CHAPTER 1

INTRODUCTION

The magnitude of HIV infection and diversity in sub-Saharan Africa is more than that seen on other continents. About 70% of the world's HIV-infected adults live in sub-Saharan Africa (UNAIDS, 2010) and over 75% of all HIV deaths since the beginning of the epidemic have occurred in Africa. In many countries, the epidemic is generalized, affecting the general population at rates seen only among high risk populations elsewhere (UNAIDS, 2000; Kristensen *et al.,* 2002).

Historically, the first HIV-1 isolated and characterized were from the United States and Europe (Barre-Sinousi *et al.*, 1983; Gallo *et al.,* 1984). In the early 1990s, it was discovered that multiple HIV-1 subtypes existed, but surprisingly the U.S. and European epidemics resulted from only subtype B (Kanki *et al.*, 1999). To date, the remarkable molecular diversity of HIV-1, maintained and sustained through mutation and recombination, has resulted in the classification of the virus into 4 phylogenetic groups (M, N, O and P). The Group M consists of at least 9 different subtypes (A, B, C, D, F, G, H, J, and K), 55 circulating recombinant forms (CRFs) and many unique recombinant forms (McCutchan, 2006; Hemelaar *et al.*, 2006, Delgado *et al.*, 2010; Fernandez-Garcia *et al.*, 2010; Korber *et al.*, 2012).

The global distribution of HIV is complex and dynamic with continental, regional and national epidemics harbouring only a subset of the global diversity (McCutchan, 2006). Africa, in particular West-Central Africa, shows the greatest molecular diversity or heterogeneity of HIV (Ishikawa *et al.,* 1996; Kanki *et al.,* 1999; Hamel *et al.,* 2007; Sankale *et al.,* 2007; Powell *et al.,* 2010). Six strains account for more than 85% of HIV infections worldwide: HIV-1 subtypes A, B, C, D, and two of the CRFs, CRF01-AE and CRF02_AG

(McCutchan, 2006; Hemelaar, 2006). Many of the known subtypes and circulating recombinant forms are still rare in the epidemic (Berger *et al*., 1998; McCutchan, 2006).

The HIV-1 epidemic in African countries is largely due to non-B HIV-1 subtypes. Most of the diverse HIV-1 subtypes and HIV-2 have been described in sub-Saharan Africa where they are associated with the largest proportion of HIV infections worldwide (Kanki *et al.,* 1986; Ishikawa *et al.,* 1996; Kanki *et al.,* 1999; Hamel *et al.,* 2007; Sankale *et al.,* 2007; Powell *et al.,* 2010). It is still poorly understood why these distinct subtypes and their epidemics have emerged or how they will evolve over time (Hemelaar *et al.,* 2006).

The predominant HIV-1 subtypes circulating in West Africa are subtypes A and AG recombinants, including the circulating recombinant form CRF02_AG (Howard *et al.,* 1994; Ishikawa *et al*., 1996; McCutchan *et al*., 1999; Esteves *et al*., 2000; Sankale *et al*., 2000; Toure-Kane *et al*., 2000; Carr *et al*., 2001; Vidal *et al*., 2003; Hamel *et al*., 2007). New subtypes, subsubtypes, and CRFs continue to be identified, and the issue of recombination has become an important consideration in tracking the global spread of HIV.

In Nigeria, multiple subtypes and CRFs of HIV-1 and HIV-2 (Olaleye *et al*., 1993; Olaleye *et al*., 1995), have been reported. Studies conducted in different parts of the country have also shown the presence of different HIV-1 subtypes including A, A1, A2, B, C, D, G and G’, many circulating recombinant forms (CRFs) like CRF01_AE, CRF02_AG, CRF06_cpx, CRF09_cpx and CRF11_cpx and some unique recombinant forms (URFs) (Abimiku *et al*., 1994; Howard *et al.,* 1994; Peeters *et al*., 2000; Odaibo *et al*., 2001a; Agwale *et al*., 2002; Odaibo *et al*., 2006; Ojesina *et al*., 2006; Sankale *et al*., 2007; Ajoge *et al*., 2011; Chaplin *et al*. 2011).

There are, however, phenotypic implications for this great genetic variation in HIV types and subtypes, with differences observed among the different variants. The phenotypic

consequences of this genetic variation suggest differences in rates of transmission, replication competence, pathogenesis, disease progression, coreceptor usage, response to antiretroviral therapy and implications for vaccine development (Kanki *et al.,* 1999; Zolla-Pazner *et al*., 1999; Kaleebu *et al.,* 2002; Peeters, *et al.* 2003; Powell *et al*., 2010; de Mendoza *et al.,* 2009; Kiwanuka *et al.,* 2008; Chaplin *et al.,* 2011).

Comparative studies on HIV-1 and HIV-2 have shown differences, including reduced pathogenic potential and slower rates of disease progression in HIV-2 (Marlink *et al*., 1988; Marlink *et al*., 1994), lower rates of and a more restricted mode of transmission for HIV-2 (Kanki, 1991; Kanki *et al*., 1992; Kanki, *et al.,* 1997), lower viral load levels in HIV-2 (Popper *et al*., 1999), promiscuous chemokine coreceptor usage in HIV-2 (Morner *et al*., 1999), response to antiretroviral therapy (Ojesina *et al.,* 2006; Chaplin *et al.,* 2011) and protection from HIV-1 superinfection in primary infection by HIV-1 (Kanki *et al*., 1996).

Among the different subtypes of HIV-1, there are also documented differences in transmission routes. Subtype B viruses have been predominantly associated with HIV-1 infection in injecting drug use and male homosexual transmission while subtypes A, C, D, F, G, CRF01_AE, CRF02_AG, and CRF09_cpx are associated with heterosexual transmission (Ou *et al*., 1993; van Harmelen *et al*., 1997; Liitsola *et al*., 2000; Hudgens *et al*., 2002; Kalish *et al*., 2002; Brodine *et al*., 2003; Herring *et al*., 2003). In an *in vitro* study with epithelial Langerhans' cells involved in heterosexual transmission of HIV-1 by vaginal intercourse, CRF01_AE grew more efficiently than subtype B (Soto-Ramirez *et al*., 1996). Subtype specific differences have also been observed in vertical transmission of HIV-1 showing reduced fitness in perinatal transmission of subtype D compared to subtypes A and C (Renjifo *et al*., 1999; Renjifo *et al*., 2001).

It has been shown that different HIV-1 subtypes have different rates of disease progression. Data from a prospective study in Senegal suggest that HIV-1 subtypes may differ in rates of progression to AIDS. It was shown that women infected with subtypes C, D and G were 8 times more likely to develop AIDS than were those infected with subtype A (Kanki *et al*., 1999). Similarly, a study in Tanzania showed that envelope subtype D is associated with faster disease progression, compared with subtype A (Kaleebu *et al*., 2002). Even sub-subtype specific differences have been observed within a subtype as shown by a study in Brazil that showed that patients infected with closely related HIV-1 serotypes may differ in the rate of progression to AIDS (Santoro-Lopes *et al*., 2000).

Other studies have shown functional distinctions in promoter architecture among HIV-1 subtypes. Functional analysis of the enhancer region within the long terminal repeat (LTR) indicates that HIV-1C isolates have $\geq$3 NFk-B binding sites, unlike other subtypes, which have only 1 or 2 sites (Montano *et al*., 2000). Comparative analysis of the long terminal repeats of HIV-1 CRF01_AE and HIV-1C isolates showed subtype-specific differences in enhancer copy numbers and sequences, as well as divergent activation in response to the cellular transcriptional activators Rel-p65, NFATc and viral Tat (Montano *et al*., 1997). Another study by Nixon *et al.* (1998) reported that HIV-1 CRF01_AE isolates contained a defective upstream NFk-B site and a unique TATA-TAR region, compared to subtype B and also demonstrated that tumor necrosis factor alpha (TNF-alpha) stimulation of the HIV-1 CRF01_AE LTR was also impaired, consistent with a defective upstream NFk-B site.

Moreover, other studies have showed that there are clinical and immunological differences in patients infected by different HIV-1 subtypes. Neilson *et al.* (1999) reported that plasma viral RNA levels were highest in women infected with subtype C virus, and such women had significantly lower CD4 lymphocyte levels than women infected with the other subtypes. Hu

and colleagues (2001) reported that higher viral loads were associated with CRF01_AE compared to subtype B in Thailand. They postulated that this difference may be as a result of inter-subtype biological differences. Multiple viral genotypes, and CRF02_AG in women have been linked with significantly higher viral load and a significantly lower CD4(+) T-cell count in early HIV-1 infection (Sagar *et al*., 2003; Sarr *et al*., 2005).

On the relationship of HIV-1 subtypes and antiviral drugs, although increasing evidence suggests that all clades of HIV probably display similar sensitivity to antiviral drugs, strains from some subtypes and/or geographical regions may have a greater propensity to develop resistance against certain drugs than other viral variants (Spira *et al*., 2003; Wainberg, 2004). Drug resistance mutations in ART drug-naïve individuals, codon usage, polymorphisms (Ojesina *et al.,* 2006) and five RT mutations have been shown to be HIV-1 subtype-specific. According to Chaplin *et al.* (2011), these subtype-specific responses to HIV therapy may have significant consequences for efforts to provide effective therapy to the populations infected with these HIV-1 subtypes.

Furthermore, coreceptor usage by different HIV-1 subtypes has been studied and results also show that some subtype-specific or subtype-dependent differences exist in type and frequency of usage of certain coreceptors. Comparative studies have revealed that the scarcity of CXCR4 usage, as well as other phenotypic characteristics of subtype C isolates, distinguishes this subtype (Bjorndal *et al*., 1999) while dual tropism for CCR5 and CXCR4 was not found among subtype D isolates (Tscherning *et al*., 1998). Ajoge *et al,* (2011) showed a higher CXCR4 corecptor usage of HIV-1G in the North-Central geopolitical zone of Nigeria based on genotypic analysis of the V3 region.

It has been shown that early in infection, HIV-1 generally uses the CCR5 chemokine receptor (along with CD4) for cellular entry (Bjorndal *et al*., 1999). In many HIV-1 infected

individuals, viral genotypic changes arise that allow the virus to use CXCR4 (either in addition to CCR5 or alone) as an entry receptor (Kupfer *et al*., 1998) and this change is associated with accelerated CD4 decline and more rapid progression of HIV-1 disease (Koot *et al*., 1992). The V3 loop of gp120 largely determines coreceptor usage and the development of CXCR4 viruses is gradual and involves the accumulation of multiple amino acid changes in the V3 (Jensen *et al*., 2003). Some investigators in different countries have shown that coreceptor usage is largely affected by HIV-1 subtype (Ping *et al*., 1999; Kaleebu *et al*., 2007; Patel *et al.,* 2008; Raymond *et al*., 2008; Duri *et al*., 2011).

JUSTIFICATION

Multiple subtypes and CRFs of HIV-1 co-circulate in different states and regions of Nigeria as reported by previous work but the current circulating strains remain largely uncharacterized. There is also a dearth of information about HIV-1 subtype and coreceptor usage with the only reported data by Ajoge and colleagues (2011). The implications of these and their relationships to virus coreceptor usage in the country remain undetermined. There is also growing evidence of increasing viral resistance (primary and secondary – fuelled by both virus mutation and non-adherence issues) to commonly used anti-retroviral drugs available in the country and there remains a possibility that CCR5-inhibitors like Maraviroc, can be used for HIV-1 antiretroviral therapy.

SPECIFIC OBJECTIVES

This study was designed to achieve the following specific objectives

1. Determine the HIV-1 subtypes circulating among HIV-1 infected patients and detect, if any, the introduction of subtypes previously not described or reported in the country
2. Examine the dynamics and evolution of HIV-1 subtypes in the study area
3. Predict virus coreceptor usage of circulating HIV-1 subtypes in the study area by V3 amino acid sequence analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 HISTORY AND ORIGIN OF HIV/AIDS

Acquired immunodeficiency syndrome (AIDS) was first recognized as a new disease 30 years ago in the United States. Clinicians in Los Angeles (Gottlieb *et al*., 1981), San Francisco (Masur *et al*., 1981) and New York (CDC, 1982a), saw young, homosexual men with *Pneumocystis carinii* (now *P. jiroveci*) pneumonia (PCP) and Kaposi's sarcoma (KS), unusual diseases for young adults not known to be immunosuppressed.

All the individuals shared a profound depletion of CD4-positive T-helper lymphocytes. In 1982, the Centers for Disease Control and Prevention (CDC) published reports of Haitians without homosexual behaviour in several states in the United States with Kaposi's sarcoma and opportunistic infections (OIs) (CDC, 1982b) and, also reported on PCP among persons with hemophilia (CDC, 1982d). Other cases were later reported in hemophiliacs, recipients of blood transfusions (CDC, 1982e), infants (CDC, 1982f), female sexual partners of infected men (CDC, 1983a; Masur *et al*., 1982), prisoners (CDC, 1983b), and Africans (Clumeck *et al*., 1983). The first case in a transfusion recipient was also reported from San Francisco in an infant in late 1982. At first, the new disease was called gay-related immunodeficiency syndrome (GRIDS), but by September of 1982, the CDC had published a case definition, using the current designation of acquired immune deficiency syndrome (AIDS) in print, and it was rapidly adopted by researchers (CDC, 1982c).

The prominence of homosexual men and injecting drug users in the early cases of AIDS suggested an agent that was both blood borne and sexually transmitted, although early speculation about the etiology of AIDS included the hypothesis that all the patients were immunosuppressed because they had a history of drug use or multiple sexually transmitted

diseases or malnutrition (the "immune overload" hypothesis) (Sonnabend *et al.,* 1983). Majority of researchers thought that the likely agent was a sexually transmitted virus that would be found in the peripheral blood.

HIV was first isolated in France in 1983 by Françoise Barré-Sinoussi in the laboratory of Luc Montaignier as lymphadenopathy-associated virus (LAV) (Barre-Sinoussi *et al*., 1983) and was confirmed as the AIDS virus in 1984 by Gallo and colleagues who published four papers in one issue of *Science*, and designated their isolate HTLV-III (Gallo *et al*., 1984). The virus was also isolated in San Francisco in 1984 by Jay Levy who named his isolate AIDS-associated retrovirus (ARV) (Levy *et al*., 1984). All three of these designations for the virus appear in the early literature. Human Immunodeficiency Virus (HIV) was chosen as the designation of the virus by the International Committee on the Taxonomy of Viruses (ICTV) in 1986. Later in 1986, HIV-2 virus was discovered in West Africa and the original virus was then known as HIV-1 (Guyader *et al*., 1987).

By 1985, serologic assays had been developed to test for HIV infection (CDC, 1984). Early trials of antiviral treatments for HIV and immune modulators met with disappointment (Byers *et al*., 1994; Mildvan *et al*., 1991; Pert *et al*., 1986; Schooley *et al*., 1990). In 1987, zidovudine (AZT) was licensed and approved by the U.S. Food and Drug Administration (FDA) for the treatment of AIDS (Fischl *et al*., 1987). Early excitement over the life-extending effects of AZT did not last long, as patients treated with this single-drug therapy began to experience disease progression leading in most cases, to death. However, a greater understanding of the epidemiology, treatment, and prophylaxis of opportunistic infections (OIs) associated with HIV-induced immune deficiency led to significant life-saving advances, particularly in the areas of infection with *Pneumocystis jiroveci* and *Mycobacterium avium* complex (MAC) (Shafer *et al.,* 1989).

Protease inhibitors (PIs) were introduced in the mid-1990s and it revolutionized the treatment of HIV and AIDS (Hammer *et al*., 1997). In the late 1990s in the United States and Western Europe, effective combination antiretroviral therapy (ART) was introduced and soon became the standard of care when it began to be noticed that morbidity and mortality associated with HIV infection was sharply declining (Palella *et al*., 1998). Patients treated with combination ART showed precipitous decreases in the amount of HIV RNA circulating in their serum, indicating interference with HIV replication. It was also noticed that after successful inhibition of viral replication, CD4 T-cell counts began to increase in treated individuals (Ho *et al*., 1995). Studies began to show the value of HIV RNA measurement (viral load) as both a predictor of disease progression and a measure of treatment success thus corroborating the understanding of the dynamic interaction between viral replication and the host immune system, (Hughes *et al*., 1997; Mellors *et al*., 1997; O'Brien *et al*., 1997).

Potent therapy was not without complications, however; and the dogma of the late 1990s, "hit early, hit hard" (CDC, 1998), became balanced by realization that long-term medication toxicity was likely among individuals who were now living longer, healthier lives with HIV infection. Once again, the paradigm of HIV treatment underwent revision, and treatment was now recommended primarily for individuals with more advanced disease (Muesing *et al*., 1985).

Although AIDS was not recognized as a new clinical syndrome until 1981, researchers examining the earlier medical literature identified cases appearing to fit the AIDS surveillance definition as early as the 1950s and 1960s (Huminer *et al.,* 1987). Frozen tissue and serum samples were available for a 15-year-old black boy from St. Louis, USA, who was hospitalized in 1968 and died of an aggressive, disseminated KS (Garry *et al*., 1988). His tissue and serum specimens were HIV-antibody positive on Western blot and antigen-positive

on ELISA. This was the first confirmed case of HIV infection in the United States. The patient had no history of travel out of the country, so it was likely that some other persons in the United States were infected with HIV as long ago as the 1960s or earlier.

The origin of HIV-1 among non-human primates has been traced to a simian virus, SIVcpz, which infected several geographically isolated chimpanzee communities in southern Cameroon. This HIV-1 progenitor probably was passed from chimpanzees to human hunters through blood borne transmission. Phylogenetic analysis of HIV-1 and related viruses from nonhuman primates suggests that three independent transmission events early in the 20th century spawned three HIV-1 groups: major (M, between 1915 and 1941), outlier (O), and non major and nonoutlier (N) (Keele *et al.*, 2006; Korber *et al.*, 2000). Although strains related to the M and N groups have been found in chimpanzees, recent evidence suggests that group O HIV-1 may have originated in gorillas, in which the closest relatives of this group have been identified (Van Heuverswyn *et al.*, 2006). It is speculated that the virus then spread among humans along the Congo River into Kinshasa, Zaire, where the earliest documented case of HIV-1 infection (with group M strain) in humans has been traced to a blood sample from 1959 (Zhu *et al.*, 1998).

In 2009, a new human immunodeficiency virus in a Cameroonian woman was identified. It is closely related to gorilla simian immunodeficiency virus (SIVgor) and shows no evidence of recombination with other HIV-1 lineages (Plantier *et al.*, 2009). This new virus seems to be the prototype of a new HIV-1 lineage that is distinct from HIV-1 groups M, N and O. HIV-2, a second type of HIV rarely found outside western Africa, originated in Sooty Mangabeys.

2.2 CLASSIFICATION AND MOLECULAR EPIDEMIOLOGY OF HIV

The Human Immunodeficiency virus (HIV) belongs to the genus *Lentivirus* in the virus family Retroviridae (Gallo *et al.,* 1983). Members of the family Retroviridae contain the enzyme reverse transcriptase which uses the viral RNA as a template for synthesis of proviral DNA. This proviral DNA is then integrated into the chromosome of the host cell from which it is replicated during the host's cell replication (Coffin, 1990). Retroviruses have been associated with many diseases, including rapid and long latency malignancies or cancers, wasting syndrome, neurological disorders, and immunodeficiency as well as prolong viraemia in the absence of any obvious ill health (Coffin, 1990) (Table 1). Members of the genus *Lentivirus* are known as slow viruses because of the slow, progressive nature of the disease following prolonged sub clinical infection (Coffin, 1990). Simian immunodeficiency virus (SIV) and Feline immunodeficiency virus (FIV) also belong to this genus of the family Retroviridae (Figure 1).

HIV is a highly variable virus and based on phylogenetic analyses and pairwise genetic distance analyses of the env/gag gene, the numerous virus strains are classified into types, groups, subtypes, sub-subtypes and circulating recombinant forms (CRFs) (Nkengasong *et al*., 1994a; Robertson *et al*., 2000; Robertson *et al.,* 1995). Several factors contribute to the extraordinary genetic heterogeneity of HIV-1: (a) error-prone viral DNA synthesis during

TABLE 1: THE MEMBERS OF THE RETROVIRIDAE FAMILY

New name	Examples	Morphology
Alpharetrovirus	Avian leukosis virus (ALV) Rous sarcoma virus (RSV)	C-type
Betaretrovirus	Mouse mammary tumor virus (MMTV) Mason-Pfizer monkey virus (MPMV) Jaagsiekte sheep retrovirus (JSRV)	B-, D-type
Gammaretrovirus	Murine leukemia viruses (MuLV) Feline leukemia virus (FeLV) Gibbon ape leukemia virus (GaLV) Reticuloendotheliosis virus (RevT)	C-type
Deltaretrovirus	Human T-lymphotropic virus (HTLV)-1, -2 Bovine leukemia virus (BLV) Simian T-lymphotropic virus (STLV)-1, -2, -3	—
Epsilonretrovirus	Walleye dermal sarcoma virus Walleye epidermal hyperplasia virus 1	—
Lentivirus	Human immunodeficiency virus type 1 (HIV-1) HIV-2 Simian immunodeficiency virus (SIV) Equine infectious anemia virus (EIAV) Feline immunodeficiency virus (FIV) Caprine arthritis encephalitis virus (CAEV) Visna/maedi virus	Rod/cone core
Spumavirus	Human foamy virus (HFV)	Immature

SOURCE: Field's online edition

reverse transcription (approximately 10^{-5} mutations/nucleotide/replication cycle) (Preston *et al.,* 1988), (b) high recombination frequencies accompanying reverse transcription (Hu and Temin, 1990; Stuhlmann and Berg, 1992), (c) the high levels of progeny virus production in vivo (10^9 particles/day; 150 to 300 replication cycles/year) (Ho *et al*., 1995), and (d) large numbers of infected individuals (Coffin, 1995; Jetzt *et al*., 2000; Mansky and Temin, 1995; Perelson *et al*., 1996).

There are two types of HIV: HIV-1 and HIV-2 (Clavel *et al*., 1986). Both types are transmitted by sexual contact, through blood, and from mother to child, and they appear to cause clinically indistinguishable AIDS. However, it seems that HIV-2 is less easily transmitted, and the period between initial infection and illness is longer in the case of HIV-2. Worldwide, the predominant virus is HIV-1 with HIV-2 concentrated in West Africa and is not found widely (Kanki *et al*., 1987; Kanki *et al*., 1986).

The strains of HIV-1 can be classified into **four groups** (Robertson *et al*., 2000). Groups refer to the very distinctive HIV-1 lineages M (for Major), N (for non-M non-O), O (outlier) (Figure 2) and P (Plantier *et al*., 2009). These four groups may represent four separate introductions of simian immunodeficiency viruses into humans. The vast majority of HIV-1 strains found worldwide and responsible for the pandemic, belong to just one of these lineages, group M as shown in figure 4 (Peeters *et al*., 2000). The M group of HIV-1 consists of at least nine discrete clades or subgroups (A, B, C, D, F, G, H, J, and K) (Figure 1) (McCutchan, 2000) and 49 circulating recombinant forms (CRF) (Table 2) (Kuiken *et al*, 2010). The HIV-1 subtypes and CRFs are very unevenly distributed throughout the world, with the most widespread being subtypes A and C.

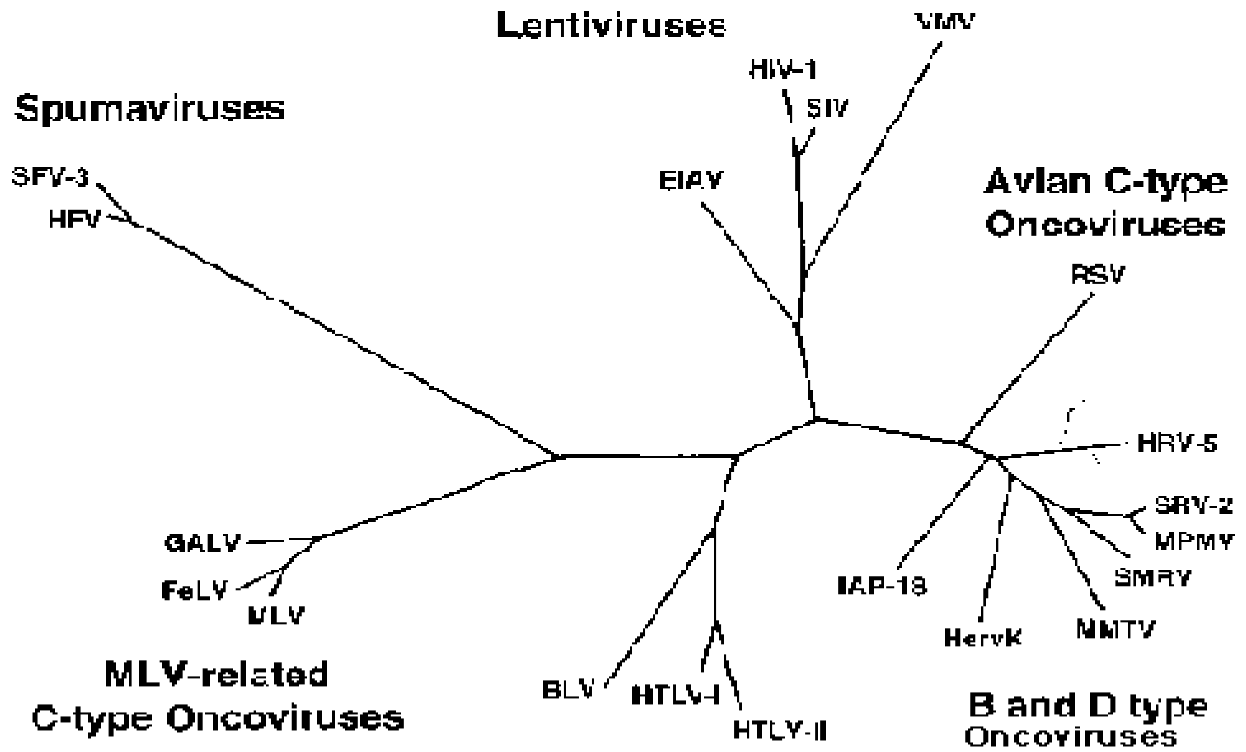

FIGURE 1: **PHYLOGENETIC TREE OF 19 ‘‘PROTOTYPIC’’ MEMBERS OF THE RETROVIRIDAE FAMILY**

SOURCE: Modified from Griffith *et al.* (1997).

- Subtype A and CRF A/G predominate in West and Central Africa, with subtype A possibly also causing much of the Russian epidemic.
- Historically, subtype B has been the most common subtype/CRF in Europe, the Americas, Japan and Australia. Although this remains the case, other subtypes are becoming more frequent and now account for at least 25% of new HIV infections in Europe.
- Subtype C is predominant in Southern and East Africa, India and Nepal. It has caused the world's worst HIV epidemics and is responsible for around half of all infections.
- Subtype D is generally limited to East and Central Africa.
- CRF A/E is prevalent in South-East Asia, but originated in Central Africa.
- Subtype F has been found in Central Africa, South America and Eastern Europe.
- Subtype G and CRF A/G have been observed in West and East Africa and Central Europe.
- Subtype H has only been found in Central Africa;
- Subtype J only in Central America; and
- Subtype K only in the Democratic Republic of Congo and Cameroon.

HIV–2 has been divided into at least 8 subtypes (group A – H) and one circulating recombinant form (CRF), AB (Table 2). Thus, recombination plays a prominent role in the emergence of a complex and dynamic HIV epidemic (Zhang *et al*. 2010). Groups A and B are epidemic. Group A spread mainly in West Africa, but also to Angola, Mozambique, Brazil, India and very limitedly to Europe or the US. Group B is mainly confined to West Africa (Taylor *et al.* 2008).

HIV-1 Group O appears to be restricted to West-central Africa and have been recovered from individuals living in Cameroon, Gabon, and Equatorial Guinea; their genomes share

approximately 65% identity with group M viruses (Gurtler *et al*., 1994; Nkengasong *et al*., 1994b; Nkengasong *et al*., 1993; Takehisa *et al*., 1999) (Figure 3). Group N HIV-1 strain - a strain discovered in 1998 in Cameroon- is extremely rare and fail to react serologically in standard whole-virus enzyme-linked immunosorbent assay (ELISA) (Roques *et al*., 2004). In 2009 a new strain closely related to gorilla simian immunodeficiency virus was discovered in a Cameroonian woman. It was designated HIV-1 group P (Plantier *et al*., 2009).

Within some subtypes, further phylogenetic structure can be identified, leading to a classification into subclades (sub-subtypes). Subtype F is subdivided into two subclades, F1 and F2 (Triques *et al*., 2000) and subtype A into three subclades A1, A2 and A3 (Gao *et al*., 2001a; Gao *et al*., 2001b; Meloni *et al*., 2004). The global distribution of HIV-1 is presented in Figure 4.

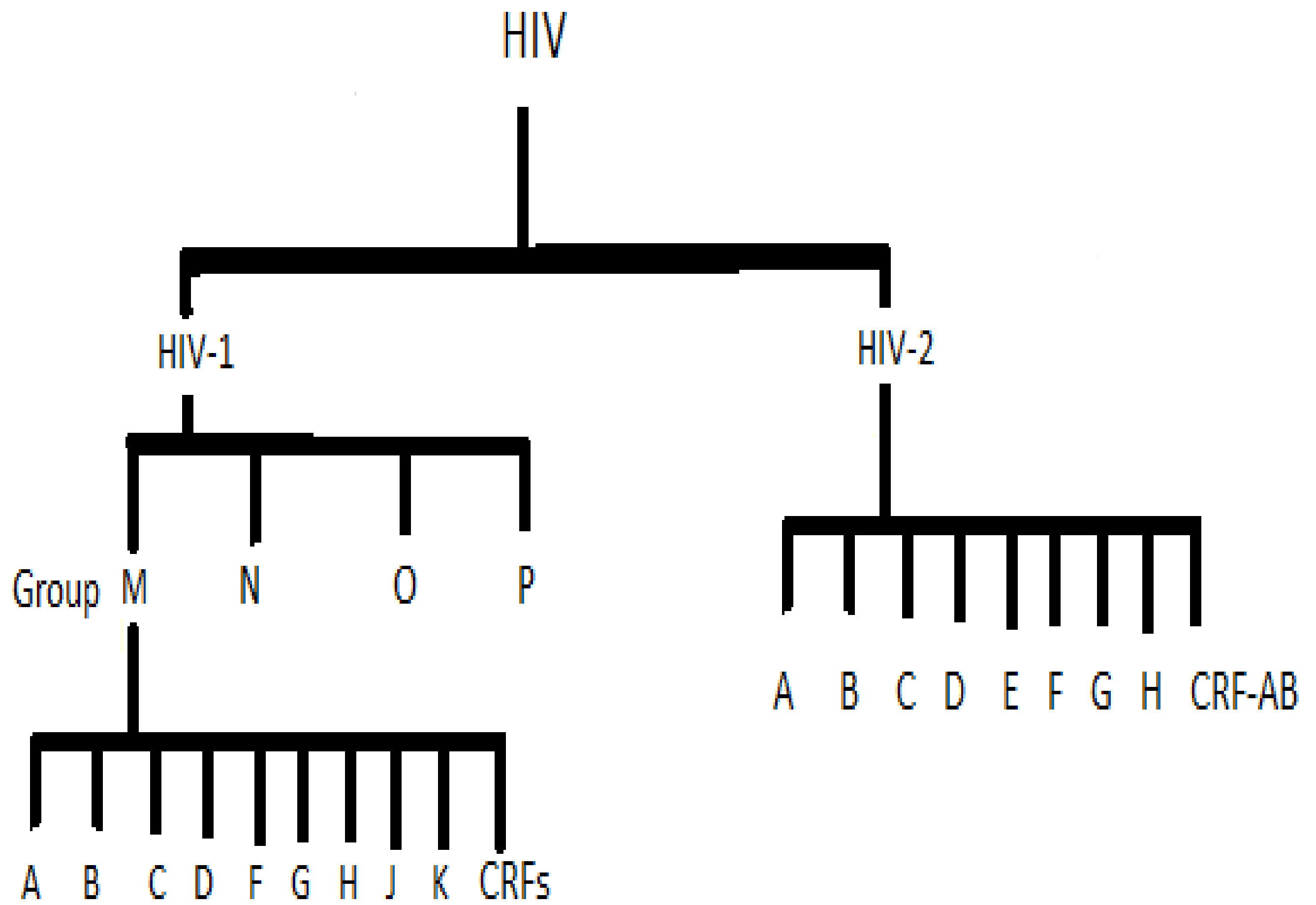

FIGURE 2: THE DIFFERENT LEVELS OF HIV CLASSIFICATION SHOWING TYPES, GROUPS, SUBTYPES AND CRFs

Source: Adapted from *http://www.avert.org*

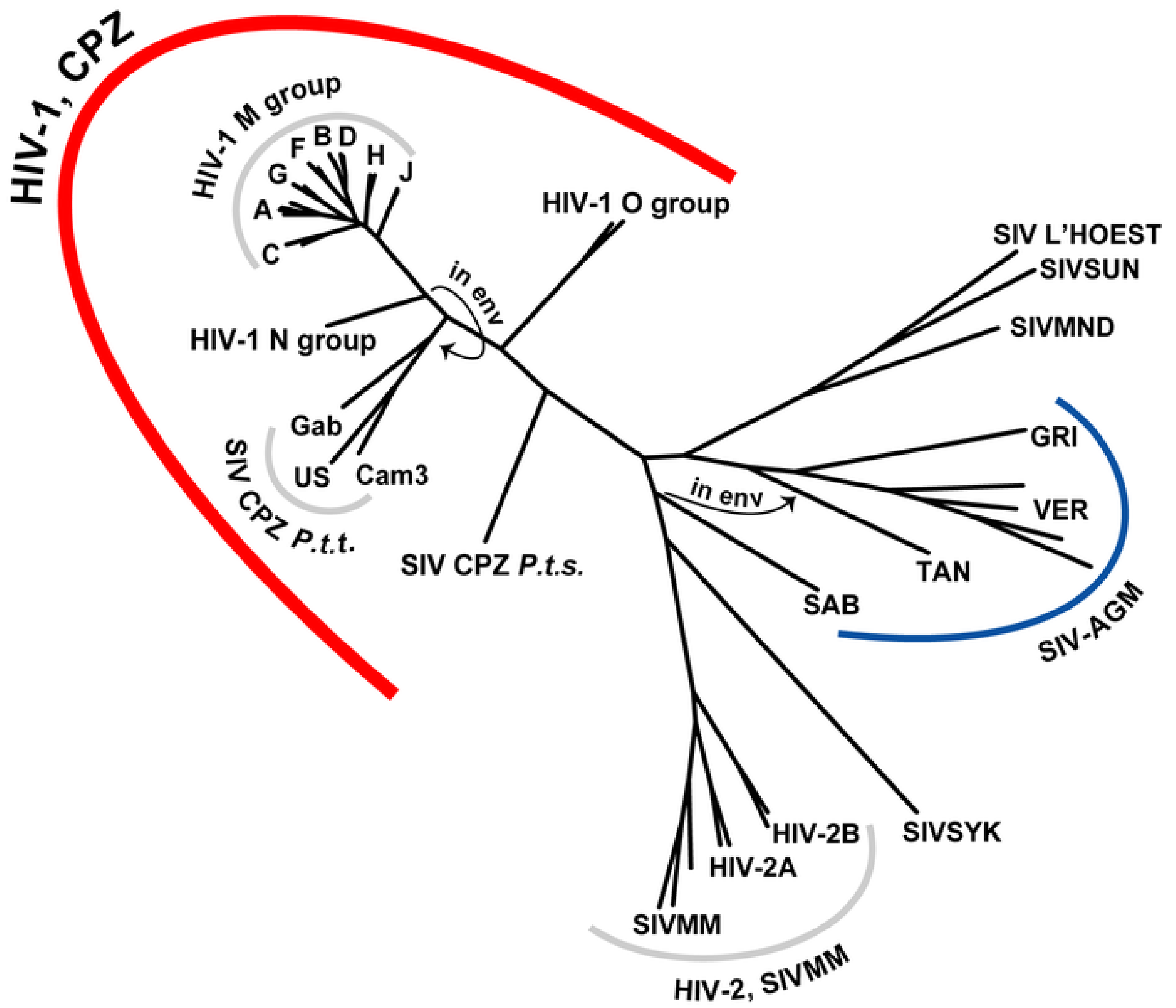

FIGURE 3: PHYLOGENETIC TREE OF THE SIV AND HIV VIRUSES

Source: http://
www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/1999compendium.html

TABLE 2: HIV CIRCULATING RECOMBINANT FORMS (CRFs)

HIV-1 CRF

Name	Reference strain	Subtypes	Author
CRF01_AE	CM240	A, E	J.K. Carr
CRF02_AG	IbNG	A, G	J.K. Carr
CRF03_AB	Kal153	A, B	K. Liitsola
CRF04_cpx	94CY032	A, G, H, K, U	D. Paraskevis
CRF05_DF	VI1310	D, F	T. Laukkanen
CRF06_cpx	BFP90	A, G, J, K	R. B. Oelrichs
CRF07_BC	97CN54	B', C	R. Wagner
CRF08_BC	97CNGX-6F	B', C	F.E. McCutchan
CRF09_cpx	96GH2911	A, G, U	F.E. McCutchan
CRF10_CD	TZBF061	C, D	I.N. Koulinska
CRF11_cpx	GR17	A, E, G, J, U	M. Peeters
CRF12_BF	ARMA159	B, F1	J.K. Carr
CRF13_cpx	96CM-1849	CRF01, A, G, J, U	K. Wilbe
CRF14_BG	X397	B, G	R. Najera
CRF15_01B	99TH.MU2079	CRF01, B	F.E. McCutchan
CRF16_A2D	97KR004	A2, D	U. Visawapoka
CRF17_BF	ARMA038	B, F1	J.K. Carr
CRF18_cpx	CU76	A1, F, G, H, K, U	M. Thomson
CRF19_cpx	CU7	A1, D, G	M. Thomson
CRF20_BG	Cu103	B, G	M. Thomson
CRF21_A2D	99KE_KER2003	A2, D	F.E. McCutchan
CRF22_01A1	02CMLT72	CRF01, A1	J.K. Carr
CRF23_BG	CB118	B, G	M. Thomson
CRF24_BG	CB378	B, G	M. Thomson
CRF25_cpx	02CM_1918LE	A, G, U	J.K. Carr
CRF26_AU	02CD_MBTB047	A, U	M. Peeters
CRF27_cpx	04FR-KZS	A, E, G, H, J, K, U	M. Peeters
CRF28_BF	BREPM12609	B, F1	R. Diaz
CRF29_BF	BREPM16704	B, F1	R. Diaz
CRF30_0206	00NE36	CRF02, CRF06	M. Peeters
CRF31_BC	04BR142	B, C	M. Soares
CRF32_06A1	EE0369	CRF06, A1	M. Adojaan
CRF33_01B	05MYKL007	CRF01, B	K.P. Ng & K.K. Tee

CRF34_01B	OUR2275P	CRF01, B	F.E. McCutchan
CRF35_AD	AF095	A, D	F.E. McCutchan
CRF36_cpx	NYU830	CRF01, CRF02, A, G	R. Powell
CRF37_cpx	NYU926	CRF01, CRF02, A, G, U	R. Powell
CRF38_BF	UY03_3389	B, F1	C. Lopez-Galindez
CRF39_BF	03BRRJ103	B, F1	M.G. Morgado
CRF40_BF	05BRRJ055	B, F1	M.G. Morgado
CRF41_CD	CO6650V1	C, D	S. Tovanabutra
CRF42_BF	luBF_13_05	B, F1	J-C. Schmit
CRF43_02G	J11223	CRF02, G	C. Brennan
CRF44_BF	CH80	B, F1	M. Thomson
CRF45_cpx	04FR.AKU	A, K, U	M. Peeters
CRF46_BF	01BR087	B, F1	S. S. Sanabani
CRF47_BF	P1942	B, F1	M. Thomson
CRF48_01B	07MYKT014	CRF01, B	Y. Takebe
CRF49_cpx	N28353	A1, C, J, K, U	T. de Silva & M. Cotten
CRF50_A1D		A1, D	G. Foster
CRF51_01B	HM021	CRF01, B	O.T. Ng
CRF52_01B	M043	CRF01, B	J. Li
CRF53_01B		CRF01, B	K.K. Tee
CRF54_01B		CRF01, B	K.K. Tee
CRF55_01B		CRF01, B	X. Han

HIV-2 CRF

Name	Reference strain	Subtypes	Author
HIV2-CRF01_AB	7312A	HIV2-A, HIV2-B	W. Sugiura

Source: ***http://www.hiv.lanl.gov/content/hiv-db/CRFs/CRFs.html***

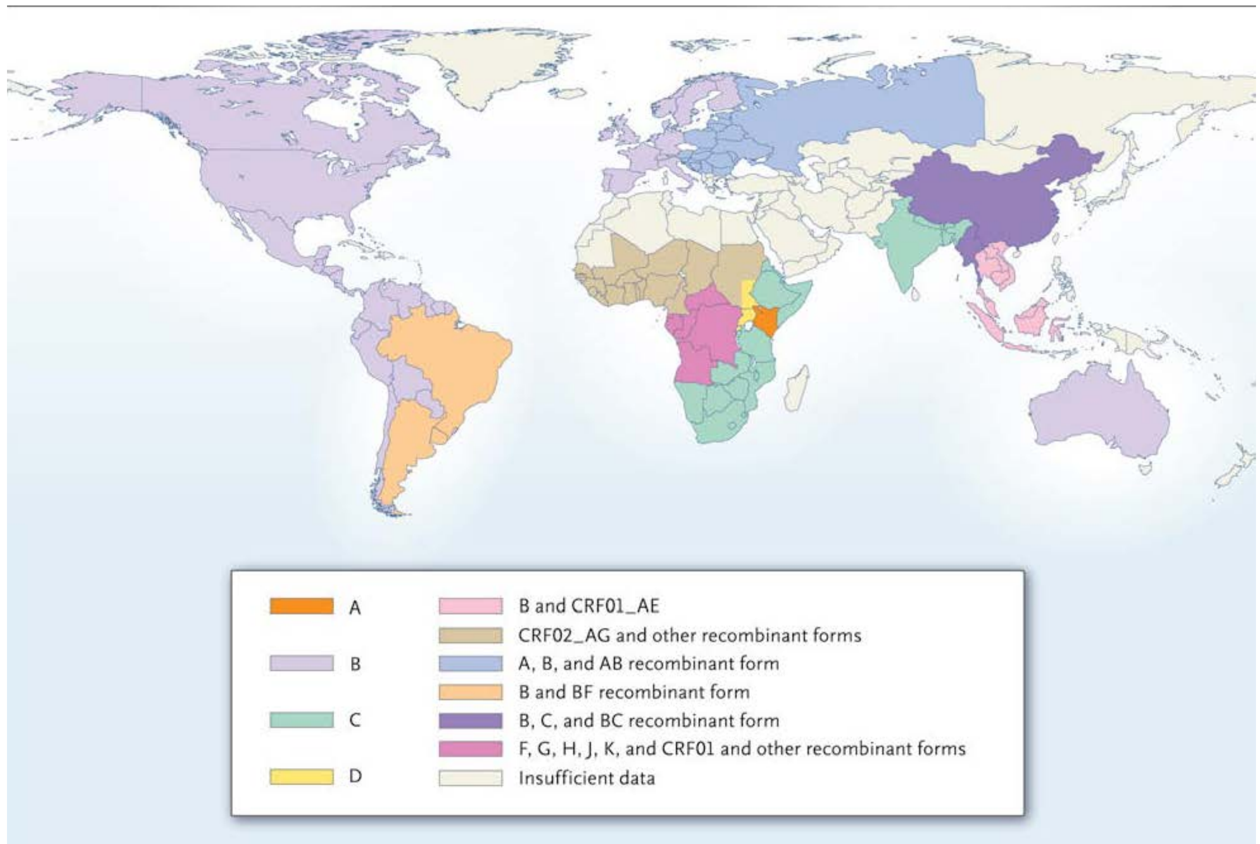

FIGURE 4: GLOBAL DISTRIBUTION OF HIV-1 SUBTYPES AND RECOMBINANT FORMS

Source: Taylor *et al.,* 2008

2.3 MORPHOLOGY OF HIV

2.3.1 HIV STRUCTURE

HIV is a spherical human retrovirus with a diameter of 120 – 200 nm (Briggs *et al*., 2003). The virions have an outer lipid bilayer (envelope) that surrounds a dense truncated cone-shaped nucleocapsid core (Benjamin *et al*., 2005).

Beneath the envelope there is a matrix protein, p17. The matrix is associated with the inner face of the bilayer (envelope) and is composed of approximately 4000–5000 copies of the N-myristoylated MA protein. Inside the matrix is an unusual conical particle (the core), whose conical outer shell (the capsid) is composed of approximately 1000–1500 copies of the viral CA protein (Briggs *et al*., 2004; Zhu *et al*., 2003).

Within the capsid is a ribonucleoprotein (RNP) particle composed of two copies of the positive-sense RNA genome (9.2kb) (Ratner *et al*., 1985), thousands of copies of the RNA binding NC protein, and approximately 250 copies each of the reverse transcriptase (RT) and integrase (IN) enzymes. The viral core also contains protease (PR), Vpu, Vif, Vpr and Nef, and some cellular factors (Hirsch and Curran, 1990; Montagnier and Clavel, 1994).

Protruding from the envelope are 72 viral glycoprotein spikes, gp160, which is made up of two component parts, gp120 and gp41, both of which are coded from the Env gene (Zhu *et al*., 2003) and play important roles in attachment and penetration of HIV into target cells.

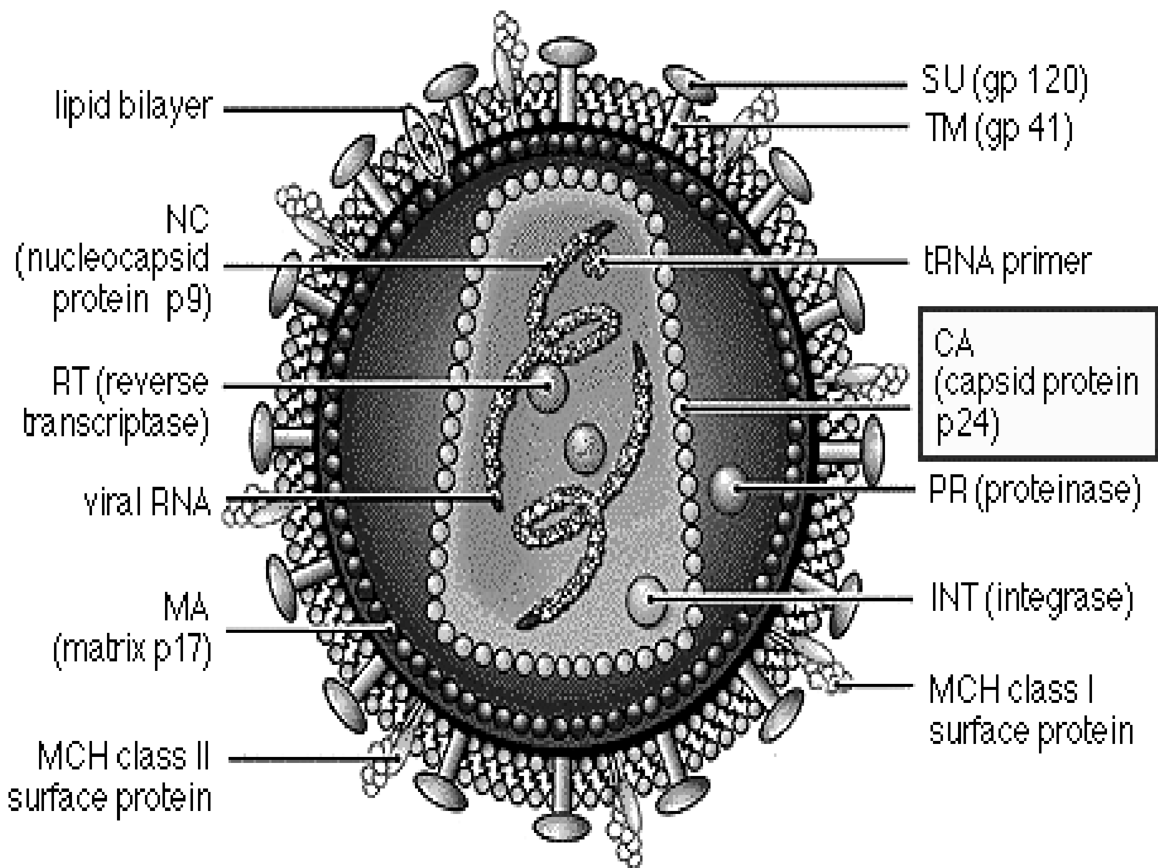

FIGURE 5: STRUCTURE OF HIV-1 VIRION

Source: Adapted from AIDS in Africa, 2007

2.3.2 HIV-1 RNA

The transcript produced from the viral promoter is approximately 9 kb long and may be thought of as a large macromolecular component of the virion containing structured subdomains throughout its length. Beginning at the 5' end, several essential regions have been defined [nucleotide numbers (nts) are approximate and vary among HIV-1 isolates] (Frankel and Young, 1998) (Figure 6):

1. The TAR hairpin (nts 1–55) is the Tat-binding site.
2. The primer-binding site (nts 182–199) is important for initiating reverse transcription by annealing to a cellular tRNALys.
3. The packaging signal or Ã (nts 240–350) binds NC and is critical for incorporation of genomic RNA into the virion (Clever and Parslow, 1997).
4. The dimerization site includes a "kissing loop" hairpin (nts 248–271) that facilitates incorporation of two genomic RNAs into the virion (Clever and Parslow, 1997; Laughrea *et al.*, 1997; Paillart *et al.*, 1996).
5. The major splice donor site (nt 290) is used to generate all subgenomic spliced mRNAs.
6. The Gag-Pol frameshifting region (nts 1631–1673) comprises a heptanucleotide slippery sequence and RNA hairpin that promote ¡1 ribosomal frameshifting, thereby translating a fused Gag-Pol polyprotein at a frequency of »5–10% (Cassan *et al.*, 1994).
7. The Rev response element (RRE) (nts 7362–7596) is the Rev-binding site.
8. Splice acceptor sites are present at several downstream regions of the RNA and allow production of a relatively large number of spliced products (the two major sites are at nts 5358 and 7971).
9. The polyadenylation signal (nts 9205–9210) is used to generate the 3' end.

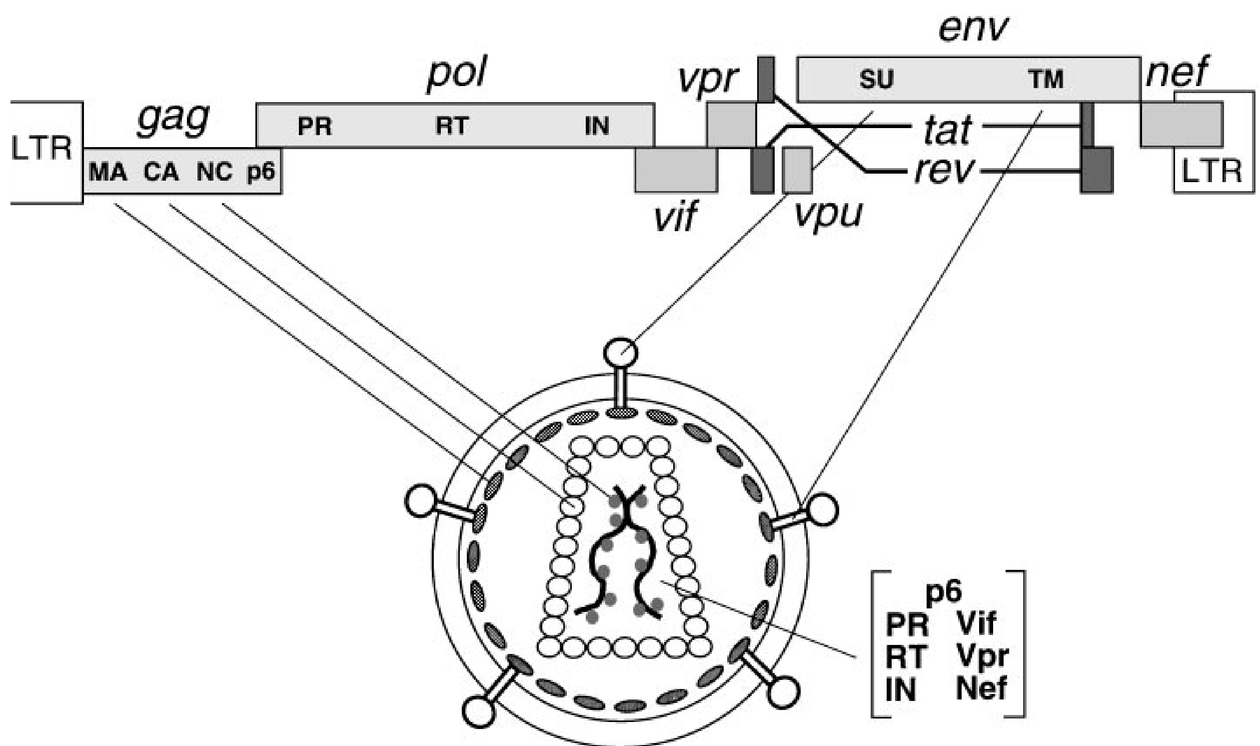

FIGURE 6: ORGANIZATION OF THE HIV-1 GENOME AND VIRION.

Source: Adapted from Frankel and Young (1998).

2.3.3 HIV-1 GENOME ORGANIZATION

With a genome of approximately ten thousand nucleotides, HIV-1 has packaged the necessary information in overlapping open reading frames to encode 15 proteins (Frankel and Young, 1998) from multiply-spliced mRNAs (Figure 7) that provide the unique characteristics to its infection (Coffin *et al.,* 1997).

Both ends of the provirus are flanked by a repeated sequence known as the long terminal repeats (LTRs), and most of the open reading frames overlap each other. The gag reading frame indicated by the provirus sequence extends from nucleotides 336-1,769 (Figure 7). The product of the pol gene is enclosed by the second large reading frame of the virus located between nucleotides 1,639-4-674 (Muesing *et al*., 1985). The Env gene product is encoded by the large open reading frame at nucleotides 5,782-8,370.

Analysis of HIV-1 transcripts revealed the presence of many differentially spliced mRNAs. The different HIV proteins are produced by specific mRNAs. The only exception is Env, which is produced together with Vpu by the same bicistronic mRNAs. Tat, Rev, Nef, and Tev proteins are produced by multiply spliced mRNAs and depend on Tat but not Rev for their expression. The other HIV-1 proteins require both Tat and Rev for efficient expression (Pavlakis, 1992).

The 5′ and 3’ untranslated regions of the HIV genome contains a noncoding, highly structured region that is involved in viral packaging, dimerization, pairing with the cellular tRNA primer for cDNA synthesis, and binding numerous viral proteins (Damgaard *et al*., 2004; Frankel and Young, 1998). Immediately downstream of this regulatory region, the HIV genome contains nine open reading frames coding for structural, regulatory and accessory genes. The HIV genome encodes a total of three structural proteins, two envelope proteins, three enzymes, and six accessory proteins (Frankel and Young, 1998).

From the 5'- to 3'-ends of the genome, after the non-coding region, are found the gag (for group-specific antigen), pol (for polymerase), and env (for envelope glycoprotein) genes, which are the structural genes that are subsequently proteolyzed into individual proteins common to all retroviruses (Purcell and Martin, 1993). The *gag* gene provides the physical infrastructure of the virus; *pol* provides the basic enzymes by which retroviruses reproduce; the *env* gene supplies the proteins essential for viral attachment and entry into a target cell.

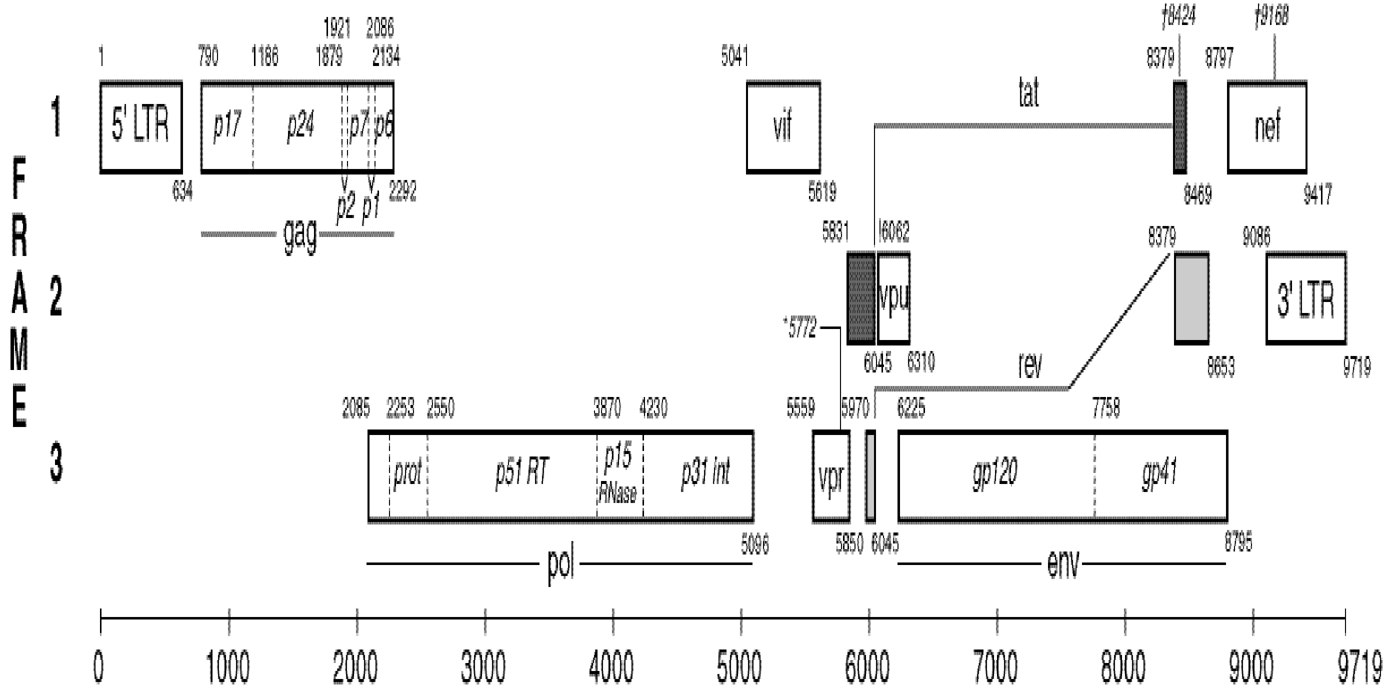

FIGURE 7: HIV-1 GENE MAP

Landmarks of the HIV-1 genome, HXB2 strain (above). Open reading frames are shown as rectangles. The gene start, indicated by the small number in the upper left corner of each rectangle, normally records the position of the a in the ATG start codon for that gene, while the number in the lower right records the last position of the stop codon. For pol, the start is taken to be the first T in the sequence TTTTTTAG, which forms part of the stem loop that potentiates ribosomal slippage on the RNA and a resulting -1 frameshift and the translation of the Gag-Pol polyprotein. The tat and rev spliced exons are shown as shaded rectangles. In HXB2, *5772 marks position of frameshift in the vpr gene caused by an "extra" T relative to most other subtype B viruses; !6062 indicates a defective ACG start codon in vpu; †8424, and †9168 mark premature stop codons in tat and nef.,

Source: Korber *et al.*, 1997 Numbering Positions in HIV Relative to HXB2CG in the database compendium,

2.4 HIV GENES AND THEIR FUNCTIONS

2.4.1 STRUCTURAL GENES

2.4.1.1 Gag

The gag gene gives rise to the 55-kilodalton (kD) *Gag* precursor protein, also called p55, which is expressed from the unspliced viral mRNA (Bryant and Ratner, 1990). After virus budding, p55 is cleaved by the virally encoded protease into four smaller proteins designated MA (matrix [p17]), CA (capsid [p24]), NC (nucleocapsid [p9]), and p6 (Gottlinger *et al.,* 1989).

Most p17 (MA) molecules remain attached to the inner surface of the virion lipid bilayer, stabilizing the particle while a subset of MA is recruited inside the deeper layers of the virion where it becomes part of the complex which escorts the viral DNA to the nucleus (Gallay *et al*., 1995). The p24 (CA) protein forms the conical core of viral particles (Franke *et al.,* 1994).

The p9 (NC) region of Gag recognizes the packaging signal of HIV (Harrison and Lever, 1992) and also facilitates reverse transcription (Lapadat-Tapolsky *et al*., 1993). The p6 polypeptide region mediates interactions between p55 and Vpr, leading to the incorporation of Vpr into assembling virions (Paxton *et al.,* 1993). The p6 region also contains a so-called late domain which is required for the efficient release of budding virions from an infected cell.

2.4.1.2 Gag-Pol Precursor

The viral protease (Pro), integrase (IN), RNase H, and reverse transcriptase (RT) are always expressed within the context of a Gag-Pol fusion protein (Jacks *et al*., 1988). During viral maturation, the virally encoded protease cleaves the Pol polypeptide away from Gag and

further digests it to separate the protease (p10), RT (p50), RNase H (p15), and integrase (p31) activities.

The HIV-1 protease (Pro) cleaves the Gag and Gag-Pol polyprotein precursors during virion maturation (Ashorn *et al*., 1990). The HIV-1 integrase (IN) protein mediates the insertion of the HIV proviral DNA into the genomic DNA of an infected cell (Bushman, Fujiwara, and Craigie, 1990).

Reverse transcriptase (RT) has both RNA-dependent and DNA-dependent polymerase activities. During the process of reverse transcription, RT makes a double-stranded DNA copy of the dimer of single-stranded genomic RNA present in the virion. RNase H removes the original RNA template from the first DNA strand, allowing synthesis of the complementary strand of DNA (Zack *et al*., 1990). The predominant functional species of RT is a heterodimer of p65 and p50.

2.4.1.3 Env

The 160 kD *Env* (gp160) is expressed from singly spliced mRNA and cleaved by a cellular protease to generate gp41 and gp120. The gp41 moeity contains the transmembrane domain of Env, while gp120 is located on the surface of the infected cell and of the virion through noncovalent interactions with gp41. Env exists as a trimer on the surface of infected cells and virions (Bernstein *et al*., 1995).

Interactions between HIV and the virion receptor, CD4, are mediated through specific domains of gp120 (Landau *et al.,* 1988). The gp41 moiety contains an N-terminal fusogenic domain that mediates the fusion of the viral and cellular membranes, thereby allowing the delivery of the virions inner components into the cytoplasm of the newly infected cell (Camerini and Seed, 1990).

2.4.2 REGULATORY PROTEINS

2.4.2.1 Tat

Tat is a transcriptional transactivator that is essential for HIV-1 replication (Ruben *et al*., 1989). Tat promotes the elongation phase of HIV-1 transcription thereby ensuring that full-length transcripts can be produced (Feinberg *et al.,* 1991; Kao *et al*., 1987). In the absence of Tat expression, HIV generates primarily short (>100 nucleotides) transcripts.

2.4.2.2 Rev

Rev is a 13-kD sequence-specific RNA binding protein (Zapp and Green, 1989) produced from fully spliced mRNAs. Rev 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 function are transcriptionally active but do not express viral late genes and consequently, do not produce virions.

2.4.3 ACCESSORY PROTEINS

In addition to the *gag*, *pol*, and *env* genes contained in all retroviruses, and the *tat* and *rev* regulatory genes, HIV-1 contains four additional genes: *nef*, *vif*, *vpr* and *vpu*, encoding the accessory proteins. HIV-2 has *vpx* instead of *vpu*. The accessory proteins represent critical virulence factors in vivo. Nef is expressed from a multiply spliced mRNA and is therefore Rev independent. In contrast, Vpr, Vpu, and Vif are the product of incompletely spliced mRNA, and thus are expressed only during the late, Rev-dependent phase of infection from singly spliced mRNAs. Most of the small accessory proteins of HIV have multiple functions.

2.4.3.1 Nef

Nef, (for *negative factor*) is the first viral protein to accumulate to detectable levels in a cell following HIV-1 infection (Kim *et al*., 1989). Nef has multiple activities, including the downregulation of the cell surface expression of CD4 (Aiken *et al*., 1994; Garcia and Miller,

1992), the perturbation of T cell activation (Baur *et al*., 1994; Luria *et al.,* 1991; Skowronski *et al.,* 1993), and the stimulation of HIV infectivity (Miller *et al*., 1994).

2.4.3.2 Vpr

The **Vpr** protein is incorporated into viral particles and approximately 100 copies of Vpr are associated with each virion (Cohen *et al*., 1990) Vpr blocks cell division (Rogel *et al.,* 1995), plays a role in the ability of HIV to infect nondividing cells by facilitating the nuclear localization of the preintegration complex (PIC) (Heinzinger *et al*., 1994) and interacts with the cellular protein uracil-DNA glycosylase (UNG) (Bouhamdan *et al*., 1996).

2.4.3.3 Vpu

Vpu is expressed from the mRNA that also encodes *env* but at levels tenfold lower than that of *env* (Schwartz *et al*., 1990). Vpu has two functions; the down-modulation of CD4 (Willey *et al*., 1992) and the enhancement of virion release (Klimkait *et al*., 1990; Schubert *et al*., 1996).

2.4.3.4 Vif

Vif is a 23-kD polypeptide that is essential for the replication of HIV in peripheral blood lymphocytes, macrophages, and certain cell lines (Strebel *et al*., 1987). Vif-defective HIV strains can enter cells but cannot efficiently synthesize the proviral DNA (von Schwedler *et al*., 1993). Vif mutant virions have improperly packed nucleoprotein cores as revealed by electron microscopic analyses (Hoglund *et al*., 1994).

2.5 REPLICATION OF HIV

2.5.1 ATTACHMENT AND PENETRATION

The process of viral entry involves fusion of the viral envelope with the host cell membrane and requires the specific interaction of the envelope with specific cell surface receptors. The two viral envelope proteins, gp120 and gp41, are conformationally associated to form a trimeric functional unit consisting of three molecules of gp120 exposed on the virion surface and associated with three molecules of gp41 inserted into the viral lipid membrane. Trimeric gp120 on the surface of the virion binds CD4 on the surface of the target cell, inducing a conformational change in the envelope proteins that in turn allows binding of the virion to a specific subset of chemokine receptors on the cell surface (Kwong *et al*., 1998) (Figure 8).

The CCR5 chemokine coreceptor binds macrophage-tropic, non-syncytium-inducing (R5) viruses, which are associated with mucosal and intravenous transmission of HIV infection. The other chemokine coreceptor, CXCR4, binds T-cell-tropic, syncytium-inducing (X4) viruses, which are frequently found during the later stages of disease (Scarlatti *et al*., 1997). The binding of surface gp120, CD4, and the chemokine coreceptors produces an additional radical conformational change in gp41 (Chan and Kim, 1998). Assembled as a trimer on the virion membrane, this coiled-coil protein springs open, projecting three peptide fusion domains that "harpoon" the lipid bilayer of the target cell. The fusion domains then form hairpin-like structures that draw the virion and cell membranes together to promote fusion, leading to the release of the viral core into the cell interior (Chan and Kim, 1998).

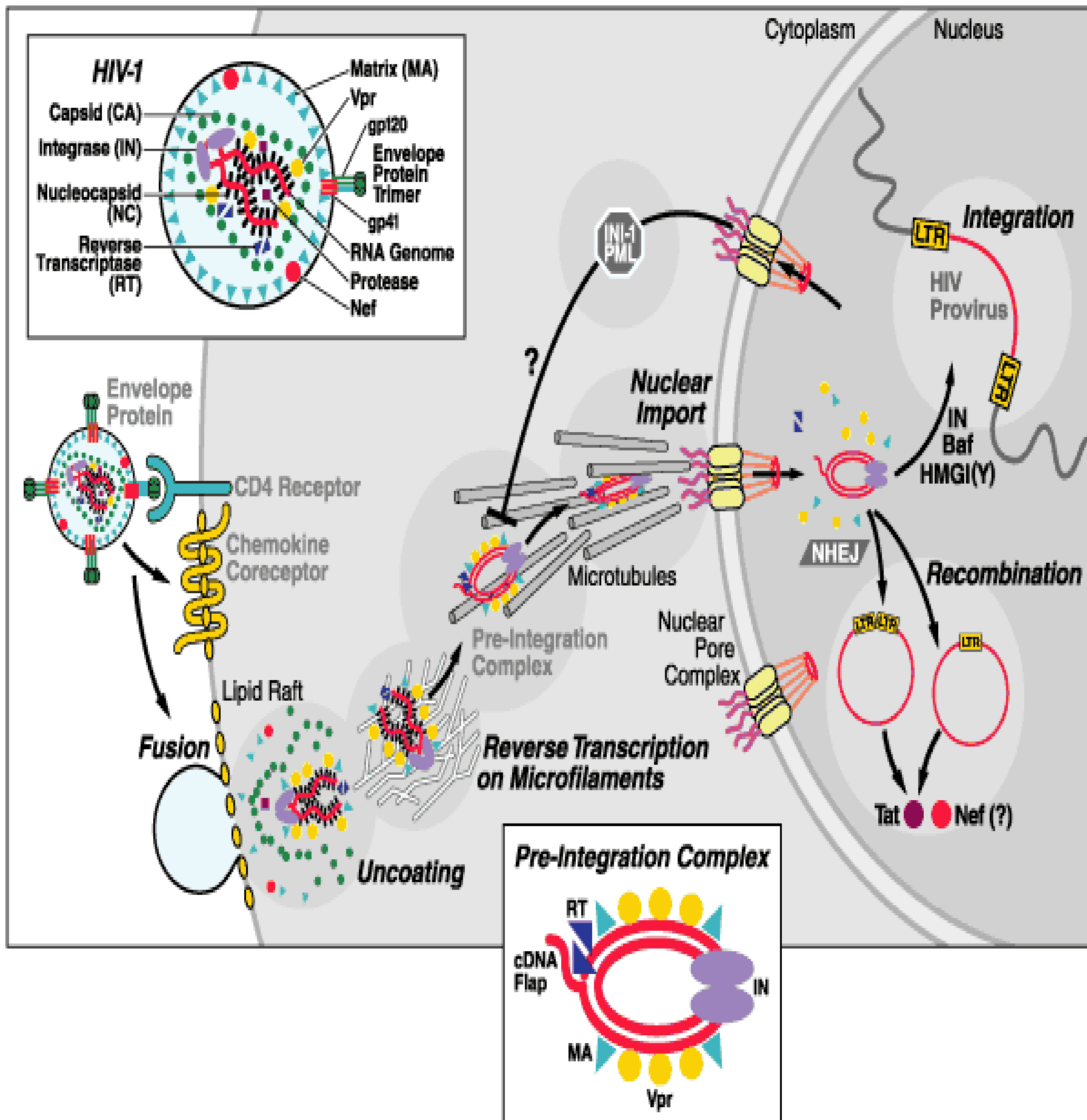

FIGURE 8: EARLY EVENTS OCCURRING AFTER HIV INFECTION OF A SUSCEPTIBLE TARGET CELL

SOURCE*: http://hivinsite (2010)*

2.5.2 UNCOATING AND CYTOPLASMIC EVENTS

The virion undergoes uncoating inside the cell while still associated with the plasma membrane (Figure 8). This process is poorly understood and may involve phosphorylation of viral matrix proteins by a mitogen-activated protein (MAP) kinase (Cartier *et al.,* 1999) and additional actions of cyclophilin A (Franke *et al.,* 1994) and the viral proteins Nef (Schaeffer *et al.,* 2001) and Vif (Ohagen and Gabuzda, 2000). Nef associates with a universal proton pump, V-ATPase (Lu *et al*., 1998), which could promote uncoating by inducing local changes in pH in a manner similar to that of the M2 protein of influenza (Takeda *et al*., 2002).

After uncoating, the viral reverse transcription complex is released from the plasma membrane (Karageorgos *et al.,* 1993). This complex includes the diploid viral RNA genome, lysine transfer RNA ($tRNA^{Lys}$) which acts as a primer for reverse transcription, viral reverse transcriptase, integrase, matrix and nucleocapsid proteins, viral protein R (Vpr), and various host proteins. The reverse transcription complex docks with actin microfilaments (Bukrinskaya *et al*., 1998). This interaction, mediated by the phosphorylated matrix, is required for efficient viral DNA synthesis. By overcoming destabilizing effects of CEM15/APOBEC3G protein, Vif stabilizes the reverse transcription complex in most human cells (Bukrinskaya *et al*., 1998; Lu *et al*., 1998; Simon *et al*., 1998).

Reverse transcription yields the HIV preintegration complex (PIC), composed of double-stranded viral cDNA, integrase, matrix, Vpr, reverse transcriptase, and the high mobility group DNA-binding cellular protein HMGI(Y) (Miller *et al.,* 1997). The PIC may move toward the nucleus by using microtubules as a conduit.

2.5.3 CROSSING THE NUCLEAR PORE

Unlike most animal retroviruses, HIV can infect nondividing cells, such as terminally differentiated macrophages (Weinberg *et al*., 1991). This requires an ability to cross the intact nuclear membrane. Integrase (Gallay *et al*., 1997), matrix (Bukrinsky *et al*., 1993), and Vpr (Heinzinger *et al*., 1994) have been implicated as key viral proteins that mediate the nuclear import of the PIC. Because plus-strand synthesis is discontinuous in reverse transcription, a triple helical DNA domain or "DNA flap" results that may bind a host protein containing a nuclear targeting signal (Zennou *et al*., 2000).

Matrix contains a canonical nuclear localization signal that is recognized by the importins alpha and beta, which are components of the classical nuclear import pathway. The HIV Vpr gene product contains at least three noncanonical nuclear targeting signals (Sherman *et al*., 2001). Vpr may bypass the importin system altogether, perhaps mediating the direct docking of the PIC with one or more components of the nuclear pore complex. The multiple nuclear targeting signals within the PIC may function in a cooperative manner or play larger roles individually in different target cells. For example, while Vpr is not needed for infection of nondividing, resting T cells (Eckstein *et al*., 2001), it enhances viral infection in nondividing macrophages (Vodicka *et al*., 1998). The finding that both matrix (Dupont *et al*., 1999) and Vpr (Sherman *et al*., 2001) shuttle between the nucleus and cytoplasm explains their availability for incorporation into new virions.

2.5.4 INTEGRATION

Inside the nucleus, the viral PIC establishes a functional provirus as shown in figure 8 by integration of double-stranded viral DNA into the host chromosome. This process is mediated by integrase, which binds the ends of the viral DNA (Miller *et al.,* 1997). The host proteins HMGI(Y) and barrier to autointegration (BAF) are required for efficient integration, although

their precise functions remain unknown (Chen and Engelman, 1998). Integrase removes terminal nucleotides from the viral DNA, producing a two-base recess and thereby correcting the ragged ends generated by the terminal transferase activity of reverse transcriptase (Miller *et al.,* 1997). Integrase also catalyzes the subsequent joining reaction that establishes the HIV provirus within the chromosome.

2.5.5 TRANSCRIPTIONAL CONTROLS AND TRANSCRIPTION

Integration to form the provirus can either lead to latent or transcriptionally active forms of infection (Adams *et al*., 1994). This transcriptional latency of HIV's explains the inability of potent antiviral therapies to eradicate the virus from the body. Moreover, despite a vigorous immune response early in infection, these silent proviruses are a reservoir that allows reemergence of HIV when the body's defenses grow weaker.

In the host genome, the 5´ LTR functions like other eukaryotic transcriptional units. It contains downstream and upstream promoter elements, which include the initiator (Inr), TATA-box (T), and three Sp1 sites (Taube *et al*., 1999). These regions help position the RNA polymerase II (RNAPII) at the site of initiation of transcription and to assemble the preinitiation complex. Slightly upstream of the promoter is the transcriptional enhancer, which in HIV-1 binds nuclear factor [kappa]B (NF-[kappa]B), nuclear factor of activated T cells (NFAT), and Ets family members (Jones and Peterlin, 1994). NF-[kappa]B and NFAT relocalize to the nucleus after cellular activation. NF-[kappa]B is liberated from its cytoplasmic inhibitor, I[kappa]B, by stimulus-coupled phosphorylation, ubiquitination, and proteosomal degradation of the inhibitor (Karin and Ben-Neriah, 2000). NFAT is dephosphorylated by calcineurin (a reaction inhibited by cyclosporin A) and, after its nuclear import, assembles with AP1 to form the fully active transcriptional complex (Crabtree, 1999). NF-[kappa]B, which is composed of p50 and p65 (RelA) subunits, increases the rates of

initiation and elongation of viral transcription (Barboric *et al*., 2001). Since NF-[kappa] B is activated after several antigen-specific and cytokine-mediated events, it may play a key role in rousing transcriptionally silent proviruses

When these factors engage the LTR, transcription begins, but in the absence of Tat described below the polymerase fails to elongate efficiently along the viral genome. In the process, short nonpolyadenylated transcripts are synthesized, which are stable and persist in cells due to the formation of an RNA stem loop called the transactivation response (TAR) element (Kao *et al*., 1987).

Tat significantly increases the rate of viral gene expression. With cyclin T1 (CycT1), Tat binds to the TAR RNA stem-loop structure and recruits the cellular cyclin-dependent kinase 9 (Cdk9) to the HIV LTR (Wei *et al*., 1998). Within the positive transcription elongation factor b (P-TEFb) complex, Cdk9 phosphorylates the C-terminal domain of RNAPII, marking the transition from initiation to elongation of eukaryotic transcription (Price, 2000). Other targets of P-TEFb include negative transcription elongation factors (N-TEF), such as the DRB-sensitivity inducing (DSIF) and negative elongation (NELF) factors (Price, 2000). The high efficiency with which the HIV LTR attracts these negative transcription factors in vivo may explain why the LTR is a poor promoter in the absence of Tat. The arginine-rich motif (ARM) within Tat binds the 5´ bulge region in TAR. A shorter ARM in cyclin T1, which is also called the Tat-TAR recognition motif (TRM), binds the central loop of TAR (Wei *et al*., 1998).

Binding of the Tat cyclin T1 complex to both the bulge and loop regions of TAR strengthens the affinity of this interaction. All of these components are required for Tat transactivation. In the presence of the complex between Tat and P-TEFb, the RNAPII elongates efficiently.

Transcription of the viral genome results in more than a dozen different HIV-specific transcripts (Saltarelli *et al*., 1996). Some are processed cotranscriptionally and, in the absence of inhibitory RNA sequences (IRS), transported rapidly into the cytoplasm (Cullen, 1998). These multiply spliced transcripts encode Nef, Tat, and Rev. Other singly spliced or unspliced viral transcripts remain in the nucleus and are relatively stable. These viral transcripts encode the structural, enzymatic, and accessory proteins and represent viral genomic RNAs that are needed for the assembly of fully infectious virions. These viral transcripts are then exported to the cytoplasm.

The nuclear export of this assembly (viral RNA transcript, Rev, and CRM1/exportin 1) depends critically on yet another host factor, RanGTP. Ran is a small guanine nucleotide-binding protein that switches between GTP- and GDP-bound states. RanGDP is found predominantly in the cytoplasm because the GTPase activating protein specific for Ran (RanGAP) is expressed in this cellular compartment. Conversely, the Ran nucleotide exchange factor, RCC1, which charges Ran with GTP, is expressed predominantly in the nucleus. The inverse nucleocytoplasmic gradients of RanGTP and RanGDP produced by the subcellular localization of these enzymes likely plays a major role in determining the directional transport of proteins into and out of the nucleus. Outbound cargo is only effectively loaded onto CRM1/exportin-1 in the presence of RanGTP. However, when the complex reaches the cytoplasm, GTP is hydrolyzed to GDP, resulting in release of the bound cargo. The opposite relationship regulates the nuclear import by importins alpha and beta, where nuclear RanGTP stimulates cargo release (Cullen, 1998).

For HIV infection to spread, a balance between splicing and transport of viral mRNA species must be achieved. If splicing is too efficient, then only the multiply spliced transcripts appear in the cytoplasm. Although required, the regulatory proteins encoded by multiply spliced

transcripts are insufficient to support full viral replication. However, if splicing is impaired, adequate synthesis of Tat, Rev, and Nef will not occur. In many non-primate cells, HIV transcripts may be overly spliced, effectively preventing viral replication in these hosts (Malim and Cullen, 1993).

2.5.6 ASSEMBLY

Virions are assembled at the plasma membrane (Figure 9) with each virion consisting of roughly 1500 molecules of Gag and 100 Gag-Pol polyproteins (Wilk *et al*., 2001), two copies of the viral RNA genome, and Vpr (Freed, 1998). Several proteins participate in the assembly process, including Gag polyproteins and Gag-Pol, as well as Nef and Env. A human ATP-binding protein, HP68 (previously identified as an RNase L inhibitor), likely acts as a molecular chaperone, facilitating conformational changes in Gag needed for the assembly of viral capsids (Zimmerman *et al*., 2002). In primary CD4 T lymphocytes, Vif plays a key but poorly understood role in the assembly of infectious virions. In the absence of Vif, normal levels of virus are produced, but these virions are noninfectious, displaying arrest at the level of reverse transcription in the subsequent target cell.

2.5.7 VIRION BUDDING

The Gag polyproteins are subject to myristylation (Gottlinger *et al.,* 1989), and thus associate preferentially with cholesterol- and glycolipid-enriched membrane microdomains (Ono and Freed, 2001). Virion budding occurs through these specialized regions in the lipid bilayer, yielding virions with cholesterol-rich membranes. This lipid composition likely favours release, stability, and fusion of virions with the subsequent target cell.

The budding reaction involves the action of several proteins, including the "late domain"(Garnier *et al*., 1999) sequence (PTAP) present in the p6 portion of Gag (Strack *et al*., 2000) (Figure 9). The p6 protein also appears to be modified by ubiquitination. The

product of the tumor suppressor gene 101 (TSG101) binds the PTAP motif of p6 Gag and also recognizes ubiquitin through its ubiquitin enzyme 2 (UEV) domain (Garrus *et al*., 2001; VerPlank *et al*., 2001). The TSG101 protein normally associates with other cellular proteins in the vacuolar protein sorting pathway to form the ESCRT-1 complex that selects cargo for incorporation into the multivesicular body (MVB) (Katzmann *et al.,* 2001). The MVB is produced when surface patches on late endosomes bud away from the cytoplasm and fuse with lysosomes, releasing their contents for degradation within this organelle. In the case of HIV, TSG101 appears to be "hijacked" to participate in the budding of virions into the extracellular space away from the cytoplasm.

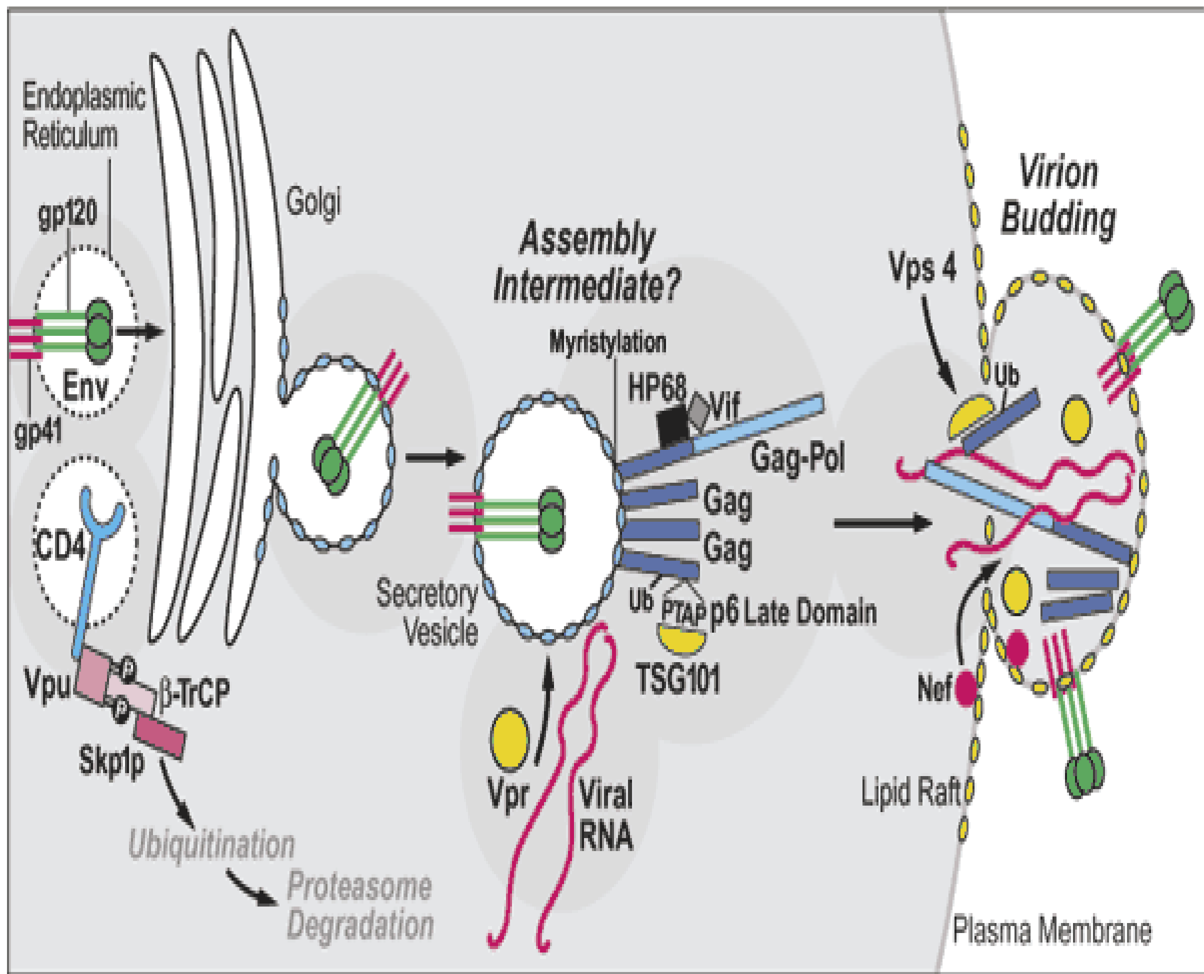

FIGURE 9: LATE STEPS IN THE ASSEMBLY OF NEW VIRIONS

SOURCE: http://hivinsite (2010)

2.6 NATURAL HISTORY OF HIV INFECTION

2.6.1 PRIMARY HIV INFECTION

Primary HIV infection is defined as the period of time from initial infection with HIV to the development of an antibody response detectable by standard serological tests. Most individuals who acquire HIV experience some symptoms of primary HIV infection (Schacker *et al*., 1996) days to weeks after infection. This acute viral syndrome of primary HIV infection is known as "seroconversion illness" and it presents with symptoms resembling those of mononucleosis (Cooper *et al*., 1985; Kahn and Walker, 1998). Symptoms may be mild or severe and may last from a few days to several weeks, with the average duration being 14 days. The presenting symptoms include fever (seen in over 75% of patients) (Vanhems *et al*., 1999), fatigue, lymphadenopathy, headache, and maculopapular rash, typically, in the trunk (Vanhems *et al*., 1999). Studies from India and Kenya by Bollinger *et al.,* (1997) and Lavreys *et al.* (2000), respectively, found more frequent reports of joint pains, night sweats, and mucosal candidiasis and less frequent rash and pharyngitis in these study populations. A more severe clinical syndrome in primary HIV infection has been associated with a more rapid subsequent clinical course of HIV disease (Pedersen *et al*., 1989).

Diagnosis of HIV during the acute seroconversion phase requires both high clinical suspicion and also an understanding of appropriate testing strategies because of the nonspecific symptoms of primary HIV infection (Schacker *et al*., 1996). Routine HIV antibody testing may be negative for several weeks or even months after exposure in the "window period"(Busch and Alter, 1995). During primary infection with HIV, plasma viral load often reaches very high levels in the range of millions of RNA copies/ml (Daar *et al*., 1991; Piatak *et al*., 1993). This high levels of viremia do not persist, however (Daar *et al*., 1991), providing evidence of a host immune response capable of bringing the infection under some

degree of control, at least in the short term. After this initial reduction of viremia, a viral "set-point" is established in each infected individual; the magnitude of which set-point correlates with the rate of progression of HIV disease (Mellors *et al*., 1995; Mellors *et al*., 1996).

During primary HIV infection, some immunological changes also take place. HIV-specific CD8+ T lymphocytes undergo a marked clonal expansion and express high levels of activation markers such as CD38 and human leukocyte antigen (HLA)-DR (Roos *et al*., 1994). The breadth and strength of this CTL response correlate positively with the degree of viral control and inversely with the rapidity of clinical progression (Borrow *et al*., 1994; Koup *et al*., 1994; Musey *et al*., 1997; Pantaleo *et al*., 1997).

CD4+ T lymphocyte counts and CD4 function may decline during primary HIV infection, occasionally to levels that allow opportunistic infections (OIs) to develop (Gupta, 1993; Pedersen *et al*., 1990; Vento *et al*., 1993). Absolute CD4 count often rebounds after the primary infection, but may not return to a normal baseline. In patients with clinical progression of HIV disease, CD4 responses against HIV itself remain particularly impaired following primary infection (Rosenberg *et al*., 1997).

2.6.2 CHRONIC HIV INFECTION

After the period of acute HIV infection a relative equilibrium between viral replication and the host immune response is reached in which individuals may have little or no clinical manifestations of HIV infection. This time between initial infection and the development of AIDS may be long, for most people averaging 10 years, even in the absence of treatment (Bacchetti and Moss, 1989).

In this period of relative clinical latency, viral replication and CD4+ cell turnover remain active. Millions of CD4+ cells and billions of virions are produced and destroyed each day. Also, most infected individuals will have progressive loss of CD4+ lymphocytes and

perturbation of immune function (Balotta *et al*., 1997; Koblin *et al*., 1999; Lefrere *et al*., 1997; Moss *et al*., 1988).

The rate of progression of infection may vary considerably between adults and infants. In adults, progression from infection to clinical AIDS is rare in the first 2 years of infection; however, reports describe rapid disease progression in infants infected by blood transfusion (van den Berg *et al*., 1994). Buchbinder *et al.* (1998) in a retrospective study of HIV infection showed that: 87% of infected individuals had developed AIDS by 17 years post seroconversion; 12% maintained a CD4+ cell count >500 cells/µl at 10 years; and, only 3% maintained a CD4+ cell count >500 cells/µL at 16 years after seroconversion (Buchbinder, 1998).

During chronic HIV infection, HIV RNA levels in plasma correlate with the rate of CD4 decline, with higher plasma viral loads predicting more rapid progression to AIDS and death (Mellors *et al*., 1995; O'Brien *et al*., 1996a). An undetectable HIV RNA level in peripheral blood is associated with stable CD4 lymphocyte counts, and increases in HIV RNA correlate with more rapid rates of CD4 cell decline (Iuliano *et al*., 1997; Mellors *et al*., 1997).

2.6.3 CLINICAL AIDS

2.6.3.1 CDC CLASSIFICATION SYSTEM FOR HIV INFECTION

According to the CDC criteria (appendix 1), AIDS is defined by either diagnosis of one of the AIDS-defining conditions, or by measurement of CD4 levels <200 cells/µl (or CD4 percentage <14%). According to this classification system, any HIV infected individual with CD4 cell count <200 cells/µl has AIDS, regardless of the presence of symptoms or opportunistic diseases (CDC, 1993; CDC, 1999).

Progression to AIDS from time of infection occurs, on average, 2 years earlier when defined by laboratory criteria (CD4 levels <200 cells/µl) compared to clinical criteria (development

of an opportunistic illness) (Longini *et al*., 1991; Osmond *et al*., 1994). Survival time from the development of AIDS varies according to the AIDS-defining event. In the Multicenter Hemophilia Cohort Study, median survival after a single AIDS-defining condition ranged from 3 to 51 months for the 10 most common conditions (Gail *et al*., 1997). The mean survival time after diagnosis of AIDS in the United States prior to the availability of antiretroviral treatment was 10-12 months (Gail *et al*., 1997).

2.6.3.2 WHO CLINICAL STAGING OF HIV AND AIDS

In resource limited settings without CD4 cell count measurements and other diagnostic and laboratory testing methods, the WHO Clinical Staging and Disease Classification System (revised in 2007) can be used. The WHO system classifies HIV disease (appendix 2) on the basis of clinical manifestations that can be recognized by clinicians with different levels of HIV expertise, and treated by clinicians in diverse setting.

This table is used to determine eligibility for highly active antiretroviral therapy (HAART) and it is categorized into 4 clinical stages, progressing from primary HIV infection to advanced AIDS. Each stage is defined by specific clinical conditions or symptoms.

2.7 DETERMINANTS IN HIV DISEASE PROGRESSION

2.7.1 HOST FACTORS

A number of host factors influence HIV disease progression and include age, coreceptor type, genetic differences and behavioural or psychological factors. Moss *et al.* (1988) in a cohort study showed that individuals who acquire HIV at an older age tend to have more rapid disease progression (Moss *et al*., 1988) and consequently, a shorter survival time (Bacchetti *et al*., 1988).

Susceptibility to HIV and disease progression is also influenced by variation in HIV coreceptor molecules. Liu *et al.* (1996) (Liu *et al*., 1996) reported that a homozygous or

heterozygous mutation in the CCR5 gene results in a mutant allele of CCR5 with a 32-base-pair deletion, CCR5-delta-32, which encodes a nonfunctional truncated protein that is not transported to the cell surface. Homozygotes for this allele exhibit a strong resistance to HIV infection whereas heterozygotes display nearly normal rates of infection with delayed progression to AIDS (10-15% of Caucasians are heterozygous, and 1% are homozygous).

Genetic differences in HLA alleles have also been shown to influence HIV disease susceptibility (Kaul *et al*., 1999; Rowland-Jones *et al*., 1998) and disease progression (Itescu *et al*., 1994; Kaslow *et al*., 1996; Kaslow *et al*., 1990; Keet *et al*., 1999; Kroner *et al*., 1995; Migueles *et al*., 2003). General HLA homozygosity (Tang *et al*., 1999) as well as the class I alleles B35 and Cw4 have been associated with accelerated progression of disease (Itescu *et al*., 1992; Jeannet *et al*., 1989; Tomiyama *et al*., 1997) while HLA B27 and B57 have been associated with long-term nonprogression of HIV disease (Kaslow *et al*., 1996). In particular, HLA B*5701 has been found to be highly overrepresented in long-term nonprogressors (Migueles *et al*., 2003). Since HLA class I alleles determine which viral epitopes can be presented to CD8 cells, a greater diversity of HLA (heterozygosity) in an individual may reflect greater options for effective cell-mediated immunity to HIV.

Behavioral or psychological host factors may also influence HIV disease progression. Rapid HIV disease progression has been reported with unprotected anal intercourse (Vittinghoff *et al*., 2001) smoking (Royce and Winkelstein, 1990), poor nutrition (Moseson *et al*., 1989) and depression (Burack *et al*., 1993).

In additon, a small subset of individuals infected with HIV (<5%) are referred to as long-term nonprogressors (LTNPs). LTNPs remain free of symptoms, achieve good control of HIV viral replication, and maintain high CD4 cell counts in the absence of antiretroviral

medications over many years of infection. In general, LTNPs appear to have strong cellular immune responses to a variety of HIV antigens (Kalams *et al*., 1999).

2.7.2 VIRAL FACTORS

HIV virions infect human cells by binding to the CD4 receptor on the cell surface and other coreceptors; CCR5 (R5) and CXCR4 (X4). M-tropic viruses preferentially infect monocytes and macrophages, using the cell surface protein CCR5 as the preferred coreceptor to enter cells, and produce a nonsyncytium-inducing (NSI) phenotype in cell culture. Conversely, T-tropic viruses preferentially infect T cells, use CXCR4 as the preferred coreceptor to enter cells, and produce a syncytium-inducing (SI) phenotype in cell culture (Burack *et al*., 1993). Dual-tropic viruses, which may use either CCR5 or CXCR4 coreceptors, also exist. M-tropic viruses are frequently found in early HIV infection, and a switch to T-tropic strains in the course of disease is associated with rapid CD4 cell depletion and rapid disease progression (Connor *et al*., 1997; Shankarappa *et al*., 1999; Tersmette *et al*., 1988).

The role of viral fitness, which refers to the pathogenicity of certain strains of HIV, on individual disease progression is just beginning to be understood. One component of viral fitness that has been studied is HIV replicative capacity (RC), which is a measure of the ability of a given virus to replicate successfully in a given environment (Caliendo *et al*., 1996; Croteau *et al*., 1997; Harrigan *et al.,* 1998; Ho *et al*., 1994; Sharma and Crumpacker, 1997). Mutations arise in the HIV reverse transcriptase and protease enzymes during the course of drug treatment that make the virus resistant to particular drugs, thus conferring a selective advantage to that subpopulation that arises from a resistant variant (Condra *et al*., 1995; Roberts, 1995). Several of these mutations have been shown to cause a reduction in replicative capacity in the absence of drug when compared to wild-type virus (Croteau *et al*., 1997; Harrigan *et al.,* 1998; Ho *et al*., 1994). Further accumulation of mutations over time

under drug selection pressure may increase the "fitness" of the drug-resistant variant by further increasing phenotypic resistance (Condra *et al*., 1995; Larder *et al.,* 1995; Molla *et al*., 1996), or by increasing replicative capacity (RC) of the resistant virus (Borman *et al.,* 1996; Ho *et al*., 1994).

There is documented evidence that HIV-1 subtype also affects the rate of disease progression. Kanki *et al.* (1999) *reported that w*omen infected with a non-A subtype were 8 times more likely to develop AIDS than were those infected with subtype A For example, faster rates of disease progression have been observed in Ugandan individuals infected with subtype D compared with subtype A isolates (Kaleebu *et al*., 2002). Additionally, rare individuals who are infected with variant HIV strains, particularly those with a defective *nef* gene product, may experience slower disease progression (Kirchhoff *et al*., 1995).

2.7.3 COINFECTIONS

Coinfections in HIV disease have been shown to influence disease progression. Oppurtunistic infections (OIs) during HIV disease not only indicates the degree of immunosuppression, but may also influence disease progression itself. When stratified by CD4 cell counts, patients with prior histories of OIs have higher mortality rates than those without prior histories of OIs (Saravolatz *et al*., 1996).

Another coinfection common in HIV disease is Hepatitis C; present in up to 40-50% of all patients in urban setting and in 90% of intravenous drug users (Sulkowski *et al*., 2000). In a study of the Swiss HIV Cohort, HCV coinfection was associated with poorer CD4 responses to ART, development of new AIDS-defining events, and increased mortality (Greub *et al*., 2000).

2.8 OPPORTUNISTIC INFECTIONS

2.8.1 BACTERIAL INFECTIONS

Bacterial infections have emerged as an important cause of morbidity and mortality in individuals infected with the human immunodeficiency virus (HIV-1). Persons with HIV-1 infection are more susceptible to bacterial infections because of defects in both cell-mediated and humoral immunity. The opportunistic bacterial infections associated with HIV are bacillary angiomatosis, pneumococcal infection, tuberculosis by M. Tuberculosis and the M. Avium complex, syphilis and other serious bacterial infections in children.

2.8.1.1 BACILLARY ANGIOMATOSIS

Bacillary angiomatosis (BA) is a disease that occurs in the later stages of HIV infection and characterized by unique vascular lesions caused by infection with small, gram-negative organisms of the genus *Bartonella* (Cockerell *et al*., 1990; Paul *et al*., 1994; Tappero *et al*., 1993). Two conceptually distinct *Bartonella*-associated syndromes have been described: bacteremia (in the absence of focal tissue vascular proliferative response) and the tissue infection (BA or BP) associated with angiogenic response resulting from infection with either *B. quintana* or *B. henselae*. Manifestations of BA include: cutaneous bacillary angiomatosis in the skin (Cockerell *et al*., 1987); osseous bacillary angiomatosis involving the bone (Knobler *et al.,* 1988); splenic and hepatic bacillary peliosis in the spleen and liver, respectively, (Perkocha *et al*., 1990); gastrointestinal and respiratory tract bacillary angiomatosis (Tuur *et al*., 1988); lymph node bacillary angiomatosis (Koehler *et al*., 1992; Perkocha *et al*., 1990); CNS bacillary angiomatosis (Spach *et al*., 1992); bacteremia (Regnery *et al*., 1992); endocarditis (Spach *et al*., 1992); and pregnancy-associated bacillary angiomatosis (Riley and Tuomala, 1992).

2.8.1.2 PNEUMOCOCCAL INFECTION

Pneumococcus is one of the most common bacterial pathogens affecting both HIV-1 infected children and adults, with the respiratory tract being the most common site of invasive pneumococcal infection (Janoff *et al*., 1992). Population-based studies have demonstrated that the incidence of pneumococcal disease, caused by *Streptococcus pneumonia,* in HIV infection is extremely high and can occur at any time during the course of the disease (Plouffe *et al.,* 1996; Redd *et al*., 1990), although with increasing incidence as HIV-1 disease progresses (Gebo *et al*., 1996; Hirschtick *et al*., 1995; Wallace *et al*., 1993). *S. pneumoniae* accounts for at least one-third of serious bacterial infections in children with advanced HIV disease (Gebo *et al*., 1996).

2.8.1.3 TUBERCULOSIS

Worldwide, tuberculosis is the most common opportunistic infection affecting HIV-seropositive individuals (UNAIDS, 1996) and it is the most common cause of death in patients with AIDS (Raviglione, Snider, and Kochi, 1995). By producing a progressive decline in cell-mediated immunity, HIV alters the pathogenesis of tuberculosis, greatly increasing the risk of developing disease in coinfected individuals and leading to more frequent extrapulmonary involvement and atypical radiographic manifestations. Although HIV-related tuberculosis is both treatable and preventable, incidence rates continue to climb in developing nations (Raviglione *et al.,* 1992) where HIV infection and tuberculosis are endemic and resources are limited with approximately 10 million people estimated to be coinfected with *M tuberculosis* and HIV (Dye *et al*., 1999). Studies have found that HIV infected patients are more likely to develop acquired drug resistance (ADR) than seronegative cases (Bishai *et al*., 1996; Bradford *et al*., 1996; Lutfey *et al*., 1996; Nolan *et al*., 1995).

2.8.1.4 MYCOBACTERIUM AVIUM COMPLEX AND ATYPICAL MYCOBACTERIAL INFECTIONS

Mycobacterium avium complex (MAC) consists of several related species of *Mycobacterium* (*M avium*, *Mycobacterium intracellulare*, and other species of *Mycobacterium* that have not been classified) that are ubiquitous in the environment, and rarely causes disease in individuals with a normal immune system. In patients with AIDS, however, it is one of the most common serious opportunistic infections (OIs) (Chaisson *et al.*, 1992; Nightingale *et al.*, 1992) occurring almost exclusively in patients with a CD4 count <50 cells/µL (Chaisson *et al.*, 1992; Nightingale *et al.*, 1992). Colonization of the respiratory or gastrointestinal (GI) tract by MAC can occur without evident morbidity; however, MAC colonization of these sites indicates that patients are at increased risk for developing disseminated MAC infection (Alisky and Schlesinger, 1998; Chin *et al.*, 1994; Hocqueloux *et al.*, 1998; Phillips *et al.*, 1999; Race *et al.*, 1998; Sheppard and Sullam, 1997). Patients with advanced HIV disease have a linear increase in the risk of developing MAC bacteremia over time, and the risk of developing disseminated MAC infection for patients surviving for 30 months after being diagnosed with AIDS was 50% (Nightingale *et al.*, 1992); the lower the absolute CD4 count the higher the risk of developing MAC.

2.8.1.5 OTHER BACTERIAL INFECTIONS

Other opportunistic bacterial infections associated with HIV infection include syphilis and other serious bacterial infections in children with HIV infection like meningitis (caused by S. *pneuminiae*, *Salmonella spp, H. Influenza*) (Krasinski *et al.*, 1988), gastroenteritis (caused by *Salmonella* spp, *Shigella* spp, *Campylobacter* spp, *Aeromonas hydrophilia*, *Vibrio* spp, *Clostridium difficile*, and enterotoxigenic, enterohemorrhagic, enteropathogenic, or enteroinvasive *E coli)*, urinary tract infections (caused by *E coli.*) (Krasinski *et al.*, 1988; Ruiz-Contreras *et al.*, 1995) *Klebsiella* spp, *Enterobacter* spp, *Enterococcus* spp,

Pseudomonas spp, *Proteus* spp, and *Morganella* spp or mixtures of organisms), skin and soft tissue infections (Geusau and Tschachler, 1997; Smith *et al*., 1994), septic arthritis (Gilbert *et al*., 1996) and osteomyelitis (NICHHD, 1991; (Roilides *et al*., 1991).

2.8.2 FUNGAL INFECTIONS

2.8.2.1 CANDIDIASIS

Mucocutaneous candidiasis occurs in 3 forms in persons with HIV infection: oropharyngeal, esophageal, and vulvovaginal disease. Oropharyngeal candidiasis (OPC) was among the initial manifestations of HIV-induced immunodeficiency to be recognized (Gottlieb *et al*., 1981; Masur *et al*., 1981) and typically affects the majority of persons with advanced untreated HIV infection. Oropharyngeal and vulvovaginal disease are the most common forms of mucocutaneous candidiasis. Up to 90% of persons with advanced untreated HIV infection develop OPC, with 60% having at least 1 episode per year with frequent recurrences (50-60%) (Bruatto *et al*., 1991; Fichtenbaum *et al*., 2000). Esophageal candidiasis occurs less frequently (10-20%) but is the leading cause of esophageal disease (Moore and Chaisson, 1996; Selik, Starcher, and Curran, 1987). Vaginal candidiasis has been noted in 27-60% of women, similar to the rates of oropharyngeal disease (Schuman *et al*., 1997). However, the incidence appears to be similar in HIV-infected and HIV-uninfected women (White, 1996).

Symptoms of OPC may include burning pain, altered taste sensation, and difficulty swallowing liquids and solids. Most persons with OPC present with pseudomembranous candidiasis or thrush and less commonly with acute atrophic candidiasis or chronic hyperplastic candidiasis (Feigal *et al*., 1991; McCarthy *et al*., 1991).

2.8.2.2 CRYPTOCOCCOSIS

Cryptococcosis, caused by *Cryptococcus neoformans,* is the cause of the most common life-threatening meningitis in AIDS patients, occurring most frequently as meningoencephalitis

(Holtzer *et al*., 1998; Michaels *et al.,* 1999; Murphy *et al*., 2001). Although the overall incidence of cryptococcosis is unknown, it is higher among patients with AIDS in Africa and Southeast Asia than in the United States, whereas it appears less frequently in Europe (Levitz, 1991). Cryptococcemia often precedes CNS invasion and typically presents as a subacute process characterized by headache, fever, and, less often, altered mental status; however, presentations characteristic of either acute or chronic meningitis can occur. Cranial nerve palsies and papilledema are the most common ocular manifestations seen in patients with cryptococcal CNS invasion (Mitchell and Perfect, 1995).

Cryptococcal pneumonia is the most frequent fungal pneumonia encountered in persons with AIDS, except in areas hyperendemic for either histoplasmosis or coccidioidomycosis. Patients with pulmonary cryptococcosis may present with cough, fever, malaise, shortness of breath, and pleuritic pain. Physical examination may reveal lymphadenopathy, tachypnea, or rales (Cameron *et al*., 1991; Meyohas *et al*., 1995; Mulanovich, Dismukes, and Markowitz, 1995).

There are also other manifestations of cryptococcosis. Cutaneous cryptococcosis is a sign of dissemination present in approximately 10% of cases and may precede life-threatening disease by several weeks (Murakawa *et al.,* 1996; Ricchi *et al*., 1991). Adrenal insufficiency may occur secondary to cryptococcal invasion of the adrenal glands (Perfect, 1989). *Cryptococcus* may lie dormant in the prostate and serve as the source of systemic relapse after completion of therapy (Dismukes, 1993). Given that most patients with cryptococcosis are fungemic, it is not surprising that there are reports of disease in many organ systems, including cryptococcal myocarditis, arthritis, and gastroenteritis (Mitchell and Perfect, 1995).

2.8.2.3 HISTOPLASMOSIS

In patients with advanced HIV infection, histoplasmosis almost always is manifested by signs of progressive disseminated disease, as opposed to the asymptomatic or limited pulmonary infection observed in the majority of healthy individuals exposed to *H capsulatum* (Wheat *et al*., 1990). In the United States, histoplasmosis has been diagnosed in 2-5% of the HIV-positive population. Significantly higher rates of infection have been described in geographic regions where this infection is endemic (Johnson *et al*., 1988; Wheat *et al*., 1990). The majority of AIDS patients with disseminated disease have CD4 counts <150 cells/µL, with a median CD4 count of 50 cells/µl (Wheat *et al*., 1990). Clinical presentations fever, weight loss, malaise, hepatomegaly, splenomegaly, skin lesions (Karimi *et al*., 2002), generalized lymphadenopathy over a period of several weeks and rare clinical manifestations including pleuritis, pancreatitis (Wheat *et al*., 1990), prostatitis, and retinitis (Couppie *et al*., 2004).

2.8.2.4 OTHER FUNGAL INFECTIONS

Other fungal opportunistic infections in HIV infection include coccidioidomycosis (Woods *et al*., 2000) endemic to the southwestern United States and certain areas of Mexico and Central and South America, penicilliosis (caused by *P marneffei*) endemic to Southeast Asia and the southern part of China (Duong, 1996), and PCP (*Pneumocystis carinii now jiroveci, pneumonia*) which is a common opportunistic disease that occurs almost exclusively in persons who have profound immunodeficiency (Armengol, 1995).

2.8.3 PARASITIC INFECTIONS

2.8.3.1 MALARIA

HIV-related immunosuppression may increase rates of malaria infection and clinical malaria disease presentation (Whitworth *et al.,* 2000; French *et al.,* 2001), with an increased risk of complicated and severe malaria and death (Chirenda *et al.,* 2000; Cohen *et al*., 2005; Grimwade *et al*., 2004). A study from Malawi showed that HIV-1 plasma viral loads were

significantly higher in patients with malaria infection than in those without, and these levels remained higher for up to 10 weeks after treatment (Kublin *et al*., 2005). The increases in viral load were greatest in those with clinical malaria, high levels of parasitemia, and relatively high CD4 counts which suggest that malaria may speed the progression of HIV disease. Another study from Uganda showed increased CD4 cell decline associated with episodes of malaria despite prompt treatment (Mermin *et al.,* 2006).

Clear evidence indicates an interaction between HIV-1 and malaria in pregnancy, causing more peripheral and placental parasitemia, increased placental HIV-1 viral load, higher parasite densities, more clinical malaria, more anemia, and increased risks of adverse birth outcomes, including death (Mwapasa *et al*., 2004; ter Kuile *et al*., 2004).

2.8.3.2 TOXOPLASMOSIS

Toxoplasmosis associated with HIV infection is typically caused by reactivation of a chronic infection and manifests primarily as toxoplasmic encephalitis. This disease is an important cause of focal brain lesions in HIV-infected patients and usually occurs in HIV-infected patients with CD4 T-cell counts <100/µl (Luft and Remington, 1992).

Toxoplasmic encephalitis has a subacute onset with focal neurologic abnormalities frequently accompanied by headache, altered mental status, and fever (Levy and Bredesen, 1988; Navia *et al*., 1986; Renold *et al*., 1992). The most common focal neurologic signs are motor weakness and speech disturbances. Patients can also present with other clinical manifestations including seizures, cranial nerve abnormalities, visual field defects, sensory disturbances, cerebellar dysfunction, meningismus, movement disorders, and neuropsychiatric manifestations (Levy and Bredesen, 1988; Navia *et al*., 1986; Renold *et al*., 1992). Diffuse toxoplasmic encephalitis is considered in patients with anti-*T gondii*

immunoglobulin G (IgG) antibodies and CD4 T-cell counts of <100/μL who present with unexplained neurologic disease (Gray *et al*., 1989).

Extracerebral toxoplasmosis, with or without concomitant encephalitis, may develop in HIV-infected patients. Ocular and pulmonary diseases are the most common presentations in patients with extracerebral toxoplasmosis (Rabaud *et al*., 1994).

2.8.3.3 MICROSPORIDIOSIS

Microsporidia are small, sporeforming, obligate intracellular protozoan parasites that are found in the intestine, liver, kidney, cornea, brain, nerves, and muscles of a variety of wild and domesticated animals (Weber *et al*., 1994). Microsporidiosis is recognized as a cause of gastrointestinal disease, renal disease, sinusitis, and keratitis in AIDS patients (Bryan, 1995; Shadduck, 1989). One study reported prevalence of microsporidiosis in AIDS patients in one select population that was referred for gastrointestinal symptoms was 39% (Kotler and Orenstein, 1994). *Enterocytozoon bieneusi* infection of AIDS patients may be as common as *Cryptosporidium* as a cause of diarrhea in AIDS patients (Orenstein *et al*., 1990; Weber *et al*., 1994; Wuhib *et al*., 1994).

Infection of the intestinal epithelium with *Enterocytozoon bieneusi* or *Encephalitozoon intestinalis* is the most common manifestation of microsporidiosis in AIDS patients (Dobbins and Weinstein, 1985; Modigliani *et al*., 1985; Molina *et al*., 1993). Clinical manifestations of disease include wasting, chronic diarrhea, and cholangiopathy and are indistinguishable from the manifestations of isosporiasis and cryptosporidiosis in AIDS patients (McWhinney *et al*., 1991). Diarrheal stools are watery and are not accompanied by blood or fever (Orenstein *et al*., 1990). Routine laboratory tests are normal, with occasional hypokalemia and hypomagnesemia. Carbohydrate and fat malabsorption are present.

2.8.3.4 CRYPTOSPORIDIOSIS, CYCLOSPORIASIS AND ISOSPORIASIS

Cryptosporidium parvum causes chronic (persistent and profuse) watery diarrhea (stool volume of 1-17 liters per day and 6-26 bowel movements per day) which develops and leads to malabsorption, malnutrition, dehydration, and cachexia in immunocompromised persons, including patients with advanced HIV disease (Janoff *et al*., 1990; Pitlik *et al*., 1983; Zar, Geiseler, and Brown, 1985). Cryptosporidiosis that persists for 4 weeks in an HIV-positive person confers a CDC-defined diagnosis of AIDS.

Cyclospora cayetanensis is an emerging worldwide cause of diarrhea in AIDS patients (Wurtz, 1994). Illness caused by *Cyclospora* is characterized by watery diarrhea, abdominal cramping, flatulence, weight loss, and nausea (Huang *et al*., 1995; Ortega *et al*., 1997; Wurtz, 1994). Symptoms typically wax and wane for several weeks and may persist for several months (Huang *et al*., 1995).

Human isosporiasis is caused by *Isospora belli*. Infection is acquired by the ingestion of oocysts in food or water contaminated with the feces of infected humans (Lindsay *et al.,* 1997). In patients with advanced HIV disease, *I. belli* is a cause of debilitating diarrhea with wasting and malabsorption (Soave, 1988; Whiteside *et al*., 1984).

2.8.4 VIRAL INFECTIONS

2.8.4.1 CYTOMEGALOVIRUS INFECTION

Cytomegalovirus (CMV) is a major cause of morbidity and mortality in patients with AIDS (Hoover *et al*., 1993; Lerner and Tapper, 1984). In patients with AIDS, progressive loss of immune function, and, in particular, loss of cell-mediated immunity, permits CMV reactivation and replication to begin. Asymptomatic excretion of CMV in urine can be detected in approximately 50% of HIV-infected individuals with a CD4 lymphocyte count <100 cells/µl (MacGregor *et al*., 1995).

Without antiretroviral therapy most HIV-infected patients would eventually develop CMV end-organ disease including chorioretinitis (Jacobson *et al*., 1988), esophagitis (Wilcox *et al*., 1990), colitis (Meiselman *et al.,* 1985), pneumonia (Shepp *et al*., 1985), and central nervous system disease including radiculopathy (Miller *et al.,* 1990) and subacute encephalitis (Hawley *et al*., 1983). Approximately 75% of injection drug users and >90% of homosexual men who are infected with HIV have detectable IgG antibodies to CMV (Jackson *et al*., 1988). Higher prevalence rates among homosexual men correlates with the increased risk of exposure associated with receptive anal intercourse (Mintz *et al*., 1983).

2.8.4.2 HERPES SIMPLEX VIRUS INFECTION

HSV-1 and HSV-2 infections are common among HIV-1 infected individuals with prevalences that approximate or exceed those in the general population (Strick *et al.,* 2006). The clinical presentation of symptomatic HSV-2 infection can vary considerably among HIV-1 infected persons. HSV reactivation among the HIV-1-infected typically presents with frequent, persistent, extensive and deep painful vesicular and ulcerative lesions of the oral and anogenital areas, particularly among those with low CD4 counts (McClelland *et al*., 2005).

Studies have shown that oral and genital shedding of HSV-1 and HSV-2 occurs more frequently among those who are also infected with HIV-1 than among HSV-infected/HIV-1-uninfected persons (Augenbraun *et al*., 1995; Kim *et al*., 2006; Schacker *et al*., 1998). Among HIV-1-infected persons, HSV mucosal shedding occurs more frequently, and with higher quantity of HSV, among those with lower CD4 counts (Augenbraun *et al*., 1995; Wright *et al*., 2003), although individuals with intermediate or high CD4 counts may also shed HSV-2 frequently (Corey *et al*., 2004).

2.8.4.3 HEPATITIS B INFECTION

In sub-Saharan Africa, where vertical and early childhood exposure are the most common modes of transmission and overall HBV prevalence is higher, the prevalence of HBV among HIV-infected individuals also is higher, at an estimated 20-30% (Hoffmann and Thio, 2007; Uneke *et al*., 2005). HIV-infected persons are half as likely as HIV-uninfected persons to spontaneously clear HBV when infected with HBV at adulthood (Bodsworth *et al.,* 1989).

The course of acute HBV may be modified in the presence of HIV infection, with a lower incidence of icteric illness and lower rates of spontaneous clearance of HBV (Gatanaga *et al*., 2000). Persons with HIV and chronic HBV coinfection have higher levels of HBV DNA and lower rates of clearance of the hepatitis B e antigen (HBeAg) (Gilson *et al*., 1997; Hadler *et al*., 1991; Piroth *et al*., 2007; Thio, 2003). Serum transaminase levels may be lower in HIV/HBV-coinfected patients than in HBV-monoinfected patients

HIV increases the risk of cirrhosis and end-stage liver disease in HBV coinfection (Thio *et al*., 2002). Liver-related disease has emerged as the leading cause of non-HIV-related mortality in parts of the world where effective antiretroviral therapy (ART) is widely available. Studies have reported that the risk of liver-related mortality has been found to be 2-3 times higher in HIV/HBV-coinfected patients than in HIV-monoinfected patients (14% vs 6%) (Bonacini *et al*., 2004; Weber *et al*., 2006). In addition, HIV coinfection is associated with more frequent flares of hepatic transaminases, which can occur with immune reconstitution inflammatory syndrome (IRIS) owing to ART, interruption of HIV/HBV treatment, or the development of resistance to HIV/HBV treatment; they also can occur spontaneously (Chauvel *et al*., 2007).

2.8.4.4 HCV INFECTION

Hepatitis C virus (HCV) infection occurs commonly among HIV-infected individuals, with approximately 20% of HIV-infected persons worldwide estimated to have concurrent chronic HCV infection (Soriano *et al.,* 2006). HCV is transmitted via percutaneous contact with HCV-infected blood, most commonly via shared injection drug use (IDU) equipment or contaminated blood product or hospital equipment. Rates of mother-to-child HCV transmission increase with maternal HIV coinfection (Mast *et al*., 2005). Heterosexual transmission of HCV also is uncommon but infectivity is increased when partners are coinfected with HIV. HCV transmission via men having sex with men (MSM) in the absence of IDU increasingly has been recognized in outbreaks in the United States and Europe (Urbanus *et al*., 2009a). The risk of HCV infection via MSM contact appears to increase with HIV coinfection, concurrent sexually transmitted diseases such as syphilis, drug use, and sex practices that may injure rectal epithelium (Luetkemeyer *et al*., 2006; Urbanus *et al*., 2009a; Urbanus *et al*., 2009b).

Although many individuals are asymptomatic at the time of HCV infection, a subset will develop acute HCV symptoms, which include fatigue, myalgia, jaundice, diarrhea, abdominal pain, and laboratory findings of elevated transaminase and increased bilirubin levels. HIV-coinfected individuals are less likely to clear HCV without treatment, with an estimated 85% developing chronic HCV after acute infection (Thomson *et al*., 2009).

The rate of HCV replication is enhanced in the presence of HIV coinfection, resulting in higher serum and liver HCV RNA levels. The rate of progression of fibrosis in HIV/HCV-coinfected patients is estimated to be 3 times higher than that in HCV-monoinfected patients (Graham *et al*., 2001), with a significantly shorter interval from the time of HCV infection to the development of cirrhosis (estimated at 7 years, vs 23 years in monoinfected patients; p <

.001) (Soto *et al*., 1997). A study of HIV/HCV-coinfected patients undergoing serial biopsies showed that 24% progressed at least 2 fibrosis stages (on a scale of 0 to 4) in an average of 3 years (Sulkowski *et al*., 2007). Some studies have found HIV/HCV-coinfection to be associated with an increased risk of death compared with HCV monoinfection, with a hazard ratio of death of 1.84 (Anderson *et al.,* 2004).

HCV infection may negatively impact CD4 cell count restoration, and cirrhosis is associated with depressed CD4 cell counts, independent of HIV or HCV infection (McGovern *et al*., 2007).

2.8.4.5 VARICELLA-ZOSTER VIRUS INFECTION

Varicella-zoster virus (VZV) is the aetiologic agent of both varicella ("chickenpox") and zoster ("shingles"). Patients with HIV disease are at risk for developing severe illness from either varicella or zoster. Progressive primary varicella, a syndrome with persistent new lesion formation and visceral dissemination, may occur in HIV-infected patients and may be life-threatening. HIV-infected patients with primary varicella are also at higher risk for secondary bacterial infection of skin lesions (Cone and Schiffman, 1984; Dolin *et al*., 1978; Quinnan *et al*., 1984; Ryder *et al*., 1986; Sandor *et al*., 1984).

Zoster eruptions in HIV-infected patients can be extensive and locally destructive, and can become secondarily infected. Zoster may also disseminate cutaneously, and has been reported as the cause of encephalitis in patients with HIV disease (Cohen *et al.,* 1988; Cohen and Grossman, 1989; Colebunders *et al*., 1988; Friedman-Kien *et al*., 1986; Gilson *et al*., 1989).

2.9 HIV ASSOCIATED MALIGNANCIES

2.9.1 ANOGENITAL NEOPLASIA

Anogenital neoplasia includes both cervical and anal cancer and their likely precursor lesions, cervical and anal squamous intraepithelial lesions (SIL). Anogenital neoplasia is an increasing problem among HIV-infected individuals and Human papillomavirus (HPV) infection is one of the most important risk factors associated with anogenital neoplasia (Vernon *et al.,* 1995). HPV types 16 and 18 are associated with high grade cervical or anal SIL and cervical and anal cancer (Pfister, 1996).

There is increased prevalence of anogenital HPV infection and SIL in HIV-infected patients because of the destruction of cell-mediated immunity in HIV infection (Nakagawa *et al*., 1997) and a direct interaction between HIV and HPV at the cellular level (Vernon *et al*., 1993).

Many studies have documented a higher prevalence of cervical HPV infection, cervical squamous epithelial lesions (CSIL), and an increase in latent infections (HPV positive without CSIL on cytology or histology) in HIV-infected women as opposed to women not infected with HIV (Sun *et al*., 1995; Sun *et al*., 1997; Wright *et al*., 1994; Wright and Sun, 1996). Anal cancer is a rare malignancy and it was reported that the risk for anal cancer in HIV-seropositive men doubled from 15 to 30-fold as an AIDS diagnosis was approached (Biggar, 1998). There is a higher prevalence of anal squamous intraepithelial lesions (ASIL) and HPV infections in HIV-Infected women and men compared to seronegative women and men, both of which correlate with the severity of immunosuppression (Hillemanns *et al*., 1996; Williams *et al*., 1994). A higher Incidence of anogenital neoplasia has been reported in HIV infected homosexual men compared to HIV seronegative homosexual men (Critchlow *et al*., 1995).

2.9.2 KAPOSI SARCOMA (KS)

In 1981, the emergence of Kaposi sarcoma (KS) among young homosexual men in New York, Los Angeles, and San Francisco heralded the beginning of the AIDS pandemic (CDC, 1981; Hymes *et al.,* 1981). Kaposi Sarcoma, caused by human herpesvirus-8 (HHV-8), is the most common AIDS-associated malignancy worldwide (Chang *et al*., 1994; Eltom *et al*., 2002).

AIDS patients are excessively at risk for developing KS (20,000 times that of the general population and 70 times that of other immunosuppressed populations) and in such patients KS disproportionately affects homosexuals (Beral *et al*., 1990). Studies have showed that HIV-infected cells can produce extracellular factors that potentiate the growth of KS tumor cells in vitro (Ensoli *et al.,* 1991; Ensoli *et al*., 1990; Ensoli *et al*., 1994; Fiorelli *et al*., 1995; Nakamura *et al*., 1988) and factors released from these cells, in turn, can promote angiogenesis in vivo (Ensoli *et al*., 1989; Miles *et al*., 1990; Salahuddin *et al*., 1988). Studies have reported that the amount of HHV-8 detected was higher in saliva from HIV-positive men compared to HIV seronegative men (Pauk *et al*., 2000). Among men and women infected with HIV and HHV-8 those with higher CD4 counts are more likely to have HHV-8 detected in the oropharynx. HIV-infected individuals not receiving ART and those with evidence of inflammation in the oral cavity also are at increased risk of HHV-8 shedding.

2.9.3 HIV-ASSOCIATED LYMPHOMAS

Approximately 75-80% HIV-associated lymphomas are classified histologically as large-cell lymphomas and the remaining 20-25% as Burkitt (or small-cell). The more commonly observed HIV-associated large-cell lymphomas are classified as either diffuse large B-cell lymphoma/centroblastic or diffuse large B-cell lymphoma/immunoblastic. Similarly, and in agreement with the WHO recommendations, HIV-associated Burkitt lymphoma is now categorized as classical Burkitt lymphoma, Burkitt lymphoma with plasmacytoid differentiation, or atypical Burkitt lymphoma (Meeker *et al*., 1991; Shiramizu *et al*., 1992).

The HIV-1 associated lymphomas are Burkitt's lymphoma, B cell lymphoma, primary effusion lymphoma (PEL), HIV-1 associated Hodgkin disease and HIV-1 associated T-cell lymphoma. A high proportion of HIV-1-associated Hodgkin disease is linked to EBV infection, implicating pathogenetic elements encoded by EBV (Dolcetti *et al*., 2001). In HIV-1 associated T-cell lymphoma prognosis is poor, and survival beyond 9 months is unusual (Shiramizu *et al.,* 1994).

For HIV-1-associated systemic lymphomas (B-cell lymphomas), widespread disease involving extranodal sites is common (Kaplan *et al*., 1989; Knowles *et al*., 1988; Ziegler *et al*., 1984). Symptoms at the time of presentation can be quite variable; 75% of patients have CD4 T-lymphocyte counts >50/mm^3 (advanced AIDS) at presentation, and many patients will not have had prior opportunistic infections (Kristal *et al*., 1988; Levine *et al*., 1991). HIV-1-associated primary CNS lymphomas usually arise de novo in patients with advanced AIDS and prognosis is uniformly poor, and the disease is an EBV opportunistic infection (Kristal *et al*., 1988).

2.10 HIV-SPECIFIC DISORDERS

2.10.1 AIDS DEMENTIA COMPLEX

AIDS dementia complex (ADC) is a neurologic condition characterized by impaired concentration and memory, slowness of hand movements, ataxia and incontinence, apathy, and gait difficulties associated with HIV-1 infection of the CNS. Pathologic examination of the brain revealed white matter rarefaction, perivascular infiltrates of lymphocytes, foamy macrophages, and multinucleated giant cells (Lipton and Gendelman, 1995).

At first, the symptoms of ADC may be indistinguishable from reactive depression. Other symptoms manifested by patients with ADC include behavioural abnormalities such as psychomotor slowing, generalized myoclonus, poor balance, tremors, brisk deep tendon jerks, loss of volition, and progression to coma (Sacktor *et al.,* 1996; Maher *et al.,* 1997),

2.10.2 SPINAL CORD DISEASES

The types of spinal cord diseases that have been observed in AIDS patients include vacuolar myelopathy, pure sensory ataxia and sensory impairment. These symptoms will need to be differentiated from other spinal cord diseases like subacute combined degeneration of the cord due to avitaminosis B12, spinal cord compression, acute inflammatory polyneuropathy, or drug-induced peripheral neuropathies (Onen, 2002).

2.10.3 WASTING SYNDROME

Wasting syndrome, also known as “slim disease” is defined by weight loss of at least 10% body weight in the presence of diarrhoea or chronic weakness and documented fever for at least one month (Onen, 2002). Some factors that contribute to weight loss include metabolic alteration, anorexia, malabsorption, hypogonadism, excessive cytokine production (cytokines such as interferon alpha, TNF –alpha, interleukin-1) and rapid bowel transit due to diarrhoea caused by opportunistic pathogens in HIV disease (Onen, 2002).

2.11 LABORATORY DIAGNOSIS OF HIV

Although HIV antibody tests are the most appropriate for identifying infection, an array of other laboratory methods can contribute to an accurate diagnosis of HIV infection, assist in monitoring the response to highly active antiretroviral therapy (HAART), and can be used to effectively predict disease outcome (Schramm *et al*., 1998). HIV isolation through viral culture, nucleic acid tests to detect viral RNA, and tests to detect p24 antigen can be used to demonstrate virus or viral components in blood, thereby verifying infection. Other methods provide an estimate of T-lymphocyte numbers (cell phenotyping). These methods are highly specific, and a positive result confirms infection.

2.11.1 SEROLOGICAL DIAGNOSIS OF HIV INFECTION

The chronologic order of the sequence of markers to identify HIV infection in serum following infection is: viral RNA, p24 antigen, and anti-HIV antibody. Viraemia increases exponentially about 2 weeks after infection and then declines to a steady state level as humoral and cell-mediated immune responses control HIV replication (Ketema *et al*., 2001). This time interval, known as the serologic "window period," is characterized by seronegativity, occasionally detectable antigenemia, viremia (as measured by RNA), and variable CD4 lymphocyte levels. Detection of specific antibody to HIV signals the end of the window period and labels the individual as seropositive.

2.11.1.1 HIV ANTIBODY ASSAYS

HIV antibody assays can be classified into screening assays and confirmatory assays. The screening assays are designed to detect all infected individuals and thus, possess a high degree of sensitivity. Confirmatory assays, on the other hand, further confirm the presence of HIV infection by excluding individuals who are not infected, and thus have a high specificity (Constantine *et al*., 1994).

2.11.1.1.1 HIV SCREENING ASSAYS

A. RAPID TESTS

Rapid assays are tests used for detecting specific HIV antibody with results available in less than 30 minutes. These tests, which have proved to be as accurate as the ELISA method when performed carefully by experienced personnel, have wide utility in a number of testing situations such as emergency rooms, labour rooms, physicians' offices, point-of-care testing, autopsy rooms, funeral homes, small blood banks, situations where immediate treatment is recommended for exposures and large-scale or community-wide HIV testing programs (CDC (1998). Importantly, these rapid assays are simple, robust, and easy to perform, easy to interpret, require little or no addition of reagents, and contain a built-in quality-control reagent to control for technical errors. Some tests can be stored at a wide range of temperatures (from 15° C to 30° C), transported easily, and are widely used in developing countries, where facilities may not be optimal, stable electricity may be unavailable, and formal education programs for laboratorians may be absent or grossly inadequate (Ketema *et al*., 2001). Several of these rapid tests have the ability to differentiate HIV-1 and HIV-2 (Schramm *et al*., 1998).

The formats of rapid assays in use include the dip-stick, dot-blot (or immunoblot) and 1-step and assays. In the dipstick rapid assay antigen is attached on the "teeth" of comblike devicese.g. Immunocomb (Schramm *et al*., 1998). Most dot-blot assays have the same procedure requiring drop-wise additions of reagents in the following sequence: buffer, sample, wash buffer, conjugate, wash buffer, substrate, and stop solution. Some assays substitute an IgG binding dye (protein A gold reagent) for the antiimmunoglobulin conjugate, thereby decreasing the procedure by a step. A positive test will produce a well-circumscribed colored dot on the solid phase surface. An example is Genie 2.

The 1-step rapid assays, also known as immunochromatographic assays, consist of a self-contained flat cartridge device which is usually plastic or paper. Whole blood, plasma, or serum or is placed at the tip of the device and allowed to diffuse along a strip that is impregnated with reagents (often protein A colloidal gold) that bind and permit visual detection of HIV antibodies; some use third-generation (antigen sandwich) technology. Popular examples include Determine (Abbott, USA), Stat Pak (Chembio, Ireland) and Uni-Gold (Trinity Biotech; Wicklow, Ireland) to mention a few (Ketema *et al*., 2001; Schramm *et al*., 1998). Disadvantages of rapid assays include a subjective interpretation and difficulty in reading if the laboratorian is color-blind.

B. ELISA (Enzyme-Linked Immunosorbent Assay)

The Enzyme-linked immunosorbent assay (ELISA) is commonly used to screen for HIV infection because of its relatively simple methodology, high sensitivity, and suitability for testing large numbers of samples, particularly in blood testing and transfusion centers (Constantine and Zink, 2005; Constantine *et al.* 2005). The use of enzyme conjugates (an antihuman immunoglobulin with a bound enzyme) that bind to specific HIV antibody and substrates (chromogens) that produce color in a reaction catalyzed by the bound enzyme are common features of all varieties of ELISAs (>40 different ELISA test kits).

The most common ELISA method used in HIV testing is the indirect ELISA method. In the indirect ELISA method HIV antigen is attached to a well of a 96-well microtiter plate (which comes already prepared by the manufacturer). Antibody in the sample for testing is allowed to react with the antigen-coated solid support, usually for 30 – 60 minutes at 37º C. After a wash step to remove unbound serum components, addition of the conjugate binds to the specific antibody that is attached to the antigens on the solid phase. After another wash step, addition of an appropriate substrate results in color development that is detected by a

spectrophotometer and is proportional to specific HIV antibody concentration in the sample. Optical density (OD) values are produced as the colored solution absorbs transmitted light, and provide an indication of the amount of color, which is proportional to the antibody concentration. A mathematical calculation, usually based on the OD of the negative controls multiplied by a factor, produces a cutoff value on which the OD of the sample is compared to determine the antibody status; samples with OD values > OD cutoff are considered antibody reactive (positive). Several indirect ELISA tests incorporate polyvalent conjugates (anti-IgG and anti-IgM) and antigen-sandwich configurations in order to increase sensitivity for detecting early infection (during seroconversion) (Constantine *et al*., 1994).

Assays in an ELISA format that have the capability to detect both HIV antibody and HIV p24 antigen simultaneously have also been developed, thereby eliminating the need to perform separate assays (Weber *et al*., 1998). These ELISAs offer advantages for decreasing the time, personnel, and costs necessary to perform each assay individually and have demonstrated a high analytical sensitivity of detection that is most likely attributed to the combination of a third-generation format (antigen sandwich) for antibody detection and the ability to simultaneously detect HIV p24 antigen (Lackritz *et al*., 1995). Some examples of these fourth-generation assays include the VIDAS HIV DUO Ultra (bioMérieux; Marcy l'Etoile, France), Enzymun-Test-HIV-Combi (Boehringer; Mannheim, Germany) (Martinez-Martinez *et al*., 1999); (Yerly *et al.,* 1999), Vironostika HIV Uni-Form II Ag/AB (Organon Teknika; Boxtel, Netherlands) (van Binsbergen *et al*., 1998; van Binsbergen *et al*., 1999), AxSYM-HIV Ag/AB (Abbott Laboratories; Abbott Park, IL), Enzygnost HIV Integral (Dade Behring; Marburg, Germany), Genescreen Plus HIV Ag-AB (Bio-Rad), and COBAS Core HIV Combi (Roche Diagnostics; Mannheim, Germany) (Hayashi *et al*., 1999; Ly, Edlinger, and Vabret, 2000; Weber *et al*., 1998).

2.11.1.1.2 HIV CONFIRMATORY ASSAYS

A. WESTERN BLOT TEST

The Western blot is the most widely accepted confirmatory assay for the detection of antibodies to HIV. Most authorities consider it the gold standard for validation of HIV results. It is based on using a nitrocellulose paper blotted with different bands of electrophoretically separated and purified HIV antigens and the subsequent identification of specific antibodies to each of the viral antigens in a set of steps similar to the ELISA methodology (Constantine, 1999).

The HIV-1 viral proteins and corresponding bands on the WB are designated p (for proteins) or Gp (for glycoproteins), followed by the relative molecular weight (x1000) are separated as follows (from top to bottom): gp160, gp120, p66, p55, p51, gp41, p31, p24, p17, and p15 (Gueye-Ndiaye, 2002; Olaleye *et al.,* 2006).

Reactivities between HIV antibodies in the individual's sample with the separated antigenic components result in band profiles. The type of profile, ie the combination and intensity of bands, which are present, determines whether the individual is considered positive for antibodies to HIV. The classification of Western blot results is determined by certain criteria. Criteria established by manufacturers include reactions to 1 gene product from each of the 3 major groups (Gag, Pol, and Env) for positivity for HIV-1 while the CDC guidelines require reactivity to at least 2 of the following antigens: gp41, p24, gp120/160 for a positive classification. A negative result is the absence of all bands or the presence of only a very weak p17 band (Gueye-Ndiaye, 2002; Olaleye *et al.,* 2006). Indeterminate classifications occur when there is reactivity to 1 or more antigens, but not fulfilling the criteria for positivity, with most indeterminate results showing only weak reactions to the gag proteins (mostly p17, p24 and/or p55).

Some individuals who exhibit indeterminate results (eg, reactivity to p24 and p55) later seroconvert, demonstrating that a p24 and p55 profile can indicate early infection. Conversely, other individuals may have the identical profile for long periods of time (years) and never seroconvert (ie, they are not infected). In fact, most indeterminate Western blot results from noninfected individuals exhibit the p24 and/or p55 profile. Therefore, an indeterminate Western blot result cannot predict early infection. The WHO and other organizations like the CDC recommend retesting persons after 1-6 months. If at all possible, the retesting of an individual at a later time should be performed in parallel with reassay of the initial sample on the same run with the same kit lot numbers and the same assay conditions to ensure that the samples can be compared directly. If an individual is retested over a period of 1 year and becomes negative or the band profiles do not progress, infection with HIV generally can be ruled out (Gueye-Ndiaye, 2002; Olaleye *et al.,* 2006).

The significance of an indeterminate Western blot result varies depending on the risk factors, clinical status of the patient, and the Western blot profile produced. For example, individuals with a history of high-risk behavior are more likely to be the ones who later seroconvert, because the chances of their being infected are high. In addition, some Western blot profiles are more suggestive of early infection (eg, p24, p31, and p55) than are others (eg, p17 only). Many initially indeterminate results that subsequently become negative or remain indeterminate probably are a result of nonspecific reactions, hypergammaglobulinemia, the presence of cross-reactive antibodies, infection by HIV-2, or infection by an unknown, but related retrovirus. There have been a few reports where autoimmune diseases (eg, systemic lupus erythematosus) can cause false-positive HIV tests, including Western blot (Jindal *et al.,* 1993). Also, it is known that some individuals with AIDS may lose reactivity to p24, and perhaps other antibodies, later in disease, so that even AIDS patients may have indeterminate Western blot results by some criteria. Ancillary tests, such as polymerase chain reaction

(PCR) and viral culture may be helpful in resolving these indeterminate results if the diagnosis is in question (Gueye-Ndiaye, 2002; Olaleye *et al.,* 2006).

Most recently developed western blot assays incorporate the use of viral lysates from HIV-1 and synthetic peptides artificially applied from HIV-2 on the same nitrocellulose strip. In this case, multiple HIV-1 antigens and 1 HIV-2-specific band (gp36 or gp41) are present on the strip. To be considered positive for HIV-2, the test must show reactions to the HIV-2-specific antigen plus a reaction to HIV-1-specific antigens.

B. INDIRECT IMMUNOFLUORESCENT ANTIBODY ASSAY

In this technique lymphocytes are infected with HIV and are fixed to a microscope slide. Serum containing HIV antibodies is added and reacts with the intracellular HIV. The slide is washed and then allowed to react with antiimmunoglobulin antibodies with a covalently bound fluorescence label attached. The reaction is visualized using a fluorescent microscope. This technique has the advantage of sometimes providing definitive diagnosis of samples that have yielded indeterminate results by Western blot analysis. Disadvantages to its use include the requirement of an expensive microscope and a subjective interpretation, thus necessitating well-trained individuals.

C. LINE IMMUNOASSAY

Another alternative to the classic Western blot and IFA confirmatory tests is the line immunoassay (LIA). In this assay, recombinant or synthetic peptide antigens are applied on a nitrocellulose strip, rather than electrophoresed as in the Western blot. This use of "artificial" antigens decreases the presence of contaminating substances derived from cell culture that can cause interference and sometimes false reactions. The use of LIA is popular in Europe. A number of reports have verified that the accuracy is equivalent to the Western blot (Tamashiro *et al*., 1993).

2.11.1.2 HIV p24 ANTIGEN DETECTION ASSAY

The p24 antigen, although transient, characteristically appears early and late during HIV infection but remains undetectable in most asymptomatic patients during the course of infection (Allain *et al*., 1986). It is found in serum as both free form or bound by anti-p24 antibody. Free p24 can be measured with enzyme immunoassays whereas detection of bound p24 requires pretreatment with an acid to dissociate the complex. Procedures to dissociate antigen-antibody complexes have improved the sensitivity of the p24 test, but antigen (Hammer *et al*., 1993). Testing for p24 antigen is of value in: detecting early HIV infection (Daar *et al*., 1991), screening blood, diagnosing infection in the newborn (Lange *et al*., 1986), and monitoring antiviral therapy (Spector *et al*., 1989).

The p24 antigen can appear as early as 2 weeks after infection and lasts 3 to 5 months (Allain *et al*., 1986) corresponding to the appearance and increase of anti-p24 antibody (Lackritz *et al*., 1995). Late in the course of infection, i.e., as progression to clinical AIDS occurs, anti-p24 antibody decreases and p24 antigen again becomes detectable (Schupbach *et al*., 1985). The decline in antibody to p24, the production of antigen, and the generation of immune complexes are most likely related to changes in viral replication (Goudsmit *et al*., 1987), because antibodies to other components (e.g., envelope glycoproteins) persist throughout infection (Constantine *et al.* 1992).

The p24 Antigen Test

The p24 antigen test detects soluble p24 antigen (viral capsid core) after viral replication. Antigen detection signals infection and positive results in seronegative individuals can be an effective means to identify early infection (Daar *et al*., 1991). A negative result for antigen does not rule out infection both because low levels of antigen are difficult to detect, and because antigenemia occurs only transiently during different stages of infection (Goudsmit *et*

al., 1987). In fact, it has been reported that the HIV antigen test is incapable of detecting 75% of blood donors who are infected but seronegative. However, the specificity of the p24 antigen test in detecting HIV infection using PCR as the gold standard was calculated from data using test kits from 2 manufacturers to be 99.9-100% (Goudsmit *et al*., 1987).

The p24 antigen test is the method of choice for detecting the presence of free antigen in supernatants in HIV viral culture, and more sensitive than the reverse transcriptase assay (Daar *et al*., 1991). The test is most useful for detecting viral antigen in culture fluids because large quantities of antigen are exuded in the supernatant fluid during viral replication. The p24 antigen test can also be performed on fluids other than those of culture and serum. Evidence determined from the testing of cerebrospinal fluid (CSF) indicates that many patients with HIV dementia and encephalopathy have detectable antigen in CSF, most likely due to active replication of the virus in cerebral tissue.

2.11.2 NUCLEIC ACID-BASED HIV-1 ASSAYS

Untreated HIV-1 infection is characterized by high-level viral production and CD4+ T-cell destruction which progresses, after a period of clinical latency, to significant net loss of CD4+ T cells and AIDS (Ho *et al*., 1995; Perelson *et al*., 1996; Wei *et al*., 1995). The absolute level of steady-state viral load is a strong predictor of the rate of disease progression and, in combination with CD4+ T-cell counts, is of great prognostic value (Katzenstein *et al*., 1996; Mellors *et al*., 1997; Mellors *et al*., 1996; O'Brien *et al*., 1996b; Ruiz *et al*., 1996; Saag *et al*., 1996). The measurement of blood plasma HIV-1 RNA concentration (viral load) using nucleic acid-based molecular diagnostic assays is the standard of care in many areas of the world with access to antiretroviral therapy (ART) and standard molecular diagnostic facilities or laboratories (Saag *et al*., 1996). Consequently, sensitive precise viral load assays are used to quantify HIV-1 RNA accurately.

Current ART guidelines advocate the use of plasma viral load testing at baseline when considering ART initiation, monitoring response to therapy periodically, and instituting a change in drug regimen when virologic failure or resistance develops.

2.11.2.1 AMPLICOR HIV-1 MONITOR V1.5 ASSAYS

There are 3 Amplicor HIV-1 Monitor v1.5 assays (Roche Diagnostics, Branchburg, NJ) currently approved for in vitro diagnostic use. The 3 viral load assays share a PCR-based target amplification strategy (Mulder *et al*., 1994) but differ in the type of equipment used, degree of automation, and method of sample preparation. Following isolation of viral RNA, reverse transcription (RT) is performed to yield single-stranded complimentary DNA (cDNA). The cDNA is amplified exponentially in repeated cycles of heating and cooling by PCR with the v1.5 primers SK145 and SKCC1B, which are complementary to highly conserved regions in HIV-1 *gag* and are optimized to yield equivalent amplification of HIV-1 group M (subgroup A-H) viruses (Michael *et al*., 1999; Triques *et al*., 1999). Viral RNA is quantified using a synthetic, noninfectious armored (protein-coated) RNA construct as the internal quantitation standard (QS), added to the specimen at a known concentration before RNA extraction. The internal QS can be differentiated from the viral target sequences and serves to compensate for variability in RNA extraction and to indicate substances in plasma that may be inhibitory to PCR amplification. External controls consisting of a high positive, low positive, and a negative control also are included in each assay. All Amplicor HIV-1 Monitor assays use 1 of 2 viral RNA extraction procedures with overlapping linear dynamic ranges: the Ultrasensitive (50 to 100,000 copies/mL) or the Standard (400 to 750,000 copies/mL) (Sun *et al*., 1998).

2.11.2.2 VERSANT HIV-1 RNA 3.0 ASSAY (BDNA)

The Versant HIV-1 RNA 3.0 assay (Bayer, Tarrytown, NY) is a bDNA sandwich nucleic acid hybridization method that quantifies plasma HIV-1 by amplifying the signal rather than the target RNA. The bDNA assay is performed in a 96-well microtiter plate format using the System 340 bDNA Analyzer (Collins *et al.*, 1997; Dewar *et al.*, 1994; Kern *et al.*, 1996). It is FDA approved for in vitro diagnostic use and has a wide linear dynamic range (75 to 500,000 copies/mL within the United States, 50 to 500,000 copies/mL outside the United States). The bDNA assay does not require viral RNA purification or PCR amplification steps. Instead, virions are concentrated by centrifugation and disrupted by detergent and proteinase K, releasing viral RNA. This lysate is incubated with 2 sets of oligonucleotides. The first set captures viral RNA, hybridizing both to conserved regions of the HIV-1 *pol* gene and to oligonucleotides bound to the microwell. The second set of oligonucleotides provides signal amplification. This set consists of 4 components: oligonucleotides with homology to both the target RNA and to preamplifier oligonucleotides (target probe), preamplifier oligonucleotides, amplifier oligonucleotides, and oligonucleotide probes bound to alkaline phosphatase (AP) for detection. Each of these components binds by hybridization to the next at multiple sites. In this way, the signal is amplified without copying the target RNA. Detection is by chemiluminescence using an AP-specific substrate. The amount of light detected is directly proportional to the amount of bound nucleic acid. The absolute quantity of HIV-1 RNA is determined from an external standard curve run on the same plate. The assay does not incorporate an internal QS.

2.11.2.3 NucliSens HIV-1 ASSAY

The NucliSens HIV-1 QT assay (bioMerieux, Boxtel, Netherlands) is based on target amplification using NASBA technology. The assay selectively and directly amplifies HIV-1 RNA in an isothermal, 1-step sandwich hybridization procedure using 2 oligonucleotide

primers, 3 enzymes, nucleoside triphosphates and the appropriate buffers (van Gemen *et al.*, 1993). First, RNA is extracted and highly purified using guanidine thiocyanate and silicon dioxide particles. The RNA is amplified by repeated cycles of synthesis and transcription off a double-stranded DNA intermediate. An oligonucleotide primer (P1) specific to a region in HIV-1 *gag* is used to synthesize cDNA from the specimen RNA template using avian myeloblastosis virus reverse transcriptase. The RNA strand is degraded by RNAse H, allowing the oligonucleotide primer P2 to bind and initiate second-strand DNA synthesis. Antisense RNA is then transcribed off the double-stranded DNA via a T7 polymerase promoter (originally incorporated by P1). This cycle is repeated, resulting in exponential amplification (10^6- to 10^9-fold). The amount of nucleic acid is determined directly by electrochemiluminescence, which is characterized by very high sensitivity and a broad dynamic range. Quantitation of HIV-1 viral load is accomplished using the NucliSens Reader by coamplification of 3 internal RNA quantitation standards, or calibrators. The calibrators are spiked into the original specimen and are coextracted and coamplified with the sample RNA.

2.11.2.4 LCx HIV RNA QUANTITATIVE ASSAY

The LCx HIV RNA Quantitative Assay (Abbott Laboratories, Abbott Park, IL) uses competitive RT-PCR for target amplification of blood plasma HIV-1 *pol* sequences followed by microparticle enzyme immunoassay. The assay uses an internal standard for each specimen that is carried through specimen preparation, amplification, and detection to control for sample preparation and amplification inhibitors, and a set of 6 calibrator/standards for quantitation. It has an extensive linear dynamic range, 50 to $1x10^6$ copies/ml for a 1 ml plasma input volume and 178 to $5x10^6$ copies/ml for a 0.2 ml plasma input volume (Johanson *et al.*, 2001).

The LCx HIV RNA Quantitative Assay can be performed with varying degrees of automation. Sample preparation may be performed manually using modified Qiagen sample preparation kits (QIAGEN, Hilden, Germany) (Johanson *et al.*, 2001), or with automation using the MagNA Pure LC instrument. Both approaches demonstrate equivalent performance characteristics (Muller *et al.*, 2004). Following sample preparation and reverse transcription, the cDNA is amplified in a competitive PCR reaction, in which primer concentration is limiting. Primers are labeled with the hapten carbazole, allowing subsequent capture via anticarbazole labeled microparticles using the LCx Analyzer. HIV- and QS-specific detection probe-conjugates are present during the PCR cycles, allowing colorimetric distinction and quantification after amplification in a microparticle enzyme immunoassay. Copy number is calculated by the LCx Analyzer, which determines the signal ratio for the HIV and QS products, and based on the independent calibration curve, the concentration is determined.

Of interest, the LCx assay can specifically amplify and quantify group O viruses (Swanson *et al.*, 2001; Swanson *et al.*, 2006). It also demonstrates superior performance in measuring group M subtype C and some circulating recombinant form (CRF) viruses (Swanson *et al.*, 2006). Numerous other studies comparing the performance characteristics of viral load assays in genetically diverse strains (Swanson *et al.*, 2006) underscore the challenges of designing amplification strategies to equally detect all viruses. With continuous generation of diversity and recombinant forms in combination with global migration, discrepant viral load results should be reevaluated in the context of group and subtype involved.

2.11.2.5 REALTIME HIV-1 ASSAY

The RealTime HIV-1 Assay (Abbott Laboratories) measures PCR product amplification in real time with a unique partially double-stranded probe design to minimize inefficient binding due to sequence mismatch at the probe binding site. The probe strands are labeled with a

fluorophore (reporter) at the 5' end, and a quencher moiety at the 3' end of the shorter, complimentary strand (Swanson *et al.*, 2006). In the presence of target *pol* sequences, the reporter probe preferentially binds, and upon release of the shorter quencher probe, fluoresces. The RealTime HIV-1 Assay uses an armored RNA internal standard introduced during the specimen preparation step, which is differentiated from the amplified target sequence by a fluorescent single-stranded oligonucleotide probe. Because the exonuclease activity of *rTth* polymerase is not required in the RealTime assay, as it is for TaqMan-based assays, the annealing temperature of the probe binding step is lower than that of the amplification steps, minimizing the impact of mismatches at the probe binding site. Sample preparation and amplification/detection steps can be automated using the *m*2000*sp* and *m*2000*rt* instruments.

2.11.3 VIRUS ISOLATION

Although isolation of HIV is the gold standard for detecting the presence of HIV in a patient's blood, virus isolation is not a requirement for diagnosis of HIV infection in clinical practice. Virus isolation is used as the standard by which new diagnostic methods are evaluated before introduction in clinical practice (Krivine *et al.,* 1990). Virus isolation is routinely used as a research tool and not as a routine diagnostic method because it is both capital and labour intensive requiring up 2-4 weeks for isolation of HIV, technical expertise and high cost for procurement and maintenance of tissue culture facilities.

HIV-1 can be isolated from saliva, CSF, brain, semen, breast milk, urine, lymphoid tissue, and plasma. Studies have reported that peripheral blood mononuclear cells (PBMCs) are the best medium for HIV isolation (Ho *et al.,* 1985; Gaines *et al.,* 1987) but isolation rate is dependent on a number of factors including the quality and quantity of the sample, the

number of infected cells in each sample and the expertise of personnel performing the virus isolation procedure (Harper *et al.,* 1986; Ulrich *et al*., 1988).

HIV infected PBMCs are co-cultivated with uninfected PBMCs, stimulated with Interleukin-2 and maintained in media supplemented with glutamine and heat inactivated fetal calf serum to which fresh culture media and supplements are added at regular intervals with aliquots of culture supernatants used to detect HIV replication by serology or quantification of HIV viral gag antigen (Renjifo, 2002).

2.12 HIV TRANSMISSION AND RISK FACTORS

The Human Immunodeficiency Virus has been isolated from various body fluids of infected individuals such as blood, seminal fluid, pre-ejaculate, vaginal secretions, cerebrospinal fluid, saliva, tears, and breast milk (Geier *et al*., 1992; Hollander and Levy, 1987; Wofsy *et al*., 1986). Moreover, HIV-1 DNA sequences have also been detected in pre-ejaculatory fluid (Ilaria *et al*., 1992). In genital fluids, HIV may be found in both cell-free and cell-associated compartments, but it is unknown which is responsible for productive infection (Alexander, 1990).

HIV is transmitted through direct contact of a mucous membrane with a bodily fluid containing HIV, such as blood, semen, vaginal fluid, preseminal fluid or breast milk. Consequently HIV transmission has been divided into three main transmission routes.

2.12.1 SEXUAL TRANSMISSION OF HIV

Sexual exposure is the primary method of spread of HIV infection worldwide and it occurs when infected sexual secretions of one partner come into contact with the genital (vaginal sex), oral (oral sex), or rectal (anal sex) mucous membranes of another partner. Heterosexual intercourse is the primary mode of HIV transmission globally and it also accounts for about

70% of overall sexual transmission in the areas of highest HIV prevalence (Gayle, 2000). Transmission of HIV occurs more frequently through penile-anal intercourse and penile-vaginal intercourse than through oral intercourse, although clear cases of transmission through oral sex exist (Rothenberg *et al*., 1998). Also female-to-female HIV transmission has been reported, but is rare (Monzon and Capellan, 1987). Sexual activity that is associated with exposure to infected blood (like menstrual blood) increases the risk of transmission, as does the presence of genital ulcers from STIs (Cameron *et al*., 1989; Greenblatt *et al*., 1988; Plummer *et al*., 1991). Serum HIV viral load is strongly associated with heterosexual transmission between HIV-serodiscordant sexual partners, where transmission was noted to be rare at viral loads <1,500 copies/ml (Quinn *et al*., 2000).

2.12.2 TRANSMISSION THROUGH BLOOD AND BLOOD PRODUCTS

HIV transmission through transfusion of contaminated blood products was recognized early in the pandemic CDC (1982). With current testing methods, the risk of acquiring HIV from transfused blood has been significantly reduced in many parts of the world.

HIV transmission can also occur through injection drug use, occupational exposure, or accidental needlesticks. A study on the risk of HIV infection from occupational needlesticks to health care workers from known HIV-positive source patients in case series performed prior to the availability of potent ART was found to be 0.33-0.5% (Cardo *et al*., 1997; Leentvaar-Kuijpers *et al*., 1990). Deep injury, injury with a visibly bloody device, or injury with a device that had been previously used in the source patient's vein or artery are factors that have been shown to increase the risk of HIV acquisition from an occupational needlestick (Cardo *et al*., 1997). However, Postexposure prophylaxis (PEP) has been associated with a reduction of HIV transmission after occupational needlestick events of approximately 80% (CDC, (1996).

2.12.3 MOTHER-TO-CHILD TRANSMISSION OF HIV

Mother-to-child transmission of HIV is the transmission of HIV from an HIV-positive mother to her child during pregnancy, labour, delivery or breastfeeding. In the absence of interventions, mother-to-child transmission rates range from 15-45% of live births to HIV-infected mothers (Connor *et al*., 1994). Different regimens of highly active antiretroviral drugs can reduce the rate of perinatal transmission by 50% or more (Connor *et al*., 1994; Guay *et al*., 1999; Lallemant *et al*., 2000; Shaffer *et al*., 1999; Chalermchokcharoenkit *et al.,* 2004). Approximately a third of all cases of mother-to-child transmission result from breast-feeding, with increasing risk of infection as the duration of breast-feeding increases (Richardson *et al*., 2003).

2.13 TREATMENT OF HIV AND AIDS

2.13.1 CHEMOTHERAPEUTIC AGENTS

The development and use of highly active antiretroviral therapy (HAART) has had a profound influence on the course of HIV-1 infection worldwide; with a marked decrease in AIDS-related mortality (WHO, 1993) observed in most regions of the world (1993; Egger *et al*., 2002; Palella *et al*., 1998). More than 20 antiretroviral drugs in four different classes are already in use for the treatment of HIV-1 infection; and many more are still in clinical development (appendix 3).

2.13.1.1 REVERSE TRANSCRIPTASE INHIBITORS

Inhibition of reverse transcriptase (RT) by substrate analogs (nucleoside and nucleotide RT inhibitors [NRTI]) and by noncompetitive inhibitors (non-nucleoside RT inhibitors [NNRTI]) constitutes the mainstay of most antiretroviral regimens, and divides reverse transcriptase into two types based on their modes of action (Levy, 1998).

The NRTI lack a 3-OH group, and once incorporated into the growing complementary DNA (cDNA) strand, they act as chain terminators, bringing reverse transcription to a halt (Patick and Potts, 1998). Another feature of the NRTI is their need for phosphorylation by intracellular nucleoside and nucleotide kinases to generate the active deoxynucleotriphosphate (dNTP) forms of these drugs (Garcia de la Hera *et al.,* 2004). The NRTIs have formed the backbone of antiretroviral therapy since the introduction of zidovudine (AZT) in 1987. Some NRTIs in clinical practice today include Abacavir (Ziagen, ABC), Didanosine (Videx, ddI), Emtricitabine (Emtriva, FTC), Lamivudine (Epivir, 3TC), Stavudine (Zerit, d4T) and Tenofovir (Viread, TDF).

The NNRTI are a chemically diverse class of drugs that occupy a potential drug-binding pocket in RT distinct from the dNTP binding site. Binding induces conformational changes that essentially inactivate RT (Esnouf *et al.,* 1995); in this respect, the NNRTI can be considered allosteric inhibitors of RT function (Stein *et al.,* 1997). Currently approved NNRTI generally are inactive against HIV-2, which lack tyrosine residues at positions 181 and 188 that are essential for drug binding (Richman and Bozzette, 1994). Drugs in this class play a central role as preferred agents for first-line regimens and for prevention of mother-to-child transmission. Examples of NNRTIs are Delavirdine (Rescriptor, DLV), Efavirenz (Sustiva, Stocrin, EFV), Etravirine (Intelence, TMC 125), Nevirapine (Viramune, NVP) and Rilpivirine (Edurant, TMC 278).

2.13.1.2 PROTEASE INHIBITORS

HIV protease is encoded by the polymerase (*pol*) gene and catalyses its own synthesis and cleavage, and that of Gag and Gag-Pol polyprotein, into functional structural polyproteins and enzymes (Wlodawer and Erickson, 1993). Inhibition of HIV-1 protease prevents processing of the Gag and Gag-Pol polyprotein precursors into their mature substituents,

which comprise the structural proteins of the virus core particle (capsid, matrix, and nucleoprotein) as well as the three virally encoded enzymes (protease, reverse transcriptase, and integrase) required for virus replication. In contrast to inhibitors of virus entry and RT, protease inhibitors do not prevent uninfected cells from becoming infected by HIV-1, but result in the release of noninfectious virions ((Flexner, 1998; Kageyama *et al*., 1995; Peeters *et al*., 1997). Most protease inhibitors are peptidic or peptidomimetic compounds designed as analogs of the cleavage sites found within the Gag and Gag-Pol precursor proteins. Because of their relatively poor oral bioavailability, most protease inhibitors are coadministered with a low dose of ritonavir, an HIV-1 protease inhibitor that inhibits the 3A4 isozyme of cytochrome P_{450} (CYP 3A4) (Kieffer *et al*., 2004); CYP 3A4 is responsible for metabolism of most of these drugs. Protease inhibitors have proved to be highly effective in the treatment of HIV-1 infection as components of initial and subsequent treatment regimens and some PIs used in clinical practice are Atazanavir (Reyataz, ATV), Darunavir (Prezista, DRV, TMC 114), Fosamprenavir (Lexiva, Telzir, FPV), Indinavir (Crixivan, IDV), Lopinavir/Ritonavir (Kaletra), Nelfinavir (Viracept, NFV), Ritonavir (Norvir, RTV), Saquinavir (Invirase, SQV) and Tipranavir (Aptivus, TPV).

2.13.1.3 FUSION INHIBITORS

The first step in the HIV life cycle is virus entry, a multistep process that involves attachment, coreceptor binding, and fusion of the cell and virus membranes. Small molecule inhibitors that block the gp120-CD4 interaction have been developed (Gurtler *et al*., 1994; Lindback *et al*., 2000).

Potent inhibition of HIV-1 replication was demonstrated in preliminary clinical trials by a small-molecule chemokine receptor antagonist that prevent binding of gp120 to CCR5 (Dorr *et al.,* 2005; (Fatkenheuer *et al*., 2005; Lalezari *et al*., 2003; Sulkowski *et al*., 2004). This

CCR5 antagonist, Maraviroc (Selzentry, Celsentri, MVC), is now in use in clinical practice for treatment of HIV infection.

The efficacy of the fusion inhibitor Enfuvirtide (Fuzeon, ENF, T-20), a synthetic 36-amino acid oligopeptide, was demonstrated in a series of randomized clinical trials (Ledergerber *et al*., 2004). This drug blocks virus fusion by preventing the formation of a six-helix bundle by two heptad repeats (HR-1 and HR-2) in the trimeric gp41 ectodomain (Wild *et al*., 1994; Williams *et al*., 2004).

2.13.1.4 INTEGRASE INHIBITORS

Following reverse transcription of HIV RNA, the resulting linear double-stranded DNA (dsDNA) molecule must be integrated into the host chromosome. The process of integration, which is catalyzed by the virally encoded integrase, is a multistep process that involves formation of a preintegration complex, nuclear importation, endonucleolytic processing of the 3'-ends of the DNA molecule, and a strand-transfer reaction that results in covalent attachment of the viral and cellular DNA.

The development of appropriate high-throughput screening assays (Lindback *et al*., 2000) has allowed identification and development of specific inhibitors of the strand-transfer reaction (Heberling *et al*., 1988). Important integrase inhibitor in the treatment of HIV-1 infection is Raltegravir (Isentress, RAL).

2.13.1.5 COMBINATION THERAPY

The treatment of HIV infection has been revolutionized by the introduction of increasingly potent combination drug therapies. Where one drug had a beneficial effect, combinations of 2 or 3 complementary drugs can reduce vireamia to levels that cannot be detected by the best viral load assays currently available. This has resulted in a dramatic decrease in the number

of AIDS-related deaths in persons using combination therapy and this highly effective approach has been termed highly active antiretroviral therapy (HAART) (Rosen, 1999).

The report of an improvement in efficacy associated with the combination of AZT (zidovudine) and 3TC (Lamivudine) (Enron *et al.,* 1995) was the first indication how using a combination of drugs with different modes of action could be in controlling HIV replication *in vivo*. Different HAART regimens currently in use in clinical practice are shown in Table 5.

2.13.2 IMMUNE MODULATORS FOR HIV TREATMENT

Although HAART has considerably altered the course of HIV infection, problems associated with drug toxicities, virus persistence in some latently-infected cells, availability of drugs to less priviledged societies and the development of resistance to HAART limit the general application of this strategy (Gotch *et al.,* 1999). Since control of HIV viremia depends on host immune response as well as exogenous intervention with antiretroviral medications, several techniques of immune modulation have been studied in an effort to enhance host immune response to HIV.

IL-2, a cytokine released by activated CD4 cells that regulates T-cell proliferation and maturation, has been studied as an immune modulator in HIV-infected patients. Administration of IL-2 to individuals with controlled HIV viremia leads to increased CD4 counts (Davey *et al*., 1999; Kovacs *et al*., 1996), expansion of both memory and naive CD4 pools (Hetch *et al.,* 2003), and decreased T-cell activation (Hetch *et al.,* 2003). IL-2 administration is accompanied by numerous adverse effects and toxicities. No clinical benefits of IL-2 have yet been demonstrated. Large clinical trials of IL-2 are underway. Other cytokines, such as IL-12 (McFarland *et al*., 1998) and IL-4 (Huang *et al*., 2000) are being studied, as is IL-2 in combination with therapeutic vaccination.

Techniques of immune manipulation through infusion of antigen-presenting cells that have been activated in vitro (Lu *et al*., 2003), infusion of expanded (Levine *et al*., 1996) or activated (Levine *et al*., 2002) CD4 cells, and (Kovacs *et al*., 1996) genetic manipulation of cells to induce anti-HIV CTL activity (Roberts *et al*., 1994) are also under investigation, but even if such resource-intensive, individualized approaches are shown to be effective, it is doubtful that they will become accessible to most people infected with HIV.

Administration of human growth hormone (hGH) as a method of enhancing CD4 recovery in HIV is another technique being studied, spurred by the observations of increased circulating naive CD4 cells and increased thymic mass, suggesting increased thymopoeisis, during hGH treatment (Napolitano *et al*., 2002).

CHAPTER 3

MATERIALS AND METHODS

3.1 COLLECTION OF BLOOD SAMPLES

3.1.1 SAMPLE AREA

Samples used for this study were HIV positive blood collected in the Department of Virology, College of Medicine, University College Hospital (UCH), Ibadan, Oyo State, from January 2004 to December 2006. Individuals who presented for HIV screening at the voluntary counseling and testing centre in the department come predominantly from Ibadan while others come from different towns and cities in Southwestern Nigeria.

Ibadan is the capital city of Oyo State and the third largest metropolitan area, by population, in Nigeria, after Lagos and Kano, with a population of 1,338,659 according to the 2006 census. Ibadan, which is also the largest metropolitan geographical area in Nigeria is located in southwestern Nigeria, 128 km inland northeast of Lagos and 530 km southwest of Abuja, the federal capital, and is a prominent transit point between the coastal region and the areas to the north (www.uch-ibadan.org.ng).

The University College Hospital is strategically located in Ibadan. It has 850 bed spaces and 163 examination couches and is primarily an ideal tertiary institution but it has appendages of community-based outreach activities at Igbo-Ora, Abedo, Okuku, Sepeteri, Elesu, Jago where the Hospital offers secondary and primary health care with referrals to UCH (www.uch-ibadan.org.ng).

3.1.2 SAMPLE COLLECTION

About 5ml of blood was collected by venepuncture from each patient into a sterile tube containing EDTA (ethylene diamine tetra acetic acid) as coagulant. Each specimen was labelled with the date of collection and laboratory identification number. Plasma was

separated from each sample and both plasma and packed cells were stored at -20ºC until analysed. Only samples that were positive for HIV-1 antibodies were analysed further for the study. Overall, samples from 85 HIV-1 positive individuals were used for the genetic characterization of HIV-1.

3.1.3 SEROLOGY

All the plasma samples were screened and confirmed for the presence of antibodies to HIV-1 in the Department of Virology using commercially available Enzyme Linked Immunosorbent Assay (ELISA) and Western blot kits, respectively. The ELISA was carried out using Genscreen Ultra HIV Ag-Ab (Bio-rad, France) test kit which detects HIV P24 antigen and antibodies while the Western blot assay was carried out using New Lav Blot 1 (Institute Pasteur, France) for detection of specific HIV-1 antibodies in the blood samples.

3.2 MOLECULAR CHARACTERISATION OF HIV-1 SEQUENCES

3.2.1 EXTRACTION OF GENOMIC DNA FROM HIV-1 POSITIVE SAMPLES

Genomic DNA was extracted from whole blood or packed cells using the QiaAmp DNA Blood Mini kit (Maryland, USA). Two hundred microlitres of blood was added to 20µl of Protease in a 1.5ml Eppendorf tube and 200µl of lysis buffer (buffer AL®) was then added after which it was pulse vortexed for 15 seconds and incubated in a water bath at 37ºC for 20 minutes. The Eppendorf tube was centrifuged briefly in a microcentrifuge to remove drops from the lid of the tube, and 200µl of absolute ethanol was added and the mixture pulse vortexed and centrifuged again at 8000rpm for 10 seconds. The mixture was transferred to a Qiagen column in a clean 2ml collection tube and centrifuged at 8000rpm for one minute. Five hundred microlitres of wash buffer (buffer AW1®) was added to the column and centrifuged at 8000 rpm for one minute, thereafter the column was transferred into a new 2ml collection tube and the previous collection tube discarded with the filtrate. Five hundred

microlitres of another wash buffer (buffer AW2®) was then added to the column and centrifuged at 14,000rpm for 3 minutes. The column was transferred into another 2ml collection tube and centrifuged again at 14,000rpm for one minute. The column was then placed in a clean labelled 1.5ml Eppendorf tube and the collection tube with the filtrate discarded. Thereafter, 200µl of pre-warmed buffer AE® was added into the column to elute the membrane-bound DNA into the 1.5ml Eppendorf tube. The column was incubated at room temperature for 5 minutes, centrifuged at 8000rpm for one minute and the eluate stored at -20°C. The extracted genomic DNA was visualized by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (Qiagen, 2005).

3.2.2 DETECTION 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 and mixing thoroughly. The mixture was heated to boiling in a microwave oven and allowed to cool down to room temperature. Thereafter, 10µl of ethidium bromide was added into the mixture before pouring into a mini gel cast (containing gel combs) and allowed to solidify. The solid agarose gel was then placed in the mini gel tank after which 800ml of TBE was poured into the mini gel tank to completely submerge the agarose gel. Five microlitres of DNA-containing solution was mixed with 3µl 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 done at 110volts using a current of 500 milliamperes for 60 minutes. At the completion of electrophoresis, the agarose gel was placed on a transilluminator and individual bands corresponding to loaded amplicon or controls were examined by UV light.

3.2.3 NESTED POLYMERASE CHAIN REACTION (PCR)

A Nested PCR was carried out on the extracted proviral DNA samples for HIV-1 subtype determination using primers that were specific for the HIV-1 env C2-V3 region. The primers were commercially synthesized by ThermoScientific Corporation, Germany.

The two sets of primers used were: WT1, **5'-GCTGGTTTTGGGATTCTAAAGTGTA-3'** (6884-6908, positions relative to HXB2) and WT2, **5'-CAATAGAAAAATTCCCCTCCACAAT-3'** (7353-7377) for the first (outer primers) round. The second round (inner primers) primers were: KK30, **5'-AATTTCTGGGTCCCCTCCTG-3'** (7318-7337) and KK40, **5'-ACA GTA CAA TGT ACA CAT GG-3'** (6954-6973) (Kanki *et al.,* 1999; Sankale *et al.,* 2007). The expected amplicon size after nested PCR was approximately 350bp. The primers were delivered lyophilized with a concentration of 10,000 picomoles per microlitre. The primers were diluted in nuclease-free deionized distilled water to 20 picomoles per microlitre and stored at -20°C in aliquots of 150µl until used.

The first round PCR was carried out by first preparing the mastermix using the AmpliTaq Gold RT-PCR/PCR kit (Applied Biosystems, USA) containing the following as previously described by Kanki *et al.* (1999):

5x PCR buffer	10µl
25mM $MgCl_2$	5µl
10 mM dNTP Mix	1µl
Primer 1 (20pm/ µl)	1µl
Primer 2 (20pm/ µl)	1µl
Taq polymerase (5 U/µl)	1µl
ddH_20	21µl

40µl

Then, 10µl of extracted proviral DNA was added into a 0.5ml MicroAmp PCR reaction tube containing 40µl of the mastermix which was then placed into an Applied Biosystems GeneAmp PCR System 9700® thermal cycler block previously turned on 30 minutes prior to beginning of amplification. The Applied Biosystems GeneAmp PCR System 9700® thermal cycler was programmed to the following reaction conditions:

Denaturation:	94^0C for 3 minutes (initial denaturation)
Amolification program (40 cycles):	45 sec at 94^0C, 45 sec at 57^0C, 45 sec at 72^0C
Final extension step:	5 min at 72^0C
Hold:	4^0C

Similarly, the second round PCR was carried out by first preparing the mastermix using the AmpliTaq Gold RT-PCR/PCR kit (Applied Biosystems, USA) containing the following as previously described by Kanki *et al.* (1999):

5x PCR buffer	10µl
25mM $MgCl_2$	5µl
10 mM dNTP Mix	1µl
Primer 1 (20pm/ µl)	1µl
Primer 2 (20pm/ µl)	1µl
Taq polymerase (5 U/µl)	1µl
ddH_20	26µl
	45µl

Then, 5µl of amplicon from the first round PCR was added into a 0.5ml MicroAmp PCR reaction tube containing 45µl of the mastermix which was then placed into the Applied Biosystems GeneAmp PCR System 9700® thermal cycler block previously running for 30

minutes prior to beginning of amplification. The Applied Biosystems GeneAmp PCR System 9700® thermal cycler was programmed to the following cycling conditions:

Denaturation:	94^0C for 3 minutes (initial denaturation)
Amplification program: (40 cycles)	45 sec at 94^0C, 45 sec at 55^0C, 45 sec at 72^0C
Final extension step:	5 min at 72^0C
Hold:	4^0C

Following the second PCR, the products were examined by agarose gel electrophoresis.

3.2.4 AGAROSE GEL ELECTROPHORESIS OF AMPLIFIED FRAGMENTS

For examination of the second round PCR amplicon, a 2% agarose gel was prepared by adding 100ml of Tris Borate EDTA (TBE) buffer into 2.0g of agarose in a Pyrex heat-resistant bottle and mixed thoroughly. The mixture was heated to boiling in a microwave oven and allowed to cool down to room temperature. Thereafter, 10µl of ethidium bromide was added to the mixture before pouring into a mini gel cast (containing gel combs) and allowed to solidify. The solid agarose gel was then placed in the mini gel tank after which 800ml of TBE was poured into the mini gel tank to completely submerge the agarose gel. Five microlitres of amplicon was mixed with 3µl 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 which was nuclease-free water. Electrophoresis was done at 110volts using a current of 500 milliamperes for 60 minutes. At the completion of electrophoresis, the agarose gel was placed on a transilluminator and individual bands corresponding to loaded amplicons or controls were examined by UV light.

3.2.5 PURIFICATION OF AMPLIFIED FRAGMENTS

Amplicons from the second round PCR were purified using ethanol precipitation method for purifying PCR products (Zeugin and Hartley, 1985). The contents of each PCR reaction tube

was added into an Eppendorf tube containing 100µl of 95% ethanol and 5µl of 3M sodium acetate of pH 4.6, and vortexed for 15 seconds. The Eppendorf tubes were then incubated at -20^{0}C for 40 minutes to precipitate the PCR products. The tubes were centrifuged for 20 minutes at 16,400rpm after which the supernatant was carefully aspirated using a Pasteur pipette, and discarded. The pellet was rinsed with 300ml of 70% ethanol, vortexed briefly and centrifuged at 16,400rpm for 5 minutes. Then the supernatant was gently and carefully aspirated and discarded, and the pellets air-dried for 5 minutes. The pellets were resuspended in 50µl of sterile nuclease free water. The purified DNA was then quantified as described below.

3.2.6 QUANTIFICATION OF DOUBLE-STRANDED DNA WITH PICOGREEN

3.2.6.1 Preparation of 1X TE, 200ng/ml DNA Standard and working PicoGreen

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

The DNA standard is supplied in a 100µg/ml dilution, 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 100µg/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.

3.2.6.2 Calibration of Fluorometer®

The fluorometer is always calibrated prior to quantification of DNA. One hundred microlitre of 200ng/ml DNA standard solution was aliquoted into a clean Eppendorf tube which was labeled as STANDARD and 100µl of 1X TE was aliquoted into another clean Eppendorf tube and labeled as BLANK. Thereafter, 100µl of working Picogreen was added into the standard and blank, vortexed gently and incubated in the dark for 2 minutes at room temperature after which 100µl each of the STANDARD and BLANK was aliquoted into individual clean mini-cuvettes. The fluorometer® was turned on and cuvette mini-adapter inserted in the space provided for it.

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> and <ENTER> buttons were depressed, one after the other, after which the instructions on the LED were followed for inserting the BLANK and STANDARD. At the end of the calibration, the <ENTER> button was pressed again to accept the calibration.

3.2.6.3 Quantification of experimental DNA

Into a clean and labeled 1.5ml Eppendorf tube 99µl of 1X TE was combined with 1µl of purified amplified proviral DNA and vortexed briefly. Thereafter, 100µl of working PicoGreen was added to the mixture and vortexed for 15 seconds. The tube was incubated in the dark at room temperature for 2 minutes after which 100µl of the mixture was transferred into a clean mini-cuvette and the mini-cuvette containing the mixture was placed in the mini-cuvette holder in the fluorometer, and the <READ> button pressed. The number appearing on the LED display was recorded and it represents the quantity of DNA in nanograms (ng) in 5µl of PCR product.

3.3 SEQUENCING OF PROVIRAL DNA

3.3.1 CYCLE SEQUENCING OF PCR PRODUCTS

The sequencing reaction was done using the dye terminator sequencing kit version 3.1 (Applied Biosystems, California, USA). Each reaction mix consisted of a mixture of the following in each well of a 96 well MicroAmp plate:

Primer	1µl
Terminator Mix	11µl
PCR product	8µl

The sequencing was carried out in both forward and reverse directions using primers KK40 and KK30, respectively, according to manufacturer's recommendations (Applied Biosystems, 2009).

3.3.2 PURIFICATION OF SEQUENCED AMPLICONS

The MicroAmp plate was removed from the thermal cycler and the plate seal removed. To each sample mixture, 20µl of deionized water and 60µl of 100% isopropanol was added, and the MicroAmp plate 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.

Thereafter, the MicroAmp plate was centrifuged at 2000g for 45 minutes immediately after which the adhesive aluminium foil was carefully removed, without agitating the pellets, and absorbent paper placed on the top of the plate and the plate inverted. Then, the MicroAmp plate was placed in the inverted position on top of the absorbent paper and centrifuged at 700g for one minute after which the samples in each well of the MicroAmp plate was resuspended by adding 20µl 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 3130xl Genetic

Analyzer (Applied Biosystems, California, USA), 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.

3.3.3 ANALYSIS OF SEQUENCED FRAGMENTS

Sequences were first checked for similarity to HIV-1 sequences in the GenBank by using BLAST (Basic Local Alignment Search Tool) program in the National Centre for Biotechnology Information (NCBI) database. Sequences were manually edited using both the Sequencing Analysis software version 5.1 (Applied Biosystems, California, USA) and MEGA 5.05 (Tamura *et al.,* 2011) software. A multiple alignment was carried out using the ClustalW programme in MEGA 5.05. with reference sequences in the 2009 HIV-1 compendium from the Los Alamos database and sample sequences (Kuiken *et al.,* 2009). Pair-wise distances and Phylogenetic trees were constructed by Maximum Likelihood using the Tamura-Nei model in MEGA 5.05. Reliability of the resulting trees was estimated from 1000 bootstrap resamplings.

3.4 MOLECULAR ANALYSIS OF THE V3 LOOP AMINO ACID SEQUENCES

The HIV-1 env C2-V3 nucleic acid sequences were translated into amino acid sequences from 2 open reading frames (ORFs) within each sequence using the MEGA 5.05 software. An alignment of the amino acid residues of the 64 HIV-1 *env* C2-V3 sequences was compared with the consensus amino acid residues of subtypes B, G, CRF_02.AG, and CRF_06cpx. The amino acid sequences were then manually examined and analyzed for N-linked glycosylation sites (NGS), V3 characteristics and crown motif, presence of basic amino acids at position 11 and 25 of V3, X4-associated mutations and maraviroc-resistance mutations. The results of the V3 loop amino acid analysis was used in the genotypic prediction of virus co-receptor usage and Maraviroc-resistant phenotype.

CHAPTER 4

RESULTS

Overall, sequence results sufficient for analysis were obtained from 64 (75.3%) of the 85 HIV-1 infected anti-retroviral therapy-naïve patients including 42 males and 43 females. The 85 HIV-1 positive patients had a median age of 37 years (range 18-58 years) and comprised of 40 seroconcordant couples and partners of 5 serodiscordant couples. The 64 HIV-1 positive patients comprised of 23 seroconcordant couples and 18 individuals which were 34 male and 30 female.

4.1 DNA QUANTITATION VALUES FOR HIV-1 PROVIRAL SEQUENCES

DNA quantitation of the sequenced proviral DNA from the 64 HIV-1 positive patients as well as the negative and positive controls are presented in Table 6. The value ranged from 35.9ng/µl to 207ng/µl for the patient samples, while the value for the negative control was 0.7ng/µl.

4.2 ANALYSIS OF THE NUCLEOTIDE SEQUENCE OF THE *ENV* C2-V3 REGION OF HIV-1

4.2.1 PHYLOGENETIC ANALYSIS OF HIV-1 *ENV* C2-V3 SEQUENCES BY MAXIMUM LIKELIHOOD

Following alignment of the approximately 350bp HIV-1 C2-V3 nucleotide sequences with each other and with sequences in the Los Alamos HIV database, a Maximum Likelihood tree was constructed using ClustalW (Figure 10 and Appendix 4). Based on the C2-V3 sequences, 2 (3.1%), 34 (53.1%), 18 (28.1%), 9 (14.1%) and one (1.6%) of the sequences were found to belong to subtypes A, G, CRF02_AG, CRF06_cpx, and CRF35_AD, respectively (Figure 11).

TABLE 3: HIV-1 PROVIRAL DNA QUANTITATION VALUES AMONG HIV-1 PATIENTS IN IBADAN

Sample ID	Value	Sample ID	Value	Sample ID	Value	Sample ID	Value	Sample ID	Value
NGIB.04.001	42.6	NGIB.04.014	149.5	NGIB.05.010	52.4	NGIB.05.023	35.9	NGIB.06.003	172.7
NGIB.04.002	53.5	NGIB.04.015	147.3	NGIB.05.011	85.3	NGIB.05.024	76.6	NGIB.06.004	168.3
NGIB.04.003	56.3	NGIB.04.016	125.9	NGIB.05.012	77.6	NGIB.05.025	131.4	NGIB.06.005	98.4
NGIB.04.004	54.5	NGIB.04.017	61.1	NGIB.05.013	59.2	NGIB.05.026	117.4	NGIB.06.006	157.6
NGIB.04.005	58.6	NGIB.05.001	75.0	NGIB.05.014	75.5	NGIB.05.027	120.8	NGIB.06.007	74.5
NGIB.04.006	30.2	NGIB.05.002	207.0	NGIB.05.015	47.8	NGIB.05.028	110.0	NGIB.06.008	134.4
NGIB.04.007	94.7	NGIB.05.003	58.8	NGIB.05.016	39.6	NGIB.05.029	103.9	NGIB.06.009	60.9
NGIB.04.008	79.5	NGIB.05.004	94.0	NGIB.05.017	121.8	NGIB.05.030	188.0	NGIB.06.010	153.3
NGIB.04.009	106.3	NGIB.05.005	132.9	NGIB.05.018	125.2	NGIB.05.031	73.9	NGIB.06.011	137.8
NGIB.04.010	53.9	NGIB.05.006	64.6	NGIB.05.019	68.6	NGIB.05.032	77.3	NGIB.06.012	130.1
NGIB.04.011	72.7	NGIB.05.007	168.2	NGIB.05.020	58.3	NGIB.05.033	106.9	NGIB.06.013	118.6
NGIB.04.012	63.8	NGIB.05.008	203.4	NGIB.05.021	133.8	NGIB.06.001	173.4	NGIB.06.014	58.2
NGIB.04.013	145.7	NGIB.05.009	46.6	NGIB.05.022	62.9	NGIB.06.002	143.5	PC	62.9
								NC	0.7

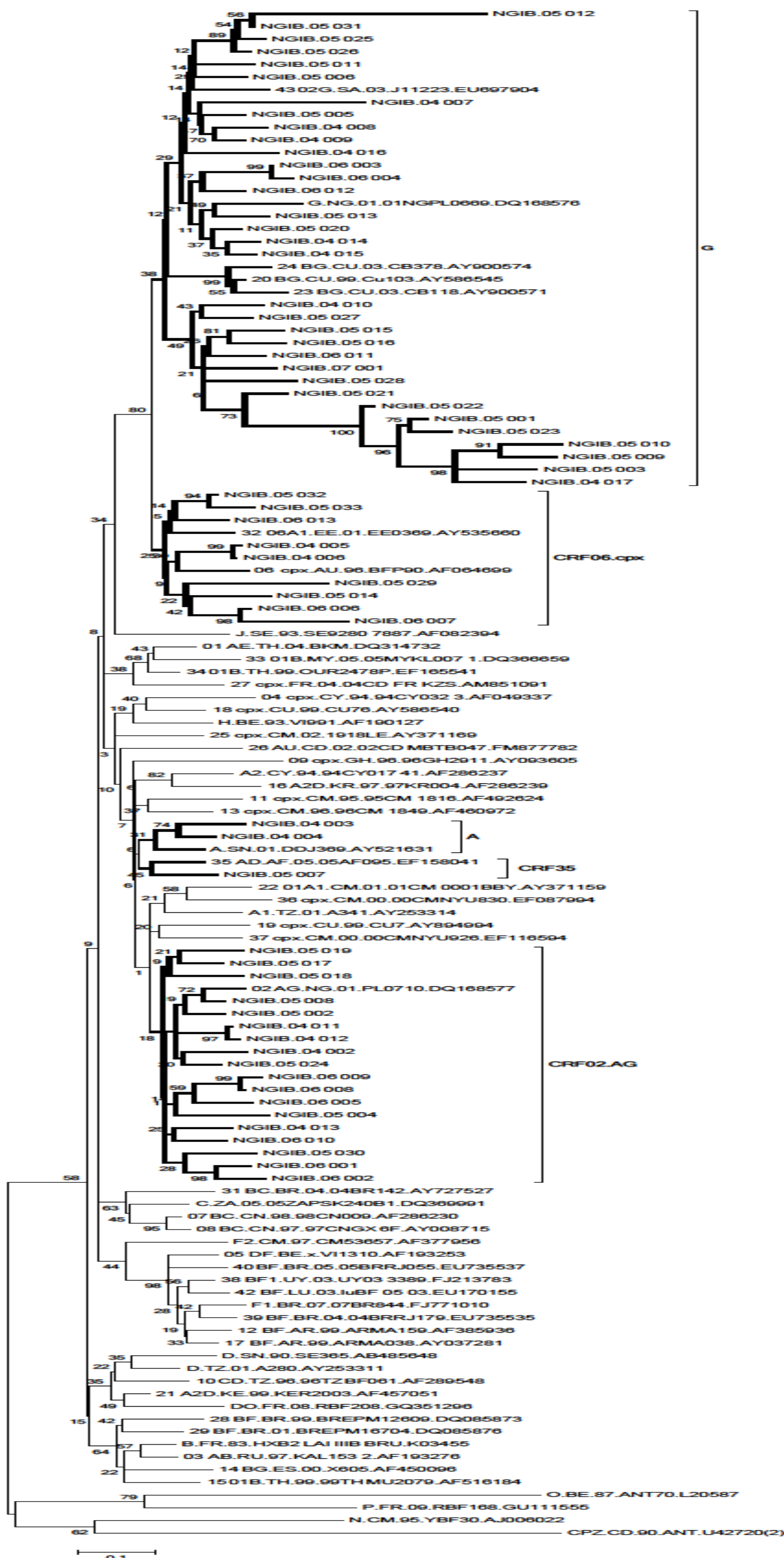

FIGURE 10: PHYLOGENETIC DISTRIBUTION OF HIV-1 SUBTYPES AND CRFs AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

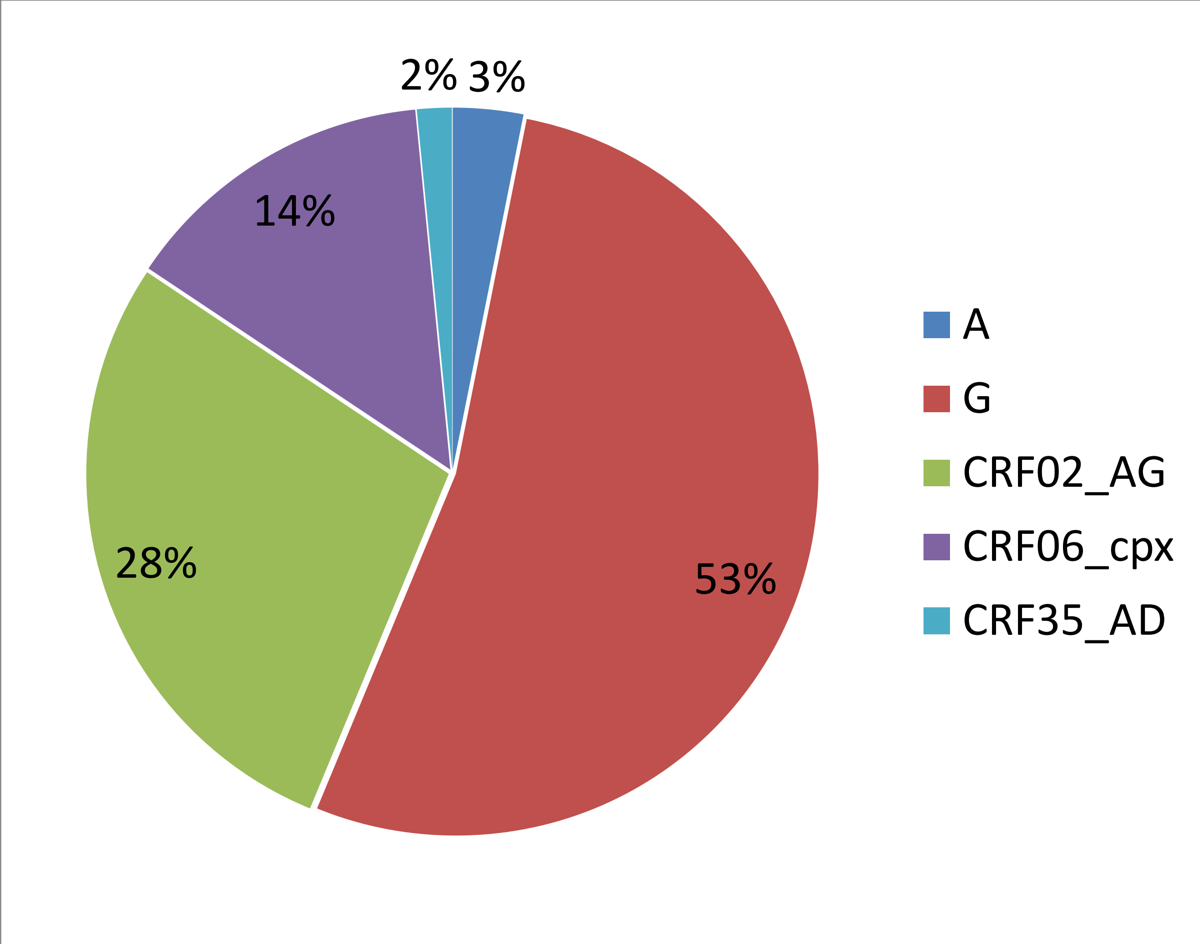

FIGURE 11: HIV-1 SUBTYPES AND CRFs AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

4.2.1.1 ANALYSIS OF SUBTYPE A SEQUENCES

The Phylogenetic analysis of the *env* C2-V3 subtype A sequences shows that the 2 subtype A viruses, NGIB.04__003 and NGIB.04__004, clustered closely with West African reference sequences from Senegal (Figure 12). Analysis of nucleotide similarities by calculating pairwise distances among the *env* C2-V3 subtype A sequences showed a similarity of 74% between NGIB.04__003 and NGIB.04__004, and a similarity of 78% and 80.8% between reference subtype A nucleotide sequence and NGIB.04__003 and NGIB.04__004, respectively.

4.2.1.2 ANALYSIS OF SUBTYPE G SEQUENCES

Further phylogenetic analysis of the 34 *env* C2-V3 subtype G sequences showed that the subtype G viruses form five different subclusters (Figure 13). Five subtype G sequences clustered with reference sequences from Estonia, two subtype G sequences clustered with a reference sequence from Sierra Leone, 13 subtype G sequences clustered with a reference sequence from Ghana and 9 subtype G sequences clustered with a reference sequence from Nigeria (Table 7). However, 5 sequences clustered on their own in the G cluster which is referred to as G’. Examination of percentage nucleotide similarities by calculating pairwise distances among the *env* C2-V3 G sequences revealed that there was similarity ranging from 64.0% to 86.8% between the reference G sequence and the 34 *env* C2-V3 G sequences from this study, giving a variation range of 22.8%. The percentage nucleotide similarity between reference subtype G and each subtype G sequence in this study is presented in Table 7.

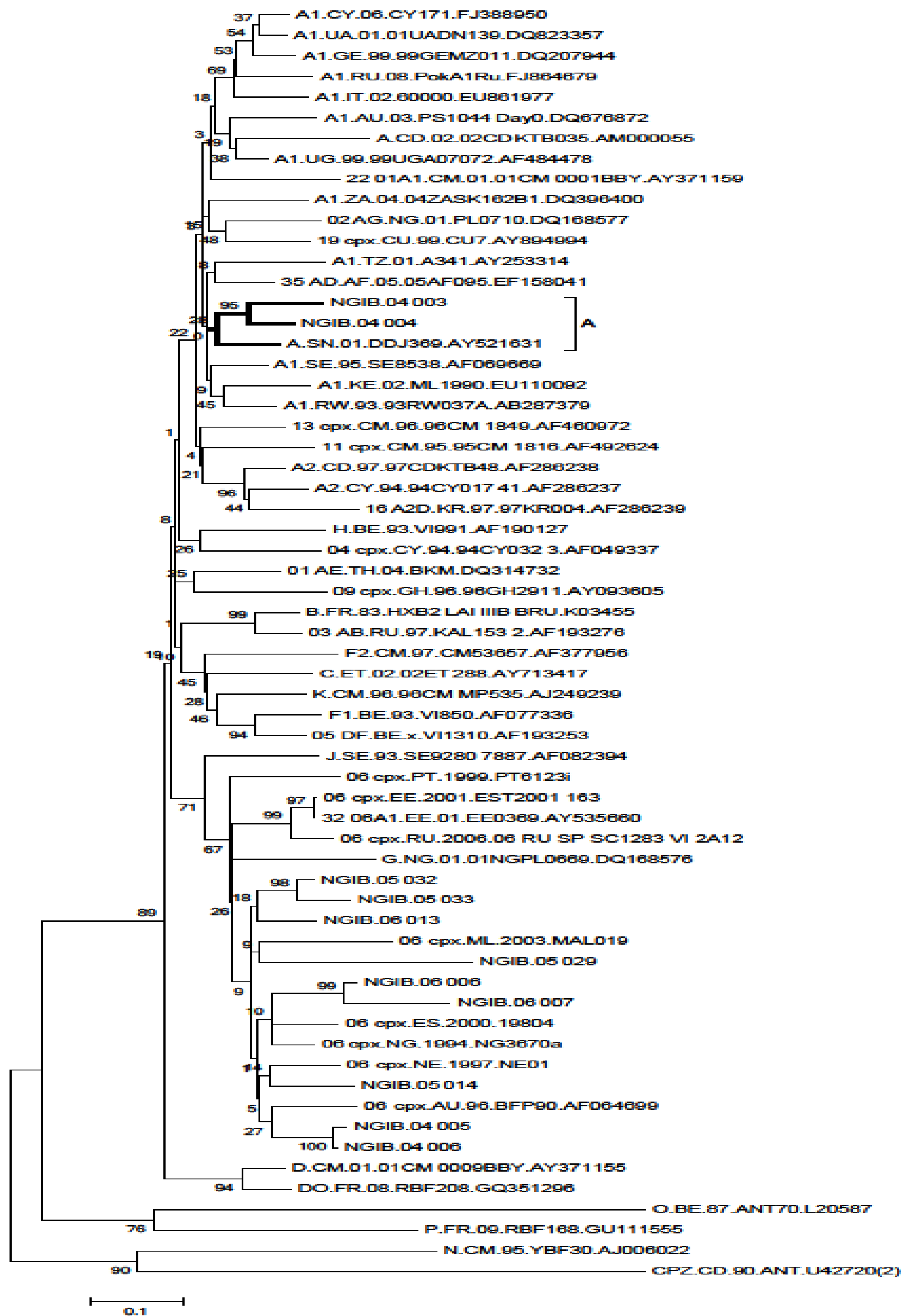

FIGURE 12: PHYLOGENETIC DISTRIBUTION OF *ENV* C2-V3 SUBTYPE A SEQUENCES AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

FIGURE 13: PHYLOGENETIC DISTRIBUTION OF *ENV* C2-V3 SUBTYPE G SEQUENCES AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

Table 7: PERCENTAGE NUCLEOTIDE SIMILARITY BETWEEN REFERENCE G AND HIV-1 *ENV* C2-V3 SUBTYPE G SEQUENCES AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

S/No	SAMPLE ID	SIM (%)	S/No	SAMPLE ID	SIM (%)
1	NGIB.04_001	84.4	18	NGIB.05_013	86
2	NGIB.04_007	75.2	19	NGIB.05_015	78.8
3	NGIB.04_008	84.4	20	NGIB.05_016	78
4	NGIB.04_009	84.4	21	NGIB.05_020	86.8
5	NGIB.04_010	82	22	NGIB.05_021	76.8
6	NGIB.04_014	86.8	23	NGIB.05_022	71.2
7	NGIB.04_015	85.6	24	NGIB.05_023	65.6
8	NGIB.04_016	80.4	25	NGIB.05_025	82.4
9	NGIB.04_017	64.8	26	NGIB.05_026	83.2
10	NGIB.05_001	66.8	27	NGIB.05_027	80.8
11	NGIB.05_003	64	28	NGIB.05_028	78.4
12	NGIB.05_005	83.2	29	NGIB.05_031	82
13	NGIB.05_006	80.8	30	NGIB.06_003	79.6
14	NGIB.05_009	64.4	31	NGIB.06_004	78
15	NGIB.05_010	62.4	32	NGIB.06_011	79.2
16	NGIB.05_011	83.6	33	NGIB.06_012	82
17	NGIB.05_012	67.6	34	NGIB.06_014	78

4.2.1.3 ANALYSIS OF CRF02_AG SEQUENCES

Further phylogenetic analysis of the eighteen HIV-1 *env* C2-V3 CRF02_AG sequences showed that the CRF02_AG viruses formed 4 different subclusters within this circulating recombinant form, clustering with reference sequences from Cyprus, Ghana, Nigeria and the United States of America (Figure 14).

The 18 CRF02_AG sequences included seven CRF02_AG sequences that clustered with a reference sequence from Cyprus, seven CRF02_AG sequences that clustered with a reference sequence from the United States of America, one CRF02_AG sequence that clustered with a reference sequence from Estonia, one CRF02_AG sequence that clustered with a reference sequence from Nigeria, and two CRF02_AG sequences which clustered with a reference sequence from Ghana (Figure 14). Analysis of nucleotide similarities by calculating pairwise distances among the *env* C2-V3 CRF02_AG sequences showed a percentage similarity ranging from 78.4% to 90.8% between the reference CRF02_AG sequence and the 18 HIV-1 *env* C2-V3 CRF02_AG sequences from this study, giving a variation range of 12.4% (Table 8).

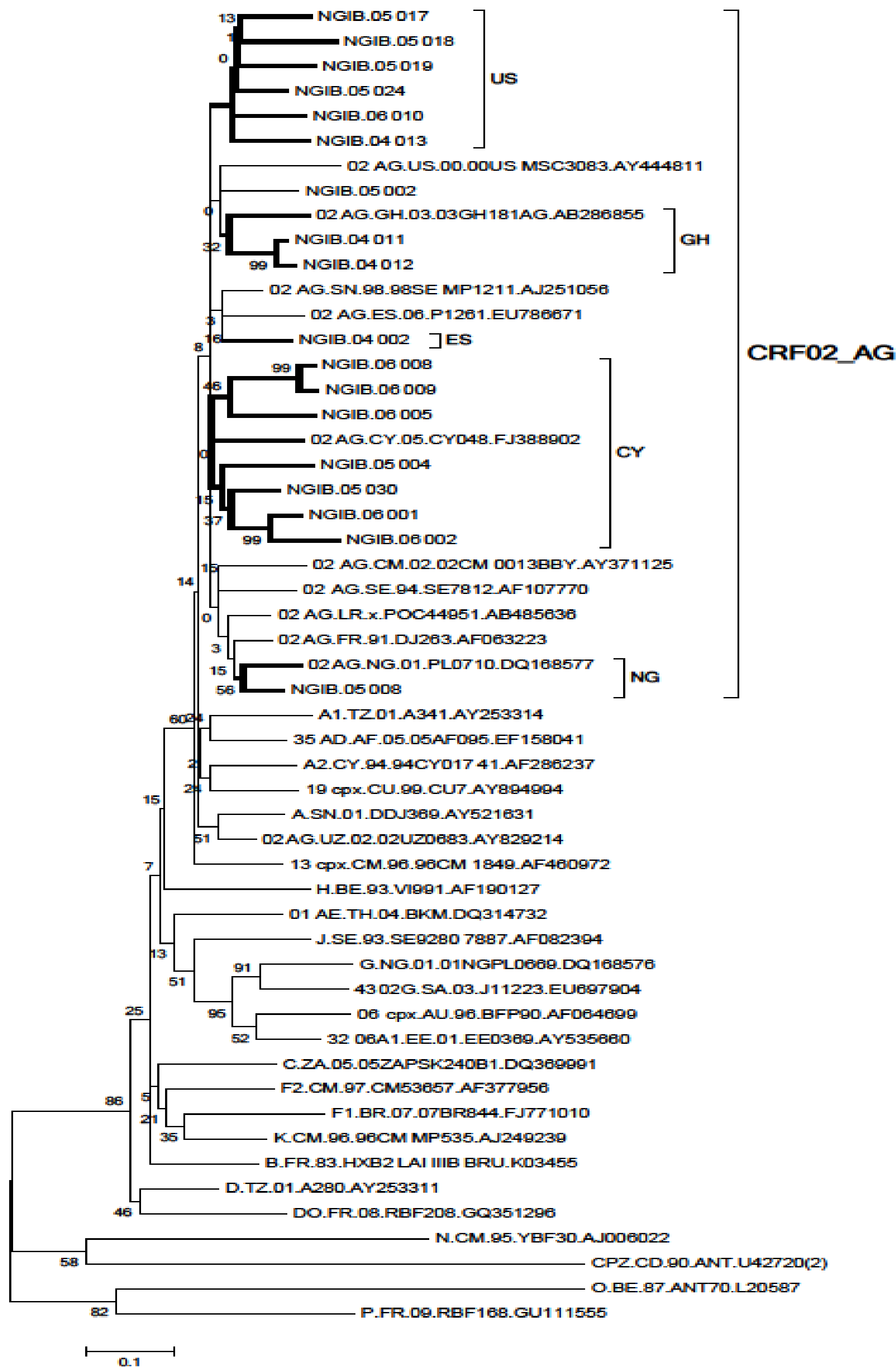

FIGURE 14: PHYLOGENETIC DISTRIBUTION OF *ENV* C2-V3 CRF02_AG SEQUENCES AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

TABLE 8: PERCENTAGE NUCLEOTIDE SIMILARITY BETWEEN REFERENCE CRF02_AG AND HIV-1 *ENV* C2-V3 CRF02_AG SEQUENCES AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

S/No	SAMPLE ID	SIM (%)
1	NGIB.04_002	88
2	NGIB.04_011	84.8
3	NGIB.04_012	84
4	NGIB.04_013	85.2
5	NGIB.05_002	87.6
6	NGIB.05_004	82
7	NGIB.05_008	90.8
8	NGIB.05_017	84.8
9	NGIB.05_018	83.6
10	NGIB.05_019	86
11	NGIB.05_024	88.4
12	NGIB.05_030	84.4
13	NGIB.06_001	81.2
14	NGIB.06_002	80
15	NGIB.06_005	82.8
16	NGIB.06_008	79.6
17	NGIB.06_009	78.4
18	NGIB.06_010	86.8

4.2.1.4 ANALYSIS OF CRF06_cpx SEQUENCES

Analysis of the nine CRF06_cpx sequences showed distinct subclusters within the CRF06_cpx cluster. Three sequences NGIB.05__032, NGIB.05__033 and NGIB.06__013 clustered together and away from the other CRF06_cpx reference sequences. Sequence NGIB.04__005 and NGIB.04__006 clustered with the Australian CRF06_cpx reference sequence while sequences NGIB.05__014 and NGIB.05__029 clustered with CRF06_cpx reference sequences from the Netherlands and Malaysia, respectively. Two sequences, NGIB.06__006 and NGIB.06__007 clustered with the Nigerian CRF06_cpx reference sequence (Figure 15).

Examining the CRF06_cpx nucleotide sequences for similarities by calculating pairwise distances among them, there was a percentage similarity ranging from 74% to 85.6% between the reference CRF06_cpx sequence and the nine *env* C2-V3 CRF06_cpx sequences from this study, giving a variation range of 11.6%. Sequence NGIB.05__029 and NGIB.04__006 had the lowest and highest sequence similarity of 74% and 85.6%, respectively, with the reference CRF06_cpx sequence (Table 9).

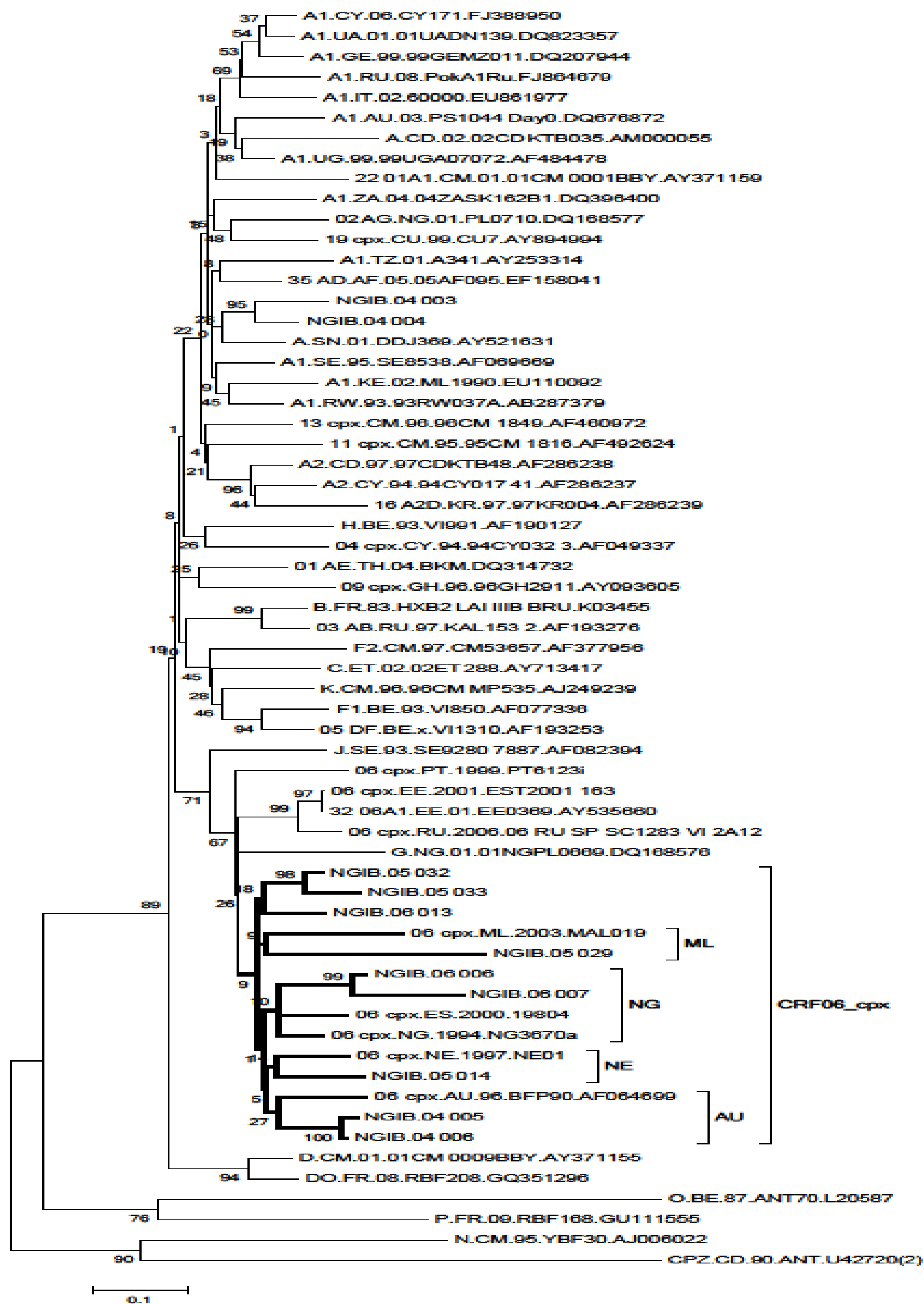

FIGURE 15: PHYLOGENETIC DISTRIBUTION OF *ENV* C2-V3 CRF06_CPX SEQUENCES AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

TABLE 9: PERCENTAGE NUCLEOTIDE SIMILARITY BETWEEN REFERENCE CRF02_AG AND HIV-1 *ENV* C2-V3 CRF02_AG SEQUENCES IN HIV-1 INFECTED PATIENTS IN IBADAN

S/No	SAMPLE ID	SIM (%)
1	NGIB.04_005	85.2
2	NGIB.04_006	85.6
3	NGIB.05_014	82.4
4	NGIB.05_029	74
5	NGIB.05_032	84.4
6	NGIB.05_033	81.6
7	NGIB.06_006	83.2
8	NGIB.06_007	78.4
9	NGIB.06_013	85.2

4.2.2 PHYLOGENETIC ANALYSIS OF HIV-1 *ENV* C2-V3 SEQUENCES BY YEAR OF SAMPLE COLLECTION

The distribution of the 64 HIV-1 *env* C2-V3 sequences from HIV-1 positive patients by year of sample collection is as follows: 17 sequences in 2004, 33 sequences in 2005, and 14 sequences in 2006. Of the 17 sequences obtained from the HIV-1 positive patients in 2004, 2 (11.7%), 9 (52.9%), 4 (23.5%) and 2 (11.7%) were subtype A, subtype G, CRF02_AG and CRF06_cpx viruses, respectively (Figure 16).

The distribution of the HIV-1 strains obtained from samples from HIV-1 positive patients in 2005 showed 20 (60.6%) HIV-1 subtype G sequences, 8 (24.2%) CRF02_AG sequences, 4 (12.1%) CRF06_cpx sequences and 1 (3.0%) CRF35_AD sequence (Figure 17).

The subtyped sequences from blood samples of HIV-1 positive patients in 2006 showed 5 (35.71%) as subtype G, 6 (42.86%) as CRF02_AG and 3 (21.43%) as CRF06_cpx viruses (Figure 18). The subtype and CRF proportion for the 3 years together is presented in Figure 19.

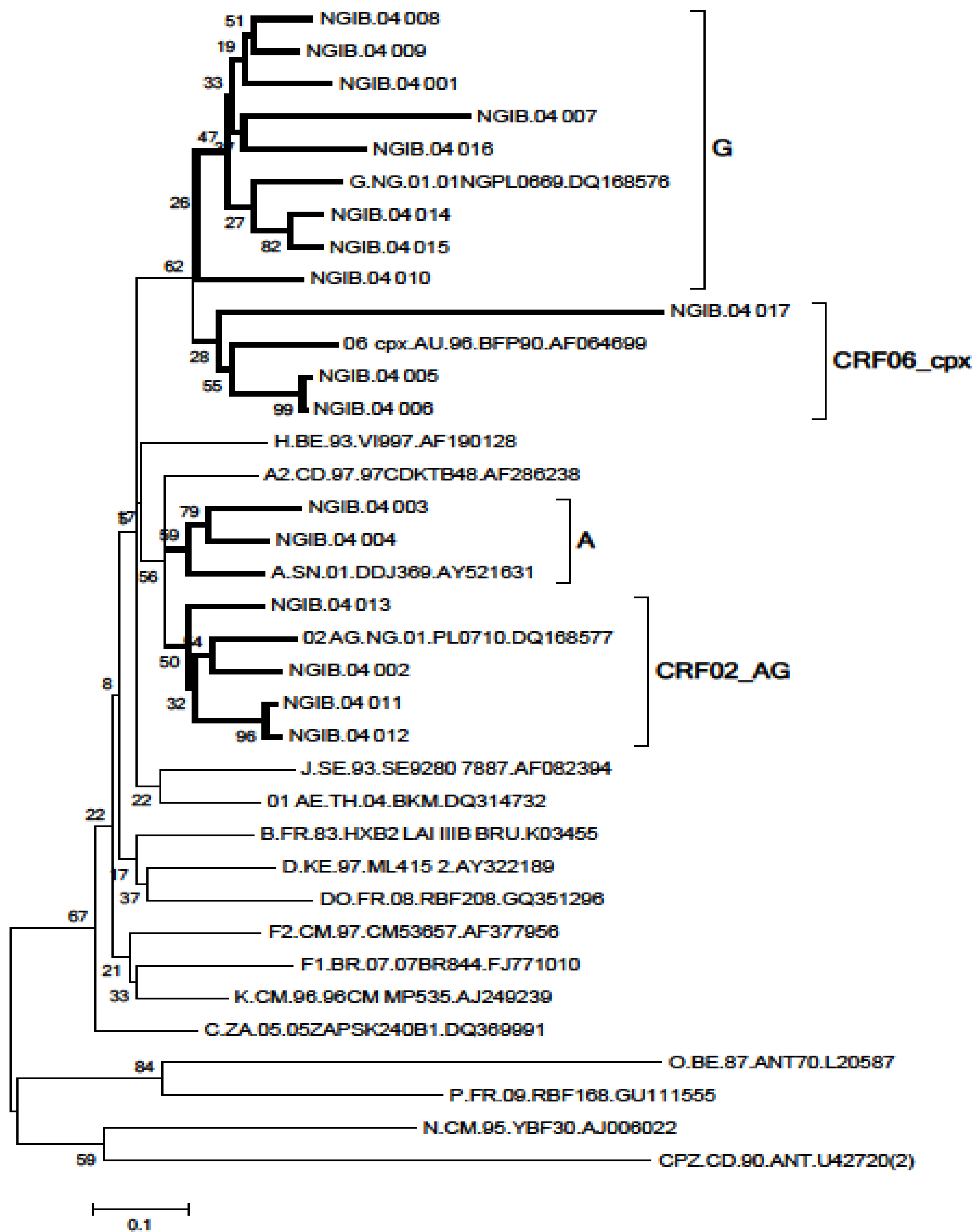

FIGURE 16: PHYLOGENETIC DISTRIBUTION OF HIV-1 *ENV* C2-V3 SEQUENCES OBTAINED IN 2004 AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

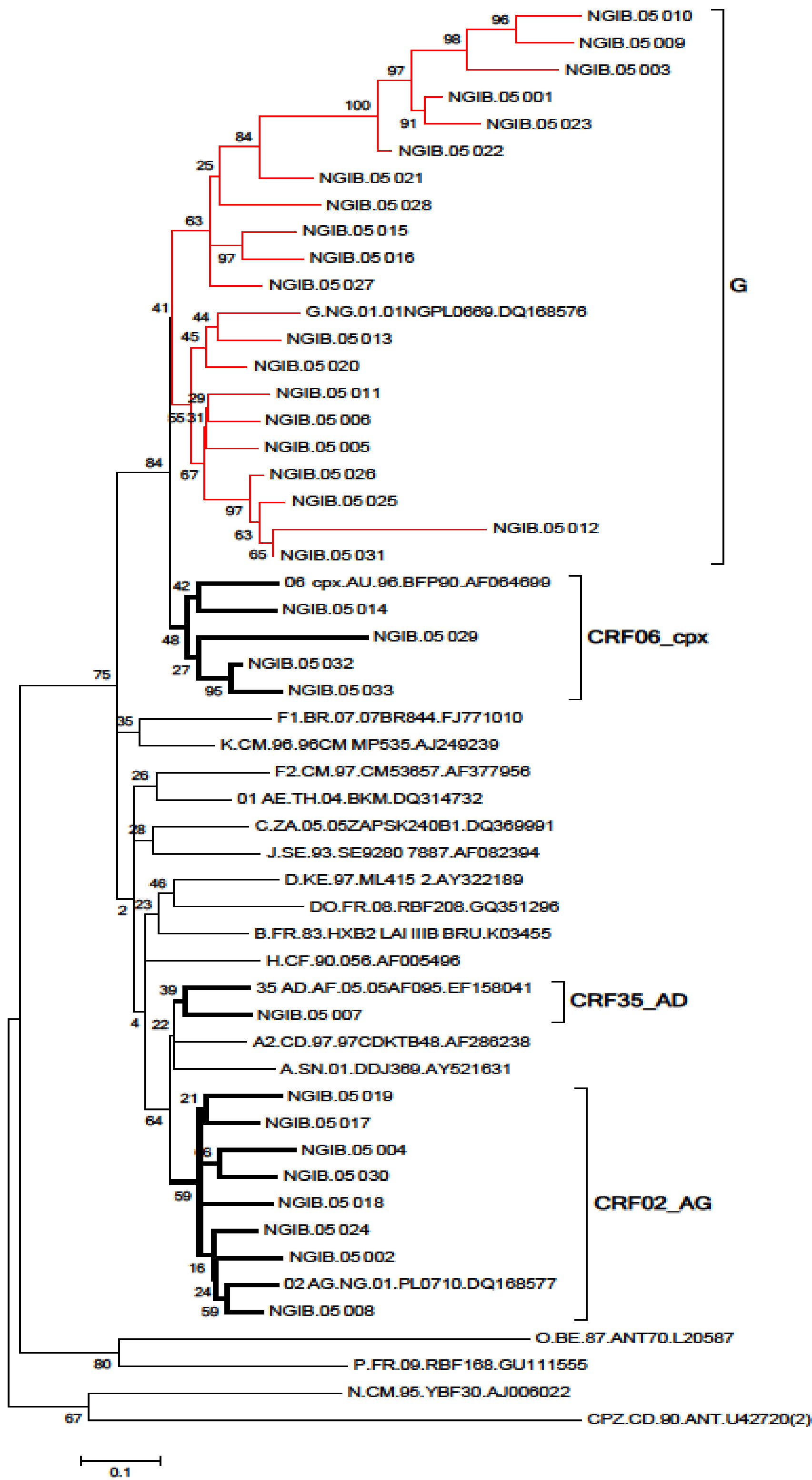

FIGURE 17: PHYLOGENETIC DISTRIBUTION OF HIV-1 *ENV* C2-V3 SEQUENCES OBTAINED IN 2005 AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

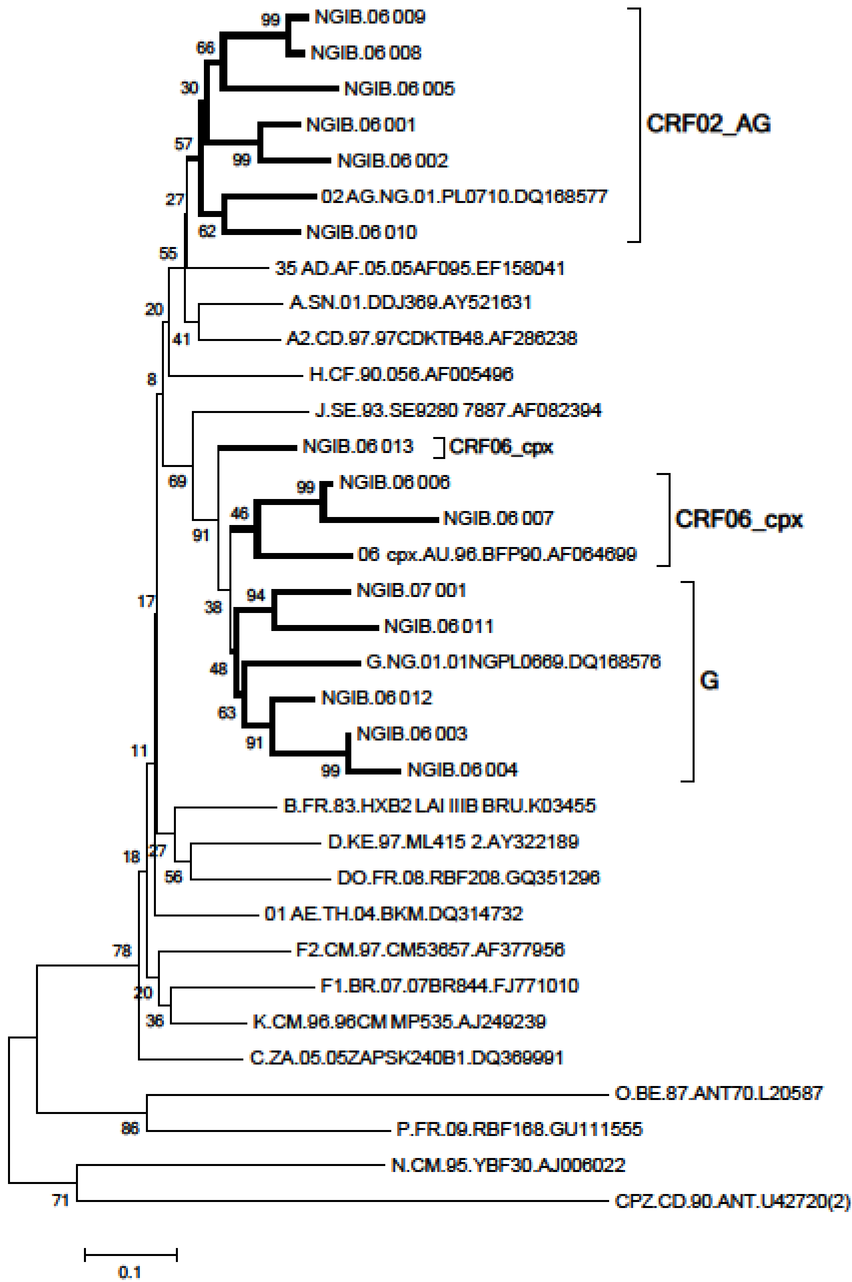

FIGURE 18: PHYLOGENETIC DISTRIBUTION OF HIV-1 *ENV* C2-V3 SEQUENCES OBTAINED IN 2006 AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

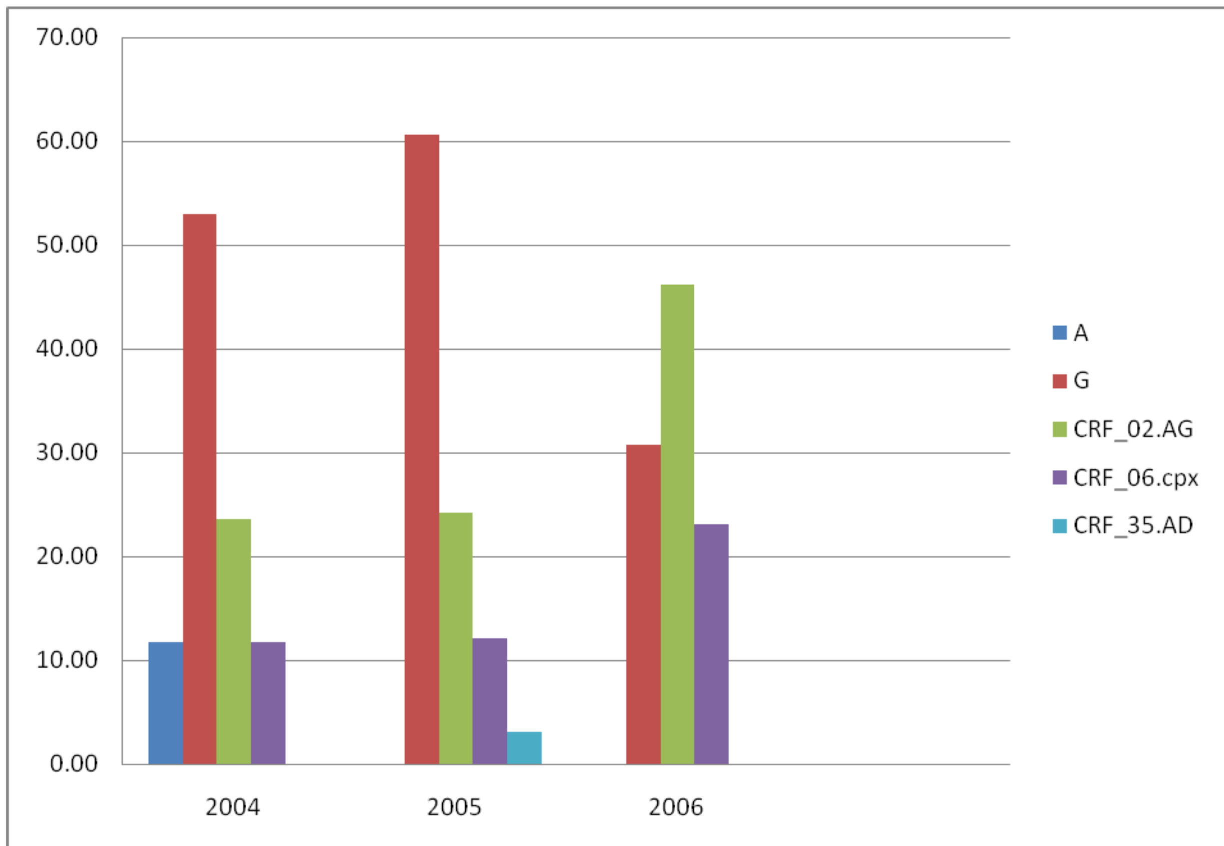

FIGURE 19: HIV-1 SUBTYPE DISTRIBUTION AMONG HIV-1 POSITIVE PATIENTS IN IBADAN BY YEAR OF SAMPLE COLLECTION

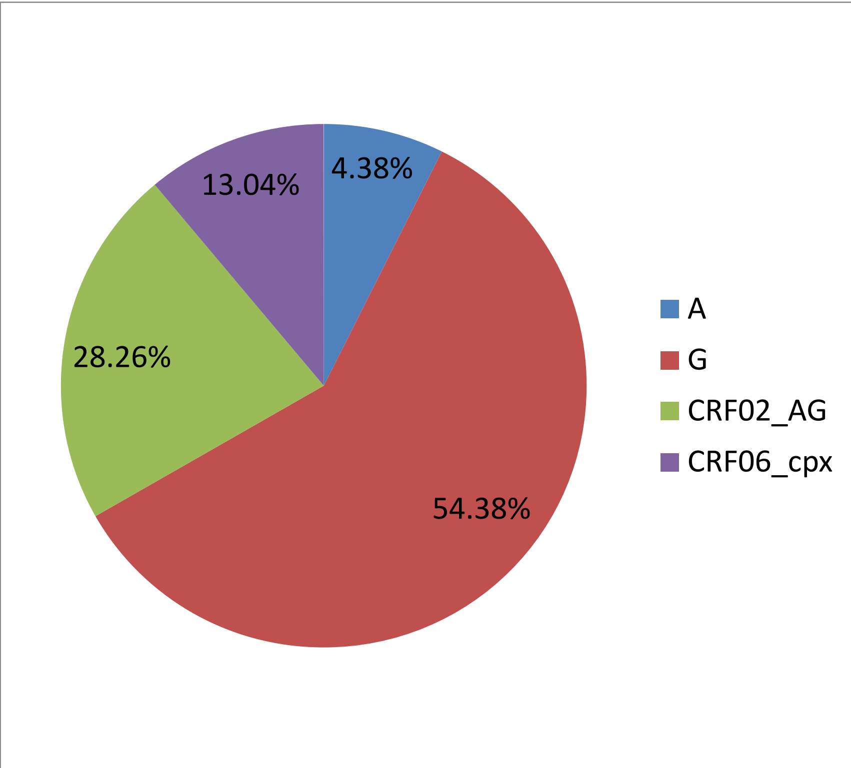

FIGURE 20: HIV-1 SUBTYPE DISTRIBUTION AMONG INFECTED COUPLES IN IBADAN

4.2.3 PHYLOGENETIC ANALYSIS OF *ENV* C2-V3 SEQUENCES OF HIV-1 INFECTED COUPLES

There were 23 HIV-1 infected concordant couples among the 64 HIV-1 positive individuals whose proviral DNA was amplified and directly sequenced. Following analysis of the aligned *env* C2-V3 nucleotide sequences of the 23 HIV-1 infected couples against themselves and sequences obtained from the Los Alamos HIV database, 2 (4.38%), 25 (54.38%), 13 (28.26%), and 6 (13.04%) belonged to subtypes A, G, CRF02_AG, and CRF06_cpx, respectively (Figure 20).

On further analysis, 18 of the 23 HIV-1 positive couples had the same HIV-1 subtype infecting both spouses. Each of the remaining 5 HIV-1 infected couples had discordant HIV-1 subtypes infecting each spouse. Of the 18 HIV-1 infected concordant couples with the same HIV-1 strain in them, one had subtype A, 10 had subtype G, 4 had CRF02_AG and, 3 had CRF06_cpx. The 5 HIV-1 infected but subtype discordant couples all have a combination of HIV-1 subtype G and CRF02_AG *env* C2-V3 sequences (Table 10).

4.2.4 PHYLOGENETIC ANALYSIS OF HIV-1 *ENV* C2-V3 SEQUENCES BY GENDER

The 64 HIV-1 positive individuals whose proviral DNA was amplified and directly sequenced comprised of 30 females and 34 males. Phylogenetic analysis of the HIV-1 *env* C2-V3 sequences among the female subjects show one (3.33%), 15 (50%), 8 (26.67%), 5 (16.67%) and one (3.33%) as subtype A, subtype G, CRF02_AG, CRF06_cpx and CRF35_AD, respectively (table 11). Similarly, among the HIV-1 *env* C2-V3 sequences of the male subjects, one (2.94%), 19 (55.88%), 10 (29.41%) and 4 (11.76%) were subtype A, subtype G, CRF02_AG and CRF06_cpx, respectively (table 12). In both sexes, HIV-1 subtype G was found to be the predominant subtype accounting for about 50% of all sequences, followed by CRF02_AG (28.04%).

4.3 ANALYSIS OF THE AMINO ACID SEQUENCES OF THE V3 LOOP REGION OF HIV-1

4.3.1 V3 LOOP CHARACTERISTICS

The V3 loop of 58 (90.6%) of the 64 HIV-1 C2-V3 sequences analyzed was 35 amino acids in length with a deletion each in all. Two V3 loop sequences have 35 amino acids each with one of them having an insertion when compared to Cons B. Three V3 loop sequences have 33 amino acids each, with 2 deletions, and one V3 loop sequence having 31 amino acids (Figure 21). All, except three HIV-1 V3 loops were limited by the two Cysteine residues responsible for the typical V3 loop configuration. Two sequences have Tyrosine and Tryptophan at their 5'cysteine position while another sequence had a tryptophan at the 3' cysteine position (Figure 21).

TABLE 10: HIV-1 SUBTYPES AMONG HIV-1 INFECTED COUPLES IN IBADAN

S/No	SAMPLE ID	Sex	SUBTYPE	HIV-1 TYPE
1	**NGIB.04.001**	**F**	**G**	**DISCORDANT**
	NGIB.04.002	**M**	**CRF_02.AG**	
2	NGIB.04.003	F	A	CONCORDANT
	NGIB.04.004	M	A	
3	NGIB.04.005	F	CRF_06.cpx	CONCORDANT
	NGIB.04.006	M	CRF_06.cpx	
4	NGIB.04.008	F	G	CONCORDANT
	NGIB.04.009	M	G	
5	NGIB.04.011	F	CRF_02.AG	CONCORDANT
	NGIB.04.012	M	CRF_02.AG	
6	NGIB.04.014	F	G	CONCORDANT
	NGIB.04.015	M	G	
7	NGIB.04.016	F	G	CONCORDANT
	NGIB.05.003	M	G	
8	**NGIB.05.001**	**F**	**G**	**DISCORDANT**
	NGIB.05.002	**M**	**CRF_02.AG**	
9	NGIB.05.005	F	G	CONCORDANT
	NGIB.05.006	M	G	
10	**NGIB.05.008**	**F**	**CRF_02.AG**	**DISCORDANT**
	NGIB.05.009	**M**	**G**	
11	NGIB.05.012	F	G	CONCORDANT
	NGIB.05.013	M	G	
12	NGIB.05.015	F	G	CONCORDANT
	NGIB.05.016	M	G	
13	NGIB.05.017	F	CRF_02.AG	CONCORDANT
	NGIB.05.018	M	CRF_02.AG	
14	NGIB.05.021	F	G	CONCORDANT
	NGIB.05.022	M	G	
15	**NGIB.05.023**	**F**	**G**	**DISCORDANT**
	NGIB.05.024	**M**	**CRF_02.AG**	
16	NGIB.05.025	F	G'	CONCORDANT
	NGIB.05.026	M	G'	
17	NGIB.05.027	F	G	CONCORDANT
	NGIB.05.028	M	G	
18	**NGIB.05.030**	**F**	**CRF_02.AG**	**DISCORDANT**
	NGIB.05.031	**M**	**G**	
19	NGIB.05.032	F	CRF_06.cpx	CONCORDANT
	NGIB.05.033	M	CRF_06.cpx	

S/No	SAMPLE ID	Sex	SUBTYPE	HIV-1 TYPE
20	NGIB.06.001	F	CRF_02.AG	CONCORDANT
	NGIB.06.002	M	CRF_02.AG	
21	NGIB.06.003	F	G	CONCORDANT
	NGIB.06.004	M	G	
22	NGIB.06.006	F	CRF_06.cpx	CONCORDANT
	NGIB.06.007	M	CRF_06.cpx	
23	NGIB.06.008	M	CRF_02.AG	CONCORDANT
	NGIB.06.009	F	CRF_02.AG	

TABLE 11: HIV-1 SUBTYPES AMONG HIV-1 INFECTED FEMALES IN IBADAN

S/No	SAMPLE ID	SUBTYPE
1	NGIB.04.001	G
2	NGIB.04.003	A
3	NGIB.04.005	CRF_06.cpx
4	NGIB.04.008	G
5	NGIB.04.011	CRF_02.AG
6	NGIB.04.014	G
7	NGIB.04.016	G
8	NGIB.05.001	G
9	NGIB.05.005	G
10	NGIB.05.007	CRF_35.AD.AF
11	NGIB.05.008	CRF_02.AG
12	NGIB.05.012	G
13	NGIB.05.014	CRF_06.cpx
14	NGIB.05.015	G
15	NGIB.05.017	CRF_02.AG
16	NGIB.05.020	G
17	NGIB.05.021	G
18	NGIB.05.023	G
19	NGIB.05.025	G'
20	NGIB.05.027	G
21	NGIB.05.030	CRF_02.AG
22	NGIB.05.032	CRF_06.cpx
23	NGIB.06.001	CRF_02.AG
24	NGIB.06.003	G
25	NGIB.06.005	CRF_02.AG
26	NGIB.06.006	CRF_06.cpx
27	NGIB.06.009	CRF_02.AG
28	NGIB.06.010	CRF_02.AG
29	NGIB.06.011	G
30	NGIB.06.013	CRF_06.cpx

TABLE 12: HIV-1 SUBTYPES AMONG HIV-1 INFECTED MALES IN IBADAN

S/No	SAMPLE ID	SUBTYPE
1	NGIB.04.002	CRF_02.AG
2	NGIB.04.004	A
3	NGIB.04.006	CRF_06.cpx
4	NGIB.04.007	G
5	NGIB.04.009	G
6	NGIB.04.010	G
7	NGIB.04.012	CRF_02.AG
8	NGIB.04.013	CRF_02.AG
9	NGIB.04.015	G
10	NGIB.04.017	G
11	NGIB.05.002	CRF_02.AG
12	NGIB.05.003	G
13	NGIB.05.004	CRF_02.AG
14	NGIB.05.006	G
15	NGIB.05.009	G
16	NGIB.05.010	G
17	NGIB.05.011	G'
18	NGIB.05.013	G
19	NGIB.05.016	G
20	NGIB.05.018	CRF_02.AG
21	NGIB.05.019	CRF_02.AG
22	NGIB.05.022	G
23	NGIB.05.024	CRF_02.AG
24	NGIB.05.026	G'
25	NGIB.05.028	G
26	NGIB.05.029	CRF_06.cpx
27	NGIB.05.031	G
28	NGIB.05.033	CRF_06.cpx
29	NGIB.06.002	CRF_02.AG
30	NGIB.06.004	G
31	NGIB.06.007	CRF_06.cpx
32	NGIB.06.008	CRF_02.AG
33	NGIB.06.012	G
34	NGIB.06.014	G

The sequence analysis of the V3 loop showed total conservation for 3 (P16, G17, and A33) out of the 35 amino acid residues (8.6% of conservation). P16 and G17 are motifs within the GPGQ motif of the V3 tip and A33 is in the base. The positions in which variation did not exceed 10% were concentrated in the base (R3-4.7%, P4-7.8%, I30-4.7%, R31-7.8%), the tip (G15-4.7%) and the stem (N6-3.1%, I26-6.3%). The most variable position was the 25th, which was the determinant for the 11/25 rule used for predicting HIV-1 coreceptor usage. The variation in the Cysteine residues limiting the V3 loop was 3.1% for C1 and 1.6% for C35.

The conserved GPGQ crown motif was the most common sequence observed at the V3 tip occurring in 76.5% (49) of the translated HIV-1 C2-V3 amino acid sequences. There was substitution from GPGQ to other V3 crown motifs like GPGL, GPGR, GPGK, APGQ and RPGQ, as shown in figure 22.

The prevalence of the various V3 crown motifs in the different HIV-1 subtypes seen in this study is shown in table 13. The GPGQ motif is the most common motif found in all subtypes and CRFs of the HIV-1 sequences translated.

4.3.2 V3 LOOP GLYCOSYLATION SITES

The NxS/Ty sequon for the potential N-glycosylation sites in the C2-V3 translated amino acid sequence is centered at position 6 upstream of the first 5'-Cysteine V3 loop residue, the 5'-Cys V3 loop residue, six amino acids downstream from the first cysteine residue and position one after the last Cysteine residue in the V3 loop.

Most potential N-linked glycosylation sites were conserved in the immediate vicinity of and within the V3 loop of the C2-V3 HIV-1 sequences analyzed. The NxS/Ty sequon for the potential N-glycosylation site centered at the 5'-Cys V3 loop residue was conserved in 46 (71.9%) of the HIV-1 C2-V3 amino acid sequences with 41 (89.1%) presenting with the NCT

(Asparagine-Cysteine-Threonine) sequon (Figure 21). Only 39 (60.9%) of the HIV-1 amino acid sequences had an N-glycosylation site centered six residues upstream of the 5'-Cys V3 loop residue.

Potential N-linked glycosylation sites located six amino acids downstream from the 5' cysteine residue in the V3 loop was conserved in 61 (95.3%) of HIV-1 infected individuals with 54 (88.5%) presenting with the NNT (Asparagine-Asparagine-Threonine) sequon. Immediately outside the V3 loop, 49 (76.6%) of the amino acid sequences had the N-glycosylation site conserved with 91.8% (45/49) presenting with the NVS (Asparagine-Valine-Serine) sequon.

Interestingly, one patient had no potential N-linked glycosylation site in the HIV-1 C2-V3 amino acid sequence analyzed. This sequence (NGIB.04__007) is a subtype G HIV-1 virus having the GPGQ V3 tip motif with a predicted X4 phenotype.

4.3.3 PREDICTION OF HIV-1 CORECEPTOR USAGE

Analysis of the four combined criteria for the genotypic prediction of HIV-1 V3 loop coreceptor usage indicates that X4 (68.8%) virus phenotype predominate in the studied group while the R5 tropic viruses were 31.2% (Figure 23).

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35	Subtype	Net Charge	Geno2Pheno (10%)	Coreceptor
Cons B	**C T R P N N N T - R K S I H I G P G R A F Y T T G E I I G D I R Q A H C**				
NGIB.04.001	C T R P G N N T - R R - Y K N G P G Q S F Y A T G D I I G D T R Q A H C	G	3	R5	R5
NGIB.04.002	C T R P N N N T - R K C - T Y G P G Q T I Y A T G A I I G E I R Q A H C	CRF_02.AG	3	X4	X4
NGIB.04.003	C T R P N N N T - R K C K N - G P G Q A F Y A T G A I T G D I I K A Y C	A	4	R5	R5
NGIB.04.004	C T R P N N N T - R K C N N - G P G Q A F Y A T G D I I G D T R K A H C	A	3	X4	X4
NGIB.04.005	C Y R P N N N T - R K Y I - I G P G Q A F Y A T G D I I G D I R Q A H C	CRF_06.cpx	2	R5	R5
NGIB.04.006	C Y R P N N N T - R K Y I - I G P G Q A F Y A T G D I I G D I R Q A H C	CRF_06.cpx	2	R5	R5
NGIB.04.007	Y - R P N - - T - - R K Y K N G P G Q T F Y A T - - - I G N I R Q A - C	G	6	X4	X4
NGIB.04.008	C T R P S N N T - R R - Y K N G P G Q A F Y A T G A V T G D I R N A H C	G	4	R5	R5
NGIB.04.009	C T R P N N N T - - R K Y R N G P G Q A F Y A T G A I I G D I R Q A H C	G	4	X4	X4
NGIB.04.010	C T R P N N N T - R K H T P - G P G Q A F F A T G D I I G D I R E A H C	G	1	R5	R5
NGIB.04.011	C S R P G N N T - - R Q Y T Y G P G Q A F Y A T G D I T G D I R Q A H C	CRF_02.AG	1	R5	R5
NGIB.04.012	C S R P G N N T - R R - Y T Y G P G Q A F Y A T G D I I G D I R Q A H C	CRF_02.AG	2	X4	X4
NGIB.04.013	C T R P G N N T - - R K Y T Y G P G Q V F Y T D - - I I G D I R Q A H C	CRF_02.AG	3	X4	X4
NGIB.04.014	C T R P N N N T - R K H K N - G P G Q M F Y A T G D I T G D I R Q A H C	G	3	R5	R5
NGIB.04.015	C T R P N N N T - - R K Y K I G P G Q T F Y A T G D I I G D I R Q A Y C	G	3	R5	X4
NGIB.04.016	C T R A N N N T - - R K Y K N G P G Q A F Y A T G D I I G D I K Q A H C	G	3	R5	X4
NGIB.04.017	C T T P N N Y T - K K D I - I G P G L A F R A K G N I I G D I R Q A H C	G	3	X4	X4
NGIB.05.001	C T R P N N Y T - K K D I - I G P G L A F R A K G N I I G D I R Q A H C	G	4	X4	X4
NGIB.05.002	C T R P N N N T - - R K Y T Y G P G Q T F Y A T G D I V G D I R Q A H C	CRF_02.AG	2	R5	X4
NGIB.05.003	C T R P N N Y T - K K D I - I G P G L A F R A K G T I I G D I K Q A H W	G	4	X4	X4
NGIB.05.004	C T R L G N N T - R R - Y T Y G P G R A F F T S G D V T G D I R K A Y C	CRF_02.AG	4	X4	X4
NGIB.05.005	C T R P G N N T - - I R Y K N G P G Q A F Y A T G K I T G D I R K A H C	G	5	X4	X4
NGIB.05.006	C T R P N N N T - R R - Y N I A P G Q A F Y T T G A I I G D I R Q A Y C	G	3	R5	R5
NGIB.05.007	C T R P N N N T - R K C - T Y G P G Q S F F A T G D I I G D I R Q A H C	CRF_35.AD	2	R5	R5
NGIB.05.008	C T R P N N N T - R K C - T Y G P G Q T F Y A T G D I I G N I R Q A H C	CRF_02.AG	3	R5	R5
NGIB.05.009	C T R P N N Y T - K K D I - I G P G L A F R A K G N I I G D I R Q A H C	G	4	X4	X4
NGIB.05.010	C T R P N N Y T - K K D I - I G P G L A F P A K G N I I G D I R Q A H C	G	3	X4	X4
NGIB.05.011	C T R P G N N T - - R K Y N N G P G Q A I Y A T G D I I G D I R Q A H C	G'	2	X4	X4
NGIB.05.012	W T R P S I N T - - R I Y R D G P G Q A V S A T V S I L V N L - I A D C	G	1	X4	X4
NGIB.05.013	C I R P N N N T - R K H K N - G P G Q T F Y T T G A I I G N I R Q A H C	G	5	R5	R5
NGIB.05.014	C I R P G N N T - K R Y I - L A P G Q V F Y A T - - I I G D I R Q A H C	CRF_06.cpx	3	X4	X4
NGIB.05.015	C T R P N N N T - - R K Y T T G P G Q A I Y A T G A I I G D I R Q A H C	G	3	X4	X4
NGIB.05.016	C S R P N N N T - - R K Y T T G P G Q A I Y A T G A I I G D I R Q A H C	G	3	X4	X4
NGIB.05.017	C T R P G N N T - R K C A Y - G P G Q A F Y T T G D I I G D I R K A H C	CRF_02.AG	3	X4	X4
NGIB.05.018	C T R P N N N T - V Q G - A C G P G Q T F Y A K D D I I G D I R Q A H C	CRF_02.AG	0	R5	R5
NGIB.05.019	C I R P S N N T - - R K F S Y G P G L K F Y T T G N I I G D I R K A H C	CRF_02.AG	5	X4	X4
NGIB.05.020	C T R P N N N T - - R K Y K N G P G Q V F Y A T G E I I G D I R Q A Y C	G	3	X4	X4
NGIB.05.021	C T R P N N N T - R K Y I - I G P G Q A L Y A T G D I I G D I R Q A H C	G	2	R5	R5
NGIB.05.022	C T R P N N Y T K K K D I - I G P G L A F R A K G N I I G D I R Q A H C	G	5	X4	X4
NGIB.05.023	C T G P N N Y T - K K D I - I G P G L A F R A K G N I I G D I R Q A H C	G	3	X4	X4
NGIB.05.024	C T R P N N N T - R K H A Y - G P G Q T F Y A T G D I I G D I R Q A H C	CRF_02.AG	2	X4	X4
NGIB.05.025	C T G P H N N T - R R - Y K D G P G Q A F Y A T G E I I G N I R Q A H C	G'	2	R5	R5
NGIB.05.026	C T R P N N N T - R R - Y K D G P G Q A F Y A T G D I I G N I R Q A H C	G'	3	R5	R5
NGIB.05.027	C T R P N N N T - - R K Y N I G P G Q A F Y A T G D I I G D I R Q A Y C	G	2	R5	X4
NGIB.05.028	C T R P G N N T - R K D - D I G P G R V F Y A T S D V V G D I R Q A H C	G	1	X4	X4
NGIB.05.029	C T R P N N N I - R K Y I P - G P G Q A F Y A T G D I T G D I R R A Y C	CRF_06.cpx	3	R5	R5
NGIB.05.030	C T R P D N N T - - R Q C A Y G P G Q V F Y A N K D I I G N I R Q A H C	CRF_02.AG	2	X4	X4
NGIB.05.031	C T R P N N N T - R R - Y R D G P G Q A F Y A T G A I V G N I R Q A H C	G	4	X4	X4
NGIB.05.032	C T R P N N N T - R K Y T H - G P G Q A F Y A T G A I I G N I R Q A H C	CRF_06.cpx	4	R5	R5
NGIB.05.033	C T R P N N N T - R R Y T H - G P G R V F Y A T G D I I G D I R R A H C	CRF_06.cpx	4	X4	X4
NGIB.06.001	C S R P S N N T - R R D - R Y G P G Q M F Y A A G E I I G D I R Q A H C	CRF_02.AG	2	X4	X4
NGIB.06.002	C T R P S N N T - R K D - R Y G P G Q M F Y A A G D I T G D I R Q A H C	CRF_02.AG	2	X4	X4
NGIB.06.003	C T R T G N N T - R R H K N - G P G Q A F Y G T G D I I G D I R K A H C	G	4	X4	X4
NGIB.06.004	C T R T G N N T - R R H K N - G P G Q A L Y G T G D I I G D I R K A H C	G	4	X4	X4
NGIB.06.005	C I R P G N N T - - R T L P Y G P G Q T F F A D - - I I G D I R Q A Y C	CRF_02.AG	1	X4	X4
NGIB.06.006	C T R P N N N T - - R K Y N L G P G Q A I Y A T G D I I G D I R Q A Y C	CRF_06.cpx	2	X4	X4
NGIB.06.007	C T R P N N N T - - K K Y T I G P G Q A N C A T G A I I G D I K Q A Y C	CRF_06.cpx	3	R5	X4
NGIB.06.008	C A R P G N N T - - R T H T Y G P G Q T F F A T G D I I G D I R Q A H C	CRF_02.AG	1	R5	R5
NGIB.06.009	C A R P G N N T - R T H T Y R R P G Q T F F A T G D I I R D I R Q A H C	CRF_02.AG	3	X4	X4
NGIB.06.010	C T R P S N N T - R K C - T Y G P G Q A F Y A T G E I T G D I R Q A Y C	CRF_02.AG	2	X4	X4
NGIB.06.011	C T R P N N N T - R R - Y N I G P G Q A I Y T T G A I I G D I R Q A H C	G	3	X4	X4
NGIB.06.012	C T R S N N N T - - R K Y K N G P G Q A F Y A T G E I I G N I R Q A H C	G	4	R5	X4
NGIB.06.013	C T R P N N N T - R K Y T H - G P G Q A F Y A T G A I I G D I R E A Y C	CRF_06.cpx	2	R5	R5
NGIB.07.001	C T R P S N N T - - R K Y N T G P G K A F Y T T G E I I G D I R Q A H C	G	3	X4	X4

FIGURE 21: V3 LOOP AMINO ACID SEQUENCE AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

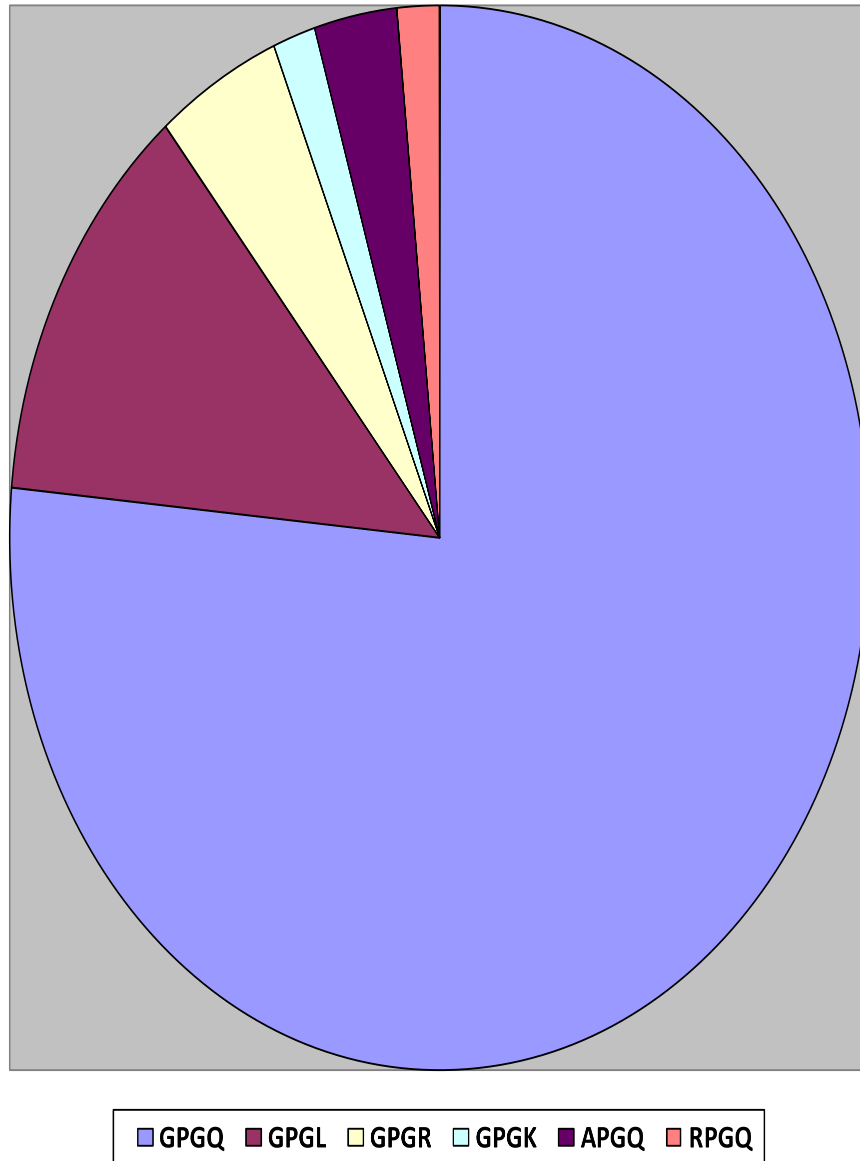

FIGURE 22: V3 LOOP CROWN MOTIF AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

TABLE 13: SUBTYPE PREVALENCE OF V3 CROWN MOTIF AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

Subtype/Motif	GPGQ	GPGL	GPGR	GPGK	APGQ	RPGQ	Total
A	2 (100%)	0	0	0	0	0	2
G	24 (70.6%)	7 (20.6%)	1 (2.9%)	1 (2.9%)	1 (2.9%)	0	34
CRF02_AG	15 (83.3%)	1 (5.6%)	1 (5.6%)	0	0	1 (5.6%)	18
CRF06_cpx	7 (77.8%)	0	1 (11.1%)	0	1 (11.1%)	0	9
CRF35_AD	1 (100%)	0	0	0	0	0	1
Total	49	8	3	1	2	1	64

On further analysis of the V3 loop amino acid sequences that met each criterion, 16 of the V3 loop amino acid sequences had Lysine residue at position +11 while only one had Arginine residue at position +11. The only V3 loop sequence with the Arginine residue at position +11 also had a lysine residue at position +25. One V3 loop amino acid sequence has a net charge of +6. Bioinformatics analysis with Geno2Pheno at 10% false positive rate (FPR) predicted 38 (including some V3 loop sequences that met the other criteria) as X4 viruses. All V3 loop sequences with 33 and 31 amino acid residues, and those not limited by the Cysteine residues, were predicted as X4 virus phenotype.

Of the 44 V3 loop amino acid sequences with predicted X4 virus phenotype, only 7 were classified as X4 based wholly on the 11/25 rule. The remaining 37 were predicted based on the presence of X4 associated mutations by the Geno2Pheno software.

All subtypes and CRFs of HIV-1 in this study, except CRF35_AD, had some V3 loop sequences with predicted X4 virus phenotype. The prevalence of predicted X4 viruses was one (50%) in subtype A, 25 (73.5%) in subtype G, 14 (77.8%) in CRF02_AG and 4 (44.4%) in CRF06_cpx. The prevalence of predicted R5 viruses was one (50%) in subtype A, 9 (26.5%) in subtype G, 4 (22.2%) in CRF02_AG and 5 (55.6%) in CRF06_cpx.

4.3.4 V3 LOOP TIP MOTIF AND PREDICTED HIV-1 CORECEPTOR USAGE

All V3 loop amino acid sequences with the GPGL, GPGK, GPGR and RPGQ motifs have an X4 predicted phenotype as shown in table 14. Also, 30 (61.2%) of V3 loops with GPGQ V3 tip motifs have predicted X4 phenotypes. Finally, it was also found that 15 (88.2%) of the 17 HIV-1 V3 amino acid sequences with Lysine or Arginine amino acid residues at position +11 have the GPGQ V3 tip motif.

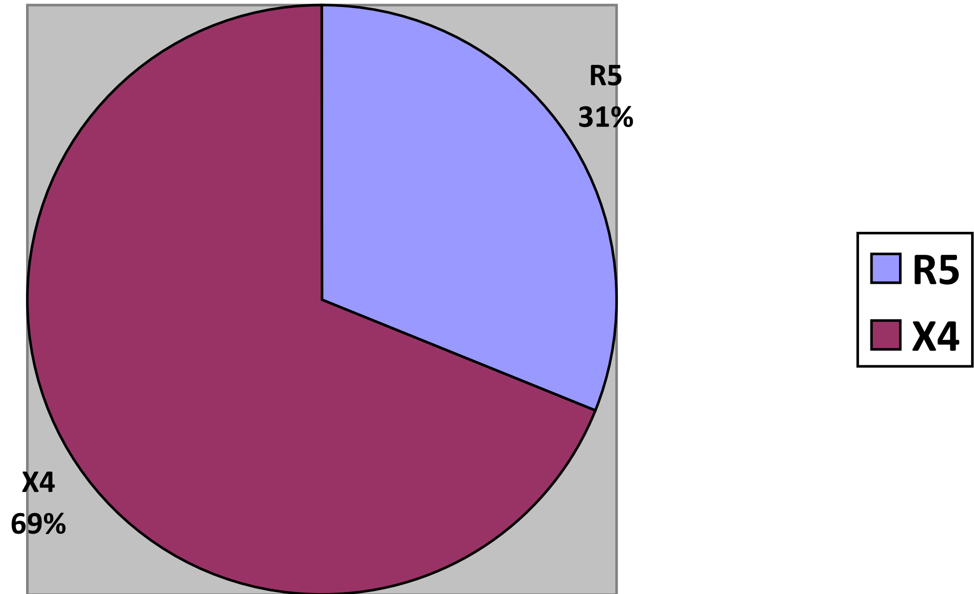

FIGURE 23: PREDICTED HIV-1 CORECEPTOR USAGE AMONG HIV-1 POSITIVE PATIENTS IN IBADAN

4.3.5 PREDICTED HIV-1 CORECEPTOR USAGE AMONG COUPLES

Of the 23 couples whose V3 loop amino acid sequences were analyzed for coreceptor usage, only 2 (8.7%) and 9 (39.1%) of the HIV-1 infected couples have concordant V3 loop amino acid sequence with predicted R5 and X4 virus phenotype, respectively (Figure 24). The remaining 12 (52.2%) had discordant coreceptor phenotypes (table 15). Of the 12 couples who had discordant coreceptor phenotypes, 8 (66.7%) and 4 (33.3%) were male and female spouses, respectively, with predicted X4 phenotypes. Conversely, 4 (33.3%) and 8 (66.7%) of the male and female spouses, respectively, have predicted V3 loop amino acid sequences with predicted R5 coreceptor phenotype.

4.3.6 V3 LOOP MUTATIONS ASSOCIATED WITH X4 CORECEPTOR USAGE

The mutations in the V3 loop associated with X4 virus phenotype in the 64 sequences were also analyzed. The mutations are N7Y, H13Y/S/T/R, A19V, F20V, A22T, T23A/R, G24E/S, E25Q/N, 127V/A and Q32K, as shown in figure 25. The predominant mutation is the H13Y/S/T/R mutation occurring in 38.6% (17/44) of the HIV-1 V3 loop amino acid sequences with predicted X4 phenotype, and 78.6% (11/14) of the CRF02_AG viruses with predicted X4 phenotype. The presence of each mutation in terms of its distribution in each subtype of HIV-1 V3 loop sequence is presented in table 15. Of the 44 V3 loop amino acid sequences with predicted X4 phenotype, 37 (80.1%) have a combination of one or more of these mutations, including 10 sequences also with Arginine or Lysine residues in position +11 and/or +25 of the V3 loop. Only 7 V3 loop amino acid sequences were predicted as X4 phenotype by the 11/25 rule.

Furthermore, examining the number of X4 mutations, without the 11/25 rule, in each V3 loop amino acid sequence with predicted X4 phenotype, 22 (59.5%), 10 (27%), 4 (10.8%) and one (2.7%) have 1, 2, 3 and 4 mutations, respectively.

TABLE 14: SUBTYPE DISTRIBUTION OF V3 LOOP TIP MOTIF IN PREDICTED HIV-1 X4 VIRUS PHENOTYPE IN IBADAN

V3 Motif	Subtype A	Subtype G	CRF02_AG	CRF06_cpx	**TOTAL**
GPGQ	1	16	11	2	30
GPGL	0	7	1	0	8
GPGR	0	1	1	1	3
GPGK	0	1	0	0	1
APGQ	0	0	0	1	1
RPGQ	0	0	1	0	1
TOTAL	1	25	14	4	**44**

	1	2	3	4	5	6	7	8		9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	Subtype	Sex	Coreceptor
Cons B	**C**	**T**	**R**	**P**	**N**	**N**	**N**	**T**	-	**R**	**K**	**S**	**I**	**H**	**I**	**G**	**P**	**G**	**R**	**A**	**F**	**Y**	**T**	**T**	**G**	**E**	**I**	**I**	**G**	**D**	**I**	**R**	**Q**	**A**	**H**	**C**			
NGIB.04.001	C	T	R	P	G	N	N	T	-	R	R	-	Y	K	N	G	P	G	Q	S	F	Y	A	T	G	D	I	I	G	D	T	R	Q	A	H	C	G	F	R5
NGIB.04.002	C	T	R	P	N	N	N	T	-	R	K	C	-	T	Y	G	P	G	Q	T	I	Y	A	T	G	A	I	I	G	E	I	R	Q	A	H	C	CRF_02.AG	M	X4
NGIB.04.003	C	T	R	P	N	N	N	T	-	R	K	C	K	N	-	G	P	G	Q	A	F	Y	A	T	G	A	I	T	G	D	I	I	K	A	Y	C	A	F	R5
NGIB.04.004	C	T	R	P	N	N	N	T	-	R	K	C	N	N	-	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	T	R	K	A	H	C	A	M	X4
NGIB.04.005	C	Y	R	P	N	N	N	T	-	R	K	Y	I	-	I	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_06.cpx	F	R5
NGIB.04.006	C	Y	R	P	N	N	N	T	-	R	K	Y	I	-	I	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_06.cpx	M	R5
NGIB.04.008	C	T	R	P	S	N	N	T	-	R	R	-	Y	K	N	G	P	G	Q	A	F	Y	A	T	G	A	V	T	G	D	I	R	N	A	H	C	G	F	R5
NGIB.04.009	C	T	R	P	N	N	N	T	-	-	R	K	Y	R	N	G	P	G	Q	A	F	Y	A	T	G	A	I	I	G	D	I	R	Q	A	H	C	G	M	X4
NGIB.04.011	C	S	R	P	G	N	N	T	-	-	R	Q	Y	T	Y	G	P	G	Q	A	F	Y	A	T	G	D	I	T	G	D	I	R	Q	A	H	C	CRF_02.AG	F	R5
NGIB.04.012	C	S	R	P	G	N	N	T	-	R	R	-	Y	T	Y	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG	M	X4
NGIB.04.014	C	T	R	P	N	N	N	T	-	R	K	H	K	N	-	G	P	G	Q	M	F	Y	A	T	G	D	I	T	G	D	I	R	Q	A	H	C	G	F	R5
NGIB.04.015	C	T	R	P	N	N	N	T	-	-	R	K	Y	K	I	G	P	G	Q	T	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	Y	C	G	M	X4
NGIB.04.016	C	T	R	A	N	N	N	T	-	-	R	K	Y	K	N	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	K	Q	A	H	C	G	F	X4
NGIB.05.003	C	T	R	P	N	N	Y	T	-	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	T	I	I	G	D	I	K	Q	A	H	W	G	M	X4
NGIB.05.001	C	T	R	P	N	N	Y	T	-	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	N	I	I	G	D	I	R	Q	A	H	C	G	F	X4
NGIB.05.002	C	T	R	P	N	N	N	T	-	-	R	K	Y	T	Y	G	P	G	Q	T	F	Y	A	T	G	D	I	V	G	D	I	R	Q	A	H	C	CRF_02.AG	M	X4
NGIB.05.005	C	T	R	P	G	N	N	T	-	-	I	R	Y	K	N	G	P	G	Q	A	F	Y	A	T	G	K	I	T	G	D	I	R	K	A	H	C	G	F	X4
NGIB.05.006	C	T	R	P	N	N	N	T	-	R	R	-	Y	N	I	A	P	G	Q	A	F	Y	T	T	G	A	I	I	G	D	I	R	Q	A	Y	C	G	M	R5
NGIB.05.008	C	T	R	P	N	N	N	T	-	R	K	C	-	T	Y	G	P	G	Q	T	F	Y	A	T	G	D	I	I	G	N	I	R	Q	A	H	C	CRF_02.AG	F	R5
NGIB.05.009	C	T	R	P	N	N	Y	T	-	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	N	I	I	G	D	I	R	Q	A	H	C	G	M	X4
NGIB.05.012	W	T	R	P	S	I	N	T	-	-	R	I	Y	R	D	G	P	G	Q	A	V	S	A	T	V	S	I	L	V	N	L	-	I	A	D	C	G	F	X4
NGIB.05.013	C	I	R	P	N	N	N	T	-	R	K	H	K	N	-	G	P	G	Q	T	F	Y	T	T	G	A	I	I	G	N	I	R	Q	A	H	C	G	M	R5
NGIB.05.015	C	T	R	P	N	N	N	T	-	-	R	K	Y	T	T	G	P	G	Q	A	I	Y	A	T	G	A	I	I	G	D	I	R	Q	A	H	C	G	F	X4
NGIB.05.016	C	S	R	P	N	N	N	T	-	-	R	K	Y	T	T	G	P	G	Q	A	I	Y	A	T	G	A	I	I	G	D	I	R	Q	A	H	C	G	M	X4
NGIB.05.017	C	T	R	P	G	N	N	T	-	R	K	C	A	Y	-	G	P	G	Q	A	F	Y	T	T	G	D	I	I	G	D	I	R	K	A	H	C	CRF_02.AG	F	X4
NGIB.05.018	C	T	R	P	N	N	N	T	-	V	Q	G	-	A	C	G	P	G	Q	T	F	Y	A	K	D	D	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG	M	R5
NGIB.05.021	C	T	R	P	N	N	N	T	-	R	K	Y	I	-	I	G	P	G	Q	A	L	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	G	F	R5
NGIB.05.022	C	T	R	P	N	N	Y	T	K	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	N	I	I	G	D	I	R	Q	A	H	C	G	M	X4
NGIB.05.023	C	T	G	P	N	N	Y	T	-	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	N	I	I	G	D	I	R	Q	A	H	C	G	F	X4
NGIB.05.024	C	T	R	P	N	N	N	T	-	R	K	H	A	Y	-	G	P	G	Q	T	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG	M	X4
NGIB.05.025	C	T	G	P	H	N	N	T	-	R	R	-	Y	K	D	G	P	G	Q	A	F	Y	A	T	G	E	I	I	G	N	I	R	Q	A	H	C	G'	F	R5
NGIB.05.026	C	T	R	P	N	N	N	T	-	R	R	-	Y	K	D	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	N	I	R	Q	A	H	C	G'	M	R5
NGIB.05.027	C	T	R	P	N	N	N	T	-	-	R	K	Y	N	I	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	Y	C	G	F	X4
NGIB.05.028	C	T	R	P	G	N	N	T	-	R	K	D	-	D	I	G	P	G	R	V	F	Y	A	T	S	D	V	V	G	D	I	R	Q	A	H	C	G	M	X4
NGIB.05.030	C	T	R	P	D	N	N	T	-	-	R	Q	C	A	Y	G	P	G	Q	V	F	Y	A	N	K	D	I	I	G	N	I	R	Q	A	H	C	CRF_02.AG	F	X4
NGIB.05.031	C	T	R	P	N	N	N	T	-	R	R	-	Y	R	D	G	P	G	Q	A	F	Y	A	T	G	A	I	V	G	N	I	R	Q	A	H	C	G	M	X4
NGIB.05.032	C	T	R	P	N	N	N	T	-	R	K	Y	T	H	-	G	P	G	Q	A	F	Y	A	T	G	A	I	I	G	N	I	R	Q	A	H	C	CRF_06.cpx	F	R5
NGIB.05.033	C	T	R	P	N	N	N	T	-	R	R	Y	T	H	-	G	P	G	R	V	F	Y	A	T	G	D	I	I	G	D	I	R	R	A	H	C	CRF_06.cpx	M	X4
NGIB.06.001	C	S	R	P	S	N	N	T	-	R	R	D	-	R	Y	G	P	G	Q	M	F	Y	A	A	G	E	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG	F	X4
NGIB.06.002	C	T	R	P	S	N	N	T	-	R	K	D	-	R	Y	G	P	G	Q	M	F	Y	A	A	G	D	I	T	G	D	I	R	Q	A	H	C	CRF_02.AG	M	X4
NGIB.06.003	C	T	R	T	G	N	N	T	-	R	R	H	K	N	-	G	P	G	Q	A	F	Y	G	T	G	D	I	I	G	D	I	R	K	A	H	C	G	F	X4
NGIB.06.004	C	T	R	T	G	N	N	T	-	R	R	H	K	N	-	G	P	G	Q	A	L	Y	G	T	G	D	I	I	G	D	I	R	K	A	H	C	G	M	X4
NGIB.06.006	C	T	R	P	N	N	N	T	-	-	R	K	Y	N	L	G	P	G	Q	A	I	Y	A	T	G	D	I	I	G	D	I	R	Q	A	Y	C	CRF_06.cpx	F	X4
NGIB.06.007	C	T	R	P	N	N	N	T	-	-	K	K	Y	T	I	G	P	G	Q	A	N	C	A	T	G	A	I	I	G	D	I	K	Q	A	Y	C	CRF_06.cpx	M	X4
NGIB.06.008	C	A	R	P	G	N	N	T	-	-	R	T	H	T	Y	G	P	G	Q	T	F	F	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG	M	R5
NGIB.06.009	C	A	R	P	G	N	N	T	-	R	T	H	T	Y	R	R	P	G	Q	T	F	F	A	T	G	D	I	I	R	D	I	R	Q	A	H	C	CRF_02.AG	F	X4

FIGURE 24: HIV-1 V3 LOOP AMINO ACID SEQUENCE IN INFECTED COUPLES IN IBADAN

4.3.7 PREDICTION OF MARAVIROC RESISTANCE

The sets of mutations in the V3 loop associated with Maraviroc-resistant phenotype in the 64 sequences were also analysed. The mutations that confer Maraviroc resistance can be differentiated into those associated *in* vivo and those associated *in vitro.*

The sets of mutations associated *in vivo* with resistance to Maraviroc are G11S+I26V, S18G+A22T, A19S+I26V, I20F+Y21I and I20F+A25D+I26V. Only 2 V3 loop sequences, NGIB05__004 and NGIB05__028, have the 120F+A25D+I26V mutation set. No other mutation set was observed in any of the sequences. Interestingly, these 2 sequences were already predicted as X4 virus phenotype. No R5 predicted virus phenotype has any of these drug resistant mutation sets.

The mutations associated *in vitro* with resistance to Maraviroc are A22T and I30V. A total of 8 V3 loop sequences have the A22T resistance mutation while none has the I30V mutation. Of the 8 V3 loop sequences with the A22T mutation, 2 and 6 are R5 and X4 virus phenotypes, respectively. In terms of HIV-1 subtype, 4 were CRF02_AG with X4 phenotypes while the remaining 4 were subtype G (2 R5 and 2 X4 virus phenotypes).

	1	2	3	4	5	6	7	8		9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	Subtype
Cons B	**C**	**T**	**R**	**P**	**N**	**N**	**N**	**T**	**-**	**R**	**K**	**S**	**I**	**H**	**I**	**G**	**P**	**G**	**R**	**A**	**F**	**Y**	**T**	**T**	**G**	**E**	**I**	**I**	**G**	**D**	**I**	**R**	**Q**	**A**	**H**	**C**	
NGIB.04.001	C	T	R	P	G	N	N	T	-	R	R	-	Y	K	N	G	P	G	Q	S	F	Y	A	T	G	D	I	I	G	D	T	R	Q	A	H	C	G
NGIB.04.002	C	T	R	P	N	N	N	T	-	R	K	C	-	T	Y	G	P	G	Q	T	I	Y	A	T	G	A	I	I	G	E	I	R	Q	A	H	C	CRF_02.AG
NGIB.04.003	C	T	R	P	N	N	N	T	-	R	K	C	K	N	-	G	P	G	Q	A	F	Y	A	T	G	A	I	T	G	D	I	I	K	A	Y	C	A
NGIB.04.004	C	T	R	P	N	N	N	T	-	R	K	C	N	N	-	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	T	R	K	A	H	C	A
NGIB.04.005	C	Y	R	P	N	N	N	T	-	R	K	Y	I	-	I	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_06.cpx
NGIB.04.006	C	Y	R	P	N	N	N	T	-	R	K	Y	I	-	I	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_06.cpx
NGIB.04.007	Y	-	R	P	N	-	-	T	-	-	R	K	Y	K	N	G	P	G	Q	T	F	Y	A	T	-	-	-	I	G	N	I	R	Q	A	-	C	G
NGIB.04.008	C	T	R	P	S	N	N	T	-	R	R	-	Y	K	N	G	P	G	Q	A	F	Y	A	T	G	A	V	T	G	D	I	R	N	A	H	C	G
NGIB.04.009	C	T	R	P	N	N	N	T	-	-	R	K	Y	R	N	G	P	G	Q	A	F	Y	A	T	G	A	I	I	G	D	I	R	Q	A	H	C	G
NGIB.04.010	C	T	R	P	N	N	N	T	-	R	K	H	T	P	-	G	P	G	Q	A	F	F	A	T	G	D	I	I	G	D	I	R	E	A	H	C	G
NGIB.04.011	C	S	R	P	G	N	N	T	-	-	R	Q	Y	T	Y	G	P	G	Q	A	F	Y	A	T	G	D	I	T	G	D	I	R	Q	A	H	C	CRF_02.AG
NGIB.04.012	C	S	R	P	G	N	N	T	-	R	R	-	Y	T	Y	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG
NGIB.04.013	C	T	R	P	G	N	N	T	-	-	R	K	Y	T	Y	G	P	G	Q	V	F	Y	T	D	-	-	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG
NGIB.04.014	C	T	R	P	N	N	N	T	-	R	K	H	K	N	-	G	P	G	Q	M	F	Y	A	T	G	D	I	T	G	D	I	R	Q	A	H	C	G
NGIB.04.015	C	T	R	P	N	N	N	T	-	-	R	K	Y	K	I	G	P	G	Q	T	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	Y	C	G
NGIB.04.016	C	T	R	A	N	N	N	T	-	-	R	K	Y	K	N	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	K	Q	A	H	C	G
NGIB.04.017	C	T	T	P	N	N	Y	T	-	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	N	I	I	G	D	I	R	Q	A	H	C	G
NGIB.05.001	C	T	R	P	N	N	Y	T	-	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	N	I	I	G	D	I	R	Q	A	H	C	G
NGIB.05.002	C	T	R	P	N	N	N	T	-	-	R	K	Y	T	Y	G	P	G	Q	T	F	Y	A	T	G	D	I	V	G	D	I	R	Q	A	H	C	CRF_02.AG
NGIB.05.003	C	T	R	P	N	N	Y	T	-	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	T	I	I	G	D	I	K	Q	A	H	W	G
NGIB.05.004	C	T	R	L	G	N	N	T	-	R	R	-	Y	T	Y	G	P	G	R	A	F	F	T	S	G	D	V	T	G	D	I	R	K	A	Y	C	CRF_02.AG
NGIB.05.005	C	T	R	P	G	N	N	T	-	-	I	R	Y	K	N	G	P	G	Q	A	F	Y	A	T	G	K	I	T	G	D	I	R	K	A	H	C	G
NGIB.05.006	C	T	R	P	N	N	N	T	-	R	R	-	Y	N	I	A	P	G	Q	A	F	Y	T	T	G	A	I	I	G	D	I	R	Q	A	Y	C	G
NGIB.05.007	C	T	R	P	N	N	N	T	-	R	K	C	-	T	Y	G	P	G	Q	S	F	F	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_35.AD
NGIB.05.008	C	T	R	P	N	N	N	T	-	R	K	C	-	T	Y	G	P	G	Q	T	F	Y	A	T	G	D	I	I	G	N	I	R	Q	A	H	C	CRF_02.AG
NGIB.05.009	C	T	R	P	N	N	Y	T	-	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	N	I	I	G	D	I	R	Q	A	H	C	G
NGIB.05.010	C	T	R	P	N	N	Y	T	-	K	K	D	I	-	I	G	P	G	L	A	F	P	A	K	G	N	I	I	G	D	I	R	Q	A	H	C	G
NGIB.05.011	C	T	R	P	G	N	N	T	-	-	R	K	Y	N	N	G	P	G	Q	A	I	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	G'
NGIB.05.012	W	T	R	P	S	I	N	T	-	-	R	I	Y	R	D	G	P	G	Q	A	V	S	A	T	V	S	I	L	V	N	L	-	I	A	D	C	G
NGIB.05.013	C	I	R	P	N	N	N	T	-	R	K	H	K	N	-	G	P	G	Q	T	F	Y	T	T	G	A	I	I	G	N	I	R	Q	A	H	C	G
NGIB.05.014	C	I	R	P	G	N	N	T	-	K	R	Y	I	-	L	A	P	G	Q	V	F	Y	A	T	-	-	I	I	G	D	I	R	Q	A	H	C	CRF_06.cpx
NGIB.05.015	C	T	R	P	N	N	N	T	-	-	R	K	Y	T	T	G	P	G	Q	A	I	Y	A	T	G	A	I	I	G	D	I	R	Q	A	H	C	G
NGIB.05.016	C	S	R	P	N	N	N	T	-	-	R	K	Y	T	T	G	P	G	Q	A	I	Y	A	T	G	A	I	I	G	D	I	R	Q	A	H	C	G
NGIB.05.017	C	T	R	P	G	N	N	T	-	R	K	C	A	Y	-	G	P	G	Q	A	F	Y	T	T	G	D	I	I	G	D	I	R	K	A	H	C	CRF_02.AG
NGIB.05.018	C	T	R	P	N	N	N	T	-	V	Q	G	-	A	C	G	P	G	Q	T	F	Y	A	K	D	D	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG
NGIB.05.019	C	I	R	P	S	N	N	T	-	-	R	K	F	S	Y	G	P	G	L	K	F	Y	T	T	G	N	I	I	G	D	I	R	K	A	H	C	CRF_02.AG
NGIB.05.020	C	T	R	P	N	N	N	T	-	-	R	K	Y	K	N	G	P	G	Q	V	F	Y	A	T	G	E	I	I	G	D	I	R	Q	A	Y	C	G
NGIB.05.021	C	T	R	P	N	N	N	T	-	R	K	Y	I	-	I	G	P	G	Q	A	L	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	G
NGIB.05.022	C	T	R	P	N	N	Y	T	K	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	N	I	I	G	D	I	R	Q	A	H	C	G
NGIB.05.023	C	T	G	P	N	N	Y	T	-	K	K	D	I	-	I	G	P	G	L	A	F	R	A	K	G	N	I	I	G	D	I	R	Q	A	H	C	G
NGIB.05.024	C	T	R	P	N	N	N	T	-	R	K	H	A	Y	-	G	P	G	Q	T	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG
NGIB.05.025	C	T	G	P	H	N	N	T	-	R	R	-	Y	K	D	G	P	G	Q	A	F	Y	A	T	G	E	I	I	G	N	I	R	Q	A	H	C	G'
NGIB.05.026	C	T	R	P	N	N	N	T	-	R	R	-	Y	K	D	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	N	I	R	Q	A	H	C	G'
NGIB.05.027	C	T	R	P	N	N	N	T	-	-	R	K	Y	N	I	G	P	G	Q	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	Y	C	G
NGIB.05.028	C	T	R	P	G	N	N	T	-	R	K	D	-	D	I	G	P	G	R	V	F	Y	A	T	S	D	V	V	G	D	I	R	Q	A	H	C	G
NGIB.05.029	C	T	R	P	N	N	N	I	-	R	K	Y	I	P	-	G	P	G	Q	A	F	Y	A	T	G	D	I	T	G	D	I	R	R	A	Y	C	CRF_06.cpx
NGIB.05.030	C	T	R	P	D	N	N	T	-	-	R	Q	C	A	Y	G	P	G	Q	V	F	Y	A	N	K	D	I	I	G	N	I	R	Q	A	H	C	CRF_02.AG
NGIB.05.031	C	T	R	P	N	N	N	T	-	R	R	-	Y	R	D	G	P	G	Q	A	F	Y	A	T	G	A	I	V	G	N	I	R	Q	A	H	C	G
NGIB.05.032	C	T	R	P	N	N	N	T	-	R	K	Y	T	H	-	G	P	G	Q	A	F	Y	A	T	G	A	I	I	G	N	I	R	Q	A	H	C	CRF_06.cpx
NGIB.05.033	C	T	R	P	N	N	N	T	-	R	R	Y	T	H	-	G	P	G	R	V	F	Y	A	T	G	D	I	I	G	D	I	R	R	A	H	C	CRF_06.cpx
NGIB.06.001	C	S	R	P	S	N	N	T	-	R	R	D	-	R	Y	G	P	G	Q	M	F	Y	A	A	G	E	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG
NGIB.06.002	C	T	R	P	S	N	N	T	-	R	K	D	-	R	Y	G	P	G	Q	M	F	Y	A	A	G	D	I	T	G	D	I	R	Q	A	H	C	CRF_02.AG
NGIB.06.003	C	T	R	T	G	N	N	T	-	R	R	H	K	N	-	G	P	G	Q	A	F	Y	G	T	G	D	I	I	G	D	I	R	K	A	H	C	G
NGIB.06.004	C	T	R	T	G	N	N	T	-	R	R	H	K	N	-	G	P	G	Q	A	L	Y	G	T	G	D	I	I	G	D	I	R	K	A	H	C	G
NGIB.06.005	C	I	R	P	G	N	N	T	-	-	R	T	L	P	Y	G	P	G	Q	T	F	F	A	D	-	-	I	I	G	D	I	R	Q	A	Y	C	CRF_02.AG
NGIB.06.006	C	T	R	P	N	N	N	T	-	-	R	K	Y	N	L	G	P	G	Q	A	I	Y	A	T	G	D	I	I	G	D	I	R	Q	A	Y	C	CRF_06.cpx
NGIB.06.007	C	T	R	P	N	N	N	T	-	-	K	K	Y	T	I	G	P	G	Q	A	N	C	A	T	G	A	I	I	G	D	I	K	Q	A	Y	C	CRF_06.cpx
NGIB.06.008	C	A	R	P	G	N	N	T	-	-	R	T	H	T	Y	G	P	G	Q	T	F	F	A	T	G	D	I	I	G	D	I	R	Q	A	H	C	CRF_02.AG
NGIB.06.009	C	A	R	P	G	N	N	T	-	R	T	H	T	Y	R	R	P	G	Q	T	F	F	A	T	G	D	I	I	R	D	I	R	Q	A	H	C	CRF_02.AG
NGIB.06.010	C	T	R	P	S	N	N	T	-	R	K	C	-	T	Y	G	P	G	Q	A	F	Y	A	T	G	E	I	T	G	D	I	R	Q	A	Y	C	CRF_02.AG
NGIB.06.011	C	T	R	P	N	N	N	T	-	R	R	-	Y	N	I	G	P	G	Q	A	I	Y	T	T	G	A	I	I	G	D	I	R	Q	A	H	C	G
NGIB.06.012	C	T	R	S	N	N	N	T	-	-	R	K	Y	K	N	G	P	G	Q	A	F	Y	A	T	G	E	I	I	G	N	I	R	Q	A	H	C	G
NGIB.06.013	C	T	R	P	N	N	N	T	-	R	K	Y	T	H	-	G	P	G	Q	A	F	Y	A	T	G	A	I	I	G	D	I	R	E	A	Y	C	CRF_06.cpx
NGIB.06.014	C	T	R	P	S	N	N	T	-	-	R	K	Y	N	T	G	P	G	K	A	F	Y	T	T	G	E	I	I	G	D	I	R	Q	A	H	C	G

FIGURE 25: MUTATION SITES IN THE V3 LOOP OF HIV-1 POSITIVE PATIENTS IN IBADAN

TABLE 15: V3 LOOP X4 ASSOCIATED MUTATIONS IN HIV-1 SUBTYPES IN IBADAN

MUTATION	Subtype A	Subtype G	CRF02_AG	CRF06_cpx	Total
N7Y	0	7	0	0	7
H13Y/S/T/R	0	5	11	1	17
A19V	0	2	2	2	6
F20V	0	1	0	0	1
A22T	0	2	4	0	6
T23A/R	0	0	2	0	2
G24E/S	0	1	0	0	1
E25Q/N	0	6	1	0	7
I27V/A	0	2	1	0	3
Q32K	1	3	3	0	4
TOTAL	1	29	24	3	57

CHAPTER 5

DISCUSSION

This study shows circulation of HIV-1 subtypes A, G, CRF02_AG, CRF06_cpx and CRF35_AD in different proportions among HIV-1 infected individuals in the study population. This data suggest that the level of diversity of the virus among the study population is complex and agrees with reports from previous molecular studies of HIV-1 diversity in Nigeria. Abimiku *et al*. (1994) and Howard *et al.* (1994) reported detection of HIV-1 subtype G in Northern Nigeria and CRF02_AG in Ibadan, respectively from HIV-1 infected patients. Peeters *et al*. (2000) found HIV-1 subtypes A and G as the major *env* subtypes responsible for the HIV-1 epidemic in Nigeria from a study conducted in 1996 in Lagos, Calabar, Kano and Maiduguri. Furthermore, Odaibo *et al.* (2001a, b) and (2006) reported detection of HIV-1 subtypes A, B, C, D, O and CRF01_AE among HIV-1 infected patients in Nigeria using a peptide based ELISA designed from consensus sequences in the V3 loop. In addition, a national molecular survey of HIV-1 strains in Nigeria involving 230 HIV-1 positive blood samples from 34 states showed the presence of *env* gp41 HIV-1 subtypes A, C, G and J (Agwale *et al*., 2002). Another study on mother-to-child transmission of different HIV-1 subtypes in Ibadan, Oyo state showed circulation of subtypes B, G and CRF02_AG (Odaibo *et al*., 2006). Sequence data from a study on the implications of HIV-1 subtypes on drug resistance and host response in treatment naïve patients in Oyo state, Nigeria yielded subtypes D, G, CRF02_AG and CRF06_cpx from reverse transcriptase and protease sequences (Ojesina *et al*., 2006). Multiple HIV-1 subtypes, sub-subtypes and circulating recombinant forms (CRFs) in Oyo state, Nigeria, were also reported by Sankale *et al*. (2007) and include A, A1, A3, C, G, CRF01_AE, CRF02_AG, CRF06_cpx, CRF09_cpx and CRF11_cpx. Another study on HIV-1 reverse transcriptase and protease sequences in patients with treatment failure, showed the presence of multiple HIV-1 strains including

subtypes A, G, CRF02_AG, CRF06_cpx and other recombinant sequences (Chaplin *et al.,* 2011). Moreover, Ajoge and colleagues (2011) in a study in North Central Nigeria which characterized the gag and *env* genes of HIV-1 isolates in 28 treatment naïve pregnant women showed HIV-1 subtypes C, F2, G, CRF02_AG and CRF06_cpx. These data show the circulation of multiple strains of HIV-1 in the country and suggest that the level of diversity of HIV-1 is complex and will have implications for diagnosis and treatment of HIV-1 infection.

Another significant finding from this study was the detection of circulation of HIV-1 CRF35_AD. This is the first report of HIV-1 CRF35_AD in Nigeria, and indeed, sub-Saharan Africa. This circulating recombinant form is a recombinant of HIV-1 subtypes A and D and it has previously been reported from countries of the Middle East (Sanders-Buell *et al*., 2007; Sanders-Buell *et al*., 2010). Analysed subregions (gag p17 and env C1-C5) and full-length sequences of this CRF were reported as largely driving the HIV-1 epidemic among intravenous drug users (IDUs) in 2 cities in Afghanistan, Kabul (Sanders-Buell *et al*., 2007) and Hirat (Sanders-Buell *et al*., 2010), respectively. A study in Iran involving the *pol* gene of HIV-1 also showed the presence of this CRF in HIV-infected patients (Soheilli *et al*., 2009). CRF35_AD has been reported from *pol*, protease and partial RT sequences among treated and treatment-naïve HIV-1 infected patients in Sindh province, Pakistan (Shah *et al*., 2011). The detection of this CRF in this study suggests its introduction into Nigeria from relationships with the Middle East through travel and migration. Travellers contribute to the spread of HIV-1 genetic diversity worldwide, and in the developing world migration of rural populations and civil wars are additional contributing factors (Thomson and Najera, 2005). It has been established that travel and migration promote the transfer of diverse viral strains between populations, and often across large distances (Perrin *et al.,* 2003; Thomson and Najera, 2005). In addition, the detection of this CRF may be an indication that there are yet

other unrecognized CRFs and URFs in the country. Thus, the complexity of the HIV-1 epidemic in Nigeria may be very well underrepresented.

The subtype prevalence of the different HIV-1 strains in this study was 3.1%, 53.1%, 28.1%, 14.1% and 1.6% for subtypes A, G, CRF02_AG, CRF06_cpx and CRF35_AD, respectively. The prevalence of different circulating HIV-1 subtypes has also been reported by some previous studies conducted in some cities, states and regions in Nigeria. The study by Peeters *et al*. (2000) showed HIV-1 subtype prevalence of 61.3% for subtype A and 37.5% for subtype G from four states in Nigeria. Odaibo and colleagues (2001a) reported HIV-1 subtype prevalence of 19.8%, 2.5%, 48.3%, 9.5%, 2.4% and 8.4% for subtypes A, B, C, D, O and CRF01_AE, respectively from the southeastern, southwestern and northern regions of the country. In another study by Odaibo *et al*. (2001b), the subtype prevalence was reported as 22.2% and 11.1 % for subtype A and CRF01_AE, respectively among seroconcordant couples; and, 44.4%, 16.2% and 33.3% for subtypes A, B and C, respectively among serodiscordant couples. A national molecular survey of HIV-1 strains in Nigeria conducted by Agwale and colleagues (2002) showed the subtype prevalence of HIV-1 as 44.8%, 0.4%, 54.2%, 0.4% and 0.4% for subtypes A, C, G, J and unclassifiable, respectively. In addition, another study on mother-to-child transmission of different HIV-1 subtypes in Ibadan, Oyo state showed subtype prevalence as B (10%), G (15%) and CRF02_AG (55.0%) (Odaibo *et al*., 2006). Sequence data for HIV-1 reverse transcriptase from a study by Ojesina and colleagues (2006) showed HIV-1 subtype prevalence as 25.7%, 57.1%, 11.4% and 5.7% for subtypes G, CRF02_AG, CRF06_cpx and others, respectively. Furthermore, sequence distribution data using *env* and *gag* genes of HIV-1 from 87 patients by Sankale *et al.* (2007) in Oyo state, Nigeria include A (2.3%), C (1.1%), G (32.2%), CRF02_AG (32.2%), CRF06_cpx (8.0%), unclassified (1.1%) and others (20%) including CRF01_AE, CRF09_cpx and CRF11_cpx. In addition, Chaplin *et al.* (2011) reported the prevalence of multiple HIV-1

strains including subtypes A (3.6%), G (37.9%), CRF02_AG (45.0%), CRF06_cpx (4.4%) and other recombinant sequences (9.2%) in their study. Ajoge and colleagues (2011) showed HIV-1 subtype distribution as C (3.6%), F2 (3.6%), G (32.1%), CRF02_AG (39.3%) and other recombinants (21.6%). The differences observed between the HIV-1 subtype prevalence results of this study and that of the aforementioned previous studies could be attributed to the differences in circulating strains with respect to locality, year of study and, type of assay used for subtype determination. Morever, these studies of HIV-1 sequence data clearly show on-going virus diversification and increasing virus complexity.

The results from this study also show that HIV-1 subtype G is the dominant (53%) or most prevalent subtype among multiple strains of circulating HIV-1 viruses in the study population. This result is consistent with a report by Agwale *et al.* (2002). However, some investigators have reported other subtypes as the predominant subtype. In West Africa, most HIV epidemics are believed to be due to subtype A and recombinant viruses including subtype A, in particular CRF02_AG (Peeters *et al*., 1998; Montavon *et al*., 2000; Sankale *et al*., 2000). Peeters *et al.* (2000) and Agwale *et al.* (2002) reported a predominance of subtype A viruses in southwestern Nigeria and subtype G viruses in Northern Nigeria while another study involving 925 blood donors in 8 states in Nigeria by Odaibo *et al.* (2001) showed the predominance of HIV-1 subtype C. HIV-1 reverse transcriptase and protease sequence analyses in patients with treatment failure in four sites in the country showed the predominance of HIV-1 CRF02_AG overall but with the predominant subtype varying among the different treatment sites (Chaplin *et al.,* 2011). The study by Ajoge and colleagues (2011) involving treatment naïve pregnant women in Northern Nigeria also showed HIV-1 CRF02_AG as the predominant circulating HIV-1 strain as well as the study by Sankale *et al.* (2007) involving 2 different cities in Oyo state. Of the 5 studies with available HIV-1 sequence data for Ibadan, Oyo state, Sankale *et al.* (2007) and Chaplin *et al.* (2011) reported

a predominance of HIV-1 subtype G, Ojesina *et al.* (2006) and Odaibo *et al.* (2006) reported a predominance of HIV-1 CRF02_AG, while Odaibo *et al.* (2001) reported a predominance of HIV-1 subtype C. This difference in HIV-1 subtype predominance observed in these studies might be as a result of the dynamic and evolving nature of the virus in Nigeria and differences in HIV-1 subtype distribution and prevalence in different localities. Morever, the distribution of HIV-1 genetic diversity with respect to geographic location is highly dynamic 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).

Among the 34 subtype G sequences, 5 (14.7%) formed a unique monophyletic subcluster referred to as G' (G prime). HIV-1 subtype G' has been reported from different molecular epidemiologic studies from different parts of Nigeria. The first report of HIV-1 subtype G' was from an hospitalized patient in Northeastern Nigeria whose HIV-1 virus was otherwise subtype G but shared an unclassified carboxyl-terminal gp41 segment with CA1-like strains from Cameroon (McCutchan *et al*., 1999). This had been noticed previously by Howard *et al.* (1994) and was further observed by Peeters and colleagues (2000) who observed three different subclusters among the subtype G envelope sequences. Additionally, the presence of the HIV-1 subtype G' subcluster has been reported by Ojesina *et al.* (2006) based on *pol* sequences and Sankale *et al.* (2007) for the *env* and *gag* regions of HIV-1. This was further corroborated by the work of Chaplin *et al.* (2011) who also demonstrated the HIV-1 subtype G' subcluster based on *pol* sequences. Additionally, the work of Ajoge and colleagues (2011) showed the presence of subtype G' and examined its evolutionary relationships and introduction into Nigeria. The relationship of this subtype G' to the prototypical subtype G for future immunological and vaccine research in Nigeria may only be determined through full-length genome amplification.

According to recent data for HIV-1 infection in West Africa, the dominant HIV-1 subtypes are A (21%), G (35%), CRF02_AG (28%), and other recombinants (14%), most of which is CRF06_cpx, leaving the other subtypes at less than 1% each (Hemelaar *et al.,* 2006). The results of this study show that subtype G accounts for 53% with subtype A and its recombinant, CRF02_AG, accounting for about 31% of circulating HIV-1 strains. Consequently, HIV-1 subtypes G and A, including CRF02_AG, make up 84% of the circulating HIV-1 strains. Except the study by Odaibo *et al.* (2001) which showed subtypes C and A as the predominant strains of HIV-1 accounting for 68% of the circulating HIV-1 strains, all other studies on HIV-1 diversity in Nigeria have shown that subtype G and subtype A, including its recombinant CRF02-_AG make up above 80% of the proportion of circulating viruses (Peeters *et al.* 2000; Agwale *et al.* 2002; Ojesina *et al.,* 2006; Sankale *et al.,* 2007; Ajoge *et al.* 2011). The predominance of HIV-1G and CRF02_AG suggests that these two stains of HIV-1 were first introduced into the country before other HIV-1 strains and with continuous transmission between and within different regions and states of the country have become predominant.

It was also detected that HIV-1 subtype and prevalence varied over the 3 years of sample collection from 2004 to 2006. Of the 5 HIV-1 strains found in this study, 3 strains (G, CRF02_AG and CRF06_cpx) were detected from HIV-1 positive blood samples every year, although in varying proportions. Subtype A was only detected among HIV-1 infected patients in 2004 while CRF35_AD was detected among HIV infected patients in 2005. Also, while the proportion or prevalence of CRF02_AG and CRF06_cpx viruses increased from 2005 to 2006, the proportion of subtype G significantly reduced. This dynamic and evolving picture of the HIV-1 epidemic during the same period is also evident from the previous reports of studies on HIV-1 genetic diversity in Nigeria. In 2000, HIV-1 subtypes G and CRF02_AG were detected in 4 states of Nigeria, with the predominant strain being CRF02_AG (Peeters

et al., 2000). In 2001, 6 subtypes were detected in the 8 states in the study by Odaibo *et al.* and included subtypes A, B, C, D, O and CRF01_AE with the predominant being subtype C (Odaibo *et al.,* 2001). In 2006, Odaibo *et al.* reported the detection of circulation of HIV-1 subtypes B, G and CRF02_AG in Oyo state with CRF02_AG as the predominant strain. Another study in Oyo state in 2006 by Ojesina and colleagues showed the detection of HIV-1 subtypes G, CRF02_AG and CRF06_cpx, with CRF02_AG again being the dominant subtype. However a study from Ibadan and Saki in Oyo state showed increasing complexity of circulating HIV-1 strains and included subtypes A, A1, A3, C, G, CRF01_AE, CRF02_AG, CRF06_cpx, CRF09_cpx and CRF11_cpx and some unique recombinant forms (URFs) with the predominant strain differing between the two cities (Sankale *et al.,* 2007). Furthermore, both studies in 2011 showed the predominance of CRF02_AG among HIV-1 strains but also with increasing viral diversity and increasing proportion of recombinant strains (Chaplin *et al.,* 2011; Ajoge *et al.,* 2011). All these studies clearly show endemicity of HIV-1 subtypes G and CRF02_AG as well as virus evolution and diversification reflected by increasing virus complexity and detection of newer strains, with increasing proportion of recombinants. Moreover, the increasing diversity of HIV-1 stains over time may suggest the introduction of newer strains of the virus and recombination between strains in circulation.

Some CRF specific characteristics were also found in this study. About 43% of the HIV-1 viruses from this study are recombinant viruses with recombinants of subtypes A and G predominating. This is consistent with reports that more than 20% of the current HIV-1 infections in Africa are caused by recombinant strains (McCutchan *et al*., 1999; van der Kuyl and Cornelissen, 2007). HIV-1 recombinants arise as a result of dual infections with HIV-1 strains which can be individual subtypes or CRFs. Although the possible consequences of the emergence of recombinant HIV-1 strains is not yet fully understood, the presence of this large number of recombinants may be another indication that HIV-1 diversification is

continuing in this locality. Also, if recombinants could have increased fitness over the parental strains as *in vitro* models suggest, and could exhibit increased pathogenicity, then future studies on HIV-1 sequence data would show increasing proportion of recombinant viruses because more people will become infected by recombinants. Moreover, multiple drug resistant (MDR) strains could recombine to produce a pan-resistant, transmittable virus (van der Kuyl and Cornelissen, 2007). In addition, if it is eventually discovered or proved that the efficacy of an HIV-1 vaccine is subtype-specific, the presence of this large number of recombinants may present some challenge to vaccine development (Sankale *et al.,* 2007).

Twenty-three HIV-1 infected seroconcordant couples were among the 64 HIV-1 infected individuals who had their viruses sequenced and subtyped. HIV-1 strains detected among the couples are subtype A (4.38%), subtype G (54.38%), CRF02_AG (28.26%) and CRF06_cpx (13.04%). Although there is a dearth of information on HIV-1 infection among couples in Nigeria, results of this study agree with the result of a previous study that showed the presence of multiple subtypes of HIV-1 in infected seroconcordant couples (Odaibo *et al.,* 2001b). However, while the most prevalent HIV-1 subtype from this study is subtype G (54.38%), the most prevalent HIV-1 subtype from the study by Odaibo and colleagues (2001b) was subtype C (82.3%). This difference in the most prevalent HIV-1 strain may be accounted for by the endemicity and emergence of different strains with respect to location over time.

Eighteen (78.3%) of the twenty-three HIV-1 infected seroconcordant couples were infected with the same HIV-1 strains and include subtype A (1), subtype G (10), CRF02_AG (4) and CRF06_cpx (3). This indicates that the various subtypes and CRFs are efficiently transmitted heterosexually in different localities. However, five HIV-1 infected concordant couples had discordant HIV-1 subtypes (G/CRF02_AG) which may be due to independent sources of

infection of each of the spouses. A similar observation had been previously reported by Odaibo *et al*. (2001b) among HIV-1 infected couples in Nigeria. The presence of different HIV-1 strains in HIV-1-positive seroconcordant couples may promote HIV genomic recombination between the different subtypes and CRFs which can lead to further genetic variability and diversification in progeny viruses (Robertson *et al.,* 1995). In addition, the detection of subtype G and CRF02_AG as the HIV-1 subtypes in these 5 HIV-1 positive seroconcordant, subtype discordant couples show to a large extent the higher prevalence of these two subtypes among the study population.

Various studies on motif analysis of the V3 region of *env* have shown certain characteristics that are strongly associated with CXCR4 usage and they include: the presence and accumulation of CXCR4-associated mutations, insertions and deletions in V3, the presence of basic amino acids at V3 positions 11 and/or 25, the loss of a glycosylation site and an increased positive amino acid charge ($\geq$ +6) (Chesebro *et al*., 1991; Chesebro *et al*., 1992; Holm-Hansen *et al*., 1995; Chesebro *et al*., 1996). In addition it has been reported that the presence of CXCR4-tropic HIV-1 isolates in infected individuals is associated with a rapid decline of CD4+ T cells, rapid disease progression, and reduced survival time after AIDS diagnosis (Fouchier *et al*., 1995).

Although, the "gold standard" for characterisation of HIV-1 tropism is a recombinant virus phenotypic entry assay, genotypic methods based on the V3 sequence have been successful with both HIV-1 subtype B and non subtype B HIV-1 subtypes using sequence-based criteria derived from HIV-1 subtype B (Jensen *et al*., 2006; Raymond *et al*., 2009; Monno *et al*., 2010). Prediction of HIV-1 coreceptor usage by genotypic methods are easier to do, more cost effective and have been found to be useful as screening strategy in medical practice (Raymond *et al*., 2008; Chueca *et al*., 2009). Interest in HIV-1 tropism is as a result of the

introduction of CCR5 inhibitors as components of antiretroviral therapy (Dorr *et al*., 2005). Interestingly, the anti-CCR5 inhibitor, Maraviroc, can be taken with or without food, and does not require refrigeration and thus, can be very suitable for resource-limited settings like sub-Saharan Africa.

The sequence characteristics of the V3 amino acid sequences of HIV-1 subtypes and CRFs found in this study show that all, except three sequences, are 34-amino acids in length. Although the typical V3 loop amino acid sequence is 35 amino acids, short length V3 amino acid sequences have been reported by other investigators (Jensen *et al*., 2003; Jensen and van 't Wout, 2003). Short length V3 amino acid sequences are frequently associated with CXCR4 usage (Jensen *et al*., 2003; Jensen and van 't Wout, 2003).

In addition, some regions of V3 loop of HIV-1 sequences analysed in this study showed sequence conservation while others showed sequence variability. Sequence conservation was observed in the base region of V3 (1-8 and 26-35) and tip (15-17) in the different subtypes while sequence variability was observed in the stem and turn regions. The sequence conservation is almost entirely consistent when considering the first 8 amino acid residues proximal to the terminal Cysteine residue in the base region across the subtypes. It has been reported that sequence conservation in the V3 region across different HIV-1 subtypes is consistent with a V3 interaction with an invariable cellular protein (Patel *et al.,* 2008). The sequence variability in the stem and turn regions is as a result of site specific mutations and consequently, most (7/10) of the X4-associated mutations detected in this study occurred in the stem and turn regions (positions 9-25) of the V3 loop. These site-specific mutations at either side of the V3 loop crown motif are important determinants of CXCR4 coreceptor usage (Chesebro *et al*., 1992; Patel *et al.,* 2008).

The majority of the V3 loop sequences of HIV-1 strains analysed in this study have the GPGQ crown motif. Studies have shown that the V3 loop of HIV-1 has a highly conserved crown motif and the GPGQ motif in particular is generally associated with African HIV isolates and it occurs irrespective of coreceptor usage (Holm-Hansen *et al*., 1995; Monno *et al.,* 2010).

GPGQ to GPGL/R/K substitution was observed in 18.8% (12/64) of the V3 loop sequences of HIV-1 subtypes and CRFs found in this study and were predicted as CXCR4-tropic which is in agreement with some studies. Coetzer *et al*. (2006) reported amino acid substitution in this conserved tetrapeptide from GPGQ to GPGR in 75% of the CXCR4 viruses in HIV-1 subtype C isolates in Zimbabwe. In another study, Duri and colleagues (2011) also reported that amino acid substitution in this V3 loop crown motif from GPGQ to GPGR is associated with CXCR4 coreceptor usage. However, HIV-1 subtype B has a GPGR crown motif irrespective of coreceptor usage (Coetzer *et al*., 2006).

Sixteen (94.1%) of the 17 V3 loop sequences with basic amino acids at positions 11 and 25 in this study have Arginine residues at position 11 only, while one sequence had Lysine and Arginine residues at positions 11 and 25, respectively. The presence of basic amino acids at these positions on the V3 loop have been reported by previous studies. Fouchier and colleagues (1995) reported a strong association between the syncytium-inducing (SI) capacity of HIV-1 and the presence of positively charged amino acid residues at positions 306 and/or 320 (positions 11 and 25 of V3) in the third variable domain (V3) of gp120 and it was confirmed in 97% of 402 primary HIV-1 isolates, indicating that the V3 genotype may be useful for prediction of the viral phenotype. Also, studies of 3D molecular modeling of the V3 loops from primary isolates whose coreceptor usage was experimentally defined have revealed a charged "patch" on the surface of V3 that correlates with coreceptor usage. This

V3 surface patch is positively charged in X4-tropic viruses and negatively charged or neutral in R5-tropic viruses, and is formed by two amino acids, at position 11 and at position 24 or 25; amino acids 11 and 24 or 11 and 25 contact each other in 3D space (Cardozo *et al*., 2007). However, a study showed that sequence variability in V3 loop among subtype C viruses was not associated with the presence of basic amino acid substitutions which is in contrast to that observed in subtype B where sequence variability is associated with basic substitutions and is a determinant of altered coreceptor usage (Ping *et al*., 1999). The presence of these basic amino acids at either position 11 and/or 25 is a determinant of CXCR4 coreceptor usage of HIV isolates.

Furthermore, amino acid sequence analysis of the HIV-1 strains in this study showed that one V3 loop sequence had a net charge of +6 and multiple amino acid deletions, including the deletion of the N-linked glycosylation site. A strong association between high V3 loop net charge and the loss of the N-linked glycosylation site in V3 loop, with the eventual development of CXCR4 coreceptor usage, was reported by Pollakis *et al*. (2001). Johnston and colleagues (2003) reported that CXCR4 viruses in HIV-1 subtype C displayed a high net charge compared to CCR5 viruses. Comparing the switch to X4 viruses among HIV-1 subtypes A and D virus isolates, Kaleebu *et al.* (2007) reported that a V3 charge of +5 and greater was highly associated with X4 virus phenotype. Sequence analysis of the V3 region by Coetzer *et al.* (2006) also reported that CXCR4-using viruses were often associated with an increased positive amino acid charge and a loss in the glycosylation site. These studies have shown that a high net charge of +5 and above in V3 loop with a loss of one or more N-linked glycosylation sites are characteristics of X-tropic viruses.

Majority (68.8%) of the V3 loop sequences of the HIV-1 subtypes and CRFs from this study had one or more X4-associated mutations including the 7 sequences that had only basic

amino acid at V3 position 11 and 25. The presence of these X-4 associated mutations in the V3 loop have been reported by other investigators. A review that compared different genotypic coreceptor usage prediction methods and analyses showed that the presence of mutations in the V3 loop is predictive and indicative of CXCR4 coreceptor usage (Lengauer *et al.*, 2007). Sing *et al.* (2007) using univariate analyses for the prediction of HIV coreceptor use from clonal HIV V3 loop sequences identified 41 V3 mutations significantly associated with coreceptor usage. Marcelin and colleagues (2009) reported that one reason for the failure of CCR5 inhibitors was the selection of resistance to CCR5 antagonists through amino acid changes in V3 loop. Since these X4-associated mutations are present in majority of the V3 loop sequences in this study, they are indicative and predictive of CXCR4 coreceptor usage.

Furthermore, other studies have shown that these X4-associated mutations in the V3 loop of *env* and the presence of basic amino acids at V3 positions 11 and 25 are strongly associated with syncytium induction and CXCR4 usage. Chesebro and colleagues in their studies on HIV infectivity and replication in different permissive cells, observed that infectivity and replication in macrophages was affected by the presence of basic amino acids in positions 11 and 25 of V3 with viruses harboring such mutations being T-cell tropic and syncytium-inducing (Chesebro *et al.,* 1991; Chesebro *et al.*, 1992; Chesebro *et al.*, 1996). These observations were also reported by Fouchier and colleagues in their studies (Fouchier *et al.*, 1992; Fouchier *et al.*, 1994; Fouchier *et al.*, 1995). Hoffman *et al.* (2002) also reported that these basic amino acids in V3 are phenotype-associated and result in the use of the CXCR4 coreceptor. Studies on HIV-1 V3 motif analysis by Jensen *et al.* (2003) also observed the association between basic amino acids in V3 and CXCR4 coreceptor usage. Raymond *et al.* (2008), Soulie *et al.* (2008) and Ajoge *et al.* (2011) in their various studies also observed that the presence of basic amino acids at V3 positions 11 and 25 was associated with CXCR4 coreceptor usage. With all these evidence from previous work, the combination of basic

amino acids at position 11 and/or 25 together with X4-associated mutations on V3 predicts CXCR4 coreceptor usage.

The four N-linked glycosylation sites (NGS) within the C2V3 region are present in different proportions in all, except one, of the sequences. Only 37.5% of the C2V3 sequences have the 4 NGS. The loss of one or more N-linked glycosylation sites has been shown to result in the development of X4 viruses (Coetzer *et al.*, 2006), assist in more efficient use of CXCR4 and might be an important factor in the switch of CCR5 to CXCR4 viruses, and therefore, virus tropism (Pollakis *et al.*, 2001; Polzer *et al.*, 2002; Nabatov *et al.*, 2004).

Overall, the predicted coreceptor usage using four combined criteria (Lengauer *et al.*, 2007; Sing *et al.*, 2007) for the V3 loop amino acid sequences of the HIV-1 subtypes and CRFs in this study was 31.2% and 68.8% for CCR5 and CXCR4, respectively, independent of CD4 and viral load status of the patients. The use of the combined criteria has been shown to increase both the sensitivity and the specificity of the prediction as reported by Raymond *et al.* (2008) and Ajoge *et al.* (2011). Since HIV-1 switches coreceptor usage from CCR5 to CXCR4 in the later stages of HIV disease and when exposed to antiretroviral medication, the HIV positive patients in this study with the CXCR4 coreceptor are in the advanced stage of infection associated with accelerated disease progression, or they might have been exposed to anti-retroviral therapy, or both (Connor *et al.,* 1997; Esbjornsson *et al.,* 2010; Wesby *et al,.* 2005; Johnston *et al.*, 2003; Westby *et al.,* 2007).

The X4 phenotype predominates in both subtype G and CRF02_AG viruses but is not the predominant predicted phenotype in CRF06_cpx. The higher usage of CXCR4 by HIV-1G from this study is in agreement with the results of a previous study and further corroborates the evidence that HIV-1G may have a higher proportion of X4 variants (Ajoge *et al.*, 2011). However, the high usage of CXCR4 by HIV-1 CRF02_AG is contrary to reports from studies

by Raymond *et al.* (2009) and Ajoge *et al.* (2011) that reported CRF02_AG HIV-1 strains to be predominantly CCR5-tropic. The prediction on CRF06_cpx shows that individuals infected with this subtype of HIV-1 may benefit from coreceptor antagonists like Maraviroc, although there is need for better characterization of HIV-1 CRF06_cpx tropism and HIV-1G in Nigeria. This study further highlights the need for more studies on phenotypic assays with corresponding genotypic testing for coreceptor usage for predominant HIV-1 subtypes in the country.

Of the 23 HIV-1 infected seroconcordant couples whose HIV-1 predicted coreceptor usage was studied, 52.2% (12) had discordant predicted virus coreceptor phenotypes, 8.7% (2) of the couples had the same R5 virus coreceptor phenotype with the remainder having predicted X4-tropic virus phenotypes. This difference in predicted virus coreceptor phenotypes in the spouses might be an indication of advanced disease in spouses with the X4 virus coreceptor phenotype (Connor *et al.,* 1997). It is well known that HIV-1 generally uses the CCR5 coreceptor early in infection except in subtype D (Huang *et al.,* 2007). Observations from this study suggest that spouses infected with the same HIV-1 strain with their partners but with the X4-tropic viruses were infected first and then transmitted it to their spouses.

In addition, 2 (10%) of the predicted CCR5-using HIV-1 from this study had the A22T mutation that confers resistance to CCR5 inhibitors like Maraviroc. The high prevalence of X4 tropic viruses and the presence of Maraviroc-resistant mutations in some R5 viruses indicate that coreceptor usage ability of patients' viral population will clearly be required before clinical administration of Maraviroc. Moreover, if future phenotypic and genotypic studies confirm the predominance of Maraviroc-resistant CCR5, and CXCR4 phenotypes in Nigeria, then the wide scale introduction and clinical application of Maraviroc in HIV-1 treatment and control will be limited.

HIV-1 genotyping results of *env* C2-V3 sequences were obtained for 64 (75.3%) of the 85 HIV-1 positive blood samples analysed. This study has shown for the first time, the circulation of HIV-1 CRF35_AD in Nigeria among multiple strains of HIV-1 including subtypes A, G, CRF02_AG, and CRF06_cpx among HIV-1 infected individuals. HIV-1G accounts for the majority of circulating strains and there was observed year to year variation in subtype prevalence and distribution among HIV-1 infected persons. The predicted coreceptor phenotypes of the different HIV-1 subtypes and CRFs show that CXCR4 viruses predominate with some CCR5-tropic viruses showing Maraviroc resistant mutations. The primers used in this study have been previously used in other molecular epidemiology studies in West Africa (Kanki *et al.,* 1999; Sankale *et al.,* 2000; Sankale *et al.,* 2007). When the distribution of the subtypes of HIV-1 in this study was examined according to the demographic of the patients no association was found between HIV-1 subtype and age or gender. Inability to amplify the target region of HIV-1 from genomic DNA in some of the patients' samples could be attributed to diversity of the virus at primer binding sites and DNA copy number (Sankale *et al.,* 2007).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

This study shows the presence of HIV-1 subtypes A and G, and 3 CRFs of HIV-1 among the study population and describes for the first time CRF35_AD in Nigeria. The data indicates multiple introduction of at least two of these, and suggests the existence of intra-territory transmission. This data also shows that the level of viral diversity is complex.

The data show a high prevalence of HIV-1 subtype with the GPGQ crown motif in the V3 region suggesting a functional use of this motif. Moreover, X4 viruses predominate in the study population and it is associated with multiple amino acid deletions and mutations in V3 and the loss of one or more N-linked glycosylation sites.

The picture of HIV-1 genetic diversity in the global pandemic continues to evolve. Identification of new variants, including circulating and unique recombinant forms, recognition of new outbreaks and changes in established epidemics, as well as characterization of growing numbers of full-length genomes indicate high dynamism and increasing complexity. The pervasive role of recombination as a major driving force in the generation of diversity in the HIV-1 pandemic is becoming evident, and it is particularly visible in areas in which different genetic forms coexist, referred to as "geographic recombination hotspots". Genetic diversity within subtypes is increasing over time and new geographically localized lineages deriving from point introductions are being recognized. Characterization of such variants may be of relevance to vaccine development and may allow the detection of intrasubtype recombination and superinfection. Recent studies supporting the correlation of HIV-1 clades to immune responses and drug resistance-associated mutations lend increasing relevance to the role of molecular epidemiology as an essential tool in understanding of the AIDS pandemic. However, knowledge on the global HIV-1 genetic

diversity and its implications is still far from adequate and a major scaling up of efforts is needed.

This study involved an approximately 350bp gene fragment from *env* and it is possible that strains characterized as one subtype or CRF may be recombinant viruses. Additional work, involving two or more genes or full-length sequencing, is needed to fully characterize the viruses circulating in this region and other parts of the country. At the molecular level, the HIV-1 epidemic in some cities and states of Nigeria have been previously characterized, thus, this study does complement and add value to the current understanding of the HIV-1 epidemic and the genetic variants of HIV-1 in Nigeria.

Continual surveillance of HIV subtypes and their phylogenetic relationships will help to identify outbreaks that are possibly caused by newly introduced variants, and, in turn, this information can help in planning of prevention programs, and can potentially contribute to halting the onward transmission of a particular outbreak. It will be appropriate to carry out genotypic prediction tests for HIV-1 coreceptor usage in HIV patients' population encompassing the various regions of the country and in the predominant circulating HIV-1 subtypes before the introduction of CCR5 antagonists in clinical administration.

In conclusion therefore, it is important to monitor the emerging genetic diversity of HIV-1 because it has implications for vaccine development, diagnosis, screening of blood products, and the selection of optimal treatment regimens. Molecular epidemiological analysis will also facilitate epidemiological investigation of transmission patterns and help define strategies for preventing the spread of infection (Hue *et al*., 2005; Peeters, 2003).

RECOMMENDATIONS

It is important to conduct extensive surveys covering all regions of the country, with a sufficient number of samples, for the following reasons: first, to fully assess the molecular epidemiology of HIV in the country; second, to understand the immune response to the various subtypes; third, to understand the transmission patterns and pathogenesis of the different virus subtypes; and lastly, to guide decisions about candidate vaccine development.

It is also important to continue the surveillance of subtypes on a systematic basis in order to understand the extent the proportions of the different subtypes will change over time.

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APPENDIX 1

CDC CLASSIFICATION SYSTEM FOR HIV-INFECTED ADULTS AND ADOLESCENTS

CD4 Cell Categries	**Clinical Categories**		
	A	**B***	**C#**
	Asymptomatic, Acute HIV, or PGL	Symptomatic Conditions, not A or C	AIDS-Indicator Conditions
(1) >500 cells/ul	A1	B1	C1
(2) 200-499 cells/ul	A2	B2	C2
(3) <200 clls/ul	A3	B3	C3

PGL = Persistent Generalized Lymphadenopathy

***Category B symptomatic conditions**

Category B symptomatic conditions are defined as symptomatic conditions occurring in an HIV-infected adolescent or adult that meets at least one of the following criteria:

- They are attributed to HIV infection or indicate a defect in cell-mediated immunity.
- They are considered to have a clinical course or management that is complicated by HIV infection.

Examples include, but are not limited to, the following:

- Bacillary angiomatosis
- Oropharyngeal candidiasis (thrush)
- Vulvovaginal candidiasis, persistent or resistant
- Pelvic inflammatory disease (PID)
- Cervical dysplasia (moderate or severe)/cervical carcinoma in situ
- Hairy leukoplakia, oral

- Herpes zoster (shingles), involving two or more episodes or at least one dermatome
- Idiopathic thrombocytopenic purpura
- Constitutional symptoms, such as fever (>38.5℃) or diarrhea lasting >1 month
- Peripheral neuropathy

 # Category C AIDS-Indicator Conditions

- Bacterial pneumonia, recurrent (two or more episodes in 12 months)
- Candidiasis of the bronchi, trachea, or lungs
- Candidiasis, esophageal
- Cervical carcinoma, invasive, confirmed by biopsy
- Coccidioidomycosis, disseminated or extrapulmonary
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis, chronic intestinal (>1 month in duration)
- Cytomegalovirus disease (other than liver, spleen, or nodes)
- Encephalopathy, HIV-related
- Herpes simplex: chronic ulcers (>1 month in duration), or bronchitis, pneumonitis, or esophagitis
- Histoplasmosis, disseminated or extrapulmonary
- Isosporiasis, chronic intestinal (>1-month duration)
- Kaposi sarcoma
- Lymphoma, Burkitt, immunoblastic, or primary central nervous system
- *Mycobacterium avium* complex (MAC) or *Mycobacterium kansasii*, disseminated or extrapulmonary
- *Mycobacterium tuberculosis*, pulmonary or extrapulmonary

- *Mycobacterium*, other species or unidentified species, disseminated or extrapulmonary
- *Pneumocystis jiroveci* (formerly *carinii*) pneumonia (PCP)
- Progressive multifocal leukoencephalopathy (PML)
- *Salmonella* septicemia, recurrent (nontyphoid)
- Toxoplasmosis of brain
- Wasting syndrome caused by HIV (involuntary weight loss >10% of baseline body weight) associated with either chronic diarrhea (two or more loose stools per day for ≥1 month) or chronic weakness and documented fever for ≥1 month

SOURCE: CENTRES FOR DISEASE CONTROL AND PREVENTION. 1993 REVISED CLASSIFICATION SYSTEM FOR HIV INFECTION AND EXPANDED SURVEILLANCE CASE DEFINITION FOR AIDS AMONG ADOLESCENTS AND ADULTS.

APPENDIX 2
WHO CLINICAL STAGING OF HIV/AIDS FOR ADULTS AND ADOLESCENTS

Primary HIV Infection
Asymptomatic
Acute retroviral syndrome

Clinical Stage 1
Asymptomatic
Persistent generalized lymphadenopathy

Clinical Stage 2: Early (mild) disease
Moderate unexplained weight loss (<10% of presumed or measured body weight)
Recurrent respiratory infections (respiratory tract infections, upper respiratory tract infections, sinusitis, tonsillitis, otitis media, and pharyngitis)
Herpes zoster
Minor mucocutaneous manifestations (angular cheilitis, recurrent oral ulcerations, papular pruritic eruptions, seborrheic dermatitis, fungal nail infections of the finger)

Clinical Stage 3: Intermediate (moderate) disease
Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations
Unexplained severe weight loss (>10% of presumed or measured body weight)
Unexplained chronic diarrhea for >1 month
Unexplained persistent fever for >1 month (>37.6ºC, intermittent or constant)
Persistent oral candidiasis (thrush)
Oral hairy leukoplakia
Pulmonary tuberculosis (current)
Severe presumed bacterial infections (e.g., pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteremia)
Acute necrotizing ulcerative stomatitis, gingivitis, or periodontitis
Unexplained anemia (hemoglobin <8 g/dL) and/or neutropenia (neutrophils <500cells/µl) and/or thrombocytopaenia (platelets <50,000cells/µl)

Clinical Stage 4: Late (severe) disease (AIDS)
Conditions where presumptive diagnosis can be made on basis of clinical signs or simple investigations HIV wasting syndrome, as defined by the U.S. Centers for Disease Control and Prevention (CDC) *Pneumocystis jiroveci* (formerly *carinii*) pneumonia
Recurrent severe or radiological bacterial pneumonia
Chronic herpes simplex infection (oral or genital, or anorectal site) >1 month duration
Esophageal candidiasis, extrapulmonary tuberculosis, Kaposi sarcoma, Central Nervous System toxoplasmosis, HIV encephalopathy
Conditions where confirmatory diagnostic testing is necessary
Cryptococcosis, extrapulmonary, Disseminated nontuberculosis mycobacteria infection

Progressive multifocal leukoencephalopathy, Candida of Trachea, bronchi or lungs

Cryptosporidiosis, Isosporiasis, Visceral herpes simplex infection, Cytomegalovirus infection (retinitis or organ other than liver, spleen, or lymphnode)
Any disseminated mycosis (eg. Histoplasmosis, coccidiomycosis, penicillosis)
Recurrent nontyphoidal salmonella septicemia, Lymphoma (cerebral or B-cell non-Hodgkin)
Invasive cervical carcinoma, Visceral leishmaniasis

SOURCE: *World Health Organization. Interim WHO Clinical Staging of HIV/AIDS and HIV/AIDS Case Definitions for Surveillance 2005*

APPENDIX 3

TABLE 5: ANTIRETROVIRAL REGIMENS RECOMMENDED FOR TREATMENT OF HUMAN IMMUNODEFICIENCY VIRUS 1 (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] /ritonavirâ€ + (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 RT inhibitors; PI, protease inhibitors.
Source: http://www.aidsinfo.nih.gov

APPENDIX 4