocapsid, Reverse transcription, cDNA incorporation into the host genome, Replication, Transcription, RNA splicing in the host cell nucleus, Translation, Post translational cleavage of viral polyproteins, Glycosylation of gp160, Phosphorylation of viral proteins, Fatty acylation of GAG, Viral assembly, Budding, Maturation of the virus particle after budding. Drugs currently being used for the treatment of HIV/AIDSare grouped into 6 major types on the basis of their mechanism of interference with steps in HIV replication. The antiretroviral drugs are as follows:

2.12.1 NUCLEOSIDE/NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTIs)

Theycompetitively attach to the active site of the enzyme reverse transcriptase thereby terminating/inhibiting DNA chain elongation.In order to produce antiviral effect, they require phosphorylation by cellular kinases after host cell's entry (Hart *et al*., 1992). They are therefore administered as prodrugs. Table2.5shows a list of some NRTIs.

TABLE 2.5: SOME EXAMPLES OF NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTIS)

Brand Name	Generic Name	Manufacturer Name	Approval Date
Combivir	lamivudine and zidovudine	GlaxoSmithKline	27-Sep-97
Emtriva	emtricitabine, FTC	Gilead Sciences	02-Jul-03
Epivir	lamivudine, 3TC	GlaxoSmithKline	17-Nov-95
Epzicom	abacavir and lamivudine	GlaxoSmithKline	02-Aug-04
Hivid	zalcitabine, dideoxycytidine, ddC (no longer marketed)	Hoffmann-La Roche	19-Jun-92
Retrovir	zidovudine, azidothymidine, AZT, ZDV	GlaxoSmithKline	19-Mar-87
Trizivir	abacavir, zidovudine, and lamivudine	GlaxoSmithKline	14-Nov-00
Truvada	tenofovir disoproxil fumarate and emtricitabine	Gilead Sciences, Inc.	02-Aug-04
Videx EC	enteric coated didanosine, ddI EC	Bristol Myers-Squibb	31-Oct-00
Videx	didanosine, dideoxyinosine, ddI	Bristol Myers-Squibb	9-Oct-91
Viread	tenofovir disoproxil fumarate, TDF	Gilead	26-Oct-01
Zerit	stavudine, d4T	Bristol Myers-Squibb	24-Jun-94
Ziagen	abacavir sulfate, ABC	GlaxoSmithKline	17-Dec-98

SOURCE: U.S. FDA, 2013

2.12.2 NON–NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTIs)

Inhibition of HIV-1 reverse transcriptase activity occurs when NNRTIs binds and induces hydrophobic pocket which is proximal to the active site of the enzyme (Tantillo *et al*., 1994). Spatial conformation of the substrate-binding site changes upon binding by NNRTIs and thisdecreases the activity of the polymerase (Spence *et al*., 1995). Table 2.6 shows examples of NNRTIs.

2.12.3 INTEGRASE INHIBITORS

Integrase catalyzes the cleaving of the 3′ ends of the viral cDNA after synthesis (knowns as 3′-end processing) followed by the insertion of these ends into the host cell DNA (known as strand transfer). Integrase inhibitors act by inhibiting the insertion of processed cDNA into the host cell DNA. As a result of this, they are known as integrase inhibitors (INIs) or integrase strand transfer inhibitors (InSTIs) (McColl and Chen, 2010). Some important integrase inhibitors include; Raltegravir (Isentress) manufactured by Merck & Co., Inc. and Dolutegravir (Tivicay) manufactured by GlaxoSmithKline (U.S. FDA, 2013).

2.12.4 PROTEASE INHIBITORS

HIV protease is needed for post-translational processsing of viral polyprotein precursors into individual functional proteins associated with the virus (Miller, 2001). They are encoded by the polymerase (*pol*) gene. Protease Inhibitors (PIs) are substrate analogues for this HIV aspartyl protease enzyme designed to attach to the enzyme's active site and prevent it from further activity. Through this action, viral maturation is inhibited resulting in lack of functional virion formation. Unlike inhibitors of virus entry and RT, PIs does not prevent infection of uninfected cells by HIV-1 rather, it causes release of non-infectious virions (Flexner, 1998). Because PIs have relatively poor oral bioavailability, they are mostly administered together with a low dose of ritonavir (booster drug).Resistance to PIs arise when patterns of mutations develop and accumulate in the active site of *protease* gene (Ammaranond and Sanguansittianan, 2012). Research have shown that 46 out of the 99 peotease amino acids are associated with PIs selection out of which 26 positions are the most common (Rhee *et al.*, 2010).Table 2.7 shows some PIs.

TABLE 2.6: SOME EXAMPLES OF NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTIS)

Brand Name	Generic Name	Manufacturer Name	Approval Date
Edurant	Rilpivirine	Tibotec Therapeutics	20-May-11
Intelence	Etravirine	Tibotec Therapeutics	18-Jan-08
Rescriptor	delavirdine, DLV	Pfizer	4-Apr-97
Sustiva	efavirenz, EFV	Bristol Myers-Squibb	17-Sep-98
Viramune	nevirapine, NVP	Boehringer Ingelheim	21-Jun-96
Viramune_XR	nevirapine, NVP	Boehringer Ingelheim	25-Mar-11

SOURCE: U.S. FDA, 2013

TABLE 2.7: SOME EXAMPLES OF PROTEASE INHIBITORS (PIs)

Brand Name	Generic Name	Manufacturer Name	Approval Date
Agenerase	amprenavir, APV (no longer marketed)	GlaxoSmithKline	15-Apr-99
Aptivus	tipranavir, TPV	Boehringer Ingelheim	22-Jun-05
Crixivan	indinavir, IDV,	Merck	13-Mar-96
Fortovase	saquinavir (no longer marketed)	Hoffmann-La Roche	7-Nov-97
Invirase	saquinavir mesylate, SQV	Hoffmann-La Roche	6-Dec-95
Kaletra	lopinavir and ritonavir, LPV/RTV	Abbott Laboratories	15-Sep-00
Lexiva	Fosamprenavir Calcium, FOS-APV	GlaxoSmithKline	20-Oct-03
Norvir	ritonavir, RTV	Abbott Laboratories	1-Mar-96
Prezista	Darunavir	Tibotec, Inc.	23-Jun-06
Reyataz	atazanavir sulfate, ATV	Bristol-Myers Squibb	20-Jun-03
Viracept	nelfinavir mesylate, NFV	Agouron Pharmaceuticals	14-Mar-97

SOURCE: U.S. FDA, 2013

2.12.5 ENTRY INHIBITORS

Penetration of HIV-1 into target cell requires a number of host cell proteins needed for various events which culminates in membrane fusion and subsequent release of the virus into the cell cytoplasm. Entry inhibitors are subdivided on the basis of the step of the entry process they inhibit.

2.12.5.1 Fusion Inhibitors

HIV life cycle begins with the delivery of the genome into host cell. Entry is a multistep process that involves attachment, coreceptor binding and cellular and viral membrane fusion. Following the discovery that fusion can only occur following interaction of 2 homologous domains located within viral gp41 and that a heterologous protein that mimicks any of these domains can attach and alter intramolecular interactions of the virus protein, peptide fusion inhibitors were designed. Important fusion inhibitor is enfuvirtide, T-20 (Fuzeon) manufactured by Hoffmann-La Roche & Trimeris (U.S. FDA, 2013).

2.12.5.2 Small-Molecule CCR5 Antagonists

Another class of entry inhibitors are the small-molecule CCR5 antagonists which binds to hydrophobic pockets located within transmembrane helices of Chemokine co-receptor-5(Dragic *et al*., 2000; Tsamis *et al*., 2003). An important CCR5 coreceptor antagonist is Maraviroc (Selzentry) manufactured by Pfizer (U.S. FDA, 2013). An assay to determine the co-receptor usage (tropism) of the HIV-1 strain in the infected patients is usually performed before initiation of Maraviroc treatment.

2.12.6 Combination therapy

HIV Treatment received a boost with the introduction of increasingly potent combination of drug therapies. This is achieved by co-administering 2 or more antiretroviral drugs that belong to different classes or types, into a single regimen (Table 2.8). The rationale behind this is that drugs working in synergy and does not form resistance simultaneously as well as have no overlapping toxicity will be able to decrease toxicity, improve efficacy and prevent emergence of resistance.Where one drug had a beneficial effect, combination of two or three complementary drugs can reduce viraemia to undetectable levels by the best currently available viral load assays.

TABLE 2.8: ANTIRETROVIRAL REGIMENS RECOMMENDED FOR TREATMENT OF HIV-1 INFECTION IN ANTIRETROVIRAL-NAIVE PATIENTS

Preferred Regimens	Regimens
NNRTI-based	Efavirenz[a] + (lamivudine or emtricitabine) + (zidovudine or tenofovir)
PI-based	Lopinavir/ritonavir (coformulation) + (lamivudine or emtricitabine) + zidovudine
Alternative regimens	
NNRTI-based	Efavirenz[a] + (lamivudine or emtricitabine) + (abacavir or didanosine or stavudine) Nevirapine + (lamivudine or emtricitabine) + (zidovudine or stavudine or didanosine or abacavir or tenofovir)
PI-based	Atazanavir + (lamivudine or emtricitabine) + (zidovudine or stavudine or abacavir or didanosine) or (tenofovir + ritonavir 100 mg/d) Fosamprenavir + (lamivudine or emtricitabine) + (zidovudine or stavudine or abacavir or tenofovir or didanosine) Fosamprenavir/ritonavir[b] + (lamivudine or emtricitabine) + (zidovudine or stavudine or abacavir or tenofovir or didanosine) Indinavir/ritonavir[b] + (lamivudine or emtricitabine) + (zidovudine or stavudine or abacavir or tenofovir or didanosine) Lopinavir/ritonavir + (lamivudine or emtricitabine) + (stavudine or abacavir or tenofovir or didanosine) Nelfinavir + (lamivudine or emtricitabine) + (zidovudine or stavudine or abacavir or tenofovir or didanosine) Saquinavir (sgc, hgc, or tablets)[c]/ritonavir + (lamivudine or emtricitabine) + (zidovudine or stavudine or abacavir or tenofovir or didanosine)
3-NRTI regimens	Abacavir + zidovudine + lamivudine- only when a preferred or an alternative NNRTI- or a PI-based regimen cannot or should not be used

[a]Efavirenz is not recommended for use in first trimester of pregnancy or in women who want to conceive or those who are not using effective contraception.
[b]Low-dose (100-400 mg/d) ritonavir.
[c]sgc = soft gel capsule; hgc = hard gel capsule; NNRTI = non-nucleoside reverse transcriptase inhibitor; PI = protease inhibitors

SOURCE: AIDSinfo(2004)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1STUDY AREA AND POPULATION

The study was carried out in three randomly selected states located in Southeastern geographical region of Nigeria. HIV treatment centers located in the three states were selected for the study. The participants included 246 HIV-1 positive patients who are receiving care and management at the antiretroviral treatment centers. The states are Delta, Anambra and Imostates.

3.1.1Delta State: The state was created out of the old Bendel State in 1991 with its capital in Asaba. The name was derived from the delta region of the River Niger.To the northwestern part, it is bounded by Edo and Ondo States; Imo and Anambra to the northeast while to the southeast, it is bounded by Rivers and Bayelsa States.It has coastline boundaries with the Bight of Benin on the Atlantic Ocean spanning approximately 122 Kilometers to the southwest.The indigenous ethnic groups in the state are Urhobo, Igbo, Isoko, Ezon and Itsekiri. According to the 2006 national census figures, it has a population of 4,098,391 people and constitutes about 2.93% of the total population of Nigeria.HIV prevalence in the state is 4.1%(FMOH, 2010; NACA, 2012).

3.1.2 Anambra state: The state is located in the Southeastern geopolitical zone of Nigeria. It derived its name from 'Oma Mbala', which is the native name of the Anambra River.It has its capital in Awka. The state is bounded by Imo and River States to the south, Delta State to the west, Kogi State to the north and Enugu State to the east.The major inhabitants of the state are the Igbo who make up about 98% of the population while the Igalas make up about 2% of the population and inhabit mainly the Northwestern part of the state (Igbofocus.co.uk). It has a population of 4,182,032 and constitutes about 2.99% of total Nigeria population according to the 2006 national census figures. The HIV prevalence of the state is about 8.7% (FMOH, 2010; NACA, 2012)

3.1.3 Imo State: This is also a state in Southeastern geopolitical zone of Nigeria. It came into existence in 1976having been created from the old Eastcentral State.Its name is derived

from the Imo River and has its capital in Owerri.The state occupies about 5,100 Km^2 and situatedwithin latitudes 4°45'N and 7°15'N, and longitude 6°50'E and 7°25'E.It shares a common boundary with Abia State to the East. To the west, it is bounded by the River Niger and Delta State while to the north and south, it is bounded by Anambra and Rivers States respectively.Imo people are mainly of Igbo ethnic nationality and have a population of 3,934,899 people who constitutes about 2.81% of the total Nigeria population according to the 2006 national census figures.It has HIV prevalence of about 3.0% (FMOH, 2010; NACA, 2012)

3.2ETHICAL APPROVAL

Ethical approval for the work was obtained from UI/UCH Ethics Committee with assigned numberUI/EC/11/0178. Approval was also obtained from the Hospital Management Boards of the respective states where samples were collected

3.3SAMPLE COLLECTION AND PROCESSING

About 5ml of venous blood was collected from each patient into EDTA (ethylene diamine tetra acetic acid) bottles with the assistance of trained phlebotomist. The blood was centrifuged,separated and both the packed cells and the plasma were stored in aliquots in sterileEppendorf tubes.The samples were labelled appropriately and then transported in cold chain to the Department of Virology, University of Ibadan, Ibadan where the packed cells and the plasma were stored at -20^{o}C and -80^{o}C respectively until further analysis.

3.4DATA COLLECTION

Pertinent demographic, clinical and epidemiological data of each patient were obtained by administration of a structured questionnaire (Appendix 1). Questionnaire was administered by nurses in the various health centers.

3.5LABORATORY METHOD

3.5.1 DNA EXTRACTION

Extraction of genomic DNA from the blood samples was done using phenol-chloroform and precipitated using ethanol in a procedure modified from Perucho's protocol (Perucho *et al*., 1981).

3.5.1.1 PRINCIPLE

This extraction procedure is based on the principle of phase separation of aqueous sample mixture and a solution containing TE-saturated phenol and chloroform by centrifugation. This centrifugation results into separation of the mixture into an aqueous phase to the upper part and an organic phase (mainly phenol) to the lower part.At about pH 7-8 i.e neutral conditions, the nucleic acid (DNA) partition into the aqueous phase. The negatively charged phosphate backbone of DNA makes it a polar molecule. Hence, DNA is more soluble in water than it is in phenol causing it to partition into the aqueous phase. On the other hand, protein partitions into the organic phase. This is because inside a non polar solvent like phenol, the less-polar residues sequestered inside the protein structures in aqueous environment, now interact with the phenol which is less-polar and so are pushed to the surface. On the other hand, some of the highly polar residues may turn over to the inside of the globular protein avoiding the unfavourable new solvent. As a result of this folding changes as a result of phenol, the phenol-favouring residues are forced to the outside thereby making the proteins more soluble in phenol than in water. DNA recovery from the aqueous phase is then achieved by ethanol precipitation.

3.5.1.2 REAGENTS

The following reagents were used

- Phenol crystals
- Chloroform
- Tris-EDTA (TE) buffer
- 3 M Sodium acetate
- Absolute ethanol
- Double distil water (ddH_2O)

The preparation of the 10x and 1x TE buffer used for the equilibration of the phenol from the 100X TEis as described in appendix 2 while the preparation of equilibrated phenol is as described in appendix 3.

3.5.1.3PROCEDURE FOR DNA EXTRACTION

Five hundred microliters (500µl) of each packed blood cells was dispensed into labelled Eppendorf tube and equal volume of equilibrated phenol/chloroform (50:50) was added. The tube was vortexed vigorously and spun in a centrifuge at top speed (16,000 rpm/25,000 rcf) for 3 minutes. The aqueous phase was aspirated into a new Eppendorf tube and equal volume of chloroform introduced. The tube was then vortexed vigorously and spun as above for removal of remaining traces of phenol. The upper aqueous phase was aspirated into a new Eppendorf tube carefully. This step of addition of chloroform to remove traces of phenol was repeated until there was no visible protein interface between the chloroform and the aqueous phase.The aqueous phase was aspirated into a freshEppendorf tube carefully. This was followed by DNA precipitation using the following procedure.

3.5.1.4PROCEDURE FOR DNA PRECIPITATION

Fifty microliters (50µl) of 3M sodium acetate (provides Na^+ needed to neutralize - charge on the PO_3^- groups on the nucleic acid) and 1 ml of 100% ethanol was added, vortexed and frozen at -80^{o}C for 40minutes or -20^{o}C for at least 2 hours. The tube was spun at full speed in a centrifuge for 30 minutes at 4^{o}C and the supernatant decanted. Three hundred microliters (300µl) of 70% ethanol was introduced into the DNA pellet, vortexed and spun for 5 minutes at 4^{o}C. The supernatant was decanted and the pellet air-dried at 37^{o}C for approximately 10 minutes. The DNA was resuspended by adding 50µl of 1X TE buffer. The tube was then vortexed, centrifuged for 10 seconds to pool the sample and the DNA stored at -20^{o}C until analysed.

3.6DETECTION OF GENOMIC DNA

A 1.5% agarose gel was prepared by adding 100ml of Tris Borate EDTA (TBE) buffer into 1.5g of agarose in a Pyrex heat-resistant bottle. The content was mixed thoroughly and microwaved to boiling and left to cool to about 45 to 50^{o}C. Three microliter (3µl) of ethidium bromide was then added into the mixture and swirled gently before pouring into a mini gel cast (containing gel combs) and allowed to solidify. The combs were carefully removed and the solid agarose gel was then placed in the mini gel tank, and TBE buffer added to completely submerge the agarose gel. Five microlitres (5µl) of the DNA was mixed with 1µl of gel loading buffer and loaded into individual wells in the agarose gel along with the positive control, 100bp DNA ladder and the negative control (nuclease free water).

Electrophoresis was run at 110volts using a current of 500 milliamperes for 60 minutes. After the electrophoresis, the agarose gel was transferred to gel documentation system to examine the presence of band by UV light.

3.7NESTED POLYMERASE CHAIN REACTION (PCR)

3.7.1 PRINCIPLE AND APPLICATION

This technique is based on the principle of DNA polymerization and amplification in an *in vitro* reaction. The enzymatic reaction occurs under repeated cycles of carefully controlled heating and cooling conditions. A thermostable DNA polymerase capable of withstanding high temperatures is employed in the process. DNA denaturation to single strands occurs at the high temperature followed by primer annealing at reduced temperature. Elongation/extension of the DNA strand by the polymerase using the dNTPs occurs when the temperature is slightly raised. At the end of about 30 to 35 cycles, millions and billions of copies of the DNA would have been synthesized which can then be detected using agarose gel electrophoresis. Nested protocol involves two PCR steps. First step involves use of outer primer pairs to amplify larger fragment of the genome while the second step involves use of inner primer pairs to amplify inner smaller part of the first product. This technique is highly sensitive and capable of detecting 1 DNA molecule in a clinical specimen.

3.7.2 AMPLIFICATION OF HIV-1 P24 GAG REGION

A nested PCR was performed on the extracted proviral DNA samples using two sets of primer sequences as published by Ajoge *et al*. (2011). The primers were employed for the amplification of 717bp of the HIV-1 *gag* region. The primers were commercially synthesized by Jena Biosciences, Germany. The primers were delivered lyophilized with a concentration of 10,000 picomoles per microlitre. The primers were diluted in nuclease-free ddH_2O to 10 picomoles per microlitre and stored at -20^{o}C in aliquots of 150µl until used. Details of the primers used are shown in Table 3.1.

3.7.2.1 First and second round PCR

The first and second round PCR were carried out by first preparing the mastermix using the Jena Biosciences kit (Jena Biosciences, Germany). The contents are as shown in Table 3.2.

Twenty microliters of master mix was transferred into 0.5ml MicroAmp PCR reaction tube and 5µL of extracted DNA (for the first round) and 5µL of amplicon from the first round PCR (for the second round) added. The tube was then placed into a thermal cycler (Applied Biosystems GeneAmp PCR System 9700®) previously turned on 30 minutes before commencement of amplification. The cycling conditions are the same for both 1st and 2nd round PCR and are as shown in the Table 3.3.

3.7.3 AMPLIFICATION OF HIV-1 PROTEASE ENCODING REGION OF *POL* GENE

A nested PCR was performed on the extracted proviral DNA samples using two sets of primer sequences as published by Ojesina *et al*. (2006). The primers were used for the amplification of 521bp of the portion of HIV-1 *Pol* gene. The primers were commercially synthesized by Jena Biosciences, Germany. The primers were delivered lyophilized with a concentration of 10,000 picomoles per microlitre. The primers were diluted in nuclease-free ddH_2O to 10 picomoles per microlitre and stored at -20^oC in aliquots of 150µl until used. Details of the primers used are shown in Table 3.4.

3.7.3.1 First and second round PCR

The first and second round PCR were carried out by first preparing the mastermix using the Jena Biosciences kit (Jena Biosciences, Germany). The contents are as shown in Table 3.5.

TABLE 3.1: DETAILS OF THE PRIMERS USED TO AMPLIFY THE HIV-1 p24 GAG REGION

Primer	Orientation	HXB2 position	Sequence (5′-3′)	Size of product
G00 (1st round)	Forward	764-782	GACTAGCGGAGGCTAGAAG	
G01 (1st round)	Reverse	2,264-2,281	AGGGGTCGTTGCCAAAGA	1518 nt
G60del3′G (2nd round)	Forward	1,173-1,197	CAGCCAAAATTACCCTATAGTGCA	
G25 (2nd round)	Reverse	1,867-1,889	ATTGCTTCAGCCAAAACTCTTGC	717 nt

TABLE 3.2: COMPONENTS OF MASTER MIX FOR FIRSTAND SECOND ROUND PCR FOR P24 GAG REGION

Reagent	Concentration in Stocks	Concentration in PCR	Volume/25µL reaction
Nuclease-free water			13 µL
PCR mix	5X	1X	5 µL
Forward primer (G00)-1st round (G60del3′G)-2nd round	10 pmol	0.4 pmol	1 µL
Reverse primer (G01)-1st round (G25)-2nd round	10 pmol	0.4 pmol	1 µL
Total volume			**20 µL**

TABLE 3.3: PCR CONDITIONS FOR 1STAND 2NDROUND REACTIONS FOR AMPLIFICATION OF HIV-1 p24 GAG REGION

Step	Temperature (^{o}C)	Duration	Number of cycles
Initial Denaturation	95	10 min	1
Denaturation	94	30 sec	35
Annealing	45	35 sec	
Extension	72	1 min	
Final Extension	72	5 min	1
Hold	4	∞	1

TABLE 3.4: DETAILS OF THE PRIMERS USED TO AMPLIFY THE 524 BP OF PORTION OF HIV-1 PROTEASE GENE

Primer	Orientation	HXB2 position	Sequence (5′-3′)	Size of product
OJ1 (1st round)	Forward	1,823-1,846	AAATGATGACAGCATGTCAGGGAG	2375 nt
OJ2 (1st round)	Reverse	4,173-4,197	TATCTACTTGTTCATTTCCTCCAAT	
OJ3 (2nd round)	Forward	2,074-2,095	AGACAGGCTAATTTTTTAGGGA	524 nt
OJ4 (2nd round)	Reverse	2,574-2,597	CATTCCTGGCTTTAATTTTACTGG	

TABLE 3.5: COMPONENTS OF MASTER MIX FOR FIRSTAND SECOND ROUND PCR FOR PROTEASE GENE

Reagent	Concentration in Stocks	Concentration in PCR	Volume/25µL reaction
Nuclease-free water			13 µL
PCR mix	5X	1X	5 µL
Forward primer (OJ1)-1st round (OJ3)-2nd round	10 pmol	0.4 pmol	1 µL
Reverse primer (OJ2)-1st round (OJ4)-2nd round	10 pmol	0.4 pmol	1 µL
Total volume			**20 µL**

Twenty microliters of master mix was transferred into 0.5ml MicroAmp PCR reaction tube and 5µL of extracted DNA (for the first round) and 5µL of amplicon from the first round PCR (for the second round) added. The tube was then placed into a thermal cycler (Applied Biosystems GeneAmp PCR System 9700®) previously turned on 30 minutes before the commencement of amplification. The thermal cycler was programmed to the cycling conditions as shown in the Table 3.6.

3.7.4 AMPLIFICATION OF HIV-1 C2-V3 ENVELOPE REGION

A nested PCR was carried out on the extracted proviral DNA samples for HIV-1 subtype determination using two sets of primer sequences as published by Kanki *et al*. (1999). The primers were used to amplify ~350 bp in the HIV-1 C2-V3 envelope region. The primers were commercially synthesized by ThermoScientific Corporation, Germany. The primers were delivered lyophilized with a concentration of 10,000 picomoles per microlitre. The primers were diluted in nuclease-free ddH_2O to 10 picomoles per microlitre and stored at -20^oC in aliquots of 150µl until used. Details of the primers used are shown in Table 3.7.

3.7.4.1 First and second round PCR

The first and second round PCR were done by first preparing the mastermix using the AmpliTaq Gold RT-PCR/PCR kit (Applied Biosystems, USA). The contents are as shown in Table 3.8as previously described by Kanki *et al*. (1999).

Twenty microliters of master mix was transferred into 0.5ml MicroAmp PCR reaction tube and 5µL of extracted DNA (for the first round) and 5µL of amplicon from the first round PCR (for the second round) added. The tube was then placed into a thermal cycler (Applied Biosystems GeneAmp PCR System 9700®) previously turned on 30 minutes before the commencement of amplification. The thermal cycler was programmed to the cycling conditions as shown in the Table 3.9.

TABLE 3.6: PCR CONDITIONS FOR 1ST AND 2ND ROUND REACTIONS FOR AMPLIFICATION OF PORTION OF HIV-1 PROTEASE GENE

Step	Temperature (°C)		Duration		Number of cycles
	1st round	2nd round	1st round	2nd round	
Initial Denaturation	94	94	5min	5 min	1
Denaturation	94	94	1 min	1 min	35
Annealing	46	45	1 min 50 sec	1 min 30 sec	
Extension	72	72	1 min 50 sec	1 min 30 sec	
Final Extension	72	72	10 min	10 min	1
Hold	4	4	∞	∞	1

TABLE 3.7: DETAILS OF THE PRIMERS USED TO AMPLIFY THE ~384 BP IN THE HIV-1 C2-V3 ENVELOPE REGION

Primer	Orientation	HXB2 position	Sequence (5′-3′)	Size of product
WT1 (1st round)	Forward	6,884-6,908	GCTGGTTTTGCGATTCTAAAGTGTA	494 nt
WT2 (1st round)	Reverse	7,353-7,377	CAATAGAAAAATTCCCCTCCACAAT	
KK40 (2nd round)	Forward	6,954-6,973	ACAGTACAATGTACACATGG	384 nt
KK30 (2nd round)	Reverse	7,318-7,337	AATTTCTGGGTCCCCTCCTG	

TABLE 3.8: DESCRIPTION OF PREPARATION OF MASTER MIX FOR 1ST& 2ND ROUND PCR FOR C2-V3 ENV REGION

Reagent	Concentration in Stocks	Concentration in PCR	Volume/25µL reaction
Distilled water			12µL
PCR buffer	10X	1X	2.5µL
$MgCl_2$	20mM	2mM	2.5µL
dNTP mix	10mM	0.2mM	0.5µL
Forward primer	10pmol	0.4pmol	1µL
Reverse primer	10pmol	0.4pmol	1µL
Taq Polymerase			0.5µL
Total volume			**20µL**

TABLE 3.9: PCR CONDITIONS FOR 1ST AND 2ND ROUND REACTIONS FOR AMPLIFICATION OF HIV-1 C2-V3 ENVELOPE REGION

Step	Temperature (°C)		Duration	Number of cycles	
	1st round	2nd round		1st round	2nd round
Initial Denaturation	94	94	5min	1	1
Denaturation	94	94	45 sec	35	40
Annealing	57	55	45 sec		
Extension	72	72	45 sec		
Final Extension	72	72	3 min	1	1
Hold	4	4	∞	1	1

3.8 DETECTION OF AMPLIFIED PRODUCT BY AGAROSE GEL ELECTROPHORESIS

3.8.1 PRINCIPLE OF AGAROSE GEL ELECTROPHORESIS

This method is extensively used in the separation of proteins, DNA and RNA. The molecules are separated by application of an electric field to move the molecules through an agarose matrix. The negatively charged molecules migrate (electrophorese) towards the positive pole (anode) and get separated according to size/length: smaller fragments move more quickly. The separated DNA fragments can be made visible by incorporating an intercalating dye/stain such as ethidium bromide and Sybrgreen into the gel. The DNA fragments take up the dye/stain as they migrate through the gel. When the gel is illuminated using ultraviolate (UV) light,the intercalated dye fluoresces with a pale pink colour.

3.8.2 PROCEDURE

A 1.5% agarose was prepared by adding 100ml of Tris Borate EDTA (TBE) buffer into 1.5g of agarose in a Pyrex heat-resistant bottle and mixed thoroughly. The mixture was microwaved to boiling point to melt the agarose and then left to cool between 34-38^{O}C, 5µl of ethidium bromide added and then poured into a mini gel cast (containing gel combs); and allowed to solidify. The solid agarose was then placed in the mini gel tank after which about 800ml of TBE buffer was poured into the mini gel tank to completely submerge the agarose gel. Five microlitres (5µl) of the amplicon was mixed with 1µl of gel loading dye and loaded into individual wells in the agarose gel along with the positive control, 100bp DNA ladder (Jena Bioscience, Germany). Electrophoresis was run at 110volts using a current of 500 milliamperes for 60minutes. After the electrophoresis, the preview of amplified product with the expected band size was observed usingBio Rad geldocumentation system.

3.9 PURIFICATION OF AMPLIFIED PRODUCTS

3.9.1 Amplicons from the second round PCR were purified using WIZARD Purification Kit Protocol (Promega). Equal volumes of membrane binding solution and PCR product were mixed in PCR tube. One SV Minicolumn was positioned inside a collection tube for each PCR amplification. The prepared PCR product was pipetted into the SV Minicolumn assembly and allowed to incubate for 1 minute at room temperature. This was subjected to centrifugation in a microcentrifuge at 16,000 x g (14,000rpm) for 1 minute. The SV Minicolumn was removed from the spin column assembly and the liguid in the collection

tube discarded. The SV Minicolumn was returned to the collection tube. The column was washed by adding 700µL of membrane wash solution (previously diluted with 95% ethanol) to the SV Minicolumn. The SV Minicolumn assembly was again centrifuged for 1 minute at 16,000 x g (14,000rpm). The liquid in the collection tube was again discarded and the SV Minicolumn returned to the collection tube. Washing was repeated with 500µL of Membrane Wash Solution and the SV Minicolumn assembly centrifuged for 5 minutes at 16,000 x g. The SV Minicolumn assembly was carefully removed from the centrifuge, the collection tube emptied and the column assembly recentrifuged for 1 minute at 6,000 x g with the microcentrifuge lid open to allow evaporation of any residual ethanol. The SV Minicolumn was gently placed in a clean 1.5ml microcentrifuge tube and 50µl of nuclease-free water applied directly to the center of the column without touching the membrane with the pipette tip. This was incubated at room temperature for 1 minute and then centrifuged at 16,000 x g (14,000rpm) for 1 minute. The SV Minicolumn was discarded and the purified DNA in the microcentrifuge tube was quantified using Fluorometer® and then stored at -20°C.

3.9.2 GELPURIFICATION OF AMPLIFIED PRODUCTS (AMPLICONS)

In agarose gels where more than one band appeared, the target amplicon was purified using gel purification technique. The target bands were carefully cut out from the gel while ensuring minimal exposition to UV radiation. This minimizesdenaturation of the DNA as a result of thymine dimerization. Thereafter, gel purification of the DNA was done using WIZARD Purification Kit (Promega)following manufacturer's procedure.

3.10QUANTIFICATION OF PURIFIED DNA WITH PICOGREEN

3.10.1 Preparation of 1X TE, DNA Standard and working PicoGreen

The Tris EDTA (TE) buffer is supplied as a 20X (20 strength) stock solution from which a 1X solution of TE, a 1:20 dilution of the TE was done by combining one portion of 20X TE stock with 19 portions of molecular-grade water. The mixture was vortexed and stored at room temperature.

The DNA standard is supplied in a 100µg/ml concentration suspended in Tris EDTA (TE). To prepare a working solution of 200ng/ml DNA standard, a 2µg/ml stock was first prepared by making a 1:50 dilution in which 10µL of the 100µL/ml solution was added to 490µl of 1X

TE, and vortexed for 15 seconds. From this, the 200ng/ml standard solution was made by combining 50µL of the 2µg/ml stock with 450µL of 1X TE, and vortexed.
To prepare the working PicoGreen, 5µL of PicoGreen stock was combined with 995µL of 1X TE.

3.10.2 Calibration of Fluorometer®(Turner Biosystems, Inc. Sunnyvale, CA, USA)

The fluorometer is always calibrated prior to quantification of DNA. One hundred microliter of 200ng/ml DNA standard solution was aliquoted into a clean Eppendorf tube previously labelled as STANDARD.Similarly, 100µL of 1X TE was aliquoted into another clean Eppendorf tube and labelled as BLANK. Thereafter, 100µL of working PicoGreen was added into the STANDARD and BLANK, gently vortexed followed by incubation for duration of 2mins in the dark at room temperature after which 100µL each of the STANDARD and BLANK were aliquoted into individual clean mini-cuvettes. The fluorometer® was turned on and cuvette mini-adapter inserted in the space provided for it, with the side labelled blue facing forward.

The <STD VAL> button on the fluorometer was pressed ensuring that the LED display reads 100 after which the <ENTER> button was pressed. Then, the <CAL> button was pressed followed by <ENTER> button. The BLANK was inserted following the prompting of the Flourometer followed by <ENTER>. At the prompting of the flourometer, the STANDARD was inserted followed by <ENTER>. Finally, the <ENTER> button was pressed again to complete the calibration process.

3.10.3 Quantification of DNA

Eppendorf tube was labelled with sample ID, 99µL of 1X TE and 1µL of the DNA transferred to the tube and then vortexed briefly. Thereafter, 100µL of working PicoGreen was introduced into the mixture and vortexed briefly. The tube with its contentswas allowed to incubate for a period of 2minsaway from light at room temperature. 100µL of the mixture was aliquoted into clean mini-cuvette avoiding bubbles. The mini-cuvette was placed into the mini-cuvette holder in the fluorometer and the value read after a few seconds and recorded. This value represents quantity of DNA in nanograms (ng) in 5µL of PCR product.

3.11SEQUENCING OF PROVIRAL DNA

3.11.1 CYCLE SEQUENCING OF PCR PRODUCTS

The sequencing reaction was carried outwith dye terminator sequencing kit version 3.1 (Applied Biosystems, California, USA). Each reaction mix consisted of a mixture of terminator ready reaction mix, template (PCR product), primer and deionized water in each well of a 96 well MicroAmp plate. The contents of the sequencing mix are as shown in table 3.10.

The sequencing was performed in both forward and reverse directions using primers G60del3′G and G25 respectively for P24 *gag* gene, OJ3 and OJ4 respectively for PR *pol* gene and KK40 and KK30 respectively for *env* C2-V3 gene.The sequencing reaction was plated in a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems, California, USA) and properly covered using Microseal plate covers. Reaction was performed using GeneAmp 9700 thermal cycler (Applied Biosystems, California, USA). Programming of the cycler follows thus; initial denaturation- 96^{o}C for 1min, denaturation- 96°C for 10secs (25 cycles), annealing- 50°C for 5secs, extension- 60°C for 4min and final hold at 4°C.

3.11.2 PURIFICATION OF SEQUENCED AMPLICONS

The MicroAmp plate was removed from the thermal cycler and the plate seal removed. To each sample mixture, 20 µL of deionized water and 60µL of 100% isopropanol were added. The MicroAmp plate was sealed with adhesive aluminium foil after which the MicroAmp plate was vortexed several times and incubated in the dark at room temperature for 15 minutes followed by centrifugation at 2000g for 45 minutes to allow the DNA to be precipitated. The adhesive aluminium foil was carefully removed, without agitating the pellets, absorbent paper placed on top of the plate and then the plate inverted. The MicroAmp plate was then placed in inverted position on top of the absorbent paper and centrifuged at 700g for 1 minute to remove any traces of the isopropanol. The samples in each well of the MicroAmp plate were resuspended by adding 20 µL of Hi-Di formamide, and the plate vortexed for 15 seconds. After vortexing, the MicroAmp plate was centrifuged for 40 seconds at 2000g. The plate was then assembled with the plate holder and placed in the position B in the ABI 3130xl Genetic Analyzer, ensuring that the plate retainer holes were aligned with the holes in the septa. The sequencer was run depending on the number of MicroAmp columns containing samples for automated assembly.

TABLE 3.10: DESCRIPTION OF THE SEQUENCING REACTION MIX

	Reagent	Volume
	Terminator ready reaction mix	8.0 µL
	Template (PCR product)	1.0-2.0 µL (depending on the conc.)
	Primer	3.2 pmol
	Deionized water	6.5-7.5 µL (depending on conc of DNA)
	Total	20.0 µL

3.11.3 ANALYSIS OF SEQUENCES

Similarity of generated sequences to HIV-1 sequences in the GenBank was first determined by BLAST (Basic Local Alignment Search Tool) program in the National Center for Biotechnology Information (NCBI) database. Sequences were manually edited using combination of CLC Genomics Workbench 7.6.2 (CLC BIO,Cambridge, MA), MEGA 6.06 (Tamura *et al*., 2013), BioEdit Sequence Alignment Editor and Sequence Scanner v1.0 (Applied Biosystems, USA) software. With all generated consensus sequences, multiple alignments were carried out using the ClustalW programme in Molecular Evolutionary Genetics Analysis (MEGA) software version 6.06with reference sequences in the 2015 HIV-1 compendium from the Los Alamos database and sample sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Pairwise and evolutionary distances were computed using the Maximum Composite Likelihood method. Reliability of the resulting trees was estimated from 1000 bootstrap resamplings.

3.12MOLECULAR ANALYSIS OF THE P24 GAG AMINO ACID SEQUENCES

After multiple alignment, the *gag* sequences were trimmed to correspond to positions 1,234 to 1,833 (600 bp; Gag residues 149 to 348) according to HXB2 coordinates and then analysed.

3.13DRUG RESISTANCE ANALYSIS OF THE PROTEASE AMINO ACID SEQUENCES

After multiple alignment, the protease sequences were trimmed to correspond to positions 2,253 to 2,549 (297 bp;*Pol* residues 57 to 155)according to HXB2 coordinates. Translation of the nucleotides to amino acid sequences was done using MEGA 6.06 software.The whole *protease*wassubjected to analysis for the identification of potential drug resistance mutations (DRMs), polymorphisms occurring at DRM sites, and subtype-specific polymorphisms. Definition of subtype-specific polymorphisms was with reference to subtype B consensus, as specific mutations which occured in the majority of sequences belonging to a particular subtype. DRMs were alsodetermined as explained in the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu)accessed on 15th September, 2016 and the latest definition of the International Antiviral Society (IAS-USA) mutation lists updated in 2015 (Wensing *et al*., 2015).Analysis of drug resistance was also done using HIV subtype B

consensus sequence as the reference sequence.The prediction of drug susceptibility of each HIV-1 isolate from each participant was done using the Stanford algorithm.

3.14ANALYSIS OF THE V3 LOOP AMINO ACID SEQUENCES AND CO-RECEPTOR USAGE

After multiple alignment, the C2-V3 *env*sequences were trimmed to corresponds to positions 7,059 to 7,217 (159 bp; *Env* residues 279 to 331)according to HXB2 coordinates.The sequences were translated into amino acid using MEGA 6.06. An alignment of the amino acid residues of the 14sequences was compared with the consensus amino acid residues of subtype B (Cons B). The amino acid sequences were then manually examined and analysed for N-linked glycosylation sites (NGS), V3 characteristics and crown motif, occurrence of basic amino acids at positions 11 and 25 of V3, X4-associated mutations and maraviroc-resistance mutations. The result was then used in the genotypic prediction of virus co-receptor usage and maraviroc-resistant phenotype. Combined criteria were used in the prediction of virus co-receptor usage and includes; (i) 11/25 rule which indentifies R or K at position 11 of V3 and/K at position 25 (Cardozo *et al*., 2007), (ii) R at position 25 of V3 and a net charge of ≥+5, or (iii) a net charge of ≥+6 (Montagna *et al*., 2014) (iv) bioinformatics analysis by comparison between Cons B and querry V3 loop sequence in Geno2Pheno online software which uses the presence of X4 specific mutations in the V3 loop as a predictor of X4 phenotype, (v) loss of a highly conserved glycosylation motif found at V3-loop positions 6–9 (Pollakis*et al*., 2001). Prediction of maraviroc-resistant phenotype was based on combinations of mutations which have been previously described to cause resistance to maraviroc *in vivo* and *in vitro* (Lewis *et al*., 2008).

CHAPTER FOUR

4.0 RESULTS

4.1. AMPLIFICATION AND SEQUENCING OF THE TARGET GENE

A total of 246 samples were included in the study,out of which219 (89.0%), 77 (31.3%) and 98 (39.8%) were successfully amplified for *gag*, *pol* and *env* genes respectively.Representative gel pictures of second round PCR amplified products of p24 *gag*,*protease* and *env* genes are shown in figure 4.1. Thirty samples in which amplification was successful in the three (*gag*, *pol* and *env*) regions were selected for sequencing. Out of these, readable sequences were obtained and analysed for 17 (56.7%), 28 (93.3%) and 14 (46.7%) of *gag* (p24), *pol* (PR) and *env* (C2-V3) regions respectively.

4.2. ANALYSIS OF THE NUCLEOTIDE SEQUENCE OF THE GAG p24, POL PR REGIONSAND ENV C2-V3OF HIV-1

4.2.1. PHYLOGENETIC ANALYSIS OF THE GAG p24 SEQUENCE OF HIV-1 BY NEIGHBOUR JOINING

Representative picture of nucleotide alignment of study and reference sequences using MEGA 6 software is shown in figure 4.2.Following alignment of the 600bp nucleotidesof the 17 *gag* sequences with each other and sequences in the Los Alamos HIV database, a Neighbour Joining tree was reconstructed using ClustalW (Figure 4.3).The clustering pattern showed that3 (17.7%), 5 (29.4%) and 7 (41.2%) of the sequences belong to subtypes A, G and CRF02_AG respectively while 2 (11.8%) did not cluster with any of the known subtypes or CRFs. Blast results of these two sequences, NG_AN.12_04 and NG_AN.12_07 from the Los Alamos HIV-1 sequence databasehowever showed that the isolates had closest similarity to HIV-1 CRF02_AG and are hereby refered to as unclassified 02AG (U^{02AG})(Figure 4.4).

4.2.1.1 ANALYSIS OF GAG p24 SUBTYPE A SEQUENCES

The phylogenetic analysis of the gag p24 subtype A sequences shows that the 3 subtype A viruses, NG_DE.12_03, NG_DE.12_06 and NG_AN.12_01, clustered closely with West African reference sequences from Senegal (Figure 4.5).Analysis of nucleotide similarities by calculating pairwise distances among the gag p24 subtype A sequences showed a similarity of 90%, 91% and 91% between reference subtype A nucleotide sequence and NG_AN.12_01, NG_DE.12_03 and NG_DE.12_06 respectively (Table 4.1).

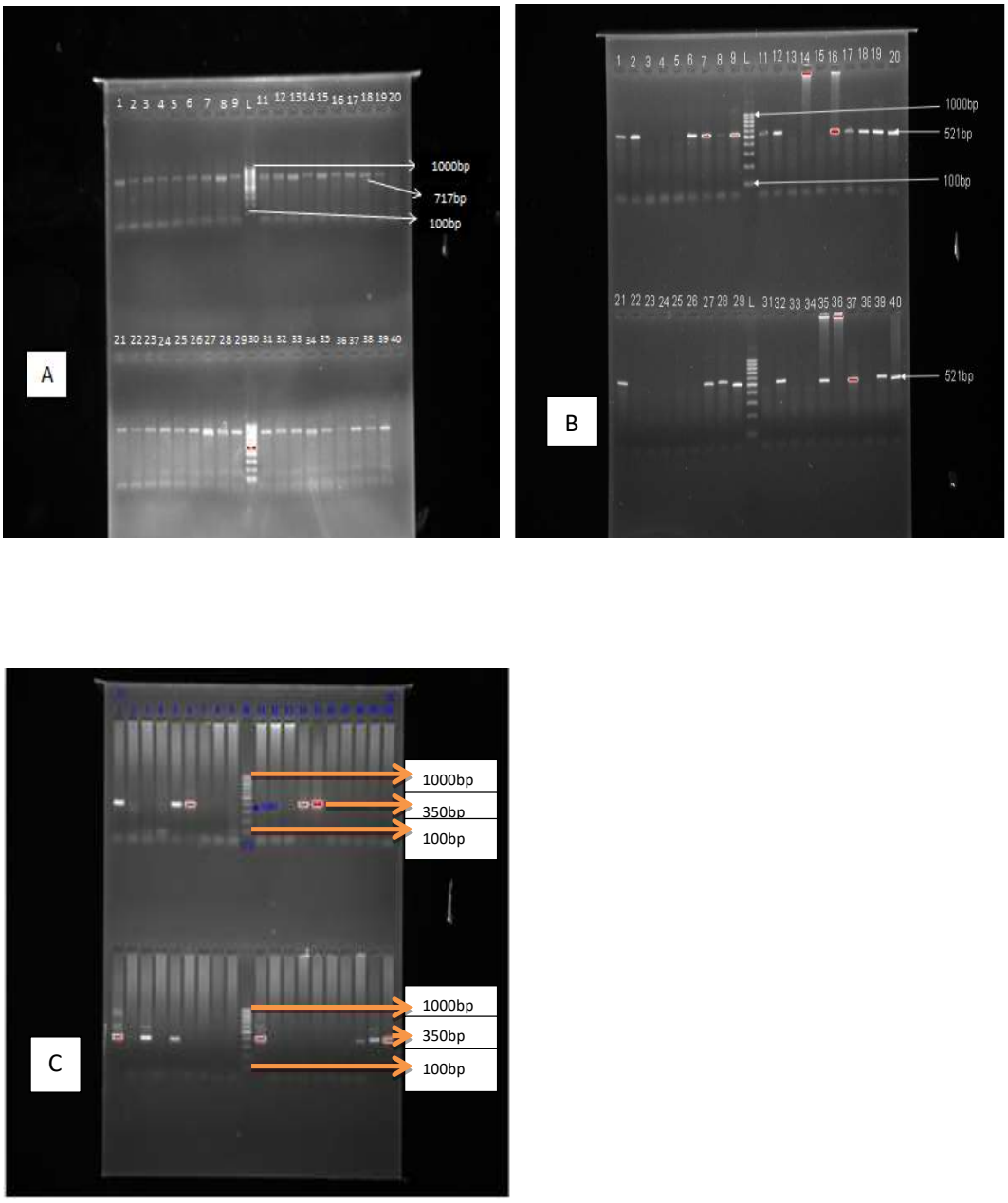


FIGURE 4.1: REPRESENTATIVE GEL PICTURE OF SECOND ROUND PCR AMPLIFIED PRODUCT OF P24 *GAG* (A), *PR* (B) AND *ENV* GENES (C) OF HIV-1 FROM INFECTED PATIENTS. For p24 *gag*; Lanes 1 and 21: Positive control, Lanes 20 and 40: Negatve control, Lanes 10 and 30: 100 bp DNA ladder, others represent samples, expected band size is 717 bp. For PR; Lanes 1 and 2: Positive control, Lanes 3 and 4: Negative control, Lanes 10 and 30: 100bp DNA ladder, others represent samples, expected band size is 521 bp. For C2-V3 *env*; Lane 1: Positive control, Lane 20: Negative control, Lanes 10 and 30: 100 bp DNA ladder, others represent samples, expected band size is 350 bp.

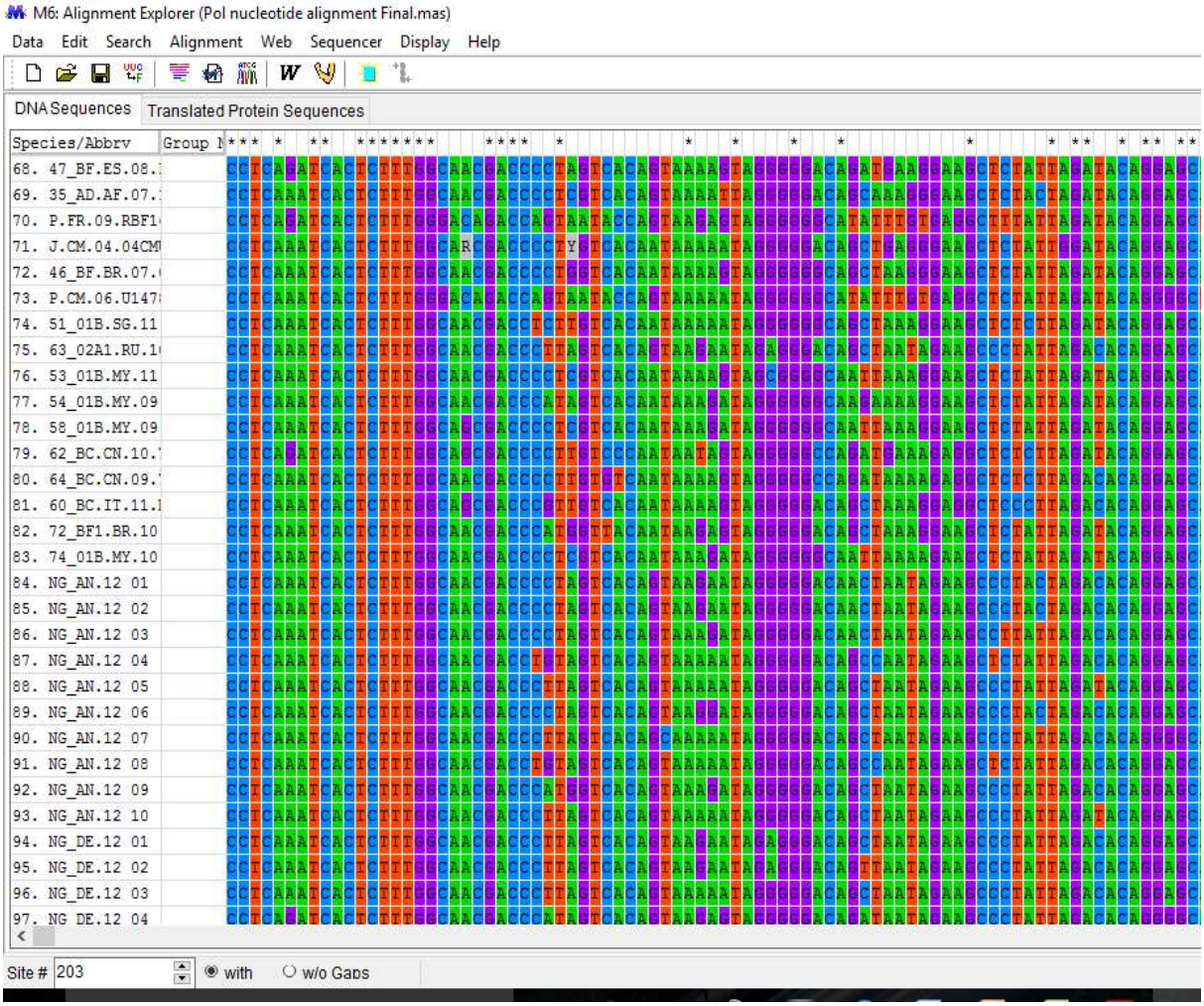


FIGURE 4.2: REPRESENTATIVE PICTURE OF NUCLEOTIDE SEQUENCE ALIGNMENT OF STUDY AND REFERENCE SEQUENCES USING MEGA 6 SOFTWARE

Key: Blue colour indicate Cytosine
Red colour indicate Thymine
Green colour indicate Adenine
Purple colour indicate Guanine

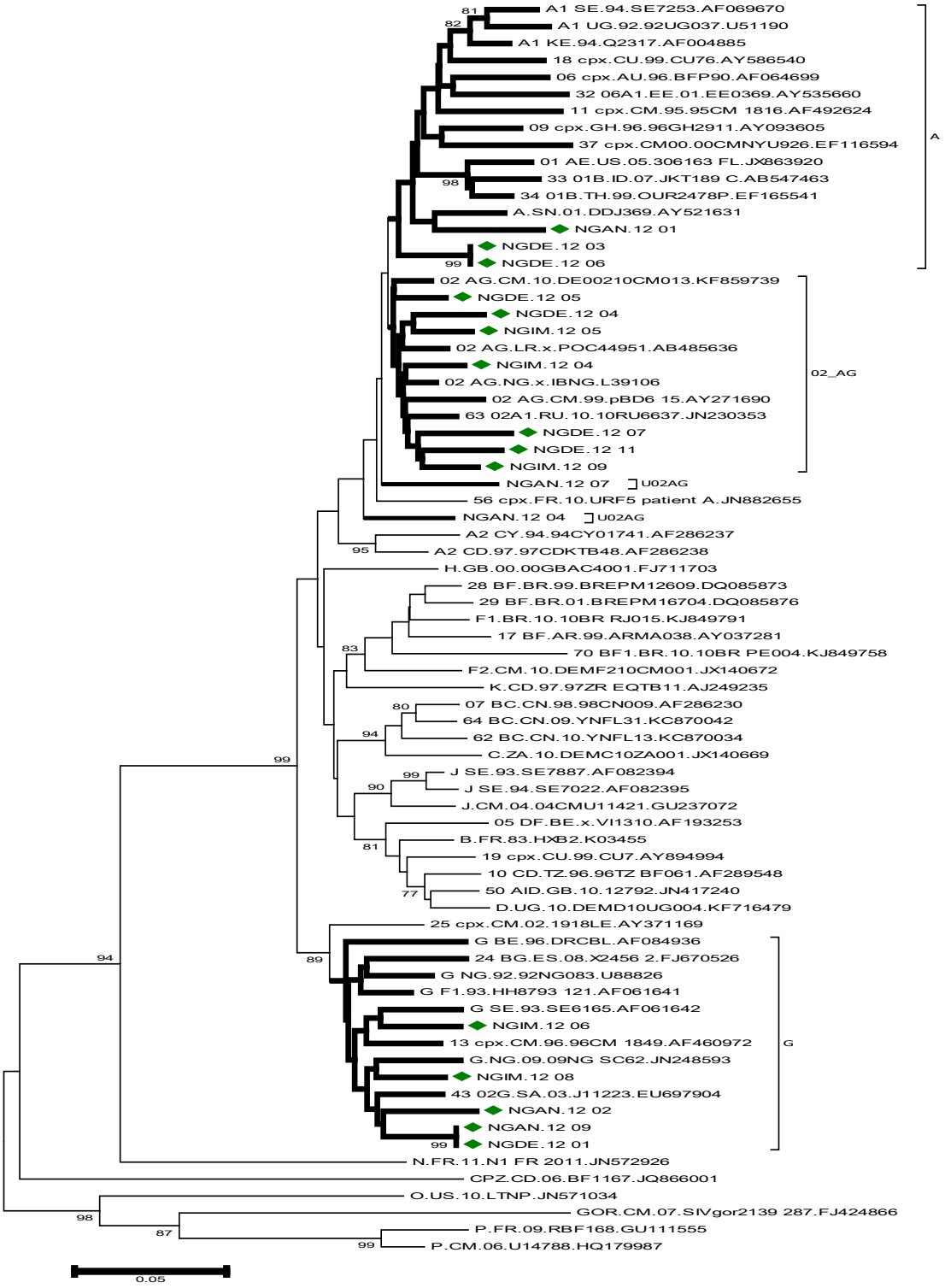


FIGURE 4.3: PHYLOGENETIC TREE USING*GAG*SEQUENCES OF HIV-1 ISOLATES AMONG PATIENTS ALIGNED WITH REFERENCE SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCES ARE INDICATED BY "◆" SYMBOL

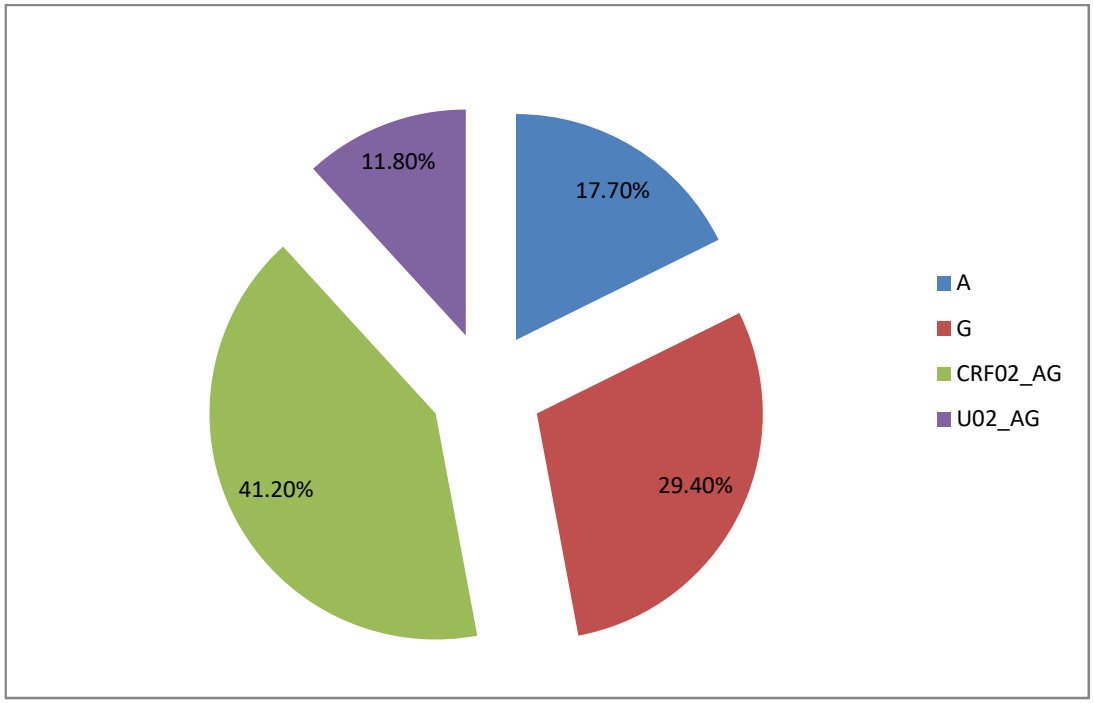


FIGURE 4.4: PROPORTIONS OF HIV-1 SUBTYPES AND CRFS AMONG HIV-1 POSITIVE PATIENTS BASED ON P24 GAG SEQUENCES

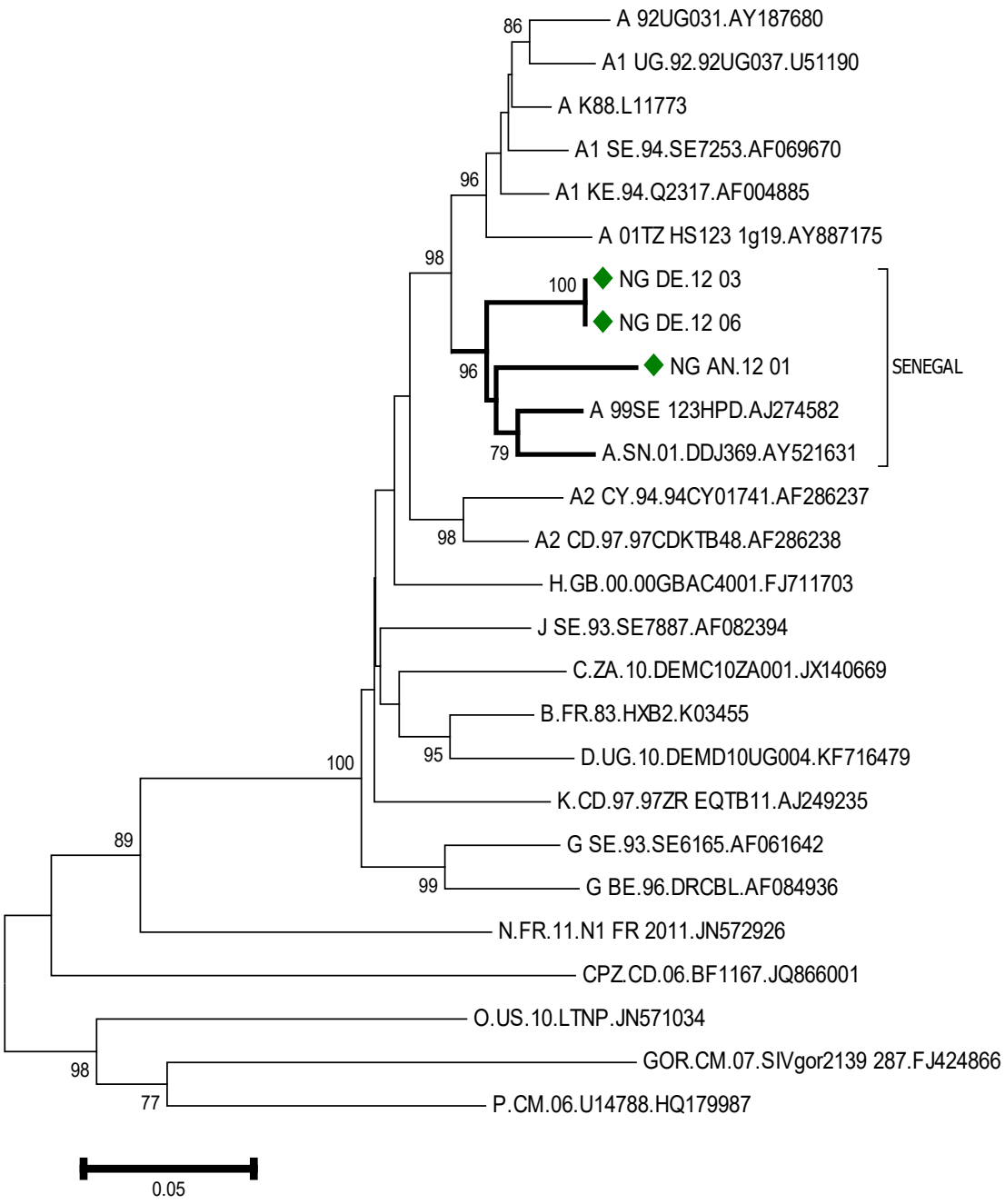


FIGURE 4.5: PHYLOGENETIC ANALYSIS OF P24 GAG SUBTYPE A ISOLATES. SEQUENCES WERE COMPARED WITH REFERENCE SUBTYPE A AND HIV-1 GROUP M SUBTYPES SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCES ARE INDICATED BY "◆" SYMBOL

TABLE 4.1: ESTIMATES OF EVOLUTIONARY DIVERGENCE OF GAG P24SUBTYPE A SEQUENCES AND REFERENCE SEQUENCE

	1	2	3	4
1. KX389608				
2. NG AN.12 01	0.10			
3. NG DE.12 03	0.09	0.08		
4. NG DE.12 06	0.09	0.08	0.00	

4.2.1.2 ANALYSIS OF GAG p24 SUBTYPE G SEQUENCES

Analysis of the 5 *gag* p24 subtype G sequences shows that the subtype G viruses formed 3 different subclusters (Figure 4.6).One subtype G sequence, NG_IM.12_06 clustered with reference sequence from Nigeria while another 1 subtype G sequence, NG_IM.12_08 clustered with reference sequence from Liberia. However, 3 sequences formed separate cluster in the G cluster and are hereby referred to as G′. Analysis of nucleotide similarities by calculating pairwise distances among the gag p24 subtype G sequences showed a similarity of 92%, 92%, 92%, 93% and 93% between NG_AN.12_02, NG_AN.12_09, NG_DE.12_01, NG_IM.12_06 and NG_IM.12_08 respectively with reference sequence. Similarity of the sequences with each other ranges from 94% to 100% (Table 4.2).

4.2.1.3 ANALYSIS OF THE GAG p24 CRF02_AG SEQUENCES

Further phylogenetic analysis of the 7 *gag* p24 CRF02_AG and 2 unclassified sequences shows that the sequences formed 7 different subclusters within this circulating recombinant forms. Sequences NG_IM.12_04, NG_IM.12_09, NG_DE.12_07, NG_DE.12_11, NG_DE.12_05 and NG_AN.12_07 clustered with reference sequences from United States of America and France, Niger, UK, Germany, Saudi Arabia and Switzerland respectively. Two sequences, NG_DE.12_04 and NG_IM.12_05 formed a separate cluster while NG_AN.12_04 did not cluster with any of the sequences (Figure 4.7). Analysis of nucleotide similarities by calculating pairwise distances among the *gag* p24 CRF02_AG and unclassified sequences showed similarities of 93%, 94%, 94%, 96%, 94%, 96%, 97%, 96% and 96% between isolates NG_AN.12_04, NG_AN.12_07, NG_DE.12_04, NG_DE.12_05, NG_DE.12_07, NG_DE.12_11, NG_IM.12_04, NG_IM.12_05 and NG_IM.12_09 respectively and reference sequence. The percentage similarity between the sequences ranged from 90% to 95% (Table 4.3).

4.2.2. PHYLOGENETIC ANALYSIS OF SEQUENCES OF THE PROTEASE REGION

Nucleotide sequences from the 28 isolates sequenced in the protease region were aligned with the sequences of representative reference strains of the different genetic subtypes and CRFs in the Los Alamos HIV database by the CLUSTALW multiple sequence alignment programs. Following the alignment, phylogenetic tree was reconstructed by Neighbour Joining distance method (Figure 4.8).

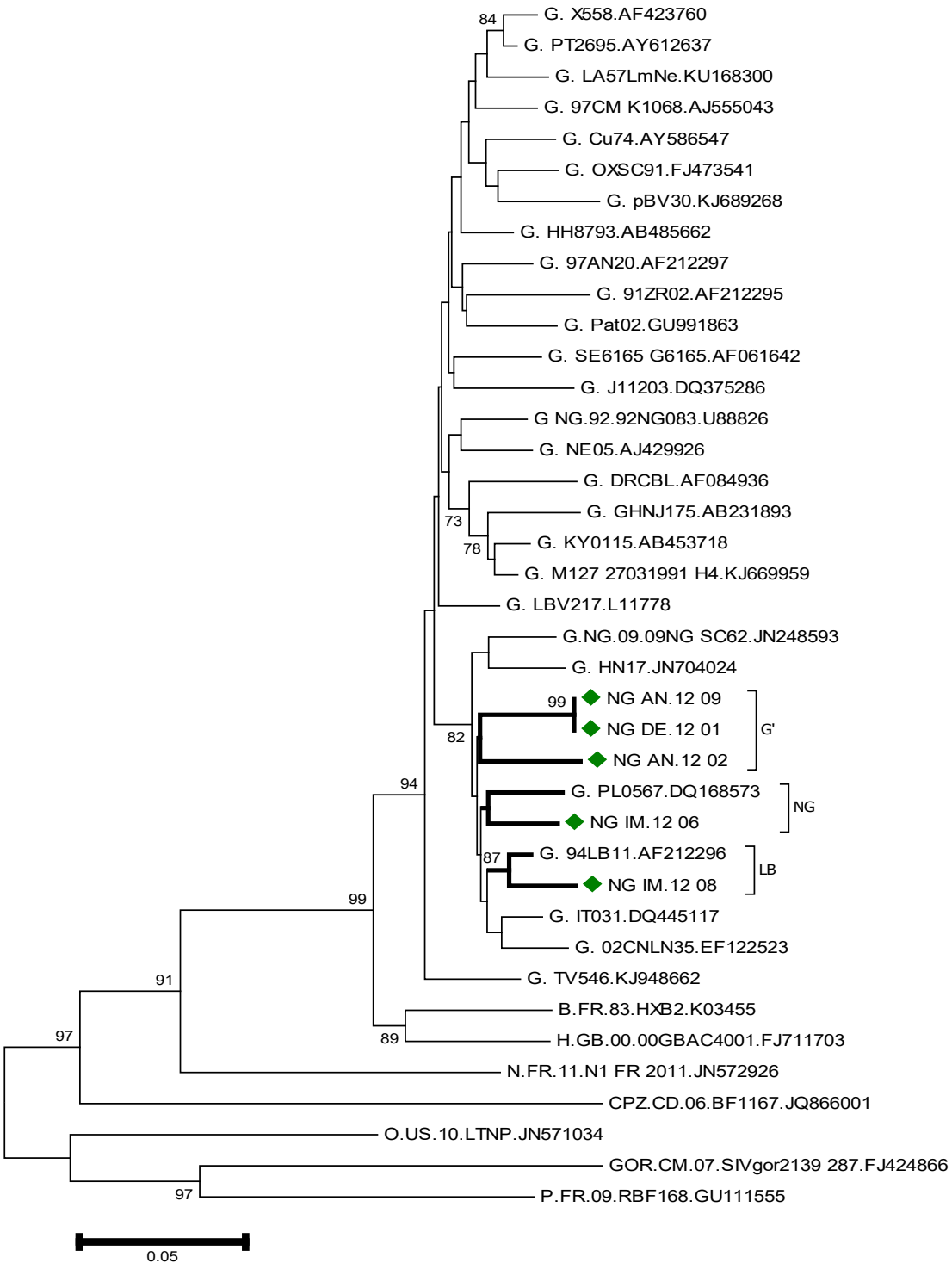


FIGURE 4.6: PHYLOGENETIC ANALYSIS OF P24 GAG SUBTYPE G ISOLATES. SEQUENCES WERE COMPARED WITH REFERENCE SUBTYPE G AND HIV-1 GROUP M SUBTYPES SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCES ARE INDICATED BY "◆" SYMBOL

TABLE 4.2: ESTIMATES OF EVOLUTIONARY DIVERGENCE OF GAG P24SUBTYPE G SEQUENCES AND REFERENCE SEQUENCE

	1	2	3	4	5	6
1. U88826						
2. NG AN.12 02	0.08					
3. NG AN.12 09	0.08	0.06				
4. NG DE.12 01	0.08	0.06	0.00			
5. NG IM.12 06	0.07	0.06	0.05	0.05		
6. NG IM.12 08	0.07	0.06	0.06	0.06	0.06	

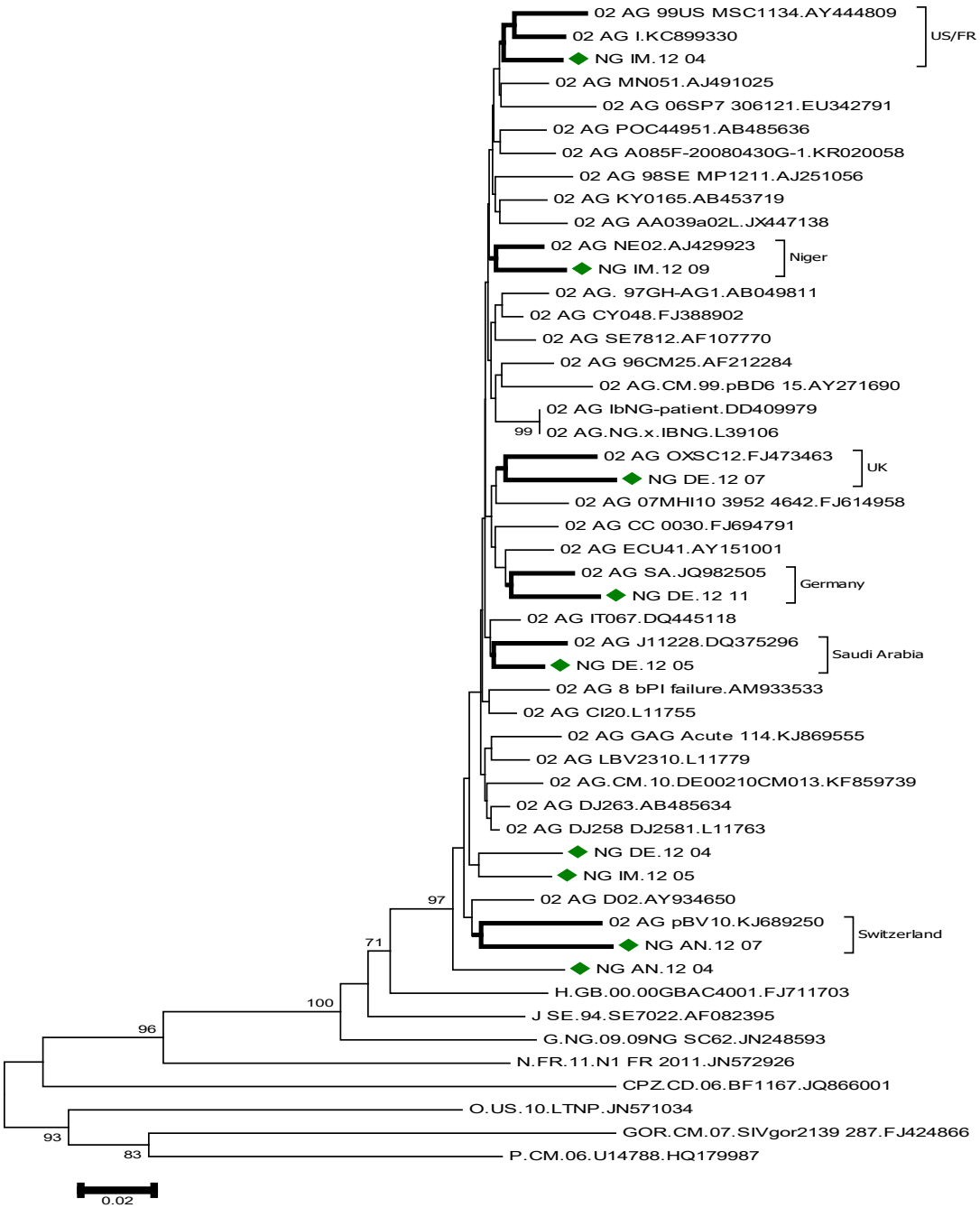


FIGURE 4.7: PHYLOGENETIC ANALYSIS OF P24 GAG CRF02_AG ISOLATES. SEQUENCES WERE COMPARED WITH CRF02_AG REFERENCE FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCES ARE INDICATED BY "◆" SYMBOL

TABLE 4.3: ESTIMATES OF EVOLUTIONARY DIVERGENCE OFGAG P24 CRF02_AG SEQUENCES AND REFERENCE SEQUENCE

	1	2	3	4	5	6	7	8	9	10
1. L39106										
2. NG AN.12 04	0.07									
3. NG AN.12 07	0.06	0.08								
4. NG DE.12 04	0.06	0.08	0.07							
5. NG DE.12 05	0.04	0.06	0.06	0.06						
6. NG DE.12 07	0.06	0.10	0.09	0.08	0.07					
7. NG DE.12 11	0.04	0.08	0.08	0.08	0.06	0.07				
8. NG IM.12 04	0.03	0.08	0.08	0.07	0.05	0.07	0.05			
9. NG IM.12 05	0.04	0.07	0.07	0.05	0.06	0.07	0.06	0.05		
10. NG IM.12 09	0.04	0.08	0.07	0.07	0.06	0.06	0.05	0.05	0.05	

FIGURE 4.8: PHYLOGENETIC TREE OF HIV-1 *POL* REGIONS AMONG THE HIV-1 INFECTED PATIENTS ALIGNED WITH REFERENCE SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCES ARE INDICATED BY "◆" SYMBOL

Ten (35.7%) and 16 (57.1%) of the 28 sequences were identified as subtype G and CRF02_AG respectively while 2 (7.1%) sequences did not cluster with any of the known subtypes or CRFs. Blast results of these two sequences, NG_DE.12_09 and NG_IM.12_06 from the Los Alamos HIV-1 sequence database however showed that the isolates had closest similarity to HIV-1 subtype G and are hereby reffered to as unclassified subtype G (U^G) (Figure 4.9). Among the CRF02_AG cluster, 3 (10.7%) formed a subcluster with CRF56_cpx while 4 (14.3%) formed another subcluster with CRF18_cpx and CRF37_cpx.

4.2.2.1 ANALYSIS OF PROTEASE SUBTYPE G AND U^G SEQUENCES

The phylogenetic analysis of the protease subtype G sequences shows that the sequences formed 6 different subclusters (Figure 4.10). Four sequences, NG_AN.12_06, NG_IM.12_08, NG_DE.12_08 and NG_DE.12_03 clustered with reference sequence from Serbia. Three sequences, NG_AN.12_01, NG_AN.12_02 and NG_AN.12_03 clustered with reference sequence from Spain. Isolate NG_AN.12_05 clustered with two reference sequences from Venezuela and Togo while NG_AN.12_09 clustered with two reference sequences from Czech Republic and Malaysia. Also sequence NG_IM.12_02 clustered with reference sequence from Liberia. Interestingly, the two sequences that clustered separately in the general phylogenetic tree (U^G) also clustered separately in this subtype phylogeny. Analysis of nucleotide similarities by calculating pairwise distances among the *pol* protease subtype G and unclassifiedsubtype G sequences showed similarity ranging from 90% to 96% between the sequences and reference sequence while the similarity ranges from 91% to 100% between the isolates with each other (Table 4.4).

4.2.2.2 ANALYSIS OF PROTEASE CRF02_AG SEQUENCES

Further phylogenetic analysis of the 16 *protease* CRF02_AG sequences shows that the sequences formed 9 different clusters within this circulating recombinant form. Four CRF02_AG isolates formed clusters with reference sequences from Burkina Faso and Algeria, 3 isolates formed clusters with reference sequences from Cameroon and Ecuador, 2 sequences each clustered with reference sequences from Cote d'Ivoire and France, 1 sequence each formed cluster with reference sequences from Senegal, Denmark, Belgium and Gabon and Nigeria. One sequence, NG_IM.12_01 clustered with CRF18_cpx and CRF37_cpx (Figure 4.11).

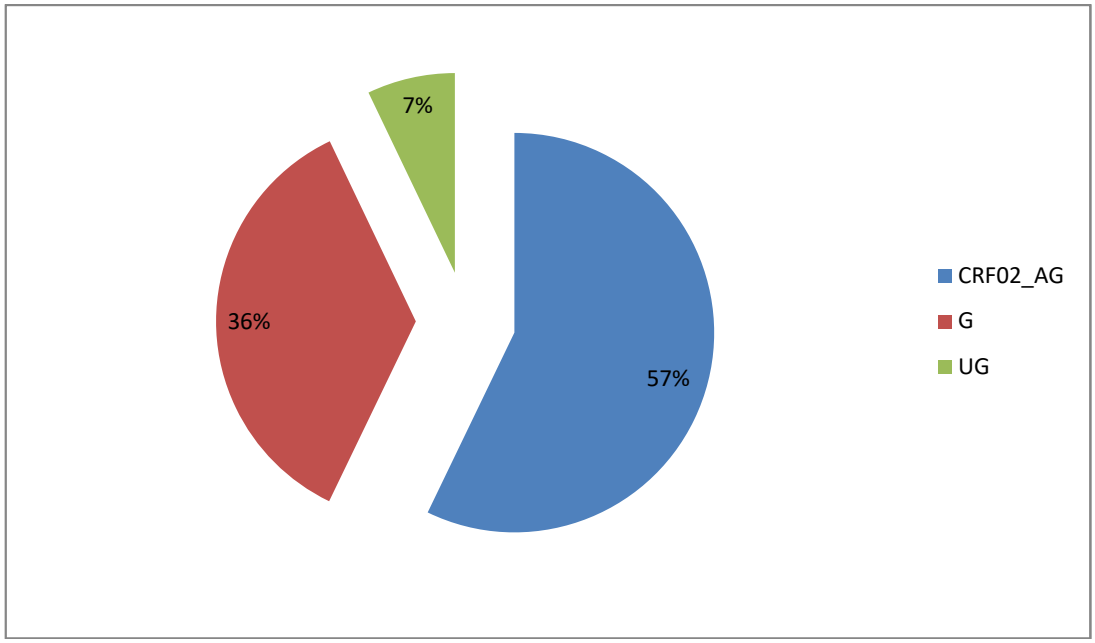


FIGURE 4.9: PROPORTIONS OF HIV-1 SUBTYPES AND CRFS AMONG HIV-1 POSITIVE PATIENTS BASED ON PROTEASE SEQUENCES

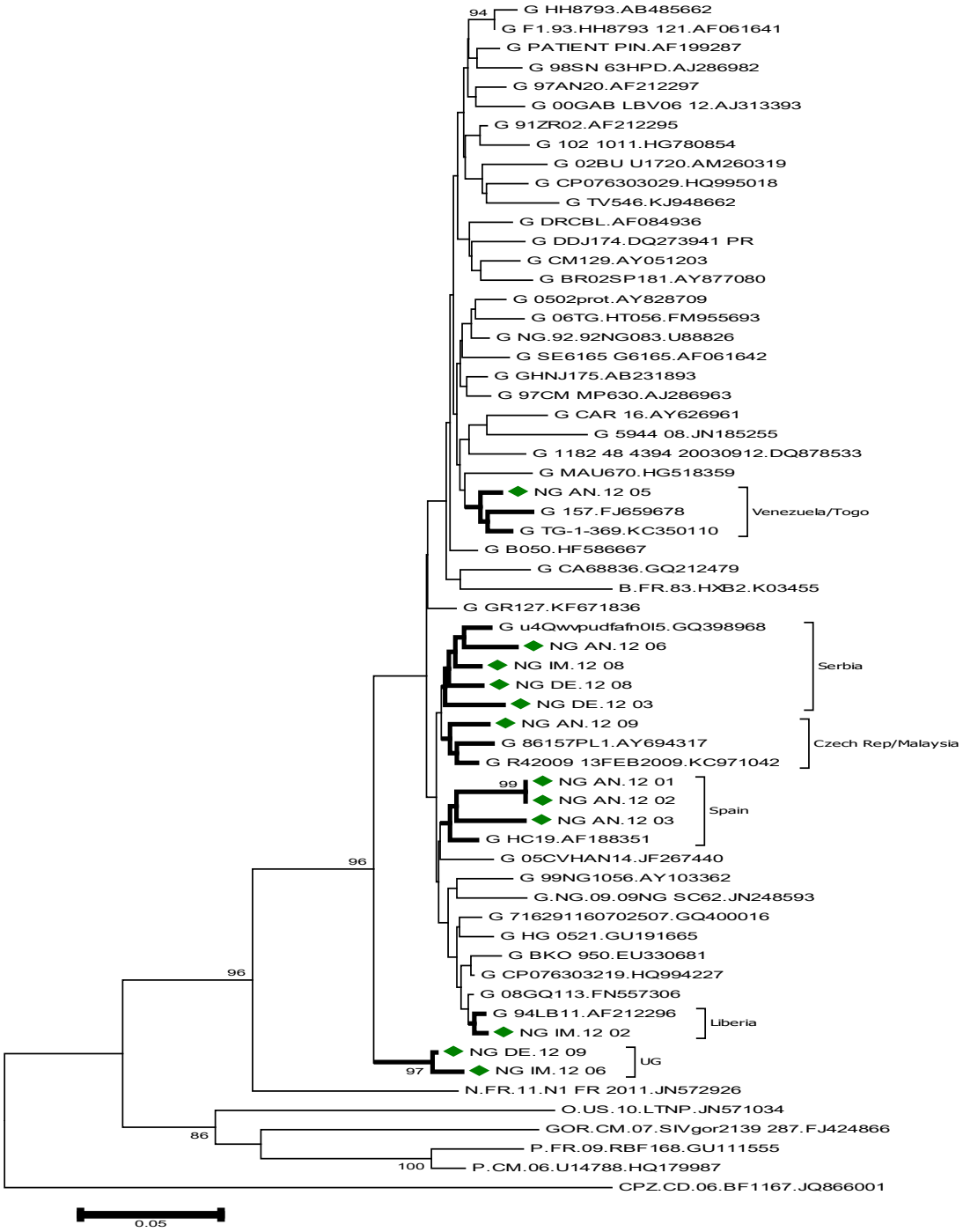


FIGURE 4.10: PHYLOGENETIC ANALYSIS OF PROTEASE SUBTYPE G AND U^{G} ISOLATES. SEQUENCES WERE COMPARED TO SUBTYPE G REFERENCE SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCES ARE INDICATED BY "◆" SYMBOL

TABLE 4.4: ESTIMATES OF EVOLUTIONARY DIVERGENCE OF PROTEASE SUBTYPE G AND U^{G}SEQUENCES AND REFERENCE SEQUENCE

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. U88826													
2. NG AN. 12 01	0.08												
3. NG AN. 12 02	0.08	0.00											
4. NG AN. 12 03	0.07	0.05	0.05										
5. NG AN. 12 05	0.04	0.09	0.09	0.08									
6. NG AN. 12 06	0.08	0.05	0.05	0.06	0.09								
7. NG AN. 12 09	0.06	0.06	0.06	0.05	0.07	0.05							
8. NG DE. 12 03	0.07	0.06	0.06	0.06	0.07	0.06	0.05						
9. NG DE. 12 08	0.07	0.06	0.06	0.06	0.08	0.05	0.05	0.05					
10. NG DE. 12 09	0.09	0.08	0.08	0.07	0.07	0.07	0.07	0.07	0.08				
11. NG IM. 12 02	0.06	0.05	0.05	0.06	0.05	0.06	0.05	0.05	0.05	0.07			
12. NG IM. 12 06	0.10	0.08	0.08	0.07	0.08	0.08	0.08	0.07	0.08	0.01	0.08		
13. NG IM. 12 08	0.06	0.05	0.05	0.04	0.07	0.03	0.04	0.04	0.04	0.06	0.04	0.06	

FIGURE 4.11: PHYLOGENETIC ANALYSIS OF PROTEASE CRF02_AG ISOLATES. SEQUENCES WERE COMPARED TO CRF02_AG REFERENCE SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCES ARE INDICATED BY "◆" SYMBOL

Analysis of nucleotide similarities by calculating pairwise distances among the PR CRF02_AG sequences showed a similarity with reference sequence ranging from 88% to 99% while the similarity of the sequences with each other ranges from 85% to 98% (Table 4.5).

4.2.3. PHYLOGENETIC ANALYSIS OF ENV C2-V3 SEQUENCE OF HIV-1

Following alignment of the C2-V3 nucleotide sequences with each other and sequences in the Los Alamos HIV database, a Neighbour Joining tree was reconstructed using ClustalW (Figure 4.12). The C2-V3 sequences alignment, showed that CRF02_AG was the most predominant strain (50.0%)followed by subtype G (35.7%). The only other subtypes identified were A and J, one (7.1%) each (Figure 4.13).

4.2.3.1 ANALYSIS OF ENV C2-V3 SUBTYPE A SEQUENCE

We went further to align the nucleotide sequence of *env* C2-V3 subtype A isolate in this study with nucleotide sequences of subtype A reference strains and then phylogenetic analysis. Interestingly, the analysis showed that the subtype A sequence in this study clustered with reference sequence from Nigeria (Figure 4.14). Analysis of nucleotide similarities by calculating pairwise distances shows 86.0% and 87.0% between NG_AN.12_01 and two subtype A reference sequences KX389608 and KX389622 respectively (Table 4.6).

4.2.3.2 ANALYSIS OF ENV C2-V3 SUBTYPE G SEQUENCES

The phylogenetic analysis of the *env* C2-V3 subtype G sequences shows that two sequences, NG_AN.12_06 and NG_IM.12_05 clustered with reference sequences from Nigeria and Taiwan, sequences NG_IM.12_06 and NG_AN.12_09 clustered with reference sequences from Cape Verde and Greece respectively while NG_IM.12_02 clustered with reference sequences from France and Angola as shown in figure 4.15.

Analysis of nucleotide similarities by calculating pairwise distances among the *env* C2-V3 subtype G sequences showed sismilarity of 80%, 89%, 89%, 89% and 80% between isolates NG_IM.12_02, NG_IM.12_06, NG_AN.12_06, NG_AN.12_09 and NG_IM.12_05 respectively and reference sequence while the similarity between the sequences with each other ranged from 71% to 91% (Table 4.7).

TABLE 4.5: ESTIMATES OF EVOLUTIONARY DIVERGENCE OF PROTEASE CRF02_AG SEQUENCES AND REFERENCE SEQUENCE

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. L39106																	
2. NG AN. 12 10	0.06																
3. NG IM. 12 04	0.02	0.05															
4. NG IM. 12 07	0.12	0.12	0.10														
5. NG IM. 12 01	0.03	0.07	0.05	0.14													
6. NG DE. 12 01	0.01	0.06	0.03	0.12	0.04												
7. NG DE. 12 06	0.04	0.07	0.04	0.13	0.05	0.04											
8. NG AN. 12 08	0.04	0.07	0.05	0.14	0.05	0.05	0.06										
9. NG AN. 12 04	0.07	0.10	0.08	0.14	0.08	0.07	0.09	0.03									
10. NG IM. 12 03	0.03	0.06	0.03	0.10	0.05	0.03	0.02	0.05	0.07								
11. NG DE. 12 07	0.03	0.06	0.03	0.12	0.05	0.03	0.03	0.05	0.08	0.03							
12. NG DE. 12 02	0.03	0.06	0.05	0.13	0.05	0.04	0.04	0.06	0.09	0.04	0.03						
13. NG DE. 12 05	0.05	0.07	0.05	0.14	0.06	0.05	0.06	0.06	0.10	0.05	0.05	0.05					
14. NG AN. 12 07	0.03	0.06	0.03	0.13	0.04	0.03	0.03	0.05	0.08	0.03	0.02	0.04	0.05				
15. NG DE. 12 04	0.06	0.09	0.06	0.14	0.06	0.06	0.06	0.07	0.09	0.05	0.05	0.06	0.07	0.05			
16. NG DE. 12 10	0.05	0.07	0.06	0.15	0.06	0.06	0.06	0.05	0.09	0.06	0.05	0.07	0.07	0.05	0.06		
17. NG IM. 12 05	0.06	0.08	0.05	0.15	0.06	0.06	0.06	0.07	0.10	0.06	0.05	0.07	0.08	0.05	0.08	0.05	

FIGURE 4.12: PHYLOGENETIC TREE OF HIV-1 V3 LOOP SEQUENCES AMONG THE HIV-1 INFECTED PATIENTS ALIGNED WITH REFERENCE SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCES ARE INDICATED BY "◆" SYMBOL

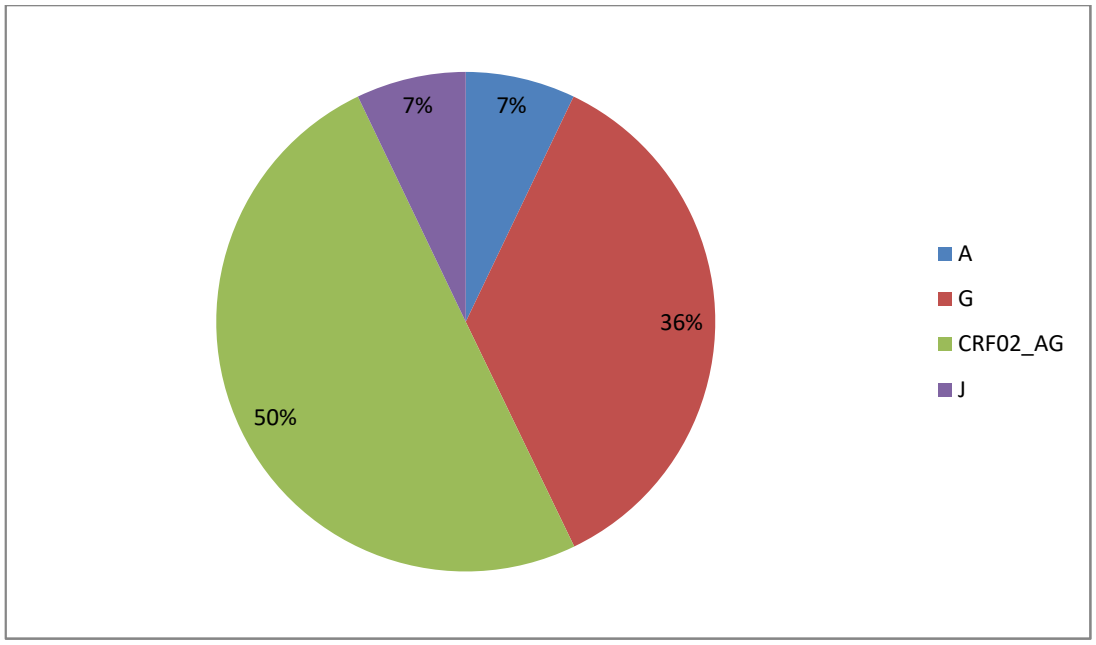


FIGURE 4.13: PROPORTIONS OF HIV-1 SUBTYPES AND CRFs AMONG HIV-1 POSITIVE PATIENTS BASED ON V3 LOOP SEQUENCES

FIGURE 4.14: PHYLOGENETIC ANALYSIS OF ENV C2-V3 SUBTYPE A ISOLATE. SEQUENCE WAS COMPARED TO SUBTYPE A REFERENCE SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCE IS INDICATED BY "◆" SYMBOL

TABLE 4.6: ESTIMATES OF EVOLUTIONARY DIVERGENCE OF ENV C2-V3 SUBTYPE A SEQUENCE AND REFERENCE SEQUENCES

	1	2	3
1. KX389608			
2. KX389622	0.11		
3. NG AN.12 01	0.14	0.13	

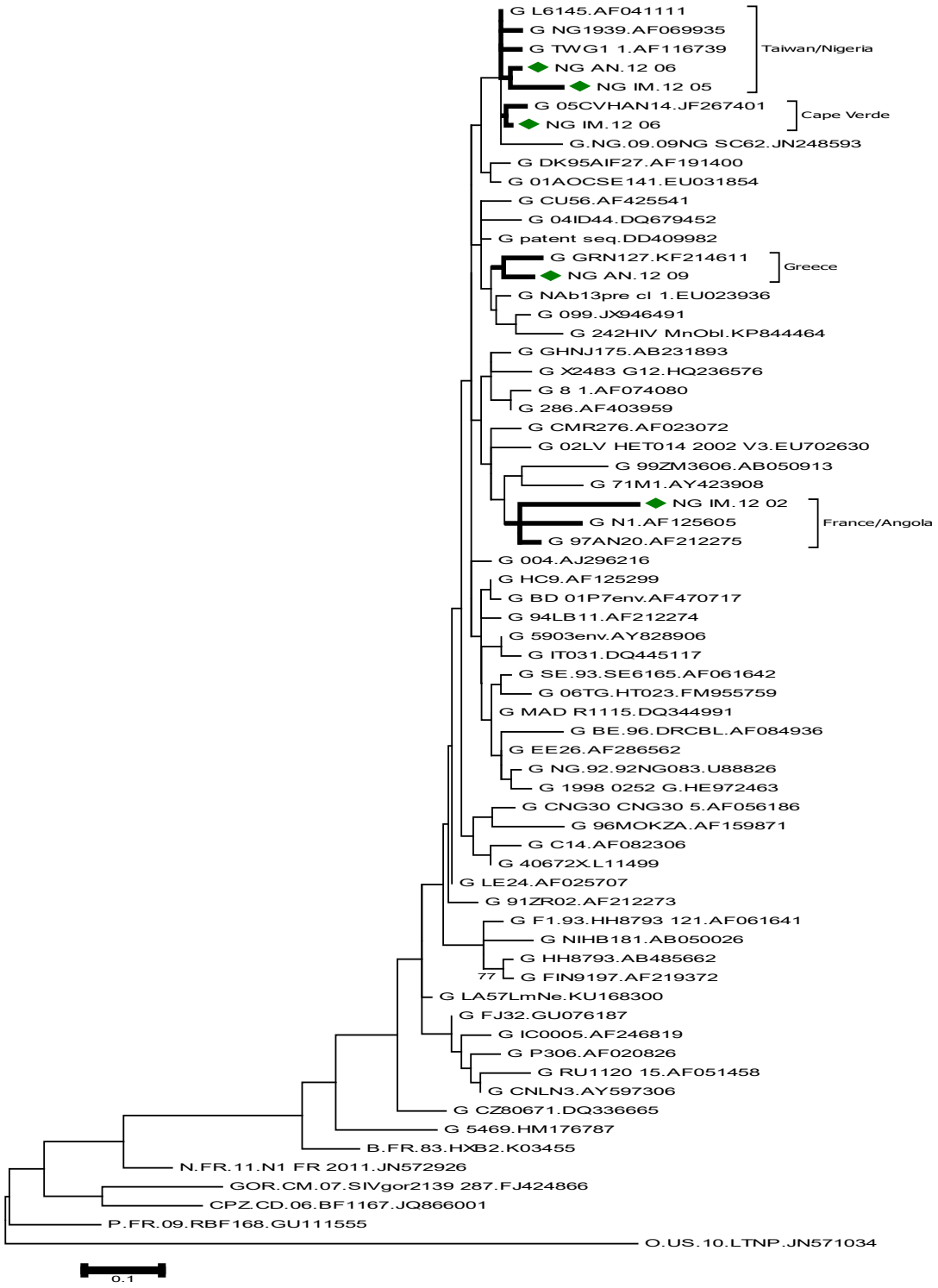


FIGURE 4.15: PHYLOGENETIC ANALYSIS OF ENV C2-V3 SUBTYPE G ISOLATES. SEQUENCES WERE COMPARED TO SUBTYPE G REFERENCE SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCES ARE INDICATED BY "◆" SYMBOL

TABLE 4.7: ESTIMATES OF EVOLUTIONARY DIVERGENCE OF ENV C2-V3 SUBTYPE G SEQUENCES AND REFERENCE SEQUENCE

	1	2	3	4	5	6
1. U88826						
2. NG IM. 12 02	0.20					
3. NG IM. 12 06	0.11	0.24				
4. NG AN. 12 06	0.11	0.22	0.09			
5. NG AN. 12 09	0.11	0.29	0.11	0.13		
6. NG IM. 12 05	0.20	0.26	0.13	0.11	0.21	

4.2.3.3 ANALYSIS OF ENV C2-V3 CRF02_AG SEQUENCES

Further analysis of the *env* C2-V3 CRF02_AG sequences shows that the CRF02_AG viruses formed 5 different subclusters with the reference sequences. Two sequences clustered with a reference sequence from Cameroon, 1 sequence clustered with reference sequences from Cameroon and Portugal, 1 sequence clustered with reference sequence from United Kingdom, 1 sequence clustered with reference sequences from Uzbekistan, Ecuador and Israel while 2 sequences clustered with a reference sequence from Niger (Figure 4.16). Examining percentage nucleotide similarities among sequences by calculating pairwise distances among the *env* C2-V3 CRF02_AG sequences showed similarity ranging from 66% to 93% between the sequences and reference sequence. Similarity between the isolate sequences with each other ranges from 57% to 91% (Table 4.8).

4.2.3.4 ANALYSIS OF ENV C2-V3 SUBTYPE J SEQUENCES

There are few HIV-1 subtype J sequences available in the Los Alamos HIV sequence database. The phylogenetic analysis of of the *env* C2-V3 subtype J sequence shows that the sequence clustered with a reference sequence from Cameroon (Figure 4.17). Analysis of nucleotide similarities by calculating pairwise distances showed similarity of 69% between reference sequence from Cameroon and *env* C2-V3 subtype J sequence from this study and similarity ranging from 58% to 64% with other African subtype J reference sequences (Table 4.9).

4.3 GENETIC DIVERSITY

In all, 17, 28 and 14 samples were sequenced and analysed for *gag* p24 gene, *pol* protease gene and *env* C2-V3 gene respectively (Table 4.10). Analysis showed that a total of 7(23.3%) of the isolates were successfully sequenced in all the 3 fragments of *gag*, *pol* and *env* genes, 15 (50.0%) were sequenced in two of the three fragments while only one fragment was sequenced in 8(26.7%) of the 30 isolates (Table 4.11).

Further analysis showed that 2 (28.6%) of the 7 isolates that were sequenced in all the 3 fragments were subtype concordant in all the regionswhile 5 (71.4%) showed discordant (mosaic) structure. Out of the 15 isolates that were sequenced in only two fragments, 11 (73.3%) were subtype concordant in both regions while 4 (26.7%) showed mosaic structure (Figure 4.18).

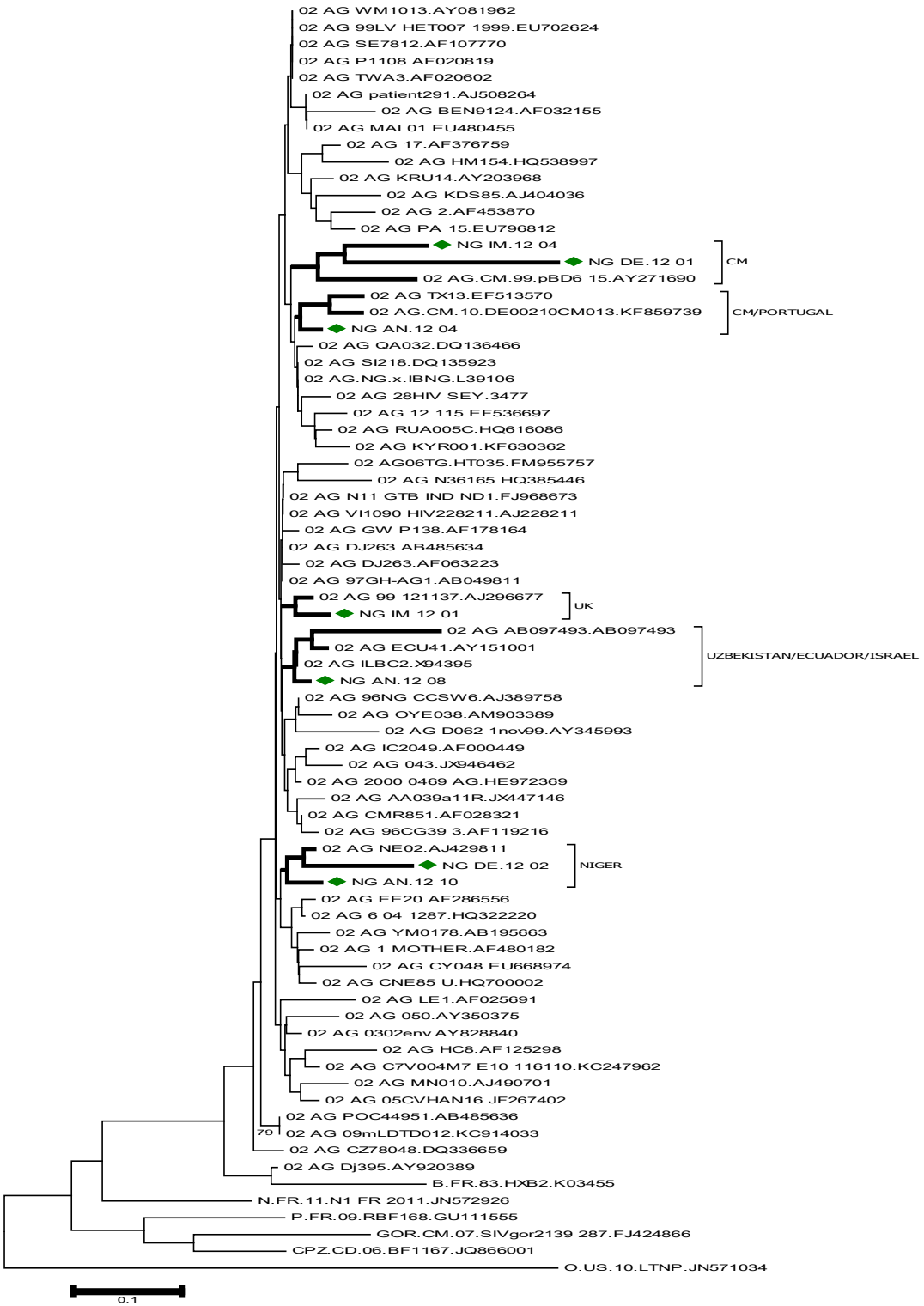


FIGURE 4.16: PHYLOGENETIC ANALYSIS OF ENV C2-V3 CRF02_AG ISOLATES. SEQUENCES WERE COMPARED TO CRF02_AG REFERENCE SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOTSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCES ARE INDICATED BY "◆" SYMBOL

TABLE 4.8: ESTIMATES OF EVOLUTIONARY DIVERGENCE OF ENV C2-V3 CRF02_AG SEQUENCES AND REFERENCE SEQUENCE

	1	2	3	4	5	6	7	8
1. L39106								
2. NG AN.12 04	0.08							
3. NG AN.12 08	0.08	0.11						
4. NG AN.12 10	0.07	0.09	0.10					
5. NG DE.12 01	0.34	0.37	0.38	0.37				
6. NG DE.12 02	0.11	0.21	0.19	0.15	0.43			
7. NG IM.12 01	0.08	0.09	0.09	0.09	0.36	0.19		
8. NG IM.12 04	0.21	0.19	0.25	0.20	0.31	0.29	0.22	

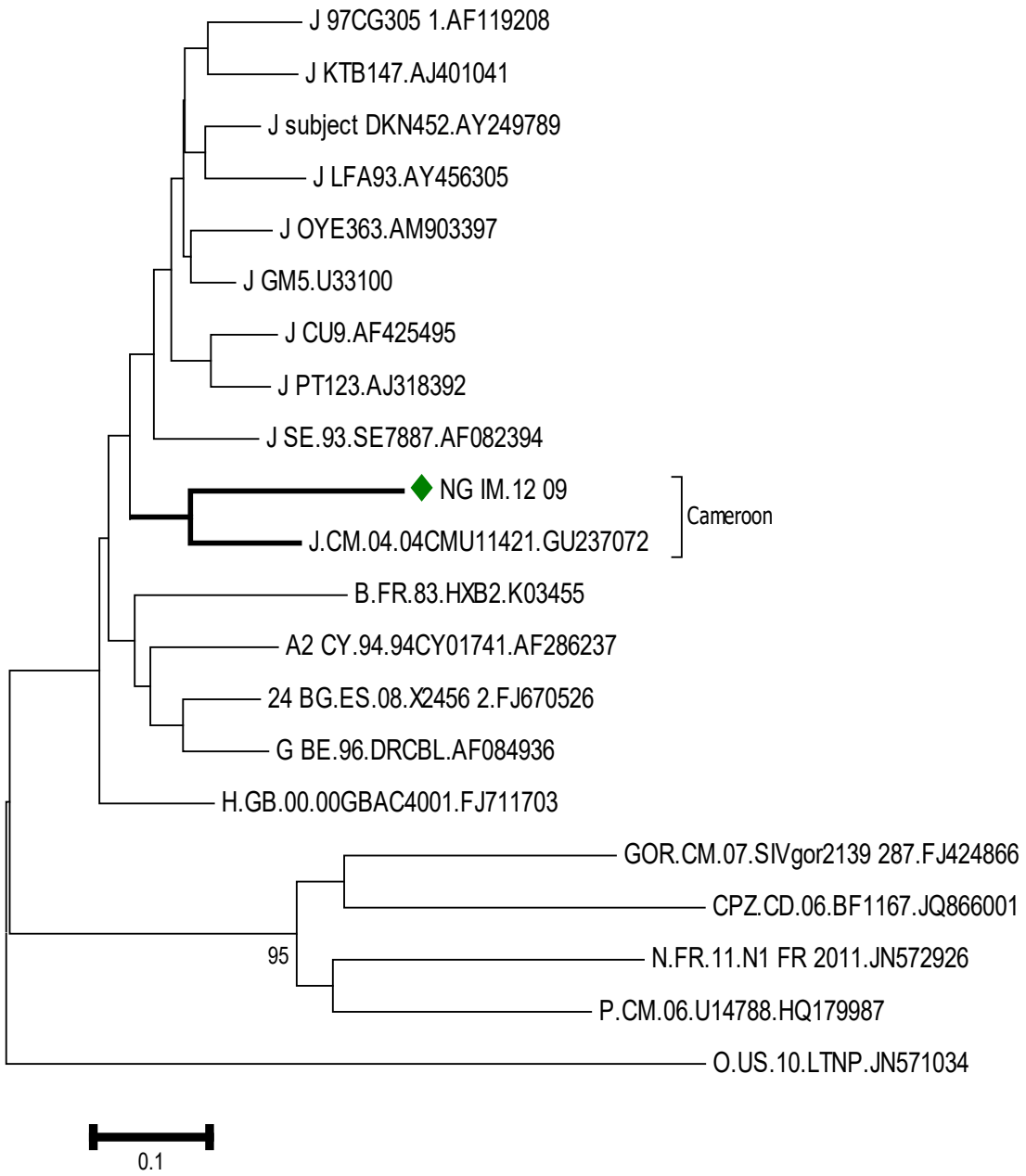


FIGURE 4.17: PHYLOGENETIC ANALYSIS OF ENV C2-V3 SUBTYPE J ISOLATE. SEQUENCE WAS COMPARED WITH SUBTYPE J REFERENCE SEQUENCES FROM THE LOS ALAMOS DATABASE (www.hiv.lanl.gov). ONLY BOOSTRAP VALUES (1000 REPLICATES) >70% ARE SHOWN. THE STUDY SEQUENCE IS INDICATED BY "◆" SYMBOL

TABLE 4.9: ESTIMATES OF EVOLUTIONARY DIVERGENCE BETWEEN ENV C2-V3 SUBTYPE J SEQUENCE AND REFERENCE SEQUENCES

	1	2	3	4	5	6	7	8
1. NG IM. 12 09								
2. GU237072	0.31							
3. U33100	0.36	0.23						
4. AY249789	0.37	0.20	0.10					
5. AM903397	0.39	0.25	0.12	0.14				
6. AJ401041	0.41	0.28	0.13	0.15	0.18			
7. AY456305	0.42	0.26	0.16	0.12	0.17	0.20		
8. AF119208	0.42	0.26	0.15	0.14	0.16	0.15	0.18	

TABLE 4.10: GENETIC DIVERSITY OF HIV-1 *GAG*, *POL* AND *ENV* GENES AMONG THE PATIENTS

S/No	Sample	*Gag* p24 gene	*Protease* gene	*Env* V3 gene
1	NG_AN.12_01	A	G	A
2	NG_AN.12_02	G	G	NS
3	NG_AN.12_03	NS	G	NS
4	NG_AN.12_04	U^{02AG}	CRF02_AG	CRF02_AG
5	NG_AN.12_05	NS	G	NS
6	NG_AN.12_06	NS	G	G
7	NG_AN.12_07	U^{02AG}	CRF02_AG	NS
8	NG_AN.12_08	NS	CRF02_AG	CRF02_AG
9	NG_AN.12_09	G	G	G
10	NG_AN.12_10	NS	CRF02_AG	CRF02_AG
11	NG_DE.12_01	G	CRF02_AG	CRF02_AG
12	NG_DE.12_02	NS	CRF02_AG	CRF02_AG
13	NG_DE.12_03	A	G	NS
14	NG_DE.12_04	CRF02_AG	CRF02_AG	NS
15	NG_DE.12_05	CRF02_AG	CRF02_AG	NS
16	NG_DE.12_06	A	CRF02_AG	NS
17	NG_DE.12_07	CRF02_AG	CRF02_AG	NS
18	NG_DE.12_08	NS	G	NS
19	NG_DE.12_09	NS	U^{G}	NS
20	NG_DE.12_10	NS	CRF02_AG	NS
21	NG_DE.12_11	CRF02_AG	NS	NS
22	NG_IM.12_01	NS	CRF02_AG	CRF02_AG
23	NG_IM.12_02	NS	G	G
24	NG_IM.12_03	NS	CRF02_AG	NS
25	NG_IM.12_04	CRF02_AG	CRF02_AG	CRF02_AG
26	NG_IM.12_05	CRF02_AG	CRF02_AG	G
27	NG_IM.12_06	G	U^{G}	G
28	NG_IM.12_07	NS	CRF02_AG	NS
29	NG_IM.12_08	G	G	NS
30	NG_IM.12_09	CRF02_AG	NS	J

Key: NS = Not sequenceable, U^{02AG} = unclassified CRF02_AG, U^{G} = Unclassified subtype G

TABLE 4.11: FREQUENCY OF OCCURRENCE OF THE DIVERSE FORMS OF THE HIV-1 ISOLATES AMONG THE PATIENTS

GAG/POL/ENV SUBTYPE/CRF	NUMBER OF SAMPLES (%)	TOTAL (%)
A/G/A	1 (3.3)	
U^{02AG}/CRF02_AG/CRF02_AG	1 (3.3)	
G/G/G	1 (3.3)	
G/CRF02_AG/CRF02_AG	1 (3.3)	7 (23.3)
CRF02_AG/CRF02_AG/CRF02_AG	1 (3.3)	
CRF02_AG/CRF02_AG/G	1 (3.3)	
G/U^{G}/G	1 (3.3)	
G/G/#	2 (6.7)	
#/G/G	2 (6.7)	
U^{02AG}/CRF02_AG/#	1 (3.3)	
#/CRF02_AG/CRF02_AG	4 (13.4)	
A/G/#	1 (3.3)	15 (50.0)
CRF02_AG/CRF02_AG/#	3 (10.0)	
A/CRF02_AG/#	1 (3.3)	
CRF02_AG/#/J	1 (3.3)	
#/G/#	3 (10.0)	
#/U^{G}/#	1 (3.3)	
#/CRF02_AG/#	3 (10.0)	8 (26.7)
CRF02_AG/#/#	1 (3.3)	

Key: #=Sequence not available, U^{02AG}= unclassified CRF02_AG, U^{G}=unclassified subtype G

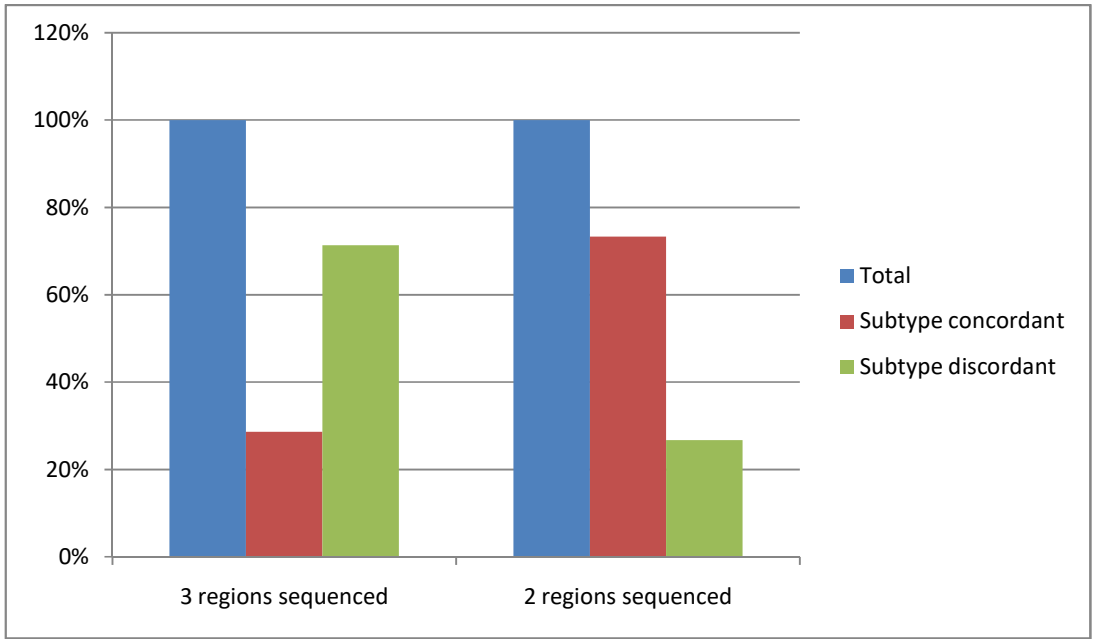


FIGURE 4.18: PERCENTAGE CONCORDANT AND DISCORDANT (MOSAIC) SUBTYPES/CRFs AMONG THE HIV-1 INFECTED PATIENTS

4.3.1 AMINO ACID DIVERSITY OF THE PROTEASE REGION

The amino acid alignment of the samples with subtype B consensus (Cons B) is shown in figure 4.19. The sequence analysis of the *Protease* showed that a total of 58 (58.6%) of the 99 amino acid positions were conserved while variation occured in 41 (41.4%) of the positions when compared to Cons B. There were no insertions or deletions in the sequence. High level of amino acid substitutions was found for amino acid positions I13-100.0%, K14-48.4%, K20-100.0%, E35-60.7%, M36-100.0%, R41-89.3%, R57-42.9%, L63-53.6%, C67-42.9%, H69-100.0%, V82-46.4% and L88-100.0%.

4.3.1.1 MUTATIONS AND POLYMORPHISMS AT PREVIOUSLY CHARACTERIZED DRUGRESISTANCE SITES

Mutations were found in all the sequences analysed. Major drug resistance mutations were detected at two PR sites (M46L and V82L) previously characterized for drug resistance in three of the sequences (Table 4.12). Polymorphisms at a known minor mutation site (K20I, M36I/L, H69K/R and L89M) were found in all the samples while L63T/P/S/Q was found in 83.3% (10/12) and 31.3% (5/16) of subtypes G/U^{G} and CRF02_AG respectively. Furthermore, a polymorphism at a known major mutation site (V82I) was detected in all subtype G/U^{G} samples.

Other common mutations among the isolates that occurred at positions not selected for drug resistance include; I13V/A which was found in all the samples, C67E/S found in all subtypes G/U^{G},E35Q in 91.7% of subtypes G/U^{G}, N37D/S/E/H in 41.7% of subtypes G/U^{G} and 25.0% of CRF02_AG respectively. Also R57K/G was found in 83.3% of subtypes G/U^{G} and 12.5% of CRF02_AG while K70R was found in 16.7% and 37.5% of subtypes G/U^{G} and CRF02_AG respectively. The frequency of occurrence of the different mutations and/or polymorphisms is shown in table 4.13.

The resistance patterns of the different isolates to the different PIs described as Susceptible, Potential-level, Low-level and intermediate-level resistant to drugs according to the Stanford algorithm are shown in table 4.14. Figure 4.20 shows the frequency of occurrence of the predicted viral susceptibility of the isolates to PIs.

FIGURE 4.19: ALIGNMENT OF PROTEASE AMINO ACID SEQUENCES OF THE ISOLATES WITH SUBTYPE B CONSENSUS (CONS B) SEQUENCE. THE LETTERS DENOTES THE AMINO ACIDS.DOTS REPRESENT SAME AMINO ACID RESIDUE WITH THE REFERENCE SEQUENCE.

TABLE 4.12: PR MUTATIONS/POLYMORPHISMS DETECTED AMONG HIV ISOLATES IN SOUTH-EASTERN NIGERIA

Sample	Subtype	Mutations/Polymorphisms
NG_AN.12_01	G	I13V, K14R, *K20I*, E35Q, M36I, R41K, K43R, **M46L**, R57K, Q61X, L63T, C67E, H69K, K70R, V82I, L89M
NG_AN.12_02	G	*K20I*, I13V, K14R, E35Q, M36I, R41K, K43R, **M46L**, R57K, L63T, C67E, H69K, K70R, V82I, L89M
NG_AN.12_03	G	I13V, *K20I*, E35Q, M36I, R41K, R57K, L63P, C67E, H69K, V82I, L89M
NG_AN.12_04	CRF02_AG	*L10V*, I13V, L19P, *K20I*, E35D, M36I, R41K, R59G, H69K, L89M, G94R, C95W, N98H
NG_AN.12_05	G	I13V, *K20I*, M36I, N37D, R41K, C67S, H69R, V82I, L89M
NG_AN.12_06	G	I13V, K14R, *K20I*, E35Q, M36I, N37S, R41K, K45R, R57K, L63S, C67E, H69K, V82I, L89M
NG_AN.12_07	CRF02_AG	I13A, *K20I*, M36I, R41K, H69K & L89M
NG_AN.12_08	CRF02_AG	*L10V*, I13V, L19P, *K20I*, E35D, M31I, R41K, H69K, K70R, L89M
NG_AN.12_09	G	L10M, I13V, *K20I*, E35Q, M36I, R41K, R57K, I62V, L63P, I64L, C67E, H69K, V82I, L89M
NG_AN.12_10	CRF02_AG	I13V, *K20I*, M36I, N37S, L63S, H69K, K70R, I72M, L89M
NG_DE.12_01	CRF02_AG	I13V, K14R, G16E, *K20I*, E35D, M36I, R41K, H69K, L89M
NG_DE.12_02	CRF02_AG	I13V, K14R, G16E, *K20I*, M36I, R41K, H69K, K70R, I72T, L89M
NG_DE.12_03	G	I13V, *K20I*, E35Q, M36I, R41K, R57K, L63P, C67E, H69K, V82I, L89M, Q92W
NG_DE.12_04	CRF02_AG	*L10I*, I13V, K14R, I15V, L19I, *K20I*, M36I, R41K, I64M, H69K, K70R, L89M
NG_DE.12_05	CRF02_AG	I13V, *K20I*, E35D, M36I, R41K, H69K, K70R, L89M, L97K, F99S
NG_DE.12_06	CRF02_AG	I13V, K14R, *K20I*, M36I, R41K, I64L, E65D, H69K, L89M
NG_DE.12_07	CRF02_AG	I13V, K14R, G17E, *K20I*, M36I, R41K, H69K, L89M
NG_DE.12_08	G	*L10I*, I13V, K14R, *K20I*, E35Q, M36I, R41K, R57K, L63Q, C67E, H69K, V82I, L89M
NG_DE.12_09	U^G	I13V, *K20I*, E35Q, M36I, N37E, L63S, C67E, H69R, V82I, L89M
NG_DE.12_10	CRF02_AG	*L10V*, T12A, I13V, I15V, L19P, *K20I*, M36I, R41K, K43R, L63P, H69K, K70R, L89M
NG_IM.12_01	CRF02_AG	*L10V*, I13V, G16E, *K20I*, E35D, M36I, R41K, I64M, H69K & L89M
NG_IM.12_02	G	I13V, K14R, *K20I*, E35Q, M36I, N37D, R41K, R57K, C67E, H69R, V82I, L89M
NG_IM.12_03	CRF02_AG	I13V, K14R, *K20I*, M36I, R41K, I64L, H69K, L89M
NG_IM.12_04	CRF02_AG	I13V, K14R, *K20I*, E35D, M36I, N37S, R41K, L63S, H69K, L89M
NG_IM.12_05	CRF02_AG	I13V, L19P, *K20I*, *L33F*, M36L, N37H, L38I, R41K, K43R, R57K, L63P, H69K, L89M
NG_IM.12_06	U^G	I13V, G17E, *K20I*, E35Q, M36I, N37E, R57K, L63S, C67E, H69R, V82I, L89M
NG_IM.12_07	CRF02_AG	*L10I*, I13V, K14R, *K20I*, M36I, N37S, R41K, L63S, H69K, I72V, P79S, **V82L**, G86L, R87L, *N88D*, L89M, T91C, Q92H, G94I, T96A, L97V, N98H
NG_IM.12_08	G	I13V, *K20I*, E35Q, M36I, R41K, K45R, R57K, L63P, C67E, H69K, V82I, L89M

Key: PI Major Resistance Mutations are in bold face, PI Minor Resistance Mutations are in italics, and other mutations are in regular face.

TABLE 4.13: FREQUENCY OF OCCURRENCE OF MUTATIONS AND/OR POLYMORPHISMS IN PROTEASE BY HIV SUBTYPE

Mutation	No. (%) of mutations		Mutation	No. (%) of mutations	
	Subtype G&U^{G} (n=12)	CRF02_AG (n=16)		Subtype G&U^{G} (n=12)	CRF02_AG (n=16)
L10V/I	1 (8.3)	6(37.5)	L63T/P/S/Q	10(83.3)	5 (31.3)
L10M	1(8.3)	-	I64L/M	1(8.3)	4(25.0)
T12A	-	1(6.3)	E65D	-	1(6.3)
I13V/A	12(100.0)	16(100.0)	C67E/S	12(100.0)	-
K14R	6(50.0)	7(43.8)	H69K/R	12(100.0)	16(100.0)
I15V	-	2(12.5)	K70R	2(16.7)	6(37.5)
G16E	-	3(18.8)	I72M/T/V	-	3(18.8)
G17E	1(8.3)	1(6.3)	P79S	-	1(6.3)
L19P	-	5(31.3)	V82I	12(100.0)	-
K20I	12(100.0)	16(100.0)	V82L	-	1(6.3)
L33F	-	1(6.3)	G86L	-	1(6.3)
E35Q	11(91.7)	-	R87L	-	1(6.3)
E35D	-	6(37.5)	N88D	-	1(6.3)
M36I/L	12(100.0)	16(100.0)	L89M	12(100.0)	16(100.0)
N37D/S/E/H	5(41.7)	4 (25.0)	T91C	-	1(6.3)
L38I	-	1(6.3)	Q92W/H	1(8.3)	1 (6.3)
R41K	10(83.3)	15(93.8)	G94R/I	-	2(12.5)
K43R	2(16.7)	2(12.5)	C95W	-	1(6.3)
K45R	2(16.7)	-	T96A	-	1(6.3)
M46L	2(16.7)	-	L97K/V	-`	2(12.5)
R57K/G	10(83.3)	2(12.5)	N98H	-	2(12.5)
I62V	1(8.3)	-	F99S	-	1(6.3)

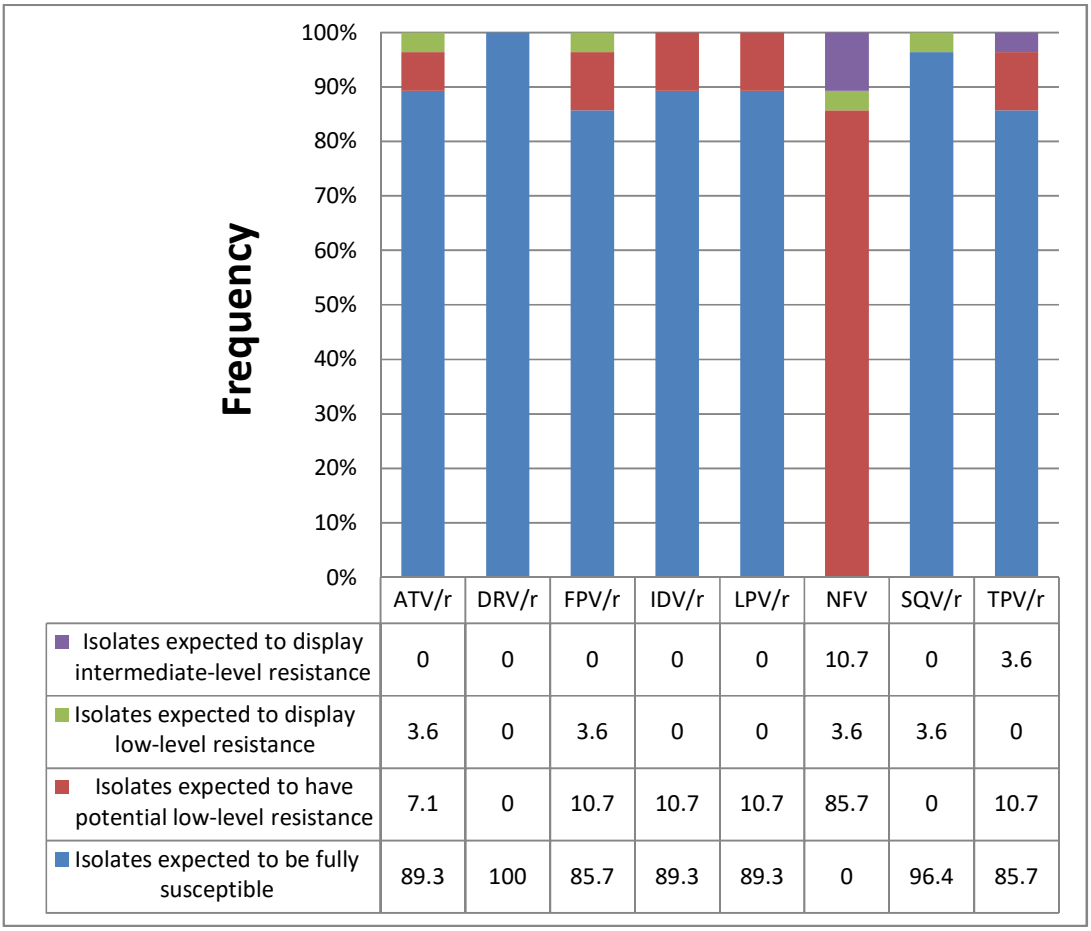
Keys: Keys: Numbers correspond to amino acid positions. The first letter corresponds to the wild-type amino acid; the substituted amino acid is coded by the last letter.

TABLE 4.14: PI RESISTANCE PATTERNS AMONG THE HIV ISOLATES FROM SOUTH-EASTERN NIGERIA

Sample	Subtype	Major mutation	Minor mutation	ATV/r	DRV/r	FPV/r	IDV/r	LPV/r	NFV	SQV/r	TPV/r
NG_AN.12_01	G	M46L	K20I	P	S	P	P	P	I	S	P
NG_AN.12_02	G	M46L	K20I	P	S	P	P	P	I	S	P
NG_AN.12_03	G	-	K20I	S	S	S	S	S	P	S	S
NG_AN.12_04	CRF02_AG	-	L10V, K20I	S	S	S	S	S	P	S	S
NG_AN.12_05	G	-	K20I	S	S	S	S	S	P	S	S
NG_AN.12_06	G	-	K20I	S	S	S	S	S	P	S	S
NG_AN.12_07	CRF02_AG	-	K20I	S	S	S	S	S	P	S	S
NG_AN.12_08	CRF02_AG	-	L10V, K20I	S	S	S	S	S	P	S	S
NG_AN.12_09	G	-	K20I	S	S	S	S	S	P	S	S
NG_AN.12_10	CRF02_AG	-	K20I	S	S	S	S	S	P	S	S
NG_DE.12_01	CRF02_AG	-	K20I	S	S	S	S	S	P	S	S
NG_DE.12_02	CRF02_AG	-	K20I	S	S	S	S	S	P	S	S
NG_DE.12_03	G	-	K20I	S	S	S	S	S	P	S	S
NG_DE.12_04	CRF02_AG	-	L10I, K20I	S	S	S	S	S	P	S	S
NG_DE.12_05	CRF02_AG	-	K20I	S	S	S	S	S	P	S	S
NG_DE.12_06	CRF02_AG	-	K20I	S	S	S	S	S	P	S	S
NG_DE.12_07	CRF02_AG	-	K20I	S	S	S	S	S	P	S	S
NG_DE.12_08	G	-	L10I, K20I	S	S	S	S	S	P	S	S
NG_DE.12_09	U^{G}	-	K20I	S	S	S	S	S	P	S	S
NG_DE.12_10	CRF02_AG	-	L10V, K20I	S	S	S	S	S	P	S	S
NG_IM.12_01	CRF02_AG	-	L10V, K20I	S	S	S	S	S	P	S	S
NG_IM.12_02	G	-	K20I	S	S	S	S	S	P	S	S
NG_IM.12_03	CRF02_AG	-	K20I	S	S	S	S	S	P	S	S
NG_IM.12_04	CRF02_AG	-	K20I	S	S	S	S	S	P	S	S
NG_IM.12_05	CRF02_AG	-	K20I, L33F	S	S	P	S	S	L	S	P

NG_IM.12_06	U^G	-	K20I	S	S	S	S	S	P	S	S
NG_IM.12_07	CRF02_AG	V82L	L10I, K20I N88D	L	S	L	P	P	I	L	I
NG_IM.12_08	G	-	K20I	S	S	S	S	S	P	S	S

ATV= Atazanavir; DRV= Darunavir; FPV= Fosamprenavir; IDV= Indinavir; LPV= Lopinavir; NFV= Nelfinavir; SQV= Saquinavir; TPV= Tipranavir; S, P, L and I indicate Susceptible, Potential-level, Low-level and intermediate-level resistant to drugs respectively



	ATV/r	DRV/r	FPV/r	IDV/r	LPV/r	NFV	SQV/r	TPV/r
Isolates expected to display intermediate-level resistance	0	0	0	0	0	10.7	0	3.6
Isolates expected to display low-level resistance	3.6	0	3.6	0	0	3.6	3.6	0
Isolates expected to have potential low-level resistance	7.1	0	10.7	10.7	10.7	85.7	0	10.7
Isolates expected to be fully susceptible	89.3	100	85.7	89.3	89.3	0	96.4	85.7

FIGURE 4.20: PREDICTED VIRAL SUSCEPTIBILITY TO PROTEASE INHIBITORS. GENOTYPIC DRUG SUSCEPTIBILITY WAS PREDICTED USING THE STANFORD HIVDB AND IAS-USA MUTATION LISTALGORITHMS

Of these virus sequences harbouring ≥1 DRM, susceptibility to boosted Darunavir was maintained in all (100%) of the isolates. Reduced HIV-1 susceptibility was predicted for boosted Atazanavir, Indinavir and Lopinavir in about 11% of sequences,Saquinavir in 4%, Fosamprenavir and Tipranavir in 14%. Reduced HIV-1 susceptibility was predicted for the only non-boosted Nelfinavir in all (100%) of the sequences due to general presence of K20I.

4.3.2 ANALYSIS OF THE AMINO ACID SEQUENCES OF THE V3 LOOP REGION

Out of the 14 V3 sequences analysed, 12 have 35 amino acids in length while the remaining two are 36 amino acids with 2 insertions each when compared to Cons B. All except two of the HIV-1 V3 loop sequences were limited by the two Cysteine residues responsible for the typical V3 loop configuration. There was deletion of the 3′ limiting Cysteine residue in two of the sequences and coincidentally these sequences are the same ones with insertions at other sites (Figure 4.21). Isolate NG_IM.12_04 had amino acids Glycine and Isoleucine inserted between amino acid positions I14 and G15 while NG_IM.12_09 had Glycine inserted between R3 and P4 as well as Isoleucine between R9 and K10 when compared to Cons B.

The sequence analysis of the V3 loop showed total conservation for 6 (C1, R3, T8, G17, G28 and R31) of the 35 amino acid residues (17.1% of conservation). C1 is the 5′ cysteine residue limiting the V3 loop while the 3′ limiting cysteine residue has variation of 14.3%. High variation was observed for amino acid positions N5-57.1%, I12-64.3%, H13-92.9%, R18-85.7%, A19-85.7%, T22-92.9% and E25-78.6%. The conserved GPGQ crown motif was the most common sequence observed at the V3 tip occurring in 71.4% (10) of the translated HIV-1 V3 loop amino acid sequences. There was substitution from GPGQ to other V3 crown motifs like GHGQ (1), RRGQ (1) and GPGR (2) as shown in figure 4.21.

4.3.2.1 V3 LOOP GLYCOSYLATION SITES AND CROWN MOTIF

Majority of the potential N-linked glycosylation sites were retained in the immediate vicinity of and within the V3 loop of the HIV-1 sequences analyzed. The NxS/T sequon for the potential N-glycosylation site centered at the 5′-Cys V3 loop residue was conserved in 11 (78.6%) of the HIV-1 C2-V3 amino acid sequences with 10 (71.4%) presenting with the NCT (Asparagine-Cysteine-Threonine) sequon while 1 (7.1%) presented with NCS (Asparaine-Cysteine-Serine) sequon.

FIGURE 4.21: V3 LOOP AMINO ACID SEQUENCE AMONG HIV-1 INFECTED PATIENTS

The potential N-linked glycosylation sites located six amino acids downstream from the 5′ cysteine residue in the V3 loop was conserved in 13 of the 14 (92.9%) sequences analysed with all presenting with the NNT (Asparagine-Asparagine-Threonine) sequon(Figure 4.22). The conserved GPGQ crown motif was the most common sequence observed at the V3 tip occurring in 71.4% (10/14) of the translated HIV-1 V3 amino acid sequences. There was substitution from GPGQ to other crown motifs like GHGQ (7.1%), RRGQ (7.1%) and GPGR (14.3%), as shown in figure 4.22.

4.3.2.2PREDICTION OF HIV-1 CORECEPTOR USAGE

Using the combined criteria for the prediction of co-receptor usage, 6 (42.9%) of the samples were predicted as X4 while the remaining 8(57.1%) were predicted as R5 viruses (Figure 4.23).Analysis of the six V3 loop amino acid sequences that met each criterion shows thatonly one sequence (NG_AN.12_08) was predicted as X4 based solely on the loss of glycosylation site. Others were predicted as X4 based on a combination of two or more criteria. Bioinformatics analysis with Geno2Pheno at 10% false positive rate (FPR) predicted 4 sequences (all of which met other criteria for X4 prediction) as X4 phenotype. NG_DE.12_01, in addition to Geno2Pheno prediction is the only sequence that met the 11/25 rulehaving Lysine at position 11 of V3 loop.

There is also substitution of its glycosylation site from NNT to GKT as well as substitution of crown motif from GPGQ to GHGQ. NG_IM.12_02 in addition to Geno2Pheno prediction also lost its glycosylation site as well as its crown motif. NG_IM.12_04 has also lost its 3′ limiting Cysteine residue in addition to Geno2Pheno prediction. NG_IM.12_09 was predicted as X4 by Geno2Pheno software. In addition, it has lost its glycosylation site, crown motif as well as 3′ limiting Cysteine residue. The only sequence that has amino acid net charge of +6 (NG_DE.12_02) also has its crown motif substituted from GPGQ (Glycine-Proline-Glycine-Glutamine) to RRGQ (Arginine-Arginine-Glycine-Glutamine) as shown in figure 4.22.

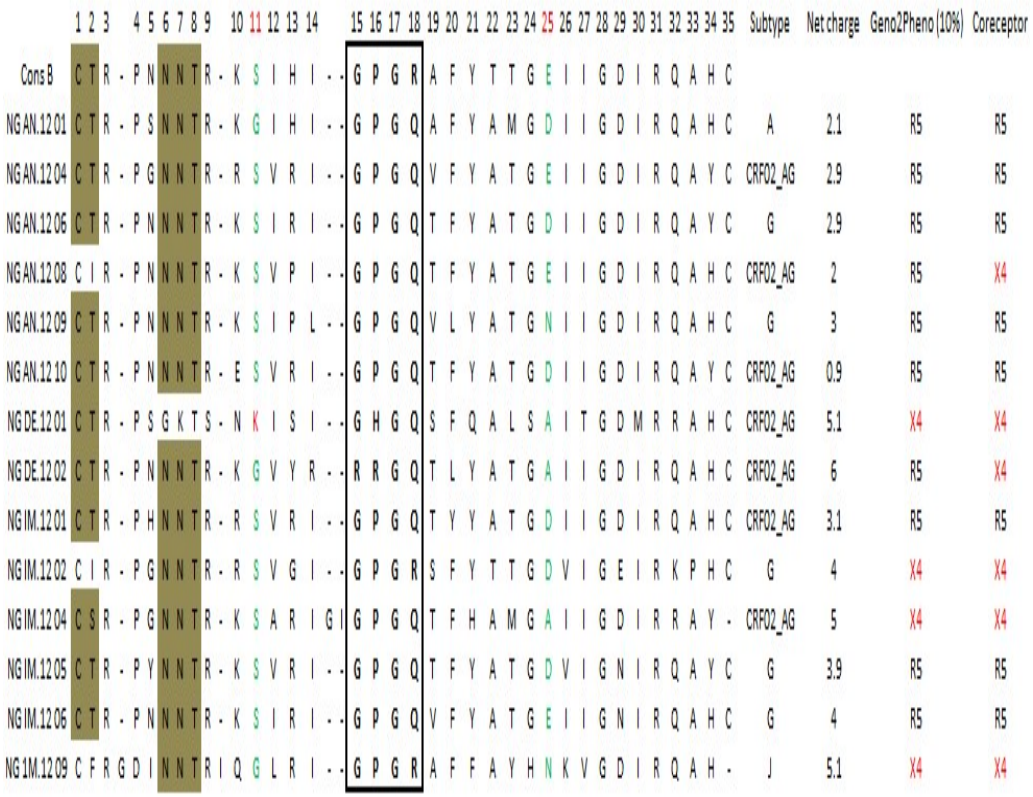


FIGURE 4.22: V3 LOOP AMINO ACID SEQUENCE, GLYCOSYLATION SITES AND CROWN MOTIF AMONG HIV-1 INFECTED PATIENTS.
The glycosylation sites are shaded while the crown motifs are enclosed in the box

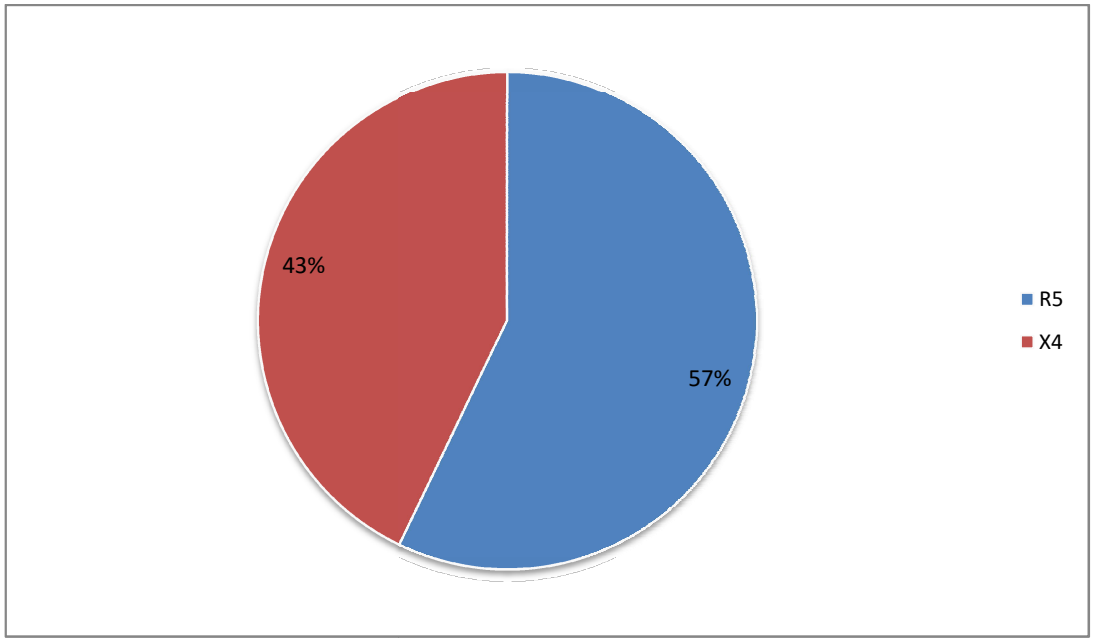


FIGURE 4.23: PREDICTED HIV-1 CORECEPTOR USAGE AMONG THE PATIENTS

4.3.2.3PREDICTION OF MARAVIROC RESISTANCE

Analysis of the sets of mutations in the V3 loop associated with Maraviroc-resistant phenotype in the 14 sequences was also carried out. Maraviroc-resistance conferring mutations are differentiated into those associated *in vivo* and those associated *in vitro*.The sets of mutations associated *in vivo* with Maraviroc resistance are G11S+I26V, S18G+A22T, A19S+I26V, I20F+Y21I and I20F+A25D+I26V. Our result showed that one sequence, NG_IM.12_02 harboured 3 (G11S+I26V, A19S+I26V and I20F+A25D+I26V) *in vivo* Maraviroc-resistance conferring mutations. Interestingly, this isolate was already predicted as X4 virus phenotype. Another sequence, NG_IM.12_05 also has 2 (G11S+I26V and I20F+A25D+I26V) *in vivo* Maraviroc-resistance conferring mutation sets. This isolate was predicted as R5 virus phenotype.

The mutations associated *in vitro* with Maraviroc resistance are A22T and I30V. Only 1 isolate, NG_IM.12_02 has the A22T resistance mutation. Curiously, this isolate also has 3 *in vivo* Maraviroc-resistance conferring mutation sets. None of the isolates has the I30V mutation. The two isolates with the Maraviroc-resistance conferring mutations belong to HIV-1 subtype G (Figure 4.24).

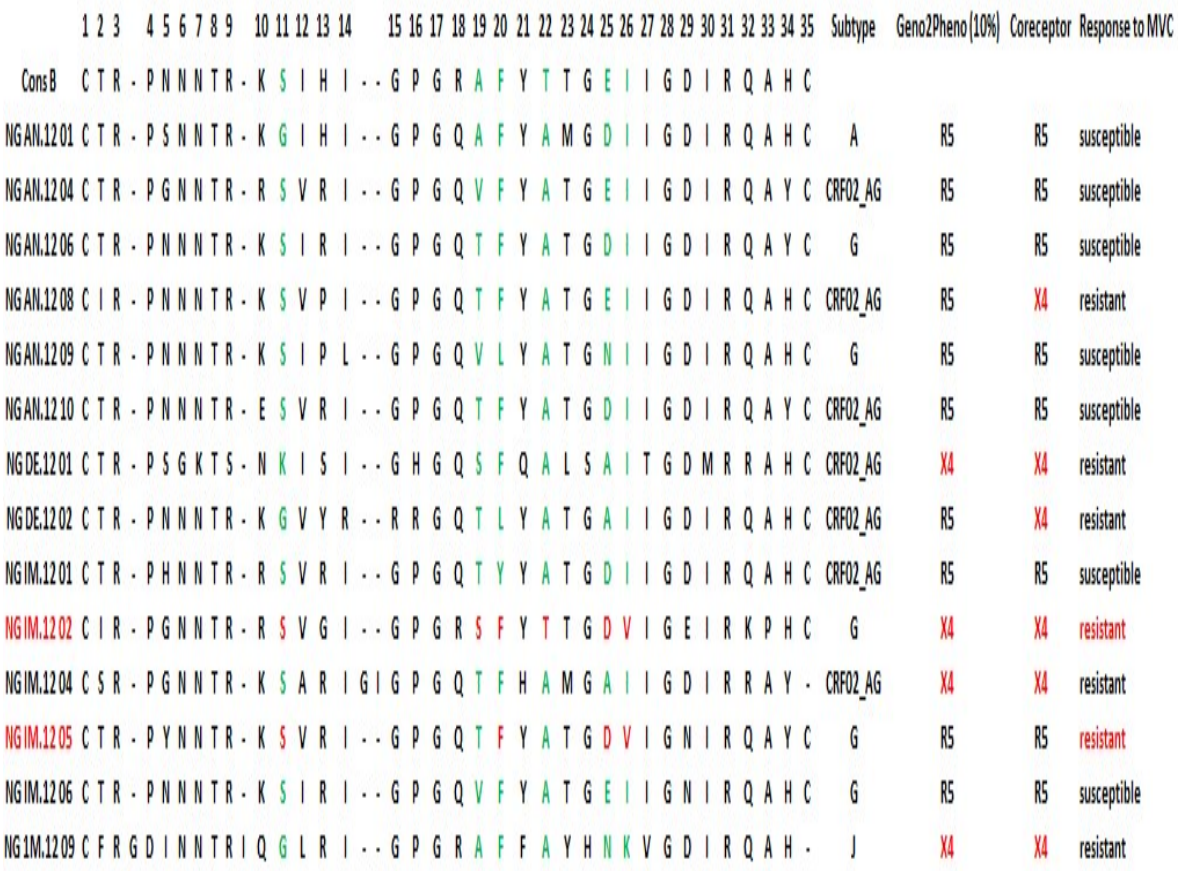


FIGURE 4.24: PREDICTION OF MARAVIROC RESISTANCE. THE RESISTANCE CONFERRING MUTATIONS ARE COLOURED RED

CHAPTER FIVE

5.0 DISCUSSION

Overall subtypes A, G, J and CRF02_AG were detected in this study. Similar subtypes have been previously reported in different proportions from various parts of the country (Odaibo *et al*., 2001; Agwale *et al*., 2002; Odaibo *et al*., 2006; Ojesina *et al*., 2006; Hawkins *et al*., 2009; Ajoge *et al*., 2011; Chaplin *et al*., 2011; Fayemiwo *et al*., 2014; Imade *et al*., 2014; Donbraye *et al*., 2015). The predominance of CRF02_AG; 41.2% in *gag* region, 57.1% in *protease* region and 50% in *env* C2-V3 region and subtype G; 29.4% in *gag* region, 35.7% in *protease* region and 35.7% in *env* C2-V3 region in this study is consistent with other studies from different parts of the country (Ojesina *et al*., 2006; Ajoge *et al*., 2011).

CRF02_AG has been reported as the predominant HIV-1 strain circulating in West Africa (Montavon *et al*., 2000).This has been corroborated by reports from different parts of Nigeria. From Ibadan, South Western Nigeria where extensive HIV-1 molecular epidemiologic studies have been carried out, Odaibo *et al*. (2006) reported the predominance of CRF02_AG (55.0%) and subtype G (15.0%) among twenty HIV-1 infected women in a study to determine mother-to-child transmission of different HIV-1 subtypes among ARV naïve infected pregnant women in Nigeria. In another study on the implications of HIV-1 subtype on drug resistance and host response in treatment naïve patients also in Oyo state, predominance of CRF02_AG and subtype G was reported(Ojesina *et al*., 2006). Furthermore, sequence distribution data from the study reporting the complexity of circulating HIV type 1 strains in Oyo state shows the predominance of CRF02_AG and subtype G with each accounting for 32.2% of the circulating strains(Sankale *et al*., 2007). More recently also, Fayemiwo *et al*. (2014) and Donbraye *et al*. (2015) in their separate studies reported CRF02_AG and subtype G as the most prevalent subtypes in Ibadan, Oyo state, Southwestern Nigeria. Similarly,in a study in Jos, Northcentral Nigeria designed to determine HIV-1 subtypes and antiretroviral drug resistance mutations for 16 infected pregnant women using part of *pol* gene, subtypes G and CRF02_AG accounted for 50.0% and 18.8% respectively while unique recombinant forms (URFs) between G and CRF02_AG, subtype C and unrelated URFs accounted for 12.5%, 6.3% and 12.5% respectively (Lar *et al*., 2007).Furthermore, a study also in Northcentral Nigeria by Ajoge and colleagues (2011) which characterized *gag* and *env* genes of HIV-1 isolates in 28 treatment naïvepregnant women showed predominance of HIV-1 CRF02_AG and subtype G. Also in a more

widespread study covering Lagos and Ibadan (Southwest), Jos (Northcentral) and Maiduguri (Northeast) Nigeria by Chaplin *et al*. (2011) which evaluated RT and PR genes of 338 samples, 45.0% of CRF02_AG, 37.9% of subtype G, 4.4% of CRF06_cpx and 3.6% of subtype A were reported while others accounted for the remaining 9.2%.The above studies all show the predominance of HIV-1 CRF02_AG in Nigeria which is believed to be largely driving the epidemic in the country. Our study also reports the circulation of HIV-1 subtype A at 17.7% and 7.1% in the regions of HIV-1 *gag* and *env* C2-V3 genes respectively. Although this subtypeaccounted for relatively small proportion in this study, it has earlier been reported together with subtype C by Odaibo and colleagues as the predominant strains accounting for 68.0% of circulating HIV-1 strains in Nigeria (Odaibo *et al*., 2001). Similarly, a national molecular epidemiologic survey of HIV-1 strains which covered 34 of 36 states of Nigeria reported a high percentage (44.8%) of HIV-1 subtype A based on *env* gp41 gene (Agwale *et al*., 2002).An interesting feature of these reports is that the studies conducted in early year 2000 showed higher proportion of HIV-1 subtype A and sometimes C in circulation in different regions of Nigeria. More recent studies including ours however, are increasingly showing predominance of CRF02_AG and subtype G in different regions of the country. Previous researchers have reported ever changing geographic distribution patterns of different HIV-1 strains with novel genetic diversity continually being generated through mutation and recombination with travel and migration promoting the transfer of diverse viral strains within and between populations over time (Gifford *et al*., 2007). Hence this changing trend in the HIV-1 epidemiology in Nigeria could be explained by either: (1) the isolates from the earlier studies may have been inaccurately classifiedas a result of the relatively older techniques used or(2) a reflection of the dynamic and evolving nature of the HIV-1 epidemic in Nigeria as a result of circulation of multiple strains.

Another significant finding of this study is the identification of HIV-1 *env* C2-V3 subtype J isolate. This is the second report of this subtype in Nigeria after it was first reported by Agwale and colleagues (2002).HIV-1 subtype J has been reported in Democratic Republic of Congo, Angola, Botswana, Cameroon, Central African Republic, Gabon, Senegal, Uganda and Zambia and some European countries like Sweden, Belgium, Spain, France, Portugal, Germany and Italy as well as United States of America and Cuba (Los Alamos HIV Database).The isolate in this study is however, closely relatedto sequence from Cameroon

which shares border with Nigeriasuggesting that the strain may have been introduced into the country by trans-border travels between these two countries. Throughout the whole world, travellers are known to be among the major contributors to the spread of HIV-1 as well as its genetic variability. This problem is further compounded in the developing world by rural-urban migration and civil wars which causes displacement of people.Interestingly also, this isolate, NG_IM.12_09 grouped with subtype J in the *env* region same as the one earlier reported by Agwale and colleagues. The *gag* region however, clustered with CRF02_AG. On further analysis using CRF02_AG subtype phylogenetic tree (Fig. 4.7), this isolate clustered with sequence from Niger republic (which also shares common border with Nigeria) while sequence was not available for classification in the *pol* region. This recombinant virus may represent a new recombinant form whose true mosaic structure can only be ascertained by full-length genome sequence analysis.

Also of importance is the identification of two isolates, NG_AN.12_04 and NG_AN.12_07(U^{02AG}s)which did not cluster with any known subtype or CRFs in the *gag* p24 phylogenetic tree (Fig. 4.3).NG_AN.12_04 was classified as CRF02_AG in both *protease* and *env* C2-V3 regions. NG_AN.12_07 was also classified as CRF02_AG in the *protease* region while sequence was not available for classification in the *env* C2-V3 region. Similarly two isolates, NG_DE.12_09 and NG_IM.12_06 (U^{G}s) also did not cluster with any known subtype or CRF in the *protease* phylogenetic tree (Fig. 4.8). In the *gag* and *env* C2-V3 trees, isolate NG_IM.12_06 grouped with subtype G while there was no available sequences for NG_DE.12_09 in the *gag* and *env* regions.Inability of these sequences to cluster with any known subtype could be as a result of one or more of the following reasons: (1) lack of enough phylogenetic information within the sequenced gene region, (2) there may be recombinant breakpoint that may evade detectionwithin the sequencedgene fragment or (3) the sequenced gene fragment may have been derived from some previously unidentified strain. This finding thus reflectsthe complex genetic variability of HIV-1 strains circulatingin Nigeria as earlier reported (Sankale *et al*., 2007).

Another striking revelation of this study is the level of discordant subtypes found in the isolates in which more than one genomic region were genotyped (Fig. 4.18). The frequent discordance among the gene fragmentsof the *gag*, *pol* and *env* regions may be as a result of high level of genetic variability of the virus in these gene regions and is a reflection of the

level of recombination occurring among the circulating virus strains. It furtherdemonstrates the complex diversity of the strains of HIV-1 circulating in South-eastern part of Nigeria as previously reported from other regions of the country.Although, the likely implications of the emergence of recombinant/mosaic HIV-1 strains is not yet fully understood, it will undoubtedly impact negatively on the development of proposed candidate HIV vaccine in Nigeria. This ugly scenario therefore is a cause for concern and calls for constant monitoring for the likely emergence of more/novel inter-subtype recombinant or even secondary recombinant strains as the epidemic progresses. HIV-1 recombinants emerge as a result of dual infections with HIV-1 strains, which can be individual subtypes or CRFs,andoccurs extensively in areas with co-circulating multiple subtypes.In Africa, it has been estimated that recombinant strains constitute >20% of HIV-1 infections (van der Kuyl and Cornelissen, 2007). Hence, our result is consistent with this earlier finding.

Mosaic viruses may have biological advantage over the parental strains as the "hybrid virus" which arose as a consequence of recombination events between two or more variants could acquire some advantage-conferring traits from the parental strains. The consequences could be in form of modified cellular tropism, altered co-receptor usage, alteration of growth kinetics, and enhancedviral replicative fitness.Mutations in the *protease* gene of viral isolates could cause development of antiretroviral drug resistance which is the bane of effective clinical management of infected individuals.Most of the currently available data on the potency of ART and the selection of drug resistance largely focused on HIV-1 subtype B, which is the predominant subtype in the western countries. In most developing countries, including Nigeria however, the circulating viruses are largely non-subtype B while subtype B plays little role in HIV epidemic in the region. With improved access to HIV-1 treatment in this region, there is a growing concern on the susceptibility and response of diverse HIV-1 strains found in this region to antiretroviral drugs (Kantor and Katzenstein, 2004).Evidence suggestthat currently available PIs could be as active against non-subtype B viruses as they are against subtype B viruses (Shafer*et al*., 1999). However, data on the genetic mechanisms of drug resistance in non-subtype B viruses and their clinical relevance remains scanty.

This study also describes the prevalence and patterns of mutations in the *protease*gene of the isolates under study. Resistance mutations in this gene can either be categorized as majoror minor.While major mutations usually emerge first in the presence of the drug and

significantly decrease drug susceptibility,minor mutations on the other hand emerge afterwards and do not significantlyaffect the virus phenotypically in the absence of other major mutations. In company of other major mutations however, they may improve the replication of the viruses (Wensing *et al*., 2015). The 10.7% prevalence of major PI resistance mutations (M46L=2 and V82L=1) found in this study is lower than 39.1% prevalence reported in a similar study which investigated patterns of drug resistance of HIV-1 among infected adults receiving antiretroviral treatment in the country (Odaibo *et al*., 2013).Other studies conducted in the country however have failed to detect any major PI resistance mutations among their subjects. One of such studies (Negedu-Momoh *et al*., 2014) investigated and assessed the level of resistance to antiretroviral drugs *vis avis* genetic diversity in both drug-experienced and naïve patients. While major drug resistance mutations were detected in the *reverse transcriptase* gene of the HIV-1 isolates, none was detected in the *protease* gene. Similarly, no such mutations was detected in a survey of transmitted drug resistance among newly detected HIV-infected ART-naïve pregnant women in North central Nigeria (Imade *et al*., 2014). Acquisition of PI resistance is known to be cumulative in nature i.e. involving gradual build-up of mutations in the setting of ongoing subjection of the virus to suboptimal, non-suppressive protease inhibitor-based regimen. Therefore the high level of IAS PR mutation detected among the patients in our study is a serious course for concern as it is a prelude to acquisition of additional PI resistance mutations.

M46L was the most predominant major PI resistance mutation (7.14% of all the isolates) found in this study with a higher rate of 16.7% among subtype G isolates. This is also similar to the report of Odaibo *et al*. (2013) which reported this mutation at a frequency of 55.6%. M46I/L is nonpolymorphic PI-selected mutation that reduces susceptibility to indinavir (IDV), nelfinavir (NFV), fosamprenavir(FPV), lopinavir **(**LPV) and atazanavir (ATV) in company of other mutations. It also reduces susceptibility to tipranavir (TPV). Although our study did not determine the phenotypic resistance pattern in the infected individuals, analysis according to the Stanford algorithm showed that M46L mutation confers potential low-level resistance to ATV/r, FPV/r, IDV/r, LPV/r, TPV/r and intermediate-level resistance to NFV to isolates in this study as shown in table 4.14.

Another major PI resistance mutation found among the isolates in this study is V82L which occurred at a rate of 6.3% among CRF02_AG isolates. Mutations at positions 82 and 88

generally co-exist and contraindicates many PIs particularly NFV (Potdar *et al*., 2011). In line with this, the only isolate with V82L mutation in this study, NG_IM.12_07, also harbors N88D mutation in addition to L10I and K20I mutations. The V82L mutation is shown to confer low-level resistance to ATV/r, FPV/r and SQV/r as well as potential low-level resistance to IDV/r and LPV/r while it confers intermediate-level resistance to NFV and TPV/r as shown in table 4.14.All the patients had preserved susceptibility to DRV/r since it is the only PI drugs analysed that was not selected by any resistant mutation. Similar observation had been reported in a more widespread study in Nigeria which examined the effect of maintaining patients on failing second line ART on the accumulation of PR mutations (Rawizza *et al*., 2013). Isolates in this study also showed high level of susceptibility (96.4%) to SQV/r. The only isolate with intermediate-level resistance to these drugs had V82L major PI resistance mutation as well as L10I and N88D minor PI resistance mutations. The V82L major resistance mutation identified in this isolate is an uncommon non-polymorphic substrate-cleft mutation that reduces susceptibility to TPV. This mutation was reported at a frequency of 22.2% in a similar study by Odaibo *et al*. (2013).

Drug resistance mutations usually emerge as a result of factors such as drug pressure,none adherence to drug prescription and drug absorption rate that lead to circulation of sub-optimal blood level of drug (WHO, 2012), resulting in failure of treatment, progression of disease and the potential for transmission of resistant virus. Transmitted drug resistance (TDR)occurs whenviruses with drug resistance mutations are transmitted horizontally from treatment-experienced patients to newly infected persons.This type of transmission has been observed continuously in countries where the use of antiretroviral drugs has been on for a long period.A 20-country survey by WHO reported the rate of transmission of drug resistance as 3.7% (Hamers *et al*., 2012).Reports also show that this rate is on the increase globally (WHO, 2012). Since all the patients in this study are drug experienced, it can be argued that the observed drug resistance mutations could have beenacquired as a result of one or more of the earlier mentioned factors rather than transmitted.It is however difficult to ascertain if the mutationsare as a result of non-adherence to medication or drug selection pressure as the level of adherence by the patients was not determined. The different degree of resistance mutations to the second line PI drugs observed among these isolates is worrisome as this is a prelude to treatment failure and further limit the choice of treatment regimen available for the patients care.This result therefore underscores the need for periodic genotypic DR testing of

patients on ART for early detection of drug resistance mutations. This will aid in the selection of appropriate treatment regimens and forestall incidences of treatment failure usually seen among these patients. The only challenge however is the high cost of carrying out these tests which may limit its implementation in resource limited settings.

Our study also revealed high levels of minor mutations in the *protease* gene of the isolates with the most frequent mutations occurring at positions L10I/Vand K20I.Earlier, similar results were reported among patients who are yet to commence antiretroviral therapy in Jos, Northcentral Nigeria in a study that investigated their HIV-1 genetic variability and the level of their accompanying antiretroviral drug resistance mutations (Anejo-Okopi*et al*., 2013).Although occurrence of this type of mutations in the absence of other forms of mutations are not known to cause high level of ARV resistance, their presenceshould be taken into account in deciding treatment options because of their potential in improving viral fitness.

Pol gene polymorphisms usually occur in non-B HIV-1 strains as genetic fingerprints that lowers their susceptibility to ARV compounds (Holguin *et al*., 2002).Other mutations/polymorphisms that occurred at very high frequencies among patients in this study include; I13V/A, K20I, E35Q, M36I/L, R41K, R57K/G, L63T/P/S/Q, C67E/S, H69K/R, V82I and L89M. Similar mutations/polymorphismsin the protease region had been reported earlier for some Nigerian isolates (Agwale *et al*., 2006; Chaplin *et al*., 2011; Rawizza *et al*., 2013). I13V/A, K20I,M36I/L, R41K, H69K/R and L89M are the consensus mutationsidentified for subtypes G, U^G and CRF02_AG while E35Q, R57K/G, C67E/S and V82I are the consensus mutations for G and U^G in this study. A study on PI-naive Nigerian HIV-patientshave earlier identified I13V, M36I and H69K as wild-type consensus mutationsfor HIV-1 subtypes G′, G, CRF02_AG, CRF06_cpx, and A (Chaplin *et al*., 2011).The same study also identified K20I as consensus for G′, G, CRF02_AG, and CRF06_cpx while V82I was identified as the consensus for G′ and G.Although our study utilized samples from drug experienced individuals,the findings are consistent with these earlier reports from drug naïve individuals.In addition, the mutations K14R, N37D/S/E/H and L63T/P/S/Q occurred in ≥ 25% of subtype G, U^G and CRF02_AG patients, at a significantly higher proportion than in subtype B. Similarly, mutations L10V/I, L19P, E35D, I64L/M and K70R appeared in ≥ 25% of CRF02_AG patients also at a significantly higher rate than in

subtype B in this study. There is already efficacy concern about treating patients infected with non-subtype B strains of HIV-1 with PI-based regimen as a result of higher frequency of polymorphism in the protease gene of non-B isolates (Wainberg, 2004).

The high propensity of HIV-1 to become resistant to different classes of antiretroviral drugs necessitated the search for more alternative regimens. CCR5 inhibitors example Maraviroc is one of the alternative antiretroviral drugs whose introduction as component of antiretroviral therapy has generated interest in HIV-1 coreceptor usage (tropism).HIV-1 generally uses CCR5 chemokine receptor in addition to CD4 receptor early in infection for entry into the target cell. Later in infection however, the ability to use CXCR4 instead of CCR5 can also arise as a consequence of viral genetic changes over time.In addition, virus clones that have the ability to use both coreceptors exist. Ability to use CXCR4 (either alone or with CCR5) correlates with rapid decline in the CD4 T-cell count,faster progression of disease and shortened survival time after AIDS diagnosis (Fouchier*et al*., 1995).The sequences responsible for determining T-cell and macrophage tropism are located primarily in the V3 loop.

Although recombinant virus phenotypic entry assay is the "gold standard" for characterization of HIV-1 tropism, genotypic methods based on analysis and characteristics of V3 amino acid sequence have been successfully used for the characterization of HIV-1 subtype B (Monno *et al*., 2010). Certain characteristics of *env*V3 that have been shown to have strong association with CXCR4 usage include: presence and accumulation of CXCR4-associated mutations, insertions and deletions in V3, the presence of basic amino acids at positions 11 and/or 25 of V3 (known as 11/25 rule), the loss of glycosylation site and an increased positive amino acid charge (≥ +6) (Holm-Hansen *et al*., 2000). Furthermore, this genotypic method has been applied and found appropriate for analyses of none subtype B HIV-1 isolates (Monno *et al*., 2010). In addition, genotypic method is easier to do, more cost effective and have been found to be useful as screening strategy in medical practice (Chueca *et al*., 2009). In view of this, this study also illustrates and interprets patterns in V3 loop sequence of the HIV-1 envelope gene of the isolates under study using genotypic methods.

Sequence characteristics of the V3 amino acid sequences of HIV-1 isolates in this study show that 12 out of the 14 isolates are 35 amino acids in length which is typical of V3 loop. Two of

the isolates had 36 amino acids with insertions at positions between 3 & 4, 9 & 10 and 14 &15 of the V3 loop, with amino acids I and G which is consistent with previous study (Coetzer *et al*., 2006). Some regions of the V3 loop of HIV-1 sequences analysed in this study showed sequence conservation while others showed sequence variability. Sequence conservation in the V3 region across different HIV-1 subtypes has been reported as being consistent with a V3 interaction with an invariable cellular protein (Patel *et al*., 2008). Sequence conservation was observed in the base region of V3 (1-9 and 26-35) and tip (15-17) in the different subtypes in this study while the stem and turn regions showed sequence variability. The sequence variability in the stem and turn regions is as a result of site specific mutations and has been identified as an important determinant of CXCR4 coreceptor usage (Kanki *et al*., 1999). Subsequently,in this study more sequence variability at either side of the V3 loop crown motif occurred among the predicted CXCR4-using viruses.

All but one of the isolates in this study had a potential *N*-glycosylation site at positions 6-8 within the V3 region. Three of the sequences in this study lack the *N*-linked glycosylation site located at the 5′ end of the V3 loop.It has been observed that most early viruses with the abilty to use CCR5 have this glycan which strongly suggest that it is needed for CCR5 interaction (Polzer *et al*., 2002). Hence all the predicted R5 isolates in this study have this glycan. Viruses that lack this glycan and are also able to use CXCR4 as coreceptor emerges following diminishing immune pressure as a result of disease progression (Pollakis *et al*., 2004). Additionally, loss of this glycan has been shown to aid in more efficient use of CXCR4 coreceptor and may enhance tropism switch from CCR5 to CXCR4 (Polzer *et al*., 2002). It is therefore not surprising that all the isolates without this glycan in this study (4/14) are predicted CXCR4-tropic.

It has been shown that the V3 loop of HIV-1 has a highly conserved crown motifand the GPGQ motif is generally associated with African HIV isolates and occurs irrespective of coreceptor usage (Chesebro *et al*., 1992). This accounted for about 71.4% of the crown motifs recorded in this study. On the other hand, GPGR crown motif is found in HIV-1 subtype B irrespective of coreceptor usage. In HIV-1 subtype C however, CCR5-tropic viruses have conserved GPGQ sequence tip while CXCR4-tropic viruses have a substitution which commonly presents as GPGR (Jensen*et al*., 2006). In this study, substitution from GPGQ to GHGQ/RRGQ/GPGR was observed in 28.6% (4/14) of the HIV-1 subtypes and CRFs

analysed in the V3 loop sequences. These isolates were predicted as CXCR4-tropic. This result agrees with previous studies. Duri *et al*. (2011) have earlier reported an association between amino acid substitution in this V3 loop crown motif from GPGQ to GPGR and CXCR4 coreceptor usage. In line with this, an earlier study in Ibadan, Nigeriaalso reported substitution in this crown motif in about 18.8% of the HIV-1 isolates predicted as CXCR4-tropic (Donbraye *et al*., 2015).

Two mutational pathways are known to cause viral escape from CCR5 antagonists namely selection of R5X4/X4-tropic viruses or development of resistance to the antagonists (Marcelin *et al*., 2009). Using the combined criteria, about 57% (8/14) of the HIV-1 isolates analysed in the V3 region in this study are CCR5-tropic and represents potential candidates for coreceptor antagonists. The remaining 43% (6/14) are CXCR4-tropic representing isolates which will not respond favourably to treatment with CCR5 antagonists. Analysis shows that 16.7% (1/6) of the predicted CXCR4-tropic viruses had Lysine (K) at position 11 of the V3 loop. The presence of this basic amino acid, Lysine or Arginine at either position 11 and /or 25 of the V3 loop otherwise known as 11/25 rule has been identified as a determinant of CXCR4 coreceptor usage of HIV isolates (Fouchier *et al*., 1995).

Further analysis of the amino acid sequences of the HIV-1 strains in this study showed that 1 V3 loop sequence had a net charge of +6 as well as substitution of GPGQ (Glycine-Proline-Glycine-Glutamine) crown motif to RRGQ (Arginine-Arginine-Glycine-Glutamine). High V3 loop amino acid net charge with a loss of one or more *N*-linkedglycosylation site have been shown to be a characteristic of CXCR4-tropic viruses (Coetzer *et al*., 2006).

Bioinformatics analysis with Geno2Pheno (a web-based software)at 10% false positive rate (FPR) predicted 4 sequences (including sequences that met the other criteria) as X4 viruses. This corroborates earlierreport in a similar study in Ibadan, Nigeria (Donbraye *et al*., 2015). This method have been extensively used by other researchers and found highly suitable. In a Brazilian studyon HIV-1 infected patients which aimed at exermining the use of Geno2Pheno programme for tropism prediction and its usefulness as an alternative method in clinical practice for screening CCR5 antagonist therapy candidates, Geno2Pheno method was compared with the trofile assay (phenotypic assay) and was found most suitable (Arruda *et al*., 2014).

Among the predicted CCR5-using isolates, one (12.5%) had the combination of mutations known to confer resistance to coreceptor antagonists. This therefore suggests that maraviroc will be relevant in the management of 87.5% of the patients infected by the CCR5-tropic viral isolates in this study while the remaining 12.5% will not be successfully managed by the drug.Contrary to previous study in North-central Nigeria (Ajoge *et al*., 2011) which predicted higher X4-tropic viruses among subtype G isolates, subtype G isolates in this study are predicted to be more of R5-tropic. This result shows that individuals infected with subtype G are more likely to benefit from coreceptor antagonists like maraviroc than those infected with CRF02_AG in this study.The occurrence of significant amount of predicted X4-using viral isolates in this study is indicative of the advanced stage of the infection among the patients as nearly all the patients are on antiretroviral therapy at the time of sampling. This is further evidenced with the occurrence of resistance conferring mutations to coreceptor antagonists among the CCR5-tropic HIV-1 isolates in this study and underscores the need for the determination of coreceptor usage ability of patients′ viral population before clinical administration of coreceptor antagonists like maraviroc.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATIONS

This study shows the presence of HIV-1 subtypes G and CRF02_AG in the three genomic regions of *gag*, *protease* and *env* C2-V3 as the predominant subtypes circulating among the study population. The study also shows the circulation of HIV-1 subtype A to a lesser extent among the study population. HIV-1 subtype J was also identified for the second time in the *env* C2-V3 genomic region suggesting trans-border transmission.In addition, there were unclassified sequences in the *gag* and *protease* regions but preliminary analysis of other regions of the isolates suggest they are new recombinant forms. This together with the high level of discordant subtypes among the isolates that were sequenced in all three and two fragments indicates that the level of viral heterogeneity in the study area is more complex than initially thought and has the potential of impacting negatively on the proposed candidate vaccine in Nigeria.

Resistance to the three common antiretroviral drug classes namely; NNRTIs, NRTIs and PIs is largely attributed to acquisition of resistance-confering mutations in the *pol* gene. Our result shows the presence of major PI-resistance mutations in a proportion of the isolates analysed and minor PI resistance mutations in all the isolates cutting across the different subtypes identified in this study. The different resistance patterns on the different PI drugs according to the Stanford algorithm were also highlighted.

Our result also shows existence of both CCR5- and CXCR4-tropic viral strains in significant proportions among the study population. X4 viruses in this study is characterized by loss of *N*-linked glycosylation sites, substitution of the GPGQ crown motif, insertions and mutations in the V3.

Three genomic regions approximating about 1.5kbp were analysed in this study which falls short of the total 9.7 kb of the entire HIV-1 genome. With this shortcoming, some sequences may not be accurately classified as evidenced by the few sequences that were designated as unclassified in this study. Additional work is therefore required involving full-length genome sequencing to fully caharacterize the viruses circulating in Southeastern parts of Nigeria.HIV-1 epidemic have been characterized to some extent in Northern and Southwestern Nigeria.

This study however adds to the knowledge of the nature of the epidemic and genetic diversity in South-eastern Nigeria.

Continuous monitoring and surveillance is needed to keep track of the epidemic and identify emergence/introduction of new/novel strains and stem outbreak of new epidemic. The information generated will help in formulation of policies and planning of preventive measures. Drug resistance analysis will also provide the much needed data that will assist in procuring the right medication and ultimately to the better management of infected persons. The identification of the different phenotypic variants in this study and few earlier studies in different parts of the country calls for more extensive study in the country before the introduction of CCR5 antagonists in clinical administration.

In conclusion therefore, continuous monitoring and surveillance of HIV-1 epidemic is necessary because of its implications for diagnosis, treatment and production of effective vaccine

RECOMMENDATIONS

More extensive and sustained study which will cover the entire country to fully identify all the circulating strains and monitor the evolving nature of the virus is recommended. Response to therapy of the various strains should also be determined to guide better management of the patients. Lastly, the data generated will guide decisions about proposed candidate vaccine in the country.

MAJOR CONTRIBUTIONS TO KNOWLEDGE

1. This study have showed that multiple subtypes of HIV-1 circulates in south-east Nigeria with predominance of subtypes G and CRF02_AG and should be considered in the design of candidate vaccine
2. This study have also showed that HIV-1 subtype J circulates in south-east Nigeria with phylogeny suggesting trans-border transmission from Cameroon
3. There are also some new recombinant forms which are yet to be fully genotyped
4. Major protease drug resistant strains of HIV-1 are circulating among patients in the region

5. Also, this study have shown that high percentage of the HIV-1 in circulation in south-east Nigeria are CXCR4-tropic and should be considered when introducing CCR5 inhibitor like maraviroc

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8.0 APPENDIX

Appendix 1

QUESTIONNAIRE

IRC Research Number: .....

MOLECULAR CHARACTERIZATION OF CIRCULATING HIV STRAINS IN SOUTH-EASTERN NIGERIA

Dear Sir/Ma,

Good day to you. I am a student majoring in Virology of the faculty of Basic Medical Sciences, College of Medicine, University of Ibadan. I am carrying a study focusing on the characterization of HIV strains circulating in eastern Nigeria. It is aimed at identifying the strains in circulation which will aid in proper diagnosis, monitoring, management, and possible vaccine development and improving the lives of those living with HIV and AIDS. It is going to take about 20 minutes to fill the questionnaire. I kindly solicit for your support and participation by answering the questions contained in the questionnaire.

Your response will be treated with utmost confidentiality.

Thanks for your anticipated cooperation and God bless you.

FOR OFFICE USE ONLY Serial No:

Please tick the box or fill the blank spaces to give the information most applicable to you.

Health facility..... Town.....

State..... Date.....

A. Socio Demographic Characteristic

1. Patient's names..... Address.....
2. Age.....
3. Sex (i) Male..... (ii) Female.....
4. Marital status (i) single....., (ii) married....., (iii) separated....., (iv) Widow....., (v) Widower.....
5. Ethnic group.....
6. Occupation.....

7. Educational qualification:

   (i) Primary.... (ii) Secondary.... (iii) Tertiary.... (iv) No formal education....

B. Clinical Data

8. When did you become aware of your status? .....
9. How often do you visit the centre? .....
10. Have you commenced antiretroviral drug treatment? .....
11. Which drug(s) are you presently on? .....
12. CD4+ count on commencement of treatment.....
13. Present CD4+ count.....

Thank you very much for your cooperation.

Appendix 2: PREPARATION OF TE BUFFER

The working strengths of TE buffer (10X and 1X) were prepared by dispensing 5ml of TE 100X concentrate into a 50ml centrifuge tube, 45ml of ddH_2O added and then mixed properly by vortexing to give 10X. Then 5ml of 10X TE buffer was dispensed into 50ml centrifuge tube, 45ml of ddH_2O added and vortexed to give 1X TE buffer. Both the 10X and 1X TE buffer were stored at room temperature.

Appendix 3: PREPARATION OF EQUILIBRATED PHENOL

The crystalline phenol bottle was brought out from freezer and allowed to thaw in the water bath at 60^oC. Twenty millilitres of phenol then was dispensed into a tube and equal volume of 10X TE buffer was added. The tube was agitated vigorously and left to stand on the bench for 10 minutes to allow the layers to separate properly. The aqueous (top) layer was aspirated and discarded using a Pasteur pipette. A second equal volume of 10X TE buffer was added to the tube, agitated vigorously, and the aqueous layer aspirated and discarded. Equal volume of 1X TE buffer was then added and the process was repeated as above. A moderate layer of 1X TE buffer was left above the phenol after the final aspiration of the second round and the pH which is expeted to be 7.8 was checked using a pH meter.