EVALUATION OF HIV TESTING METHODS AND DEVELOPMENT OF HIV TESTING ALGORITHMS IN LAGOS, NIGERIA

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, UNIVERSITY OF LAGOS, IN FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.) IN THE DEPARTMENT OF MEDICAL MICROBIOLOGY AND PARASITOLOGY, COLLEGE OF MEDICINE OF THE UNIVERSITY OF LAGOS, NIGERIA.

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CERTIFICATION

This is to certify that the thesis titled "**Evaluation of HIV Testing Methods and Development of HIV Testing Algorithms in Lagos, Nigeria**"

SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES
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for the award of the degree of

DOCTOR OF PHILOSOPHY (Ph. D.)

is a record of original research carried out by

BADARU, SIKIRU OLANREWAJU SAIDINA

in the

DEPARTMENT OF MEDICAL MICROBIOLOGY AND PARASITOLOGY, COLLEGE OF MEDICINE OF THE UNIVERSITY OF LAGOS (CMUL), IDI-ARABA, LAGOS.

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DECLARATION

We hereby declare that the thesis titled "**Evaluation of HIV Testing Methods and Development of HIV Testing Algorithms in Lagos, Nigeria**" is a record of original research work carried out by **BADARU, Sikiru Olanrewaju Saidina**, in the Department of Medical Microbiology and Parasitology, College of Medicine of the University of Lagos, Nigeria.

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DR. WELLINGTON A. OYIBO

DEDICATION

THIS THESIS IS DEDICATED WITH LOVE, TO THE MEMORY OF MY PARENTS LATE **AL-HAJJ SIKIRU KEHINDE BADARU & LATE MRS. KHADIJAH ABIKE BADARU (NEE – OLUTOGUN)** AND ALL OTHER DEPARTED SOULS THAT HAD LABOURED FOR THE PROSPERITY OF HUMANITY

THIS WORK IS ENTIRELY DEVOTED FOR **ALLAH (THE ONE AND ONLY GOD ALMIGHTY)** – As quoted: "**Say: verily my worship, my sacrifice, and my living and my dying are all for Almighty God (Allah), the Lord of the Universe....**" (Qur'an 6: 162-166); and quoted: "**The earth is the Lord's, and the fullness thereof; the world, and they that dwell therein....**" (Psalm 24: 1-10)

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Is the reward of goodness anything other than goodness ..... (Q55: 60-61)

Blessed are the poor in spirit: for theirs is the kingdom of heaven... ... (St. Matthew 5: 3-16)

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ABSTRACT

The absence of regulatory control of HIV test kits in use, non-availability of HIV testing algorithm and absence of regular and constant evaluation programme for HIV testing kits stimulated the authors' curiosity to carry out both operational and experimental studies on the commercially available HIV test devices circulating in the Nigerian market. A scientific approach to address the setbacks in HIV testing methods contending with HIV serodiagnosis and surveillance, as well as determination of blood's HIV status for blood transfusion in Nigeria, was formulated in this study. The study is focused on operational evaluation of commercially available HIV test kits, the development and evaluation of two serial HIV testing serologic algorithms as well as the detection and use of HIV complement fixing (CF) antibody titres as a prognostic marker for HIV/AIDS.

The sensitivity, specificity, reproducibility, positive predictive value (PPV), negative predictive value (NPV) of 14 HIV test kits involving 5 enzyme-linked immunosorbent assay (ELISA) and 9 simple-rapid (S/R) assay types were evaluated in two phases of this study. The ease of performance and suitability for use were also assessed in the two phases. The first phase represented 3 ELISA and 6 S/R test kits commonly used during the period of the first evaluation programme while the second phase signified the 2 latest ELISA and 3 S/R test kits available during the period of the evaluation programme. The two phases revealed the importance of periodical and update evaluation for diagnostic kits in the country as Vironostika Uni-Form II HIV Ag/Ab (a 4th generation ELISA kit in 2nd phase) was the final ELISA diagnostic kit of choice at the

expense of SUB-Recombigen HIV-1/HIV-2 (a 2nd generation ELISA kit in 1st phase) in the study.

The outcome of the first phase showed that SUB-Recombigen and Determine HIV-1 & 2 (a rapid immunochromatographic assay) were the diagnostic kits of choice for ELISA and S/R assays respectively while that of the second phase demonstrated that Vironostika and Capillus HIV-1/HIV-2 (a rapid latex agglutination assay) were also respective ELISA and S/R assays' diagnostic kits of choice. They all had sensitivities, specificities, reproducibilities, PPV and NPV of over 99.5%. Both the ease of performance and suitability for use of these kits of choice were better than any of the other kits assessed.

A rapid-based serial HIV testing algorithm (using Determine and Capillus as candidate kits) was developed for 2,500 patients of which 20.5% were seropositive with 442 HIV-1(17.7%), 12 HIV-2 (0.5%) and 58 HIV-1 & 2 (2.3%) confirmed serostatus. Of the 2,500 samples used in the algorithm, concordant negatives were 1,988 (79.5%), concordant positives were 509 (20.4%) while discordant negatives were 3 (0.12%). The algorithm was suitable for rural/field settings, [most especially for the voluntary counselling and testing (VCT) programme and emergencies in the remote localities], hospitals and clinics.

An ELISA/rapid-based serial HIV testing algorithm (using Vironostika and Determine as candidate kits) was developed for 2,500 patients, of which 20.5% were seropositive with 442 HIV-1(17.7%), 12 HIV-2 (0.5%) and 58 HIV-1 & 2 (2.3%) confirmed serostatus. Of the 2,500 samples used in the algorithm, concordant negatives were 1,976 (79%), concordant positives were 524 (21%) while discordant negatives were

12 (0.5%). The algorithm was suitable for early turnout of HIV confirmatory results in blood banks and referral centres with electricity-constraint. It would also be suitable for HIV surveillance study.

Use of CF antibody titres for measuring the progression from healthy carriers to symptomatic HIV/AIDS patients was demonstrated successfully in the study. There was a significantly higher frequency of the symptomatic patients with CF antibody titre > 1:64 in the study. The complement fixation test (CFT) can therefore be used to monitor the commencement of treatment for the people living with HIV/AIDS (PLWHA) that cannot afford the expensive HIV RNA viral load determination and CD4 T cells count methods.

The 20.5% HIV infection rate obtained in the study illustrated a generalized epidemic among the patients referred to the Central Public Health Laboratory (CPHL). Although the entire patients' population originated from all the states of the Federation and Federal Capital Territory (FCT), the 5.0% HIV prevalence in Nigeria reported during the period of the study, still needs gold standard kits (Vironostika, Determine & Capillus) and methods (ELISA/rapid-based serial HIV testing algorithm with standard probability sampling/rapid-based serial HIV testing algorithm in VCT) to ascertain the actual situation and identify the circulating strains of HIV in the country.

CHAPTER ONE
GENERAL INTRODUCTION

1.0 GENERAL INTRODUCTION

1.1 BACKGROUND

Human immunodeficiency virus (HIV) as an evasive agent, is one of the most deadly disease causing agents. A member of the Family Retroviridae and sub-family: Lentivirinae, HIV is the only causative agent for acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983; Levy, 1986). Acquired immune deficiency syndrome (AIDS) has become one of the major diseases that attract global concern by virtue of its spread and scourge.

Transmission of HIV can occur through contact with infected body fluid and can be greatly influenced by the amount of infectious virus in the body fluid and the extent of contact with the fluid (Levy, 1993). HIV enters the body system through vulnerable surfaces close to the blood capillaries, where it localizes in susceptible hosts and multiplies, thereafter establishing itself in the host cell (Pope and Haase, 2003). Consequently, it is widely disseminated to the lymph nodes and induces a rapid turnover of infected lymphocytes (Schnittman and Fauci, 1994). Cells with pre-integration HIV have shorter half-life decay, are invisible to the immune system and have a decay half-life in the order of 48 months (Pilcher *et al.*, 2004).

Many HIV particles are later released from the decayed host cells into the blood system where they migrate to several tissues. During their migration, the body's immune system becomes alerted with induction of HIV-1-specific immune response characterized by polyclonal activation of B lymphocytes, production of neutralizing antibodies, binding of immune complexes to follicular dendritic cells, synthesis and secretion of various cytokines, activation of Th-1 cells, and stimulation of cytotoxic responses including T-

cell-, NK cell-, and antibody-dependent cell-mediated activities (Schwartz and Nair, 1999). While these responses can significantly reduce the viral load in the peripheral blood, generally they are unable to completely clear the infection. Excess antibodies from these responses are detectable by enzyme immunosorbent assay (ELISA) or any other serological assay. Many of these virions however escape these antibodies and thereby have access to many other susceptible host cells (Levy, 1993).

During this process, excess antibodies now circulate in the body system and these are now detected by subjecting the patients' serum or plasma to laboratory diagnosis (Levy, 1989). The process of carrying out an initial detection of HIV antibodies by laboratory methods (such as ELISA, simple rapid assays, etc.) is referred to as HIV screening while the final analysis by similar but more specific laboratory methods (such as Western blot (WB), Line immunoassay (LIA), Immunofluorescent assay (IFA), polymerase chain reaction (PCR), etc.) is referred to as HIV confirmatory test.

Conscious of the need to advise Member States on the laboratory diagnosis of HIV, the World Health Organization (WHO) has since 1988 been providing countries with objective assessments of operational characteristics of commercially available HIV test kits (WHO, 2002). Based on their advice, the Federal Ministry of Health (FMOH) established the National AIDS and STD Control Programme (NASCP) in 1989, which took up the challenges on the assessment of the operational characteristics of commercially available HIV test kits in the country as one of her objectives (FMOH/NASCP, 1990).

In spite of the activities of NASCP aimed at controlling the influx of the commercially available HIV test kits in the Nigerian market, many of such test kits still

find their way into circulation without thorough evaluation. This problem became complicated with the upsurge of many institutions and non-governmental organizations (NGOs) interested in the control of the spread of HIV/AIDS in Nigeria. Such institutions and NGOs were opportuned to order for just any commercially available test kit without accreditation and without verifying the differences between diagnostic and research kits. The NASCP has therefore not been able to monitor all necessary operational characteristics and research tools for selecting commercially available HIV test kits.

In spite of early awareness of the FMOH on the implications of the spread of HIV/AIDS in the country since 1986 (NASCP/FMOH, 1990), the HIV sentinel seroprevalence in the country was consistently on the rise between 1986 and 2000 (FMOH/NASCP, 2002, Badaru *et al.*, 2002) until it is now temporarily stabilized at 5.0% (FMOH/NASCP, 2004). This temporary stability was brought about through intervention of both FMOH by setting up a National Action Committee for AIDS (NACA) and the WHO/United Nation Programme on HIV/AIDS (UNAIDS) as well by empowering many NGOs, most especially the Family Health International (FHI) to forestall the spread. The WHO/UNAIDS agenda on voluntary counselling and testing (VCT) was adopted. Consequently, development of new testing strategies for detecting HIV infections and monitoring the AIDS disease trends rapidly progressed with the evolution of new effective drugs, treatment of people living with HIV/AIDS (PLWHA) and advocacy towards behavioural change in the prevention of HIV.

1.2 STATEMENT OF THE PROBLEMS

The bases of controlling the spread are early detection of antibodies to HIV in infected individuals; adequate counselling for non-infected (seronegative) individuals on

remaining seronegative to avoid contracting HIV, and adequate/persistent public awareness of the effects of HIV/AIDS and its preventive measures. It is rather unfortunate that Nigeria is yet to accomplish these objectives due to the following problems. Firstly, the HIV testing technology lacks standard testing designs (algorithm) for rural and referral settings in the country. Secondly, the issue of influx of just any commercially available HIV test kit in the country has not been addressed by relevant agencies, namely; NACA and the National Agency for Food and Drug Control and Administration (NAFDAC), etc. Thirdly, these relevant agencies have not been updating and publishing approved diagnostic HIV test kits for national use. Such lack of control on test kits would result in a preponderance of false negative and positive results thereby revealing an HIV carrier as healthy individual or vice versa. Even if symptoms manifested, they mimicked other diseases such that clinical diagnosis would be wrongly done. Such an individual in turn could spread the infection ignorantly and not seek early monitoring and treatment in spite of his/her awareness. Fourthly, the high mutation rate of HIV particles calls for regular and constant assessment/evaluation of HIV testing kits used in the country to enable us detect new HIV strains in circulation. These problems have been portraying a danger of further upsurge and spread of the disease beyond epidemic threshold in the country, if not resolved in time.

1.3 PURPOSE OF THE STUDY

The absence of regulatory control of HIV test kits use, non-availability of HIV testing algorithm and absence of regular and constant evaluation programme for HIV testing kits stimulated the authors' curiosity to carry out both operational and

experimental studies on the commercially available HIV test devices circulating in the Nigerian market. At the end of the study, the importance of certification of test kits as diagnostic kits and periodical evaluation of these test kits for update would be realized. Suitable testing methods for both rural and urban centres in the country would be developed as gold standards. And finally, an alternative prognostic marker for HIV would be adopted for monitoring PLWHA. These would assist greatly in investigating the simple methods of monitoring the disease trends of the PLWHA and highlight the need to vigorously tackle the situation on ground to gain firm control.

1.4 RATIONALE OF THE STUDY

With increasing VCT centres and the flooding of the Nigerian market with all sorts of HIV testing kits as diagnostic kits across the country, there is an urgent need to evaluate the sensitivity, specificity, ease of performance, suitability and reproducibility of these kits. The assessment would elucidate the importance of updating diagnostic kits periodically at these centres and all laboratories across the country for government's approval. The assessments, also called operational evaluation of diagnostic kits, would ensure the use of suitable diagnostic kits for preliminary testing for HIV infection as a gold standard in the country. The suitable kits should be able to recognise the antibodies to most recent local strains of HIV circulating in the country.

For both rural and referral centres in the country, there is need for HIV testing designs or formats (algorithms) acceptable through scientific and rational analysis for screening and confirming the HIV status of individuals and blood specimens. The algorithms, when developed, would be suitable for both local and referral settings in the

country. The provision of such alternative effective testing methods, that is an equivalent of the WB or HIV RNA confirmatory tests, would be beneficial especially in rural or remote settings to avoid delay and reduce the cost of doing special confirmatory tests involving WB or/and HIV RNA tests. The cost of high manpower skill, special equipments, electricity supply, etc., that may not be required if serial or parallel testing strategies of two or more rapid/ELISA kits were done in place of the confirmatory tests, would be added advantages.

Early detection of progression of HIV infection to AIDS is of paramount importance for good management of PLWHA. A pilot study, such as that implemented at the National referral centre could be one of the indicators for such need.

Finally, the cost and technicalities involved in the standard prognostic markers for the disease trend of PLWHA, namely – CD4 counts and viral load, largely account for difficulties in monitoring the disease trend of PLWHA in poor resource settings. Nigeria, being one of such countries, needs alternative prognostic markers. Such markers must meet up with the standards established by CD4 counts (Cyflow) and viral load (RNA/DNA Quantiplex – Roche). For easy HIV/AIDS clinical diagnosis in this part of the tropical world, common HIV/AIDS symptoms need to be identified and compared with other parts of the world. This study is therefore focused on these key areas of need so as to intensify efforts in the control of spread of HIV/AIDS as well as to conduct the follow up of HIV/AIDS disease trends in PLWHA.

1.5 FRAMEWORK OF TESTING METHODS

The frame work of this study is anchored on the importance of accurate and early detection of HIV in individuals (i.e., the testing method) as a first step towards

appropriate management using available antiretroval drugs (ARVs). By the same token, individuals that are negative when tested in VCT centres with standard algorithm using very sensitive and specific kits would be counselled on how to maintain their seronegativity, and thereby prevent further spread of HIV infections to unexposed or uninfected individuals.

Conventional HIV testing method: Initial test (involving ELISA or Simple/Rapid tests) and confirmatory test (involving WB, IFA, LIA, virus isolation and PCR).

HIV testing strategy I: Only initial test (involving ELISA or Simple/Rapid tests) to determine the status of HIV in an individual (UNAIDS/WHO, 2001).

HIV testing strategy II: Initial test (involving ELISA or Simple/Rapid tests) and confirmatory test (involving ELISA or Simple/Rapid tests but of different principle or different coated antigen) to determine the status of HIV in an individual (UNAIDS/WHO, 2001).

HIV testing strategy III: Initial test (involving ELISA or Simple/Rapid tests) and two confirmatory tests (involving ELISA or Simple/Rapid tests but of different principle and different coated antigen) to determine the status of HIV in an individual (UNAIDS/WHO, 2001).

HIV testing strategy II (algorithm) was therefore adopted in the development of a suitable testing method in Nigeria based on the purpose of diagnosis, HIV prevalence and the proportion of PLWHA in the country.

1.6 SCOPE OF THE STUDY

This study was designed to focus on HIV serodiagnosis in Nigeria. The test kits enrolled for evaluation studies were strictly the commonly circulating commercially-available ones in Nigerian market. The testing methods were specifically designed for the entire Nigerian communities (both rural and urban) with cognisance of the topographical, climatic and socio-economic factors in the country. The designed strategies or algorithms were tested in a small population of Nigerian mostly residing Lagos. The maximum sample size was 2,500 while the sample type was only venous blood of maximum (10ml) volume. Two separate, independent but collaborative institutions were used as sites for the study and were both located in Lagos, Nigeria. The diagnostic and prognostic parameters of HIV infection were only studied. Some of these parameters are sensitivity, specificity, reproducibility, NPV and PPV.

1.7 OBJECTIVES

The general objectives were to evaluate commercially available HIV testing kits and develop standard algorithms suitable for rural and referral centres in Lagos, Nigeria.

The following specific objectives drawn for the study were to:

1. Evaluate the sensitivity, specificity, reproducibility and ease of performance as well as negative predictive value (NPV), positive

predictive value (PPV) and suitability of simple rapid (S/R) and ELISA kits for local and referral HIV diagnostic centres.

2. Develop algorithms for HIV screening and confirmatory tests that will be suitable for the local (especially rural areas) and referral centres, and use the algorithms for detecting the rate of HIV infection among patients attending the Central Public Health Laboratory (CPHL) in Lagos, Nigeria between 1997 and 1999.
3. Detect both anti-p24 and complement fixing (CF) antibodies seropositive levels with respect to their relationships with symptoms and progression of HIV/AIDS in the individuals involved in the study.
4. Determine the proportion and distribution of HIV-1 & 2 serotypes (including those occurring in mixed infections) circulating among Nigerians based on serological approaches.

CHAPTER TWO
LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND

In the early 1980s, reports of the unusual occurrence of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma in previously healthy young men in the United States led to the recognition of AIDS as a new disease syndrome (Hymes *et al.*, 1981; Levy and Ziegler, 1983). Further studies indicated that this disease was also found in Haiti and Africa. Specifically, Kaposi's sarcoma (KS) was one of the first clinical manifestations of AIDS epidemic (Hymes *et al.*, 1981; Abrams *et al.*, 1984).

The first indication that AIDS could be caused by a retrovirus came in 1983, when Barre-Sinoussi and co-workers at the Pasteur institute recovered a reverse-transcriptase - containing virus from the lymph node of a man with persistent lymphadenopathy syndrome (LAS). Since enlarged lymph nodes are observed during several viral infections, many physicians believed initially that LAS resulted from infection with a known human virus such as Epstein-Barr virus (EBV) or Cytomegalovirus (CMV). In addition, the characteristics desired for the retrovirus recovered by the Pasteur institute group (Barre-Sinoussi *et al.*, 1983) included some reported for the human T-cell leukaemia virus (HTLV). Thus, many investigators decided initially that lymph- node isolate was a member of this already recognized retrovirus group. In support of this conclusion was the concomitant publication of a paper by Gallo and co-workers (1983) on the isolation of HTLV from individuals with AIDS in the same issue of *Science* Journal reporting the recovery of the LAS agent.

HTLV as the etiological agent of AIDS, however, seemed unlikely because of its close cell association and its known poor replication in cell culture (Poiesz *et al.*, 1980;

Miyoshi *et al.*, 1981). Since haemophiliacs with AIDS had been reported (CDC, 1982), how could this type of virus be transmitted by cell free plasma products such as Factor VIII? Moreover, HTLV does not kill lymphocytes; in fact, it often immortalizes them into continuous growth (Poiez *et al.*, 1980). Thus, the characteristic loss of CD4 lymphocytes in AIDS patients (Gottlieb *et al.*, 1981; Mildvan *et al.*, 1982) could not be explained by an HTLV infection. This virus the LAS agent was therefore distinct from other known human retroviruses, such as the human T-cell leukemia virus type-1 (HTLV-1) and a rarer virus also isolated from leukocytes, HTLV-II.

Further studies in 1984 by Montagnier and co-workers clarified these questions in relation to the LAS agent. The results indicated that this human retrovirus, although similar to HTLV in infecting CD4 lymphocytes, had quite distinct properties. The virus, later called lymphadenopathy-associated virus (LAV), grew to substantial titre in CD4 cells and eventually caused cell death (Montagnier *et al.*, 1984) instead of establishing the cells in culture as does HTLV. These observations on LAV provided important evidence supporting the potential etiological role of a retrovirus in AIDS. Several other laboratories were also searching for the agent responsible for this immune deficiency syndrome, and in early 1984, Gallo and associates reported the characterization of another human retrovirus distinct from HTLV- 1 & 2 that they called HLTV-III (Gallo *et al.*, 1984; Popovic *et al.*, 1984). It was isolated from the peripheral blood mononuclear cells (PBMC) of adult and paediatric AIDS patients. These workers noted the lymphotropic and cytopathic properties of the virus and reported that HTLV-III cross-reacted with some proteins of HTLV-I and HTLV-II, particularly the core p-24 protein

(Gallo *et al.*, 1984). Thus, they believed it merited inclusion in the HTLV group, even through the newly isolated virus was cytolytic and did not transform lymphocytes.

Levy and colleagues (1984) also reported at that time the identification of retroviruses they named the AIDS-associated retroviruses (ARV). These viruses were recovered from AIDS patients from different known risk groups, as well as from other symptomatic and some healthy people. Finding ARV in asymptomatic individuals indicated for the first time a carrier state for the AIDS virus. ARV shared some cross-reactivity with the French LAV strain when examined by immunofluorescence techniques (Levy *et al.*, 1984). The ARV differ from HTLV-I and HTLV-II in that they replicate quickly and to high titres in PBMC and T-lymphocytes cultures, causing distinct cytopathic effects; they are non-oncogenic; and they demonstrate unique antigenic and molecular properties, but were similar, in every respect, to the other newly identified retroviruses. Most importantly, infection by these viruses, as described in 1984, was not limited to AIDS patients. They were also recovered from individuals with other clinical conditions, including lymphadenopathy. This later observation supported the conclusion that LAS was part of the disease syndrome.

Within a short time, the three prototype viruses (LAV, HTLV-III, and ARV) were recognized as members of the same group of retroviruses, and their properties identified them as Lentivirinae (Table 1). Their proteins were all distinct from those of HTLV- 1 & 2; their genomes showed only remote similarities to the genome of this agent, no more than that of chicken retroviruses (Rabson and Martin, 1985). Thus, the initial cross-reactivity of HTLV-III with HTLV- 1 & 2 proved incorrect. The AIDS viruses had many properties distinguishing them from HTLV (Table 2). For all these reasons, in 1986, a

Table 1: Characteristics common to lentiviruses

Clinical
1. Association with a disease with a long incubation period
2. Association with immune suppression
3. Involvement of the hematopoietic system
4. Involvement of the central nervous system
5. Association with arthritis and autoimmunity
Biological
1. Host-species-specific
2. Exogenous and non-oncogenic
3. Cytopathic effect in certain infected cells, e.g., syncytia (multinucleated cells)
4. Infection of macrophages – usually noncytopathic
5. Accumulation of unintegrated circular and linear forms of viral cDNA in infected cells
6. Latent or persistent infection in some infected cells
7. Morphology of virus particle by electron microscopy: cone-shaped nucleoid
Molecular
1. Large genome (≥9 kb)
2. Truncated *gag* gene: several processed *gag* proteins
3. Envelope gene is highly glycosylated
4. Polymorphism, particularly in the envelope region
5. Novel central open reading frame in the viral genome that separates the *pol* and *env* regions

Source: Levy (1994)

Table 2: Comparison of HIV and HTLV

Characteristic	HIV	HTLV
Retrovirus genus	Lentivirus	HTLV/BLV
Genome size (kb)	9.8	9.0
Core morphology	Cone	Cuboids
Accessory genes	6	2
Infects $CD4^+$ lymphocytes	++[a]	++
Infects $CD8^+$ lymphocytes	–	+
Wide tissue tropism	+	+
Causes syncytium formation	++	+
Cytotoxic	++	–
Transforms cells	–	++[b]
Replicates to high titres	++	–
Mostly cell associated	–	++
Can exist in a latent state	+	+
Association with immune deficiency	++	+
Association with neurological disorders	++	++[c]

[a]Plus signs (+) indicate relative presence of each characteristic.

[b]T-cell leukemia.

[c]Tropical spastic paresis.

Source: Levy (1994)

subcommittee of the International Committee on Taxonomy of viruses recommended that this new group of viruses variously referred to as LAV, HLTV-III, and ARV are named human Immunodeficiency virus (HIV) (Coffin *et al.*, 1986).

The HTLV-III strain has been confirmed to be the same virus as the LAV strain (Chermann *et al.*, 1991; Guo *et al.*, 1991; Chang *et al.*, 1993). The Pasteur Institute group had sent LAV to the National Institute of Health (NIH) where it appears to have contaminated a culture in the NIH laboratory (Chang *et al.*, 1993). This occurrence explains the unique molecular similarities between HTLV-III and LAV, in contrast to the sequence diversities observed with ARV-2 (now called HIV-1_{sf2}) and other strains (Benn *et al.*, 1985; Muesing *et al.*, 1985).

HIV isolates were subsequently recovered from the blood of many patients with AIDS, ARC, and neurological syndromes, as well as from the PBMC of several clinically healthy individuals (Levy *et al.*, 1985; Salahuddin *et al.*, 1985). It was also suggested that the various strains of the virus be designated by a code with geographically informative letters and sequential numbers placed in brackets or as a subscript (for example, HIV-1_{sf33} for San Francisco Isolate 33) (Coffin *et al.*, 1986). Thus, widespread transmission of this agent was appreciated, and its close association with AIDS and related illness strongly supported its role in these diseases.

Soon after the discovery of HIV-1, in 1986, a separate virus type of HIV, human immune deficiency virus type 2 (HIV-2), was isolated from AIDS patients and seropositive asymptomatic individuals in West Africa, particularly the Cape Verde Islands and Senegal (Clavel *et al.*, 1986). Other HIV-2 strains were subsequently isolated from individuals from Guinea- Bissau, Gambia, and Ivory Coast (Clavel *et al.*, 1987;

Kong *et al.*, 1988). HIV-2 has since become increasingly prevalent in Europe, the United States, and South America (O'Brien *et al.*, 1992 and Weiss *et al.*, 1992), as well as more recently in India (Grez *et al.*, 1994). HIV-2, after cloning and sequence analysis, differed by more than 55% from the previous HIV-1 strains isolated and was antigenically distinct (Clavel *et al.*, 1987). While HIV-2 isolates exhibit several biological characteristics in common with HIV-1, HIV-2 is two times less efficient at being sexually transmitted and 10 times less efficient at being transmitted vertically (Levy, 1994).

Both HIV types can lead to AIDS, although the pathogenic course of HIV-2 appears to be longer (Weiss *et al.*, 1992). Up till recently, both home and abroad, the genetic variation of HIV is extremely high, with rapid turnover of HIV virions, and infected persons maintaining a substantial viral burden during the entire course of infection (Hu *et al.*, 1996), HIV is now the most significantly emerging infectious pathogen of this century (Essien *et al.*, 1997; Esu-Willams *et al.*, 1997; WHO, 1998).

2.2 EPIDEMIOLOGICAL PATTERNS OF HIV INFECTION

The global epidemic of AIDS has become one of the greatest public health emergency concerns of the century. The latest statistics provided by the Joint United Nations Programme on HIV/AIDS and World Health Organization (UNAIDS/WHO) record 39.5 million PLWHA worldwide (Table 3). This can be stratified into 37.2 million adults, of whom 17.7 million are women, and 2.3 million children under the age of 15. Observing the trend from 2001 to date, the global AIDS epidemic has been on a gradual rise (Figure 1). The total number of recently HIV-infected cases with HIV/AIDS

Table 3: Global summary of the AIDS epidemic December, 2006

Number of people living with HIV in 2006		
	Total	**39.5 million (34.1–47.1 million)**
	Adults	37.2 million (32.1–44.5 million)
	Women	17.7 million (15.1–20.9 million)
	Children under 15 years	2.3 million (1.7–3.5 million)
People newly infected with HIV in 2006		
	Total	**4.3 million (3.6–6.6 million)**
	Adults	3.8 million (3.2–5.7 million)
	Children under 15 years	530 000 (410 000–660 000)
AIDS deaths in 2006	Total	**2.9 million (2.5–3.5 million)**
	Adults	2.6 million (2.2–3.0 million)
	Children under 15 years	380 000 (290 000–500 000)

The ranges around the estimates in this table define the boundaries within which the actual numbers lie, based on the best available information.

Source: UNAIDS/WHO (2006)

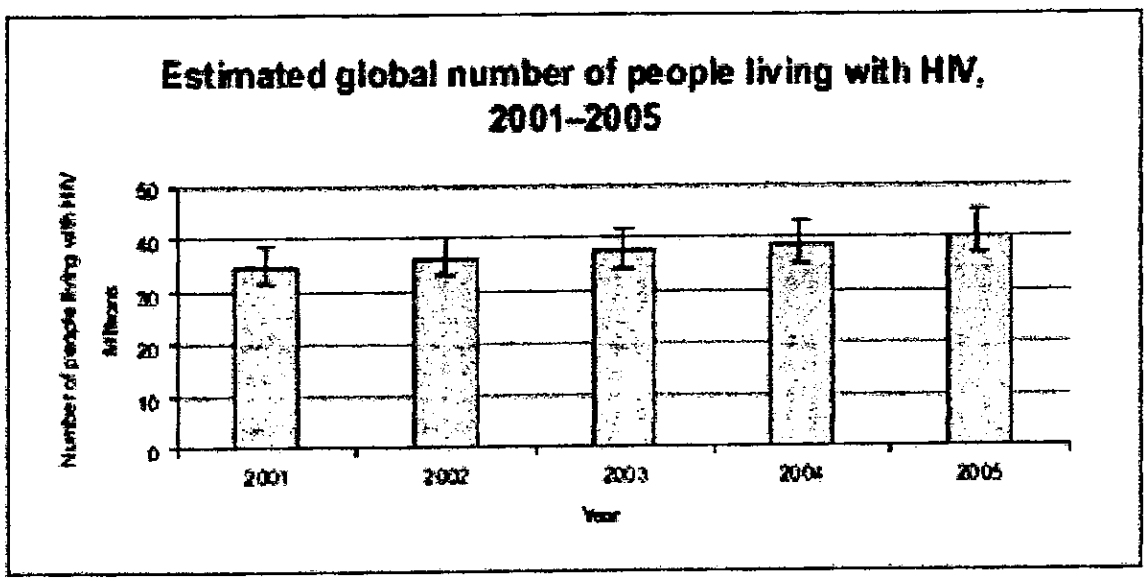

Figure 1: Estimated global number of people living with HIV, 2001-2005

Source: UNAIDS/WHO (2005)

worldwide in 2005 amounted to 4.9 million involving 4.2 million adults and 700,000 children (UNAIDS/WHO, 2005). Information on the global epidemiological situation showed that as at December 2006, the world total cumulative number of reported AIDS Death was 2.9 million (2.5-3.5 million) from 10 HIV regions around the World with cumulative adult prevalence of 1.0% and ranges from 0.1% to 5.9% in various HIV regions (Table 4)).

Sub-Saharan Africa, which has maintained the hardest hits since 1999, still has 24.7 million PLWHA atop others with 2.8 million newly HIV-infected, 5.9% adult prevalence and 2.1 million adult and child deaths due to AIDS. Although there is a drop in adult prevalence from 6.0% in 2004 to 5.9%, the impact of the scourge in this region is still of great global concern (although one million less than in 2004). Two thirds of the world's total of PLWHA are in Sub-Saharan Africa, as are 77% of all women with HIV (UNAIDS/WHO, 2006). Hence, the data portray the alarming rate at which HIV/AIDS spreads across the Sub-Saharan Africa, the region has just over 10% of the world population, but harbours more than 60% of all PLWHA.

HIV, since its discovery, has been spreading wildly globally but with certain patterns based on the world regional distribution. Worldwide, three different epidemiological patterns were observed during the 1980s (FMOH/NASCP, 1992 and Jasny, 1993). Pattern 1, seen in North America, Western Europe, and Australia, where AIDS was first reported in 1981, implying that HIV infection had probably been present since the 1970s, was characterized by spread principally via intercourse among male homosexuals, and to a lesser extent, by needle sharing among intravenous drug users (IDUs). In recent years HIV has risen increasingly in the female population, and

Table 4: Regional HIV and AIDS statistics and features, 2004 and 2006

	Adults and children living with HIV	Adults and children newly infected with HIV	Adult (15–49) prevalence (%)	Adult and child deaths due to AIDS
Sub-Saharan Africa				
2006	24.7 million (21.8–27.7 million)	2.8 million (2.4–3.2 million)	5.9% (5.2%–6.7%)	2.1 million (1.8–2.4 million)
2004	23.6 million (20.9–26.4 million)	2.6 million (2.2–2.9 million)	6.0% (5.3%–6.8%)	1.9 million (1.7–2.3 million)
Middle East and North Africa				
2006	460 000 (270 000–760 000)	68 000 (41 000–220 000)	0.2% (0.1%–0.3%)	36 000 (20 000–60 000)
2004	400 000 (230 000–650 000)	59 000 (34 000–170 000)	0.2% (0.1%–0.3%)	33 000 (18 000–55 000)
South and South-East Asia				
2006	7.8 million (5.2–12.0 million)	860 000 (550 000–2.3 million)	0.6% (0.4%–1.0%)	590 000 (390 000–850 000)
2004	7.2 million (4.8–11.2 million)	770 000 (480 000–2.1 million)	0.6% (0.4%–1.0%)	510 000 (330 000–740 000)
East Asia				
2006	750 000 (460 000–1.2 million)	100 000 (56 000–300 000)	0.1% (<0.2%)	42 000 (26 000–64 000)
2004	620 000 (380 000–1.0 million)	90 000 (50 000–270 000)	0.1% (<0.2%)	33 000 (20 000–49 000)
Oceania				
2006	81 000 (50 000–170 000)	7100 (3400–54 000)	0.4% (0.2%–0.9%)	4000 (2300–6600)
2004	72 000 (44 000–150 000)	6000 (3900–61 000)	0.3% (0.2%–0.6%)	2900 (1600–4600)
Latin America				
2006	1.7 million (1.3–2.5 million)	140 000 (100 000–410 000)	0.5% (0.4%–1.2%)	65 000 (51 000–84 000)
2004	1.5 million (1.2–2.2 million)	130 000 (100 000–320 000)	0.5% (0.4%–0.7%)	53 000 (41 000–69 000)
Caribbean				
2006	250 000 (190 000–320 000)	27 000 (20 000–41 000)	1.2% (0.9%–1.7%)	19 000 (14 000–25 000)
2004	240 000 (180 000–300 000)	25 000 (19 000–35 000)	1.1% (0.9%–1.5%)	21 000 (15 000–28 000)
Eastern Europe and Central Asia				
2006	1.7 million (1.2–2.6 million)	270 000 (170 000–820 000)	0.9% (0.6%–1.4%)	84 000 (58 000–120 000)
2004	1.4 million (950 000–2.1 million)	160 000 (110 000–470 000)	0.7% (0.5%–1.1%)	48 000 (34 000–66 000)
Western and Central Europe				
2006	740 000 (580 000–970 000)	22 000 (18 000–33 000)	0.3% (0.2%–0.4%)	12 000 (<15,000)
2004	700 000 (550 000–920 000)	22 000 (18 000–33 000)	0.3% (0.2%–0.4%)	12 000 (<15 000)
North America				
2006	1.4 million (980 000–2.2 million)	43 000 (34 000–65 000)	0.6% (0.6%–1.1%)	18 000 (11 000–26 000)
2004	1.2 million (710 000–1.9 million)	43 000 (34 000–65 000)	0.7% (0.4%–1.0%)	18 000 (11 000–26 000)
TOTAL				
2006	39.5 million (34.1–47.1 million)	4.3 million (3.6–6.6 million)	1.0% (0.9%–1.2%)	2.9 million (2.5–3.5 million)
2004	36.9 million (31.9–43.8 million)	3.9 million (3.3–5.8 million)	1.0% (0.8%–1.2%)	2.7 million (2.3–3.2 million)

Source: UNAIDS/WHO (2006)

heterosexual transmission is rapidly occurring, especially among teenagers. The annual incidence of AIDS was projected to rise to the peak in the mid-1990s. The pattern of HIV transmission in the Americas is changing and the epidemic is rapidly spreading to heterosexual populations, with new infections occurring primarily among those 15 to 25 years old (WHO, 1998).

Pattern II, seen in sub-Saharan Africa, is characterized by heterosexual transmission, with infection being equally common in males and females, and correspondingly high incidence of perinatal transmission to infants. Massive economic problems including inflation and unemployment have led to urban immigration, breakdown of traditional tribal values, and sexual promiscuity especially involving casual liaisons between female prostitutes and males separated from their families. Prevalence of HIV infection in central and East Africa has escalated alarmingly during the past decade, and the situation appears certain to deteriorate further (WHO, 1998; UNAIDS/WHO, 2005).

Pattern III applies to Asia, Eastern Europe, North Africa and the Middle East. The virus was not found in these areas until the early to mid 1980s. However, an alarming change became apparent in 1988 when an outbreak caused by HIV-1 genotype B (resembling the United States strains) occurred in IDUs in the Golden Triangles (at the junction of China, India, Myanmar, and Thailand). Then, in 1989, it became clear that female prostitutes in India and Thailand had become infected with genotype A (resembling African strains) and this genotype has since spread rapidly by heterosexual intercourse. It is predicted that by the turn of the century the number of cases in Asia will exceed the number in Africa (WHO, 1998; UNAIDS/WHO, 2005).

In much of sub-Saharan Africa, knowledge about HIV transmission routes is still low (Figure 2). Generally, women are less well-informed about HIV than men; this is also true of rural areas compared with those living in cities and towns (WHO, 1998; UNAIDS/WHO, 2005). There are three sub-regions in Sub-Saharan Africa: South Africa, East Africa, Central and West Africa (Figure 3). South Africa still remains the epicentre of global AIDS epidemic (UNAIDS/WHO, 2005). There is a slight decline in HIV prevalence in this sub region (Figure 3). Although there is a rise on the awareness of AIDS epidemic, specific knowledge about HIV is still inadequate. However, changes in sexual behaviour appear to have contributed to the declines in HIV prevalence.

East Africa still provides most hopeful indications that serious AIDS epidemic can be reversed. The countrywide drop in prevalence seen in Uganda since the mid 1990's is now observed to be mimicked in the urban parts of Kenya, where infection levels are dropping, even quite steeply in some places (Figure 3). The Central and West African sub regions historically have been less severely affected than other parts of sub-Saharan Africa, but the epidemic levels vary in scale and intensity from one part to another as well as from country to country. The national adult HIV prevalence is yet to exceed 10% in any West African country, and there is consistent evidence of significant changes of prevalence among pregnant women in recent years (Figure 3). Central Africa, especially Cameroon may be experiencing more serious epidemic very soon with the new rise in prevalence among the households in 2004.

Nigeria has been known after South Africa and India, to be the third country in the world that harbours the largest population of (between 3.2 and 3.6 million) PLWHA since 2004 (UNAIDS/WHO, 2004). In the entire country, an estimated number of

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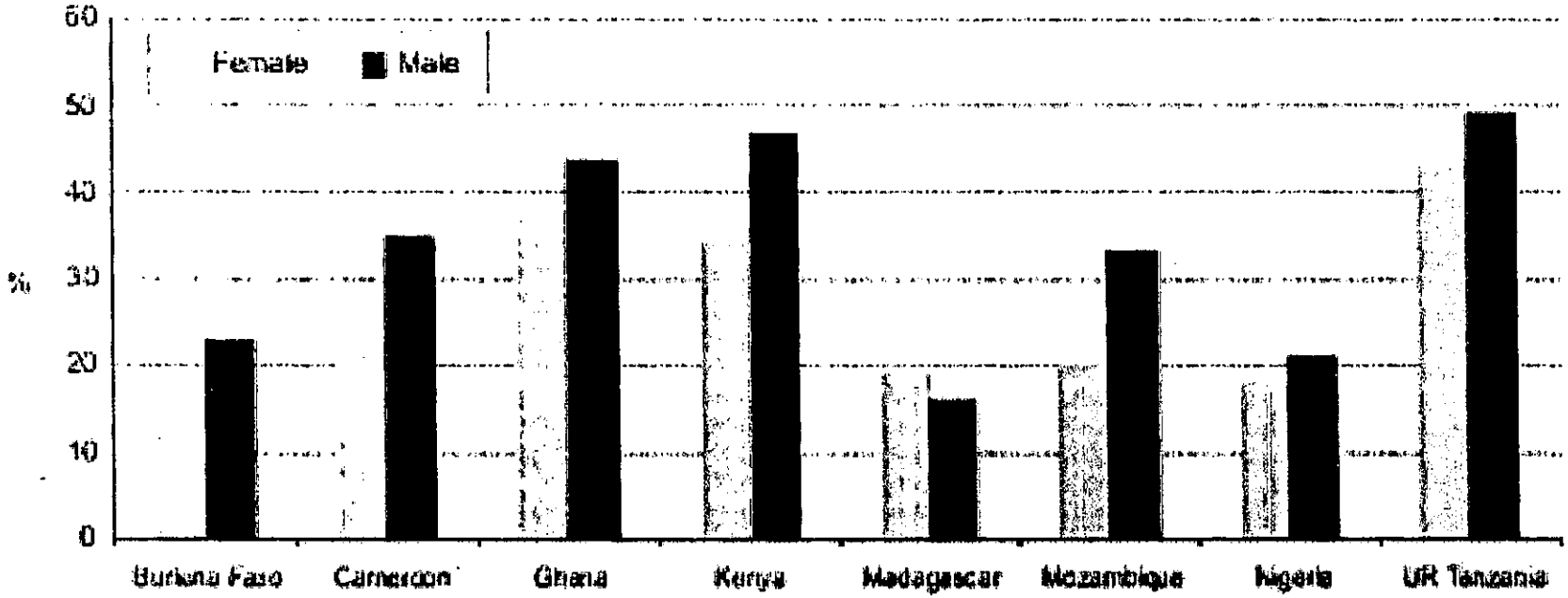


*Using condoms and limiting sex to one faithful, uninfected partner, who reject the two most common local misconceptions about HIV transmission, and who know that a healthy looking person can transmit HIV.

Figure 2: Percentage of young people aged 15-24 years who correctly identified major ways of preventing the sexual transmission of HIV*, 2003

Source: UNAIDS/WHO (2005)

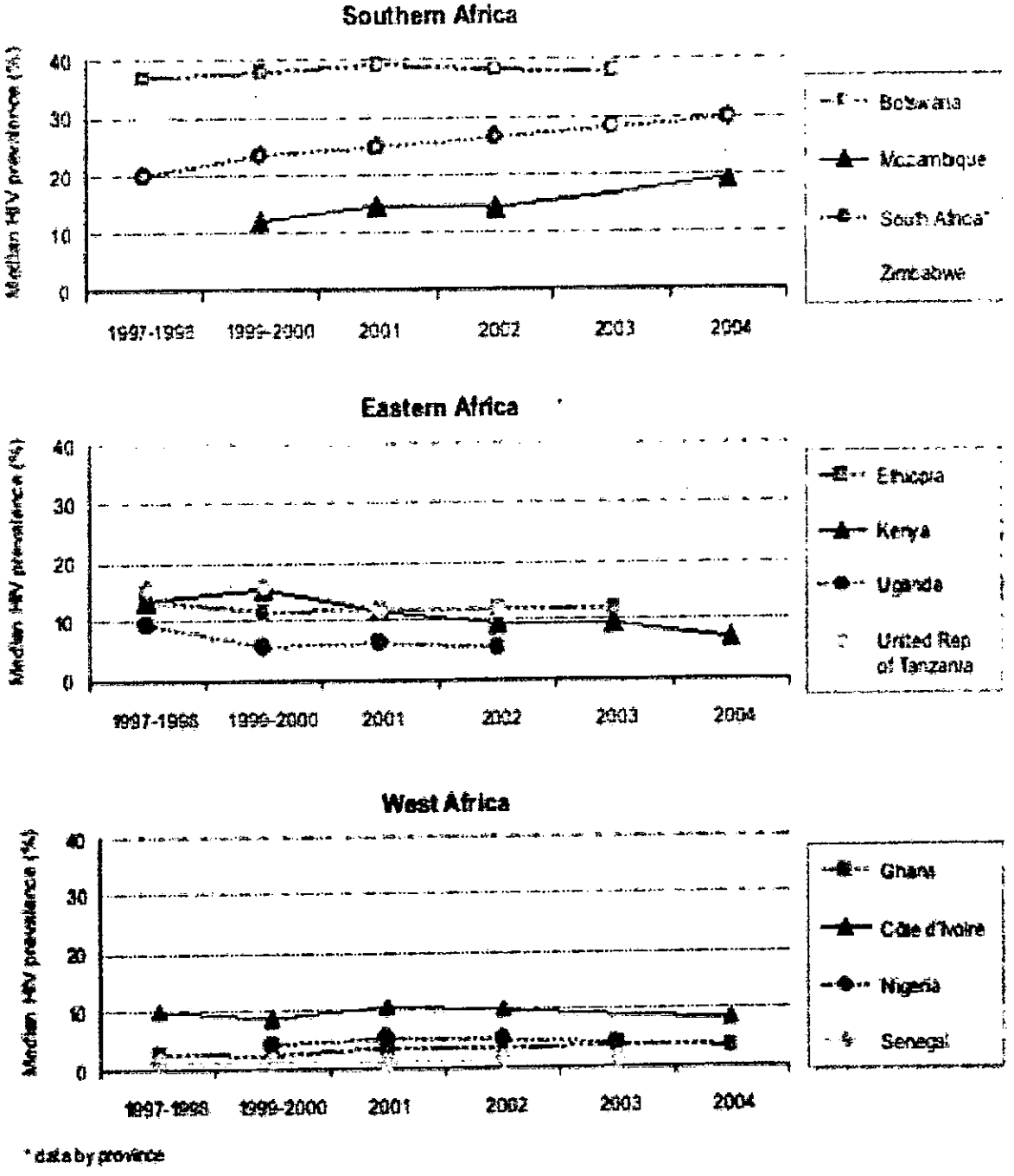


Figure 3: HIV prevalence among pregnant women attending antenatal clinics in Sub-Saharan Africa, 1997/98-2004

Source: UNAIDS/WHO (2005)

PLWHA as at the end of December, 2003 was 3.6 million (2.4-5.4 million) with 3.5 million of adults (of which 1.9 million were women) and 290,000 children aged below 15 years (Table 5). With about 1.2% sero-prevalence in 1992 (FMOH/NASCP, 1992) and about 100 million population, HIV/AIDS epidemic has rapidly increased to estimated adult prevalence rate of 5.4% out of 127,117,000 population (UNAIDS/WHO, 2004). From 484 reported AIDS cases in 90's, the number rose continuously from 719 cases in 1993 to 18,490 AIDS cases in 1998 (UNAIDS/WHO, 2004). It later declined from 16,188 cases in 1999 to 3,661 in 2001 (UNAIDS/WHO, 2004), while 310,000 Nigerian adults and children were estimated to have died of AIDS in 2003. The indelible effect of the HIV/AIDS epidemic has caused the estimated number of orphans to level at 1.8 million less than 17 years of age.

2.2.1 Epidemiological Outline of HIV in Nigeria

Information about HIV seroprevalence among antenatal clinic (ANC) attendees has been available since the mid-1980s from Nigeria (UNAIDS/WHO, 2004). However, case reporting from more than one or two sites per year did not begin until 1992. Between 1993 and 1994, ten major urban sentinel sites reported HIV seroprevalence among ANC women. By 1988-1990, 1% of ANC women tested in Lagos were HIV positive. Among sentinel sites in major urban areas, median HIV seroprevalence reached 4% by 1993-1994 and stayed at that level in 1999 and 2001. Seroprevalence among 16 sentinel sites in 2001 ranged from 1% in Lagos (Ikeja) to 14% in Abuja (Nyanya).

By 1991-1992, 20 sentinel sites from 10 states outside of the major urban areas reported HIV seroprevalence from sentinel surveillance of antenatal women (Table 6). The number of sentinel sites increased to 69 in 2001, and then to 125 in 2003 (Figure 4).

Table 5: Estimated number of adults and children living with HIVAIDS as at the end of December, 2003

These estimates include all people with HIV infection, whether or not they have developed symptoms of AIDS, alive at the end of 2003:

Adults and children	3,600,000		
Low estimate	2,400,00		
High estimate	5,400,000		
Adults (15-49)	3,300,000	Adult rate (%)	5.4
Low estimate	2,200,000	Low estimate	3.6
High estimate	4,900,000	High estimate	8.0
Children (0-15)	290,000		
Low estimate	170,000		
High estimate	500,000		
Women (15-49)	1,900,000		
Low estimate	1,200,000		
High estimate	2,700,000		

Estimated number of deaths due to AIDS

Estimated number of adults and children who died of AIDS during 2003:

Adults and Children	310,000
Low estimate	200,000
High estimate	490,000

Estimated number of orphans

Estimated number of children who have lost their mother or father or both parents to AIDS and who were alive and under age 17 at the end of 2003:

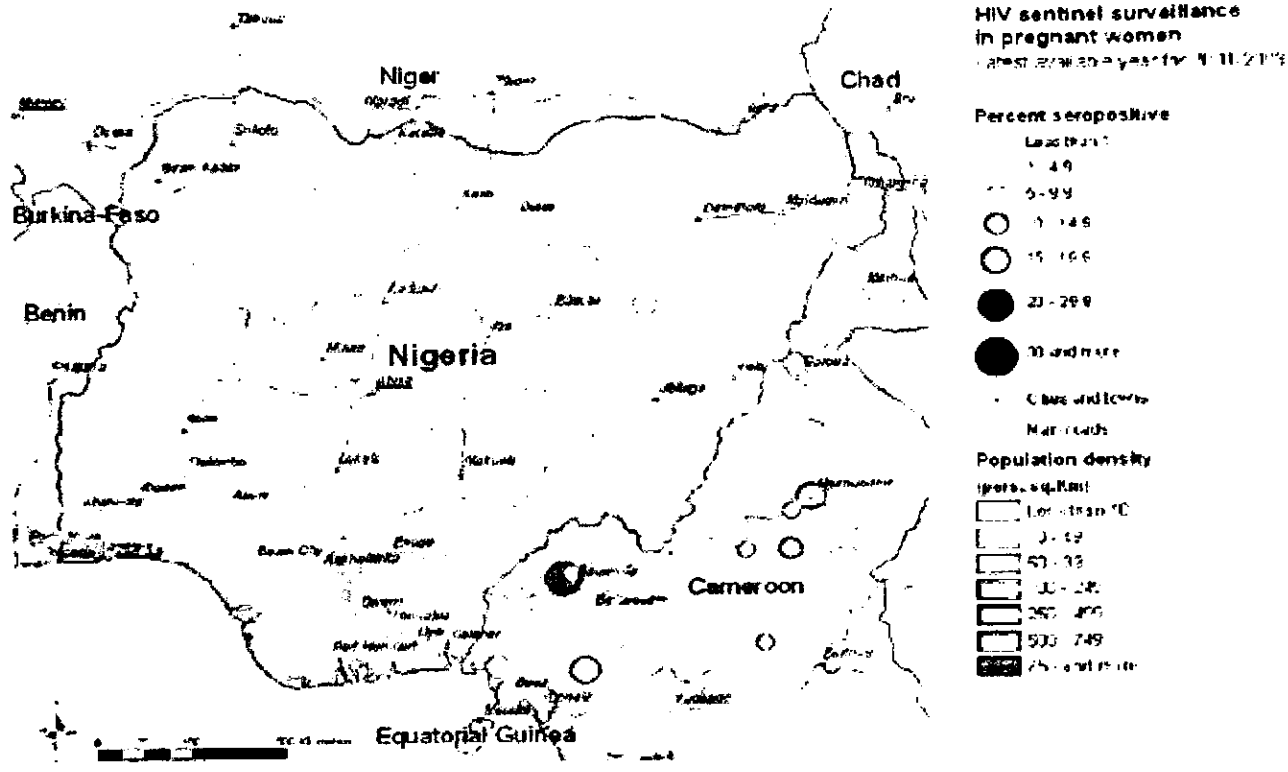
Current living orphans	1,800,000
Low estimate	1,200,000
High estimate	2,600,000

Source: UNAIDS/WHO (2004)

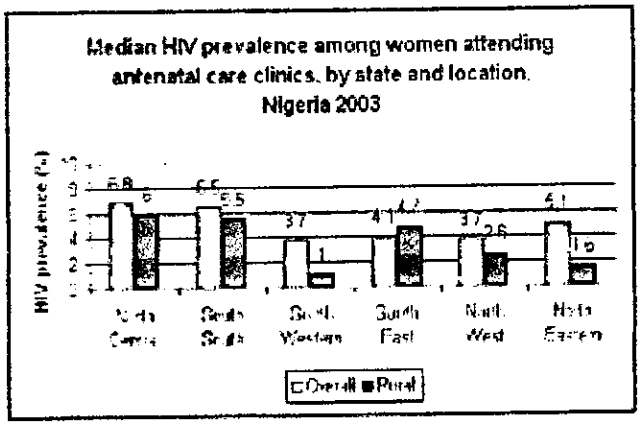
Table 6: HIV prevalence in different populations in Nigeria

Group	Area		1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003
Pregnant women	Major urban areas	N-Sites			2.00		7.00	8.00		10.00	9.00				10.00		18.00		
		Minimum			0		3.80	0		6.21	0				2.67		1.33		
		Median			0.50		5.15	0.65		3.68	1.40				4.50		4.17		
		Maximum			1.00		5.50	3.41		10.00	1.61				6.00		14.32		
	Outside major urban areas	N-Sites	1.00	1.00	1.00	2.00	1.00	21.00	1.00	53.00	52.00				63.00		70.00		
		Minimum	0	1.60	0	0	1.60	0	0.67	0	0				0.54		1.08		
		Median	0	1.60	0	0.06	1.60	0.50	0.67	2.00	2.42				4.33		5.22		
		Maximum	0	1.60	0	0.10	1.60	5.77	0.67	13.22	16.67				21.00		15.00		
Sex workers	Major urban areas	N-Sites			1.00			1.00	1.00	6.00	1.00	1.00							
		Minimum			1.71			9.04	15.30	9.80	81.73	30.50							
		Median			1.71			9.04	15.30	22.35	81.73	30.50							
		Maximum			1.71			9.04	15.30	46.90	81.73	30.50							
	Outside major urban areas	N-Sites	2.00	2.00	1.00	1.00		13.00		16.00	14.00	2.00							
		Minimum	0.21	0.49	0	4.20		0		0	6.78	46.90							
		Median	0.37	1.25	0	4.20		12.00		21.34	20.50	54.70							
		Maximum	0.52	1.97	0	4.20		54.79		57.80	67.74	62.60							
Injecting drug users																			
STI patients	Major urban areas	N-Sites						3.00	1.00	6.00	2.00					2.00			
		Minimum						0	6.15	2.00	1.17					7.20			
		Median						0.67	6.15	5.00	2.07					8.35			
		Maximum						14.91	6.15	8.91	3.03					9.50			
	Outside major urban areas	N-Sites	1.00	1.00		1.00		11.00		21.00	20.00					8.00			
		Minimum	0.49	1.28		1.68		0		0	1.14					5.00			
		Median	0.49	1.28		1.68		1.74		7.00	12.45					15.65			
		Maximum	0.49	1.28		1.68		22.42		31.10	68.74					23.00			
Men having sex with men																			
Tuberculosis patients	Major urban areas	N-Sites	1.00		1.00	1.00		6.00		5.00	3.00					5.00			
		Minimum	2.60		2.00	0.00		0		0	0					4.20			
		Median	2.60		2.00	0.00		2.50		6.00	3.75					12.40			
		Maximum	2.60		2.00	0.50		14.29		20.00	7.37					20.00			
	Outside major urban areas	N-Sites						9.00		16.00	23.00	2.00				9.00			
		Minimum						0		1.00	0	12.99				9.90			
		Median						1.02		3.52	9.00	14.14				23.10			
		Maximum						5.45		25.00	30.24	18.50				35.10			

Source: UNAIDS/WHO (2004)



Trends in HIV prevalence among antenatal clinic attendees



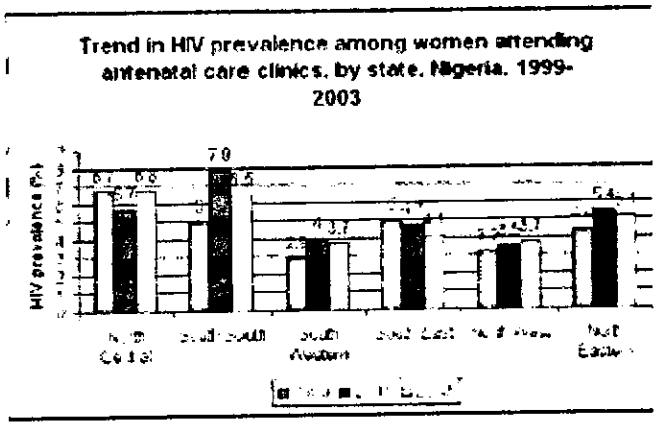


Figure 4: HIV sentinel surveillance in pregnant women – trends in HIV prevalence among the antenatal clinic attendees (2001-2003)

Source: UNAIDS/WHO (2004)

Median HIV seroprevalence among ANC women tested at these sites increased from <1% in 1991-1992 to 4.6% in 2003. Seroprevalence among 125 sentinel sites in 2003 ranged from 0% in 10 sites to >10% in 12 sites with 17% HIV seroprevalence in antenatal women tested in Badagry, Lagos. HIV seroprevalence among 15-24 year old women was 5.7% in 1999, 5.9% in 2001 and 5.4% in 2003 (UNAIDS/WHO, 2004).

Testing of commercial sex workers for HIV status in Lagos began in 1988-1989 (UNAIDS/WHO, 2004). Two percent of commercial sex workers tested at that time were HIV positive but this number has increased to 15% in 1993. Median HIV seroprevalence among 7 sentinel sites in 1994 and 3 sentinel sites in 1995 were 29% and 33%, respectively. In 1996, 31% of commercial sex workers tested in Lagos were HIV positive. In 1986-1987, <1% of commercial sex workers tested in Maiduguri (Borno State) were HIV positive; while 4% of commercial sex workers tested were HIV positive by 1989-1990. In 1992, 14 sentinel sites outside of the major urban centres were reporting information on HIV seroprevalence among commercial sex workers. At that time, a median of 11.5% of commercial sex workers tested were HIV positive. The seroprevalence among these sites ranged from 0% to 52% of commercial sex workers tested. By 1995, median seroprevalence among 17 sentinel sites was 24%. Seroprevalence ranged from 0% to 68% of commercial sex workers tested. In 1996, median HIV seroprevalence was 55% for 2 sites (Table 6).

In major urban areas from 1992 to 1995, median HIV seroprevalence among STD clinic patients tested ranged from 1% to 6%. Median HIV seroprevalence from 22 sentinel sites outside of the major urban areas was 7% in 1993-1994 (UNAIDS/WHO, 2004). From 20 sentinel sites in 1995, HIV seroprevalence ranged from 1% to 70% of

STD clinic patients tested with median HIV seroprevalence of 12.5%. In 2000, 11% of soldiers tested in an unspecified area were HIV positive. In 1993-1994, only 4% of long distance truck drivers tested in Anambra State were HIV positive (Table 6).

Finally, median HIV prevalence among pregnant women appears to have levelled at around 4% recently. Although HIV prevalence among pregnant women varies (from a low prevalence of 2.3% in the South West to a high prevalence of 7% in the North Central parts), stable trends are evident at almost all the antenatal clinics surveyed since the mid-1980s. The only exception is Cross River State, where infection levels rose from 4% in 1993-1994 to 12% in 2003 (FMOH/NASCP, 2004).

2.3 MODE OF TRANSMISSION

The transmission of a virus can be greatly influenced by the amount of infectious virus in a body fluid and the extent of contact an individual has with that body fluid (Levy, 1994a). Epidemiological studies conducted during the period 1981-82 first indicated that the major routes of transmission of AIDS were intimate sexual contact and exchange of contaminated blood (Jaffe *et al.*, 1983). The syndrome was initially described in homosexual and bisexual men and intravenous drug users (Gottlieb *et al.*, 1981; Masur *et al.*, 1981 and Siegal *et al.*, 1981), but its occurrence from heterosexual activity was soon recognized as well (Harris *et al.*, 1983). Moreover, it became evident that blood or blood products could transmit the agent easily (CDC, 1982) and, mothers could transfer the causative agent to new-born infant (Ammann *et al.*, 1983).

There are therefore three principal modes of transmission of HIV, as worked out commendably quickly (within a couple of years after the recognition of the first cases of AIDS) in Los Angeles: sexual intercourse, exchange of blood, and perinatal transmission.

The reasons for these 3 principal means of transmission-via blood, sexual contact and maternal-child (Stoneburner *et al.*, 1990; Brookmeyer, 1991) can be explained to a great extent by the relative concentration of HIV in various body fluids (Table 7).

2.3.1 Sexual Transmission of HIV

AIDS was first identified as a disease that appeared to be transmitted by a sexual route. A high prevalence among homosexual men was initially reported. Subsequent studies indicated spread by heterosexual activity, which now is responsible for the majority of infections worldwide (Stoneburner *et al.*, 1990; Nkowane, 1991; Mann *et al.*, 1992). The largest groups of individuals infected with HIV were exposed to the virus through unprotected sexual contact on a regional scale; however, there is a difference in the incidence of infection depending on sexual preference. For example, in a recent study in Germany, the incidence of infection by male heterosexual contacts is five times lower than that by male homosexual contacts (Mann *et al.*, 1992). In contrast, in sub-Saharan Africa, the incidence of infection by heterosexual contracts is much higher than that by homosexual contacts (Nkowane, 1991).

2.3.2 Mother-to-Child Transmission (MTCT) of HIV

Perinatal infection currently accounts for about 10% of all HIV infections (Fauci, 1988) while the transmission of HIV from mother to child appears to occur in 25 to 30% of children born to HIV- positive mothers (Cowan *et al.*, 1988; Scarlatti *et al.*, 1991; McIntyre, 2002). This prevalence is based primarily on Polymerase Chain Reaction (PCR) and virus culture studies: Diagnosis by serology is difficult since maternal antibodies are present in infants at birth (Oxtoby, 1990 and Rogers *et al.*, 1991).

Table 7: Isolation of HIV from body fluid

Fluid	No. with virus isolation/total no.	Estimated quantity of HIV[a]
Free virus in fluid		
Plasma	33/33	1–5,000[b]
Tears	2/5	<1
Ear secretions	1/8	5–10
Saliva	3/55	<1
Sweat	0/2	—[c]
Feces	0/2	—
Urine	1/5	<1
Vaginal and cervical	5/16	<1
Semen	5/15	10–50
Milk	1/5	<1
Cerebrospinal fluid	21/40	10–10,000
Infected cells in fluid		
PBMC	89/92	0.001–1%[b]
Saliva	4/11	<0.01%
Bronchial fluid	3/24	NK[d]
Vaginal and cervical fluid	7/16	NK
Semen	11/28	0.01–5%

[a] For cell-free fluid, quantities are given as infectious particles per milliliter; for infected cells, quantities are the percentage of total cells infected.

[b] High levels associated with symptoms and advanced disease.

[c] —, no virus detected.

[d] NK, not known.

Source: Levy (1994)

Transmission occurs in three different periods *in utero*, intrapartum, and postpartum (Rossi, 1992 and Lyman *et al.*, 1993). Transplacental infection contributes to the major means of transmission (*in utero* transmission), usually during the third trimester, although there have been cases in which this infection occurred in the first trimester (Chandiwani *et al.*, 1991). HIV-seropositive mothers are more infectious during the clinical disease, in which the second peak viremia appears (Barnett and Levy, 1991).

At delivery, infants can become infected through the birth canal – ascending infection (Menez-Baustista *et al.*, 1986). Firstborn twins experience more trauma during delivery and, are more often infected than second-born twins (Goedert *et al.*, 1991). Transmission by neonate's ingestion of maternal blood or other fluids, or by breaks in the neonatal skin and subsequent exposure to infected blood or secretions (McIntyre, 2002).

Breast-feeding is not, however, a major route of transmission. In fact, in developing countries, the advantages of breast-feeding by infected mothers in decreasing childhood mortality far outweigh the slight chances of infecting the child (Barnett and Levy, 1991). In addition, breast-feeding greatly delays the onset of AIDS in children who are already infected with the virus (Fujino *et al.*, 1992). Breast milk among others however still contributes to MTCT of HIV (Mofenson and McIntyre, 2000). Finally, the risk of MTCT is affected by numerous factors – maternal, foetal, viral and behavioural (Table 8).

2.3.3 Transmission by Blood and Blood Products

Blood transfusion and the administration of blood products such as factors VIII to haemophiliacs accounts for only 3-5% of all past and present HIV infections and declined

Table 8: Risk factors associated with overall risk of mother-to-child transmission of HIV

	Strong evidence	Intermediate evidence	Limited evidence
Maternal factors	High viral load	Chorioamnionitis	Frequent unprotected sexual intercourse
	Immuno-deficiency	Anaemia	
	Viral characteristics	Vitamin A deficiency	Multiple sexual partners
	Advanced disease	Sexually transmitted infections	Drug use involving injection
	HIV infection acquired during pregnancy or breastfeeding	Smoking	
Obstetric factors	Vaginal delivery (compared with elective caesarean section)	Invasive procedures	Episiotomy
Infant factors	Prematurity	Lesions of skin and/or mucous membranes (oral thrush)	
	Breastfeeding		

Source: McIntyre (2002)

rapidly (to about 1 in 225,000 in the United States) following the introduction in 1985 of routine screening procedures in blood banks (Peterson *et al.*, 1992). Before the screening of blood, transfusion recipients and haemophiliacs could be infected by HIV present in blood and blood products such as factors VIII and IX because transmission by contaminated blood and its products is the most efficient way of acquiring HIV infection. The efficiency of transmission of HIV infection by transfusion is greater than 90% because both free infectious virus and infected cells were present in blood, and HIV infected cells appeared to be more numerous than free virus (Levy, 1994a). The chance of transmitting infections particularly by HIV-containing cells, could then be even greater (Levy, 1993b). Even if a latently infected cell was transferred, it could serve as a source of HIV transmission, following its activation in the new host (Margolick *et al.*, 1987).

2.4 BIOLOGY OF HIV INFECTION

The aetiologic agent of acquired immunodeficiency syndrome (AIDS) is known as Human immunodeficiency virus (HIV) based on the recommendation of the International Committee on Taxonomy of Viruses (Coffin *et al.*, 1986). Human immunodeficiency virus (HIV) is a human retrovirus consisting of a noncomplementary pair of single coding or positive (+) strands of RNA enclosed within an inner, nucleocapsid, protein core surrounded by lipid bilayer envelope (Schwartz and Nair, 1999). Infections with lentiviruses typically show a chronic course of disease, a long period of clinical latency, persistent viral replication and involvement of the central nervous system. Visna infections in sheep, simian immunodeficiency virus (SIV) infections in monkeys, or feline immunodeficiency virus (FIV) infections in cats are typical examples of lentivirus infections (Barre-Sinoussi, 1996).

There are two types of HIV namely; Human immunodeficiency virus type 1 (HIV-1) and Human immunodeficiency virus type 2 (HIV-2). HIV virions contain virus nucleocapsid, which consists of (a) the major capsid protein, (b) embedded in the capsid, or nucleocapsid with which the (c) three enzymes namely reverse transcriptase (RT) (which is the viral RNA-dependent DNA polymerase), protease, and integrase, as well as (d) the nucleocapsid (NC) protein (p-9, p-6/7) are closely associated. Reverse transcriptase is the hallmark of a retrovirus and this enzyme is capable of transcribing its genomic RNA into double-stranded DNA (Barre-Sinoussi, 1996). The genomic size of HIV is about 9.8 kB, with open reading frames coding for several viral proteins.

2.4.1 Structure of the HIV virion

Viewed by electron microscopy, HIV-1 and HIV-2 have the characteristics of a lentivirus which reveal a sphere with cone-shaped core composed of the viral p-25 (or p-24) *Gag* protein, surrounded by spherical knobs of lipid bilayer envelope. This DNA copy of the retroviral genome is called a **"provirus"**.

Using electron microscopy, HIV-1 and HIV-2 resemble each other strikingly. However, they differ with regard to the molecular weight of their proteins, as well as in having differences in their accessory genes. HIV-2 is genetically more closely related to the SIV found in sootey mangabeys (SIVsm) rather than HIV-1 and therefore has a very close phylogenetic link with SIV. Both HIV-1 and HIV-2 replicate in $CD4^+$ T cells and are regarded as pathogenic in infected persons although the actual immune deficiency may be less severe in HIV-2 infected individuals (Barre-Sinoussi, 1996).

HIV-1 can be used for structural description of both types since they are very similar. HIV-1 viral particles have a diameter of 100 nm and are surrounded by a

lipoprotein membrane (Figure 5). Each viral particle contains 72 glycoprotein complexes which are integrated into this lipid membrane and are each composed of trimers of an external glycoprotein gp120 and a transmembrane spanning protein gp41. The bonding between gp120 and gp41 is only loose and therefore gp120 may be shed spontaneously within the local environment. Glycoprotein gp120 may also be detected in the serum (Oh *et al.*, 1992), as well as within the lymphatic tissue of HIV-infected patients (Sunila *et al.*, 1997). Various parts of the virion have their specific functions (Table 9).

2.4.2 The organization of HIV viral genome

The genomic size of HIV is about 9.8 kB, with open reading frames coding for several viral proteins (Figures 6; Table 9). The primary transcript of HIV is a full - length viral mRNA consisting of 3 main genes: the *Gag*, *Pol* and *Env* – **gag** means "group-antigen", **pol** represents "**pol**ymerase" and **env** is for "**env**elope" (Figure 6). The "classical" structural scheme of a retroviral genome is: 5'LTR-gag-pol-env-LTR 3'. The LTR ("long terminal repeat") regions represent the two end parts of the viral genome that are connected to the cellular DNA of the host cell after integration and do not encode for any viral proteins. These genes are translated into the *Gag*, *Pol* and *Env* proteins. The *gag* and *env* genes code for the nucleocapsid and the glycoproteins of the viral membrane; the *pol* gene codes for the reverse transcriptase and other enzymes. In addition, HIV-1 contains in its 9kB RNA six genes (*vif, vpu, vpr, tat, rev* and *nef*) that contribute to its genetic complexity. *Nef, vif, vpr* and *vpu* were classified as accessory

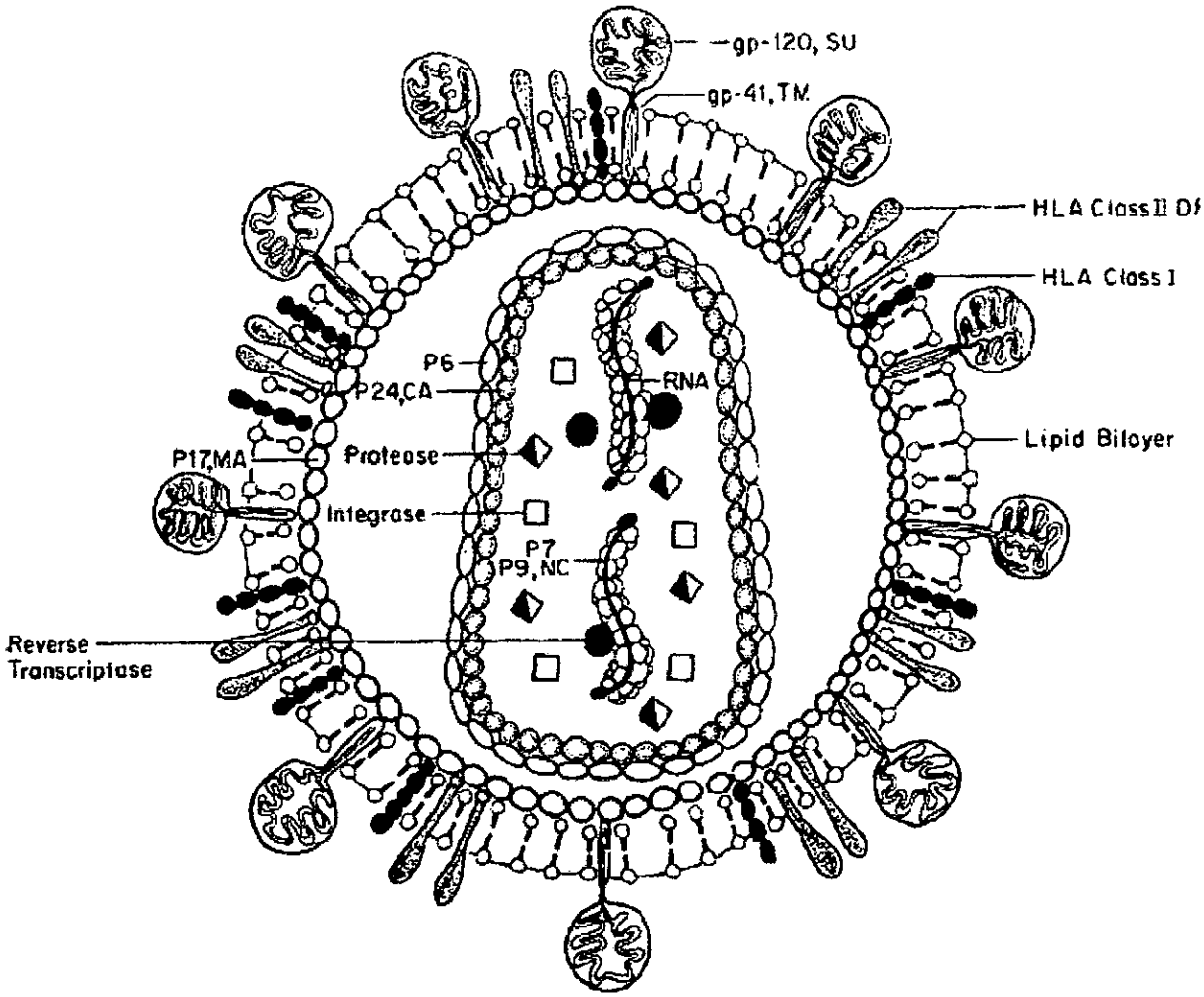


Figure 5: HIV-1 virion.

The mature HIV-1 virion measures approximately 1,000 Å in diameter. The HIV-1 genome consists of two identical, noncomplementary sense (+) strands of RNA, each approximately 9.2 kb long, packaged in a cone-shaped, protein core composed of 24 kDa capsid (CA) proteins (p24), surrounded by a unique protein, p6, that regulates release of the virus from the cell. This, in turn, is surrounded by p17 matrix (MA) proteins, and the entire virion is enclosed in a phospholipid bilayer envelope derived from the host cell membrane. The viral enzymes, RT, integrase, and protease, are packaged with the RNA in the core. The surface (SU) and transmembrane (TM) glycoproteins, gp120 and gp41, respectively, form a noncovalent complex. Also incorporated into the envelope are various host proteins including HLA Class I and II DR molecules.

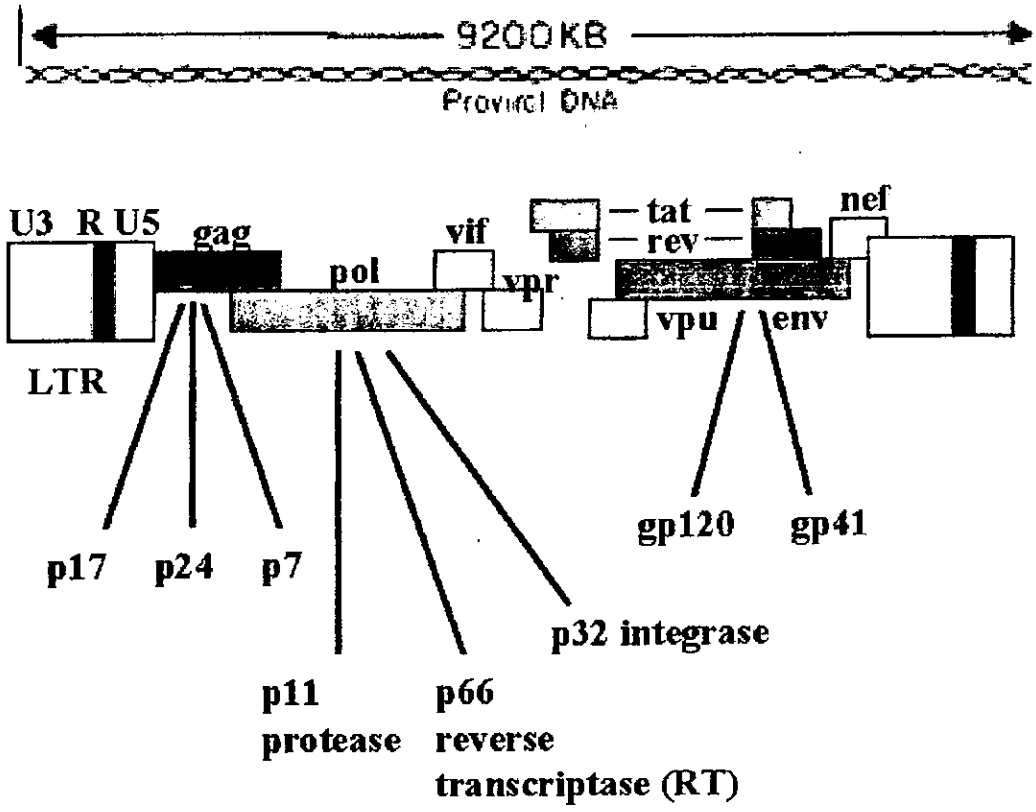
Source: Schwartz and Nair (1999)

Table 9: HIV proteins and their functions

Proteins	Size (kDa)	Function
Gag	p25 (p24)	Capsid (CA) structural protein
	p17	Matrix (MA) protein, myristoylated
	p9	RNA-binding protein (?)
	p6	RNA-binding protein (?); helps in virus budding
Polymerase (Pol)	p55, p63	RT, RNase H (inside core)
Protease (PR)	p15	Posttranslation processing of viral proteins
Integrase (IN)	p11	Viral cDNA integration
Envelope	gp120	Envelope surface (SU) protein
	gp41 (gp36)	Envelope transmembrane (TM) protein
Tat[b]	p14	Transactivation
Rev[b]	p19	Regulates viral mRNA expression
Nef[b]	p27	Pleiotropic, including virus suppression; myristoylated
Vif[b]	p23	Increases virus infectivity and perhaps cell-cell transmission; cysteine protease (?)
Vpr[b]	p18	Helps in virus replication; transactivation (?)
Vpu[c]	p15	Helps in virus release
Vpx[d]	p15	Helps in infectivity
Tev[b]	p26	Tat and Rev activators

[b] Not found associated with the virion; Vpx, not certain.
[c] Only present with HIV-1. Expression appears regulated by Vpr.
[d] Only encoded by HIV-2. May be a duplication of Vpr.

Source: Levy (1994)



Gene	Main Functions
gag	core proteins
pol	enzymes
env	envelope proteins
tat	positive regulator
rev	differential regulator
vif	infectivity factor
vpr	transcriptional activator
vpu	efficient virus budding
nef	negative regulator

Figure 6: HIV-1 genes and their locations

Source: Adapted from – Schwartz & Nair (1999) & Hoffmann & Kamp (2003)

genes in the past, as they are not absolutely required for replication in vitro. However, the regulation and function of these accessory genes and their proteins have been studied and characterized in more detail within the last years. The accessory genes, *nef, tat* and *rev*, are all produced early in the viral replication cycle (Levy, 1994; Schwartz & Nair, 1999; Hoffmann & Kamp, 2003).

2.4.3 Classification of HIV

Kingdom:	- (Regarded as independently non-living entity)
Division:	- (same as above)
Class:	Virus
Order:	RNA virus
Group:	Group VI (ssRNA-RT)
Family:	*Retroviridae*
Genus:	*Lentivirus*
Species:	***Human immunodeficiency virus 1***
Species:	***Human immunodeficiency virus 2***

2.4.4 Phenotypic characteristics of HIV

Human immunodeficiency virus is highly heterogeneous, consisting of different strains and subtypes (apart from being HIV-1 and HIV-2) due to the following biological, serological and molecular features:

- Cellular tropism
- Replication kinetics
- Level of virus production
- Cytopathicity
- Plaque- or syncytium-forming ability
- Latency and inducibility
- Sensitivity to neutralization /enhancing antibodies

- Genetic structure

Cellular tropism – HIV-1 & 2 have different strains and subtypes based on the ability to grow or preference for certain established human T-cell, B-cell, macrophages and monocytes (Fenyo *et al.*, 1988).

Replication kinetics – reproducible differences in replication and time of virus release have been observed in cells from 10 different Caucasian, Asian and black donors infected with 3 different strains of HIV-1 in peripheral blood mononuclear cells (PBMC) culture (Evans *et al.*, 1987).

Level of virus production – at the peak of replication, optimum virus titres may be high or low depending on strain types. Some low-titre viruses have been found to replicate rapidly and certain slow-replicating viruses can eventually grow to high titres after prolong period in culture (von Briesen *et al.*, 1990).

Cytopathicity – variability in inducing cytopathic effects by the relative ability of HIV strains to infect and replicate and to modulate CD4 expression (Cheng-Mayer *et al.*, 1988). It varies from lack of cytopathology, to mild or strong syncytium formation and cell-to-cell fusion (Cheng-Mayer *et al.*, 1988a).

Plaque- or syncytium forming ability – using established T-cell lines, syncytium-inducing (SI) and non-syncytium-inducing (NSI) strains have been described (Tersmette *et al.*, 1989), and differences in plaque-forming ability of HIV-1 strains have been reported (Tateno and Levy, 1988). The plaque assay is very useful in detecting highly cytopathic HIV strains using MT-4 cell lines.

Latency and inducibility – some HIV isolates enter into latent or silent state in vitro much more rapidly than others. For instance, early isolates from an asymptomatic

individual will enter a latent state in established T-cell lines, whereas later isolates from the same person with AIDS replicate efficiently in the cells (Folks & Bednarik, 1992). Sensitivity to neutralizing and enhancing antibodies – certain HIV-1 & 2 strains are more sensitive to neutralization antibodies than others (e.g., SF2) while some strains are more susceptible to enhancing antibodies than the others (e.g., SF128A). Enhancement results in doubling or tripling of virus replication in $CD4^{+}$T cells and microphages while neutralization causes biostatic virus replication or viricidal actions in these cells (Robinson *et al.*, 1988; Weiss *et al.*, 1988).

Genetic structure – differences in cell tropism, replication kinetics, virus titres, cytopathology, latency and inducibility as well as sensitivity to neutralization and enhancing antibodies are mirrored in the genetic sequences of the viruses. Fidelity is several folds higher with RNA than DNA, suggesting that most mutations occur with the DNA template-DNA primer (Haseltine and Wong-Staal, 1991; Boyer *et al.*, 1992).

On the above characteristics, HIV can be broadly divided into two phenotypes, one inducing syncytium formation among infected cells and the other does not (Fouchier *et al.*, 1996). Macrophage tropic (M-tropic) strains of HIV-1, or non-syncytia-inducing strains (NSI) use the beta-chemokine receptor CCR5 for entry, and are thus able to replicate in macrophages and CD4+ T cells (Coakley *et al.*, 2005). The normal ligands for this receptor, RANTES, macrophage inflammatory protein (MIP)-1-beta and MIP-1-alpha, are able to suppress HIV-1 infection *in vitro*. This CCR5 coreceptor is used by almost all primary HIV-1 isolates regardless of viral genetic subtype.

Indeed, macrophages play key role in several critical aspects of HIV disease. They appear to be the first cells infected by HIV in the central nervous system (CNS). In

tonsils and adenoids of HIV-infected patients, macrophages fuse into multinucleate giant cells that produce copious amounts of virus. T cells tropic (T-tropic) isolates, or syncytia-inducing (SI) strains replicate in primary $CD4^+$ T cells as well as in macrophages and use the alpha-chemokine receptor, CXCR4, for entry (Coakley *et al.*, 2005). The alpha-chemokine, SDF-1, a ligand for CXCR4, suppresses replication of T-tropic HIV-1 isolates by down regulating the expression of CXCR4 on the surface of these cells. Viruses that use only the CCR5 receptor are termed R5, those that only use CXCR4 are termed X4, and those that use both, X4R5. However, the use of coreceptor alone does not explain viral tropism, as not all R5 viruses are able to use CCR5 on macrophages for a productive infection (Coakley *et al.*, 2005).

Recognizing that the principal targets of HIV are $CD4^+$ T cells and macrophages, in 1984 researchers established that the CD4 molecule was the primary receptor for virus entry into susceptible target cells. However, additional HIV receptors have been described including galactosylceramide on oligodendrocytes and Schwann cells in the brain and epithelial cells of the intestines. Immunoglobulin Fc receptors on neutrophils, monocytes, basophils, mast cells, eosinophils, platelets, and B lymphocytes also may facilitate binding of HIV (Schwartz & Nair, 1999). Antibodies made against various HIV antigens can bind to infectious virions or infected cells that, in turn, are bound by cellular Fc receptors. The bound complex can then be internalized by the cells and subsequently infect them independently of the CD4 receptor. There is current evidence indicating that CD4 alone is insufficient for optimal entry and fusion of HIV. This is based on the observation that murine cells genetically manipulated to express the human CD4 receptors are not permissive for HIV infection.

Further, studies have shown that HIV grown in laboratory with transformed T-cell lines could infect only primary T cells and could not infect monocytes and macrophages. These variants were designated T tropic. By contrast, primary HIV isolates could infect only monocytes and macrophages but not T-cell lines and hence were called macrophage (M) tropic. In addition there are some viruses that can infect both macrophages and T cells. These variations in cell tropism appear to be due to sequence differences in the V3 region of the envelope glycoprotein, gp120. When gp120 binds to CD4, changes occur in both molecules producing a new conformation that requires additional points of attachment for effective viral entry (Coakley *et al.*, 2005).

Thus, supplementary, species-specific cell surface factors may be necessary for optimal virus fusion with the membrane. Such non-CD4 factors have been differentially named by various investigators as second receptors, co-receptors, fusion receptors, accessory receptors, or alternate receptors (Levy, 1996). Recently, considerable interest has been directed to the identification and cloning of other putative coreceptors for HIV, yielding some surprises (Alkhatib *et al.*, 1996; Schmidtmayerova *et al.*, 1996). A member of the seven transmembrane G-protein-coupled receptor family, termed leukocyte-derived seven transmembrane domain receptor (LESTER) or alternatively called fusin was found to act as a co-receptor for T-tropic HIV strains only. When fusin was introduced into HIV-resistant mouse cells expressing human CD4, they became readily susceptible to HIV infection.

Recently, this co-receptor for T-tropic strains of HIV, fusin, was found to be identical to the chemokine receptor CXCR-4. The natural ligand for CXCR-4 is the chemokine, stromal cell-derived factor 1 (SDF-1). Chemokines are chemo-attractant

cytokines, many of which have long been known to play a role in mediating allergic reactions. SDF-1 can block the ability of CXCR-4 to serve as a co-receptor for T-tropic strains of HIV. Furthermore HIV-infected patients with SDF-1 gene variants were found to have a delay in the onset of AIDS. CXCR-4 was also shown to be the primary receptor for HIV-2. To reflect their co-receptor requirement, the T-cell tropic virus strains that require CXCR-4 have recently been renamed X4 viruses (Coakley *et al.*, 2005).

The subsequent discovery of a co-receptor specific for M-tropic virus strains was not unexpected. This M-tropic HIV co-receptor was recognized to be the previously described chemokine receptor CCR5. Therefore, the M-tropic virus strains that require the CCR5 receptor for entry have been named R5 viruses (Coakley *et al.*, 2005). Other chemokine receptors, such as CCR2b and CCR3, also can serve as co-receptors for some HIV strains. The β-chemokines - regulated upon activation, normal T-cell expressed and secreted (RANTES), and macrophage in- flammatory protein-1α (MIP-1α) and MIP-1β, are ligands for CCR5. CCR5 receptor binding by these chemokines inhibited M-tropic virus infection of $CD4^+$ cells (Alkhatib *et al.*, 1996). RANTES is the most active inhibitor of HIV replication. Moreover, the absence of CCR5 on a cell has been associated with resistance to HIV infection.

The role of CCR5 as co-receptor for HIV *in vivo* is supported further by a report of resistance to HIV infection with R5 viruses by individuals who are homozygous for a 32-bp CCR5 gene deletion (D32 CCR5) (Coakley *et al.*, 2005). Independent confirmation of the role of chemokine receptors as co-receptors for HIV derives from previous studies showing that HIV replication in CD41 cells could be suppressed by soluble factors secreted from CD81 cells. There is a current controversy over whether these HIV

suppressor factors are the β-chemokines RANTES, MIP-1α, and MIP-1β or are a unique HIV inhibitor (Alkhatib *et al.*, 1996).

Although monocytes, B lymphocytes, mast cells, and fibro- blasts produce β-chemokines in low amounts, activated $CD8^+$ cells are the most potent producers (Schmidtmayerova *et al.*, 1996). The role of the β-chemokines in HIV infection has become even more complicated in view of a recent report showing that they stimulate HIV replication in macrophage cultures (Schmidtmayerova *et al.*, 1996). While there may be discrepancies between the various reports on the effect of β-chemokines on HIV, it is evident that they have significant regulatory effects on virus replication. Further studies are necessary to elucidate the specific mechanisms underlying these observations. Perhaps they will lead to new therapeutic strategies using inexpensive synthetic blockers of the chemokine receptors or ways to regulate the natural production of chemokines in vivo.

Lastly, there is evidence for another cellular HIV fusion domain that binds to envelope gp41 permitting entry of the viral core into host cells. A recent study also identified proteins secreted by $CD8^+$ from AIDS patients that could block the infection of peripheral blood mononuclear cells by both M-tropic and T-tropic isolates (Coakley *et al.*, 2005).

2.4.5 Life cycle of HIV

The life cycle of HIV is invariably the replicative cycle of HIV involving the following stages: viral entry to the cell, viral replication and transcription, and finally,

viral assembly and release. Viral entry begins with binding of HIV to target cells. This process involves interaction between glycoprotein gp 120 on the HIV virion and its receptor, CD4 on the target cell, provokes conformational changes in gp 120, the V3 loop, which binds to a cytokine receptor on the target cell, such as CCR5 or CXCR4 depending on the strain of HIV (Figure 7). Without a co-receptor, fusion will not occur, explaining HIV selectivity for susceptible $CD4^+$ T cells only.

The change in the gp 120's shape also exposes a portion of the gp 41 glycoprotein, which was previously buried in the viral membrane and loosely bound to gp 120. A fusion peptide within gp 41 causes the fusion of the viral envelope and the host-cell envelope, allowing the capsid to enter the target cell. The exact mechanism by which gp 41 causes the fusion is still largely unknown (Chan and Kim, 1998; Wyatt & Sodroski, 1998). Once HIV has bound to the target cell, the HIV RNA and various enzymes, including but not limited to reverse transcriptase (RT), integrase and protease, are injected into the cell.

As the content of the virus enters the host-cell's cytoplasm, molecules of virus-derived enzyme, RNA-dependent DNA polymerase or RT, liberates the single-stranded (+)RNA from the attached viral proteins and copies it into a negatively sensed viral complementary DNA of 9kb pairs (cDNA) (Figure 7). This process of reverse transcription is extremely error prone through substitution of incorrect bases, accounting for high mutation rate of HIV. The reverse transcriptase then makes a complementary DNA strand to form a double-stranded viral DNA intermediate (vDNA). This new vDNA is then transported into cell nucleus. The integration of the proviral DNA into the host

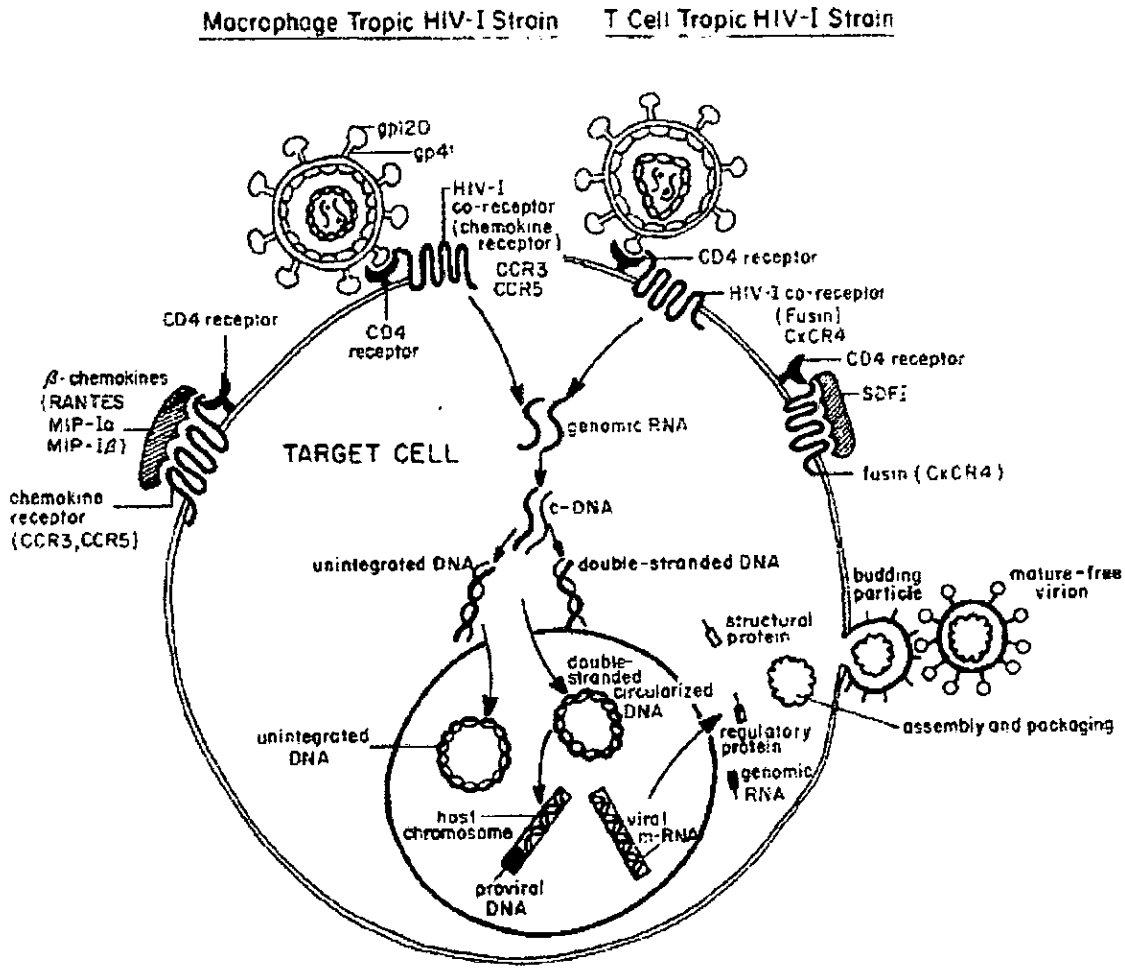


Figure 7: The life cycle of HIV-1

In addition to CD4, the primary HIV-1 receptor on the surface of susceptible cells, fusion and entry of virus is also facilitated by coreceptors. The coreceptor for the T-cell-tropic laboratory strain of HIV-1 is a G-protein-coupled protein called fusin; it also is the CXCR4 chemokine receptor. Primary M-tropic HIV-1 clinical isolates use the chemokine receptors CCR3 and CCR5 as coreceptors. The natural ligands for T- and M-tropic viruses are SDF-1 and the β-chemokines, respectively. By competing for the virus coreceptors, the chemokines block efficient entry of the virus into susceptible cells. After HIV-1 enters the cell, the genomic RNA is reverse transcribed into double-stranded DNA. The DNA is circularized and integrated into the host's genome as a latent provirus. When the latently infected cell is immunologically activated, transcription occurs followed by viral protein synthesis. After intracellular assembly, mature virions are budded from the cell membrane causing lysis of the infected cell.

Source: Schwartz & Nair (!999)

genome is carried out by another viral enzyme called integrase. This is called the latent stage of HIV infection (Zheng *et al.*, 2005).

To actively produce virus, certain transcription factors need to be present in the cell. The most important is called Necrotic factor kappa B (NF-kB) and it is present, once the T cell becomes activated. This means that those cells most likely to be killed by HIV are in fact those currently fighting infection. Initially the integrated provirus is copied to mRNA which is then spliced into smaller chunks. These small chunks produce the regulatory proteins Tat (which encourages new virus production) and Rev. As Rev accumulates it gradually starts to inhibit mRNA splicing (Pollard & Malim, 1998). At this stage the structural proteins Gag and Env are produced from the full-length mRNA. Additionally, the full-length mRNA is actually the virus genome, so it binds to the Gag protein and is packaged into new virus particles.

Interestingly, HIV-1 and HIV-2 appear to package their RNA differently; HIV-1 will bind to any RNA appropriately, whereas HIV-2 will preferentially bind to mRNA only which was used to create the Gag protein itself. This may mean that HIV-1 is better able to mutate (HIV-1 causes AIDS faster than HIV-2 and is the majority species of the virus).

The final step of the viral cycle, assembly of new HIV-1 virions, begins at the plasma membrane of the host cell. The Env polyprotein (gp 160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by protease and processed into the two HIV envelope glycoproteins gp 41 and gp 120. These are transported to the plasma membrane of the host cell where gp41 anchors the gp 120 to the membrane of infected cell. The Gag (p55) and Gag-Pol (p16) polypeptides also

associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. During maturation, HIV proteases (proteinases) cleave the polypeptides into individual functional HIV proteins and enzymes. The various structural components of the virus then assemble to produce a mature HIV virion (Gelderblom, 1997). The virus is then able to infect another cell (Figure 7).

2.4.6 Pathogenesis of HIV infection

Although the course of HIV-1 infection can vary widely from patient to patient, a generalized clinical course (Figure 8) is hereby illustrated. Fifty to 70% of patients develop an acute mononucleosis or flu-like syndrome within 3 to 6 weeks of primary infection, characterized by high levels of viremia and a significant drop in the absolute number of $CD4^+$ cells in the peripheral blood. The acute viremia is followed by activation of $CD8^+$ T cells, which may be a response to control virus replication by cytotoxicity against infected target cells or through production of a soluble mediator that can suppress viral replication.

During this stage, HIV is widely disseminated to the lymph nodes and induces a rapid turnover of infected lymphocytes (Schnittman & Fauci, 1994; Koito *et al.*, 1995). It has been estimated from a mathematical model that the rate of virus production during active infection with HIV-1 is 10.3×10^9 virions per day, and productively infected cells have a life-span of 2.2 days (Feinberg & McLean, 1997; Perelson *et al.*, 1996). The extraordinarily high production of virions and the disproportionate daily loss of $CD4^+$ cells led to what has become known as the "sink model" for CD4+ cell loss (Balter, 1997).

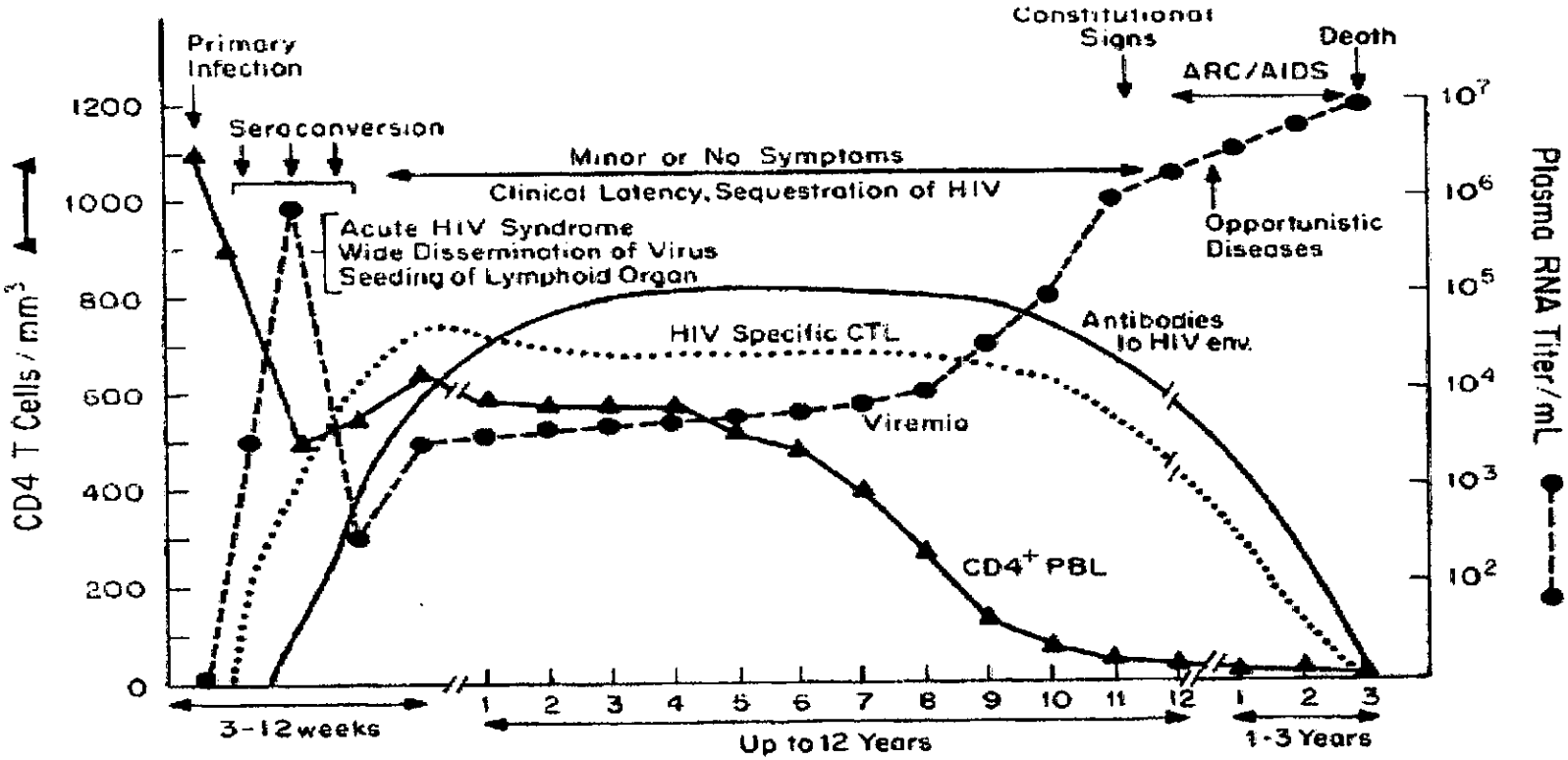


Figure 8: National history of HIV infection Although reliable clinical data are available for only the past 12 years, it is predicted that approximately 95% of HIV-1-infected patients will progressively develop AIDS within 15 years of infection. The first few weeks after primary infection are characterized by an acute mononucleosis or flu-like illness. During this stage seroconversion usually occurs and is associated with a rapid increase in circulating viral titers. This is followed by virus dissemination to lymphoid organs. Adaptive host immunity, including neutralizing antibodies and cytotoxic T lymphocytes (CTL) against virus-infected cells, subsequently develops. While these responses may temporarily control the infection, generally they are unable to eliminate the virus. The patient then enters the stage of clinical latency or the asymptomatic period. A progressive decrease in $CD4^+$ peripheral blood lymphocytes (PBL) continues during the latency stage. When CD4 counts fall below 200/mm^3, the disease usually enters a symptomatic phase, characterized by opportunistic infections and other AIDS-defining conditions.

Source: Adapted from Pantaleo *et al.* (1993)

Infection with HIV-1 induces a number of host responses including polyclonal activation of B lymphocytes, production of neutralizing antibodies, binding of immune complexes to follicular dendritic cells, synthesis and secretion of various cytokines, activation of Th-1 cells, and stimulation of cytotoxic responses including T-cell-, NK cell-, and antibody-dependent cell-mediated activities. While these responses can significantly reduce the viral load in the peripheral blood, generally they are unable to completely clear the infection. This early phase of HIV infection is followed by an intermediate stage or clinical latency that can last for several years (up to 12 years in some patients) and is characterized by gradual deterioration of immune responses and depletion of peripheral CD4+ cells.

Although the level of infectious virus in the peripheral blood is usually relatively low during this period, viral titres do not reach a peak and stay at a nadir as previously thought. Rather, they rise steadily as the absolute number of peripheral $CD4^+$ T cells decreases. Viral replication continues at a very high rate in the lymph nodes, resulting in partial disruption of the germinal centres and the follicular dendritic cells. This is followed by massive production of cytokines such as tumour necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-6 (IL-6) and a shift in the predominance of Th-2 over Th-1 responses. A decline of $CD4^+$ T cells below an absolute count of 50/ml is associated with a significant deterioration of cell-mediated immunity.

The terminal stage of HIV infection is marked by a complete collapse of the immune system. This is accompanied by various AIDS-defining illnesses, including constitutional signs and symptoms, cachexia, dementia, a variety of opportunistic infections, and/or neoplasia. Total disruption of the germinal centres and complete

disintegration of the follicular dendritic cells of the lymph nodes and the release of free virions into the circulation may be seen at this point.

Earlier studies implicated a variety of mechanisms in the progressive depletion of the absolute number of $CD4^+$ cells in the peripheral blood of patients with HIV infection. These included syncytium formation between infected and uninfected $CD4^+$ cells, selective infection or destruction of memory T cells, killing of uninfected cells by an autoimmune mechanism, Fc receptor-mediated antibody-dependent cellular cytotoxicity, and cell-mediated destruction of HIV-infected cells by cytotoxic T lymphocytes and/or NK cells. More recently another mechanism, programmed cell death or apoptosis, has been proposed to account for some of the $CD4^+$ cell depletion observed in the peripheral blood of AIDS patients (Gouen & Montagnier, 1993).

Apoptosis is a physiological, cellular suicide mechanism in which cell death occurs naturally during the maturation of specific tissues or organs. Apoptosis is characterized by several morphologic and biochemical features including cell shrinkage, condensation of chromatin, and cleavage of DNA into oligonucleosome-sized (180- to 200-bp) fragments. These oligonucleosome fragments are the basis of the so-called DNA ladder formation on agarose gel electrophoresis that is a characteristic of apoptosis. Apoptosis is not restricted to only $CD4^+$ cells in HIV infection: $CD8^+$ T lymphocytes and B lymphocytes also undergo apoptosis (Muro-Cacho *et al.*, 1995).

In HIV infection, apoptosis can be activated directly or indirectly. In indirect killing, CD4 molecules on uninfected cells can be cross-linked with gp120 of the virus envelope. This can prime the cell to undergo DNA fragmentation and subsequent programmed cell death when it encounters major histocompatibility complex (MHC) class II molecules on homologous cells or superantigens derived

from various micro organisms. Regarding direct induction of apoptosis, HIV-infected cells can induce this process in uninfected cells. The activation of various genes, including fas/ApoI (CD95), Bcl-2, several protooncogenes, and tumour suppressor genes, and cytokines and transcription factors are involved in apoptosis. In HIV infection, apoptosis regulated by the expression of *fas–Bcl-2* has been associated with the depletion of the $CD45RO^+$ T cell subset (Boudet *et al.*, 1996).

Several functional subpopulations of $CD4^+$ T-helper (Th) lymphocytes have been described including Th-1, Th-2, $CD45RA^+$ (naive), and $CD45RO^+$ (memory) cells. Activated Th-1 cells produce cytokines, such as gamma interferon, IL-2, and IL-12 that facilitate cell-mediated immunity. However, cytokines produced by Th-2 cells, such as IL-4, IL-5, IL-6, and IL-10, enhance humoral immune responses. Recent evidence suggests that a shift in $CD4^+$ subpopulations from Th-1 to Th-2 predominance is associated with disease progression and clinical deterioration of HIV-infected patients (Clerici & Shearer, 1992).

This hypothesis is supported by an animal model wherein mice lacking a Th-2 response resist the development of a murine form of AIDS. While this theory of Th-1 to Th-2 cell switching is not accepted by all AIDS researchers, clinical trials are under way using exogenously administered cytokines, such as IL-2, to expand the pool of $CD4^+$ T cells and their repertoire of T-cell receptors. Although $CD45RA^+$ and $CD45RO^+$ markers are down regulated in HIV-1 infection, (Bruunsgaard *et al.*, 1995) an increase in the $CD45RO^+$ subset of both $CD4^+$ and CD8+ T cells has been associated with progression of disease (Benito *et al.*, 1997)). HIV has been shown to preferentially infect the RO^+ (memory) subset of $CD4^+$ lymphocytes (Helbert *et al.*, 1997; Spector *et al.*, 1994).

2.5 CLINICAL SPECTRUM OF HIV INFECTION/ AIDS

The clinical expression of HIV infection is not only very diverse, but may also vary in different populations according to the relative frequency of other endemic, potentially opportunistic infections. Different classification systems for HIV-related illnesses have been proposed, e.g., the Walter Reed classification system and the Centres for Disease Control (CDC) classification system (Tables 1-4) (CDC, 1987; 1992). Patient with AIDS-related complex (ARC) are included in group IV or categories B (Table 2) of CDC classification system. Different definitions of ARC have been proposed, but most of these require AIDS-type abnormalities to be shown in laboratory tests - such definitions may not be applicable in some African countries, where laboratory facilities are often lacking.

Patients with AIDS and ARC have similar symptoms and signs, but immunological defects are less severe in ARC. Signs and symptoms observed in ARC patients include unintentional weight loss, malaise, fatigue and lethargy, anorexia, abdominal discomfort, diarrhoea, fever, night sweating, headache, amenorrhoea, popular pruritic rash, lymphadenopathy, and splenomegaly (Benenson 1990; Stewart, 1993, Castro *et al.*, 1992). These symptoms and signs are frequently intermittent, disappearing spontaneously for certain periods. By definition, ARC patients do not have opportunistic infections or malignancies (Stewart, 1993). The AIDS is at the most severe end of the clinical spectrum of HIV infection (CDC, 1985). It is characterized by the presence of opportunistic infections and tumours as a result of profound cellular Immunodeficiency.

The CDC classification system is valuable for epidemiological purposes, but is less helpful for patient management since it does not put HIV-related clinical

Table 10: 1993 Revised Classification System for HIV Infection and Expanded AIDS Surveillance Case Definition for Adolescents and Adults.

	Clinical Categories [b,c]		
$CD4^+$ T-cell Categories[a]	**A Asymptomatic, Acute (primary) HIV or PGL[d]**	**B Symptomatic, not (A) or (C) Conditions**	**C AIDS-Indicator Conditions[e]**
1. ≥500/μl	A1	B1	**C1**
2. 200-499/μl	A2	B2	**C2**
3. <200/μl AIDS-indicator T-cell count	**A3**	**B3**	**C3**

[a] See Table 4

[b] See Table 2

[c] The shaded cells illustrate the expanded AIDS surveillance case definition. Persons with AIDS-indicator conditions (category C) as well as those with CD4+ T-lymphocyte counts <200/μl (categories A3 or B3) were reportable as AIDS cases in the United States and territories effective January 1, 1993.

[d]PGL, persistent generalized lymphadenopathy. Clinical category A includes acute (primary) HIV infection.

(Source: Levy, 1994)

Table 11 Clinical Categories

CATEGORY A

- Category A consists of one or more of the conditions listed below in an adolescent or adult (≥13 years) with documented HIV infection. Conditions listed in categories B and C must have occurred.
- Asymptomatic HIV infection
- Persistent generalised lymphadenopathy
- Acute (primary) HIV infection with accompanying illness or history of acute HIV infection.

CATEGORY B

Category B consists of symptomatic conditions in an HIV-infected adolescent or adult that is not included among conditions listed in the clinical category C and that meet at least one of the following criteria: (1) the conditions are attributed to HIV infection or are indicative of a defect in cell-mediated immunity, or (ii) the conditions are considered by physicians to have a clinical course or to require management that is complicated by HIV infection. Examples of conditions in clinical category B include, but are not limited to,

- Bacillary angiomatosis
- Candidiasis, oropharyngeal (thrush)
- Candidiasis, vulvovaginaliasis; persistence, frequent, or poorly responsive to therapy
- Cervical dysphasia (moderate or severe)/cervical carcinoma in situ
- Constitutional symptoms, such as fever (38.5°C) or diarrhoea lasting > 1 month
- Hairy leukoplakia, oral
- Herpes Zoster (shingles), involving at least two distinct episode or more than one dermatome
- Idiopathic thrombocytopenic purpura
- Listeriosis
- Pelvic inflammatory disease, particularly if complicated by tubo-ovarian abscess
- Peripheral neuropathy

For classification purposes, Category B conditions take precedence over those in category A. For example, someone previously treated for oral or persistent virginal candidiasis (and who has not developed category C disease) but who is now asymptomatic should be classified in clinical category B

(Source: Levy, 1994)

Table 12: Conditions Included In the 1993 AIDS Surveillance Case Definition

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CATEGORY C

Category C includes the clinical conditions listed in the AIDS surveillance case definition Table 3. For classification purposes, once a category C condition has occurred the person will remain in category C.

- Candidiasis of bronchi, trachea, or lungs
- Candidiasis, oesophageal
- Cervical cancer, invasive*
- Coccidioidomycosis, disseminated or extrapulmonary
- Cryptosporidiosis, chronic intestinal (> 1 month's duration)
- Cytomegalovirus disease (other than liver, spleen, or nodes)
- Cytomegalovirus retinitis (with loss of vision)
- Encephalopathy, HIV-related
- Herpes simplex: chronic ulcer(s) (> 1 month's duration);
- or bronchitis, pneumonitis, or esophagitis
- Histoplasmosis, dissemination or extrapulmonary
- Isosporiasis, chronic intestinal (> 1 month's duration)
- Kaposi's sarcoma
- Lymphoma, Burkitt's (or equivalent term)
- Lymphoma, immunoblastic (or equivalent term)
- Lymphoma, primary, of brain
- *Mycobacterium* tuberculosis, any site (pulmonary* or extrapulmonary)
- *Mycobacterium*, other species or unidentified species, disseminated or extrapulmonary

* Added in the 1993 expansion of the AIDS surveillance case definition.

(Source: Levy, 1994)

Table 13: Other Definitions in HIV Infection – $CD4^+$T- Lymphocyte Categories

CD4 T-cell category	$CD4^+$ T cells per μl	$CD4^+$ percentage
1	≥500	≥29
2	200-400	14-28
3[a]	<200	<14

[a]Diagnosis of AIDS

Source: Levy (1994)

manifestations in well defined groups according to prognosis (Berkley *et al.*, 1989). The Walter Reed classification system is probably a better prognostic staging system; however it relies heavily on results of lymphocyte typing and skin testing, which either cannot be performed in most African countries or are difficult to interprete. Ideally, it would be useful to have a staging system for HIV that refers only on clinical symptoms and signs, and on easily diagnosable HIV-related diseases.

The WHO addressed these issues during a consultation on staging criteria of HIV infection, convened in Geneva on 24th—26th July 1989. As a result of that consultation, a draft proposal for clinical staging of HIV infection was developed and a preliminary validation exercise was conducted to assess the feasibility of the proposed system (Piot *et al*, 1992). Since the use of survival time as validation criterion would have required a long prospective study, it was decided first to conduct a world wide cross-sectional study in which clinical conditions were correlated without laboratory markers already known to reflect disease progression (particularly $CD4^+$ counts), using the latter as surrogates for survival. This validation exercise involved data on 907 HIV-antibody-positive patients collected in 26 clinical centres representing all parts of the world. The result of this validation exercise were reviewed by a technical working group that met in Geneva on 21st-23rd February, 1990, and the present proposal was developed. Therefore, a list of clinical markers of prognostic significance was assembled hierarchically into the following four prognostic categories:

1. Asymptomatic/Persistent Generalized Lymphadenopathy (PGL)
2. Early (Mild) Disease.
3. Intermediate (Moderate) Disease and
4. Late (severe) Disease (basically equivalent to AIDS).

In addition, the following performance scale (a modification of Eastern Co-operative Oncology Group Score) was incorporated into the system.

1. Asymptomatic Normal Activity;
2. Symptomatic, normal activity
3. Bedridden < 50% of the day; and
4. Bedridden > 50% of the day

The proposed clinical staging system for HIV infection and AIDS disease is primarily based on clinical criteria. Symptoms, signs, and diseases should be defined according to medical judgement. Patients who should be confirmed HIV-antibody-positive and 13 years of age or older, are clinically staged (category 1-4) on the basis of the clinical condition or performance score (Table 5).

A further refinement of the system would include, in addition to the "clinical axis", a laboratory axis. The laboratory axis, if available, will subdivide each clinical category into 3 strata (A, B, C), depending on the number of $CD4^+$ lymphocytes per mm^3 (>500, 200-500, <200). If $CD4^+$ count are not available, total lymphocytes should be explored as an alternative laboratory marker, also in 3 different strata (2,000; 1000-2,000; <1,000). If laboratory values are not available, patients could be classified as 1x, 2x, 3x, or 4x or simply as 1,2,3, or 4. An advantage of the system is that it provides useful reference framework for comparing results of investigations in different parts of the world, at the same time stimulating research needed to understand their prognostic significance in the absence of definitive information on the prognostic value. Another advantage is the unnecessary call for re-classification of the patients when additional laboratory information is obtained.

Table 14: Proposed Clinical Staging System for HIV Infection and Disease.

Clinical Stage 1:

1. Asymptomatic.
2. Persistent generalised lymphadenopathy (PGL)

Performance scale 1: asymptomatic normal activity.

Clinical Stage 2:

3. Weight loss <10% of body weight
4. Minor mucocutaneous manifestations (seborrhoea dermatitis, prurigo, fungal nail infections recurrent oral ulceration, and angular cheilitis).
5. Zoster within the last 5 years
6. Recurrent upper respiratory tract infections (bacterial sinusitis).

And/or Performance scales 2: symptomatic, normal activity

Clinical Stage 3:

7. Weight loss > 10% of body weight.
8. Unexplained chronic diarrhoea for more than 1 month.
9. Unexplained prolonged fever (intermittent or constant), for more than 1 month
10. Oral candidiasis (thrush)
11. Oral hairy leukoplakia
12. Pulmonary tuberculosis within the past year
13. Severe bacterial infections (pneumonia, pyomyositis)

And/or Performance scale 3 bedridden <50% of the day during the last month.

Clinical Stage 4:

14. HIV wasting syndrome, as defined by CDC
15. *Pneumocystis carinii* pneumonia
16. Toxoplasmosis of the brain
17. Crptosporidiosis with diarrhoea for more than 1 month
18. Cryptococcosis, extrapulmonary.
19. Cytomegaloviruc (CMV) disease of an organ other than liver, spleen, or lymph nodes.
20. Herpesvirus infection, mucocutaneous for more than 1 month, or visceral any duration.
21. Progressive multifocal leukoencephalopathy (PML)
22. Any disseminated endemic mycosis (Histoplasmosis, Coccidioidomycosis).
23. Candidiasis of the oesophagus, trachea, or lungs
24. Atypical mycobacteriosis disseminated.
25. Non-typhoid salmonella septicaemia.
26. Extrapulmonary tuberculosis.
27. Lymphoma.
28. Kaposi's sarcoma.
29. HIV Encephalopathy, as defined by CDC

And/or Performance scale 4: bedridden >50% of the day during the last month.

Note: Both definitive and presumptive diagnosis is acceptable

(Source: Piot *et al.*, 1992)

HIV seroconversion illness

Early infection with HIV, an acute infectious mononucleosis-like illness may occur with a variety of symptoms and signs, including lethargy, malaise, fever, sore throat, myalgia, anorexia, sweating, arthralgia, headache, diarrhoea, nausea, generalized lymphadenopathy, a macular eruption involving the trunks and arms and thrombocytopenia (Fauci, 1988; Benenson, 1990; Piot *et al.*, 1992). Occasionally, patients may develop a transitory aseptic meningo-encephalitis, or polyneuritis. The acute illness generally occurs 1-30 days after exposure and resolves in 3-21 days, but conflicting data have been reported concerning the proportion of HIV-infected individuals who developed clinical manifestations during sero-conversion. Examination of the blood reveals a temporary reduction in $CD4^+$ (and $CD8^+$) T cell count, followed by a predominant or transient $CD8^+$ lymphocytosis (Haseltine, 1991).

Asymptomatic infection

With few exceptions, the patients recover from the primary (sero-conversion) illness within 2-3 weeks, and the majority go on to enjoy at least 5 years of relatively good health (Benenson 1990). Persistent generalized lymphadenopathy (PGL), otherwise known as lymphadenopathy syndrome (LAS), may or may not become apparent towards the end of this period and does not have any clear prognostic relevance (Gruter *et al.*, 1991, Fauci, 1993). Reflecting the polyclonal activation of the immune system, autoimmune conditions may occur during this period (Clumeck *et al.*, 1984). These diseases include the Guillan-Barre syndrome, chronic demyelinating neuropathy, idiopathic thrombocytopenia, Reiter's syndrome, polymyositis, cranial nerve palsy, and Sjogren's syndrome. Also during this phase, the patient is probably infective, since HIV can be isolated from peripheral blood lymphocytes e.g., PBMC. Immunoglobulin G (IgG) antibody to HIV can be demonstrated in serum and in some

cases, in cerebrospinal fluid CSF. Immunological tests show that the numbers of lymphocytes subpopulations and the function of these lymphocytes are normal (Levy, 1993).

Symptomatic HIV infection

The most common clinical manifestations in patients with HIV infection are weight loss and weakness. The major groups of clinical manifestations are systemic manifestations, cutaneous manifestations, gastrointestinal manifestations, respiratory manifestations, neurological manifestations, lymphadenopathy, haematological manifestations, renal manifestations, cardiac manifestations, manifestations in the reproductive system, adrenal insufficiency, hepatic disease and arthritis (Colebunders *et al.*, 1987; Levy, 1993).

Systemic manifestations: Weight loss is often the first sign of HIV infection, in some patients this may be the only sign initially (Kotler *et al*, 1985; Benenson, 1990; Macallan *et al* 1993). After being treated for an intercurrent infection, some patients gain weight for a certain period, but generally such periods do not last long. Accelerated weight loss in an HIV-infected patient is a sign of disease progression (Fauci, 1993). Some AIDS patients lose over 40% of their body weight, and most of them die in extreme cachexia. Weakness (asthenia) and loss of appetite (anorexia) are very frequently present, but can be absent even in the presence of severe weight loss (Colebunders *et al.*, 1987; Berkley *et al.*, 1989; Chlebowski *et al.*, 1989). Approximately 50% of AIDS patients have episodes of fever lasting more than 1 month (Colebunders *et al.*, 1987).

Cutaneous manifestations: Many patients with HIV infection have cutaneous lesions. Since some of these manifestations (particularly in combination with other HIV- related signs and

symptoms) are fairly specific for HIV infection, recognition may lead to an early diagnosis of AIDS (Golden *et al.*, 1992; Herndier *et al.*, 1994; Durden and Elewiski, 1997). Other skin lesions are often non-specific and, importantly, may be a sign of systemic disease. Diagnosis is often impossible without scrapping or biopsy for microbiological and histopathological examination. The skin of AIDS patients may become dry and atrophic. The hair may become thin and lose its colours and natural curly architecture.

Gastro-intestinal manifestations: Along with the CNS disorders observed during some acute HIV infections, bowel symptoms have been reported soon after infections, but subside (Tindall and Cooper, 1991). The subsequent chronic malabsorption and diarrhoea, occurring several years, have generally been attributed to the opportunistic infections in the bowel as a result of immune deficiency (Kotler *et al.*, 1984; Sharpstone and Gazzard, 1996). However, HIV might be directly involved and therefore portrays the clinical symptoms and signs. Persistent diarrhoea lasting more than 1 month is one of the major complaints of AIDS patients, occurring in about 40% of cases; colitis which may be due to invasive infection with CMV or ulcerative Kaposi's sarcoma (KS) of the colon, may cause severe abdominal cramps and distension; while oesophagitis or dysphagia (oesophageal disorder) is a common complaint of patients with HIV infection.

Respiratory manifestations: Persistent cough of more than 1 month's duration is found in approximately one-third of African AIDS patients. It may be due to pulmonary tuberculosis (Johnson & Sonnenberg, 1990) which occurs particularly frequently in African patients with HIV infection. In patients with *Pneumocystis carinii* pneumonia (Serwadda *et al.*, 1989), the cough is generally non productive and may be accompanied by dyspnoea. Atypical mycobacteria, *Cryptococcus*

neoformans, Candida albicans, Streptococcus pneumonia, and *Haemophilus influenzae* may also occasionally cause pneumonia in AIDS patients. Cough may be caused by lymphoid intestinal pneumonitis, but this condition occurs mainly in HIV-infected children (Joshi, 1996). Haemoptysis and pleural effusion are mainly caused by tuberculosis or Kaposi's sarcoma (Mugerwa *et al.*, 1996). An accurate diagnosis of respiratory disease in AIDS patients is useful since many causes are treatable. Investigations should include a chest X-ray and Ziehl-Neelsen [Acid fast Bacilli (AFB)] staining of sputum.

Neurological manifestations: Central nervous system (CNS) manifestations may result from opportunistic infections or from primary involvement by HIV of the brain, spinal cord or peripheral nerves (Micheals *et al.*, 1988, Price *et al.*, 1988). Though CNS infections including syphilis, bacterial meningitis, brain abscesses, and tuberculosis are seen in HIV-infected individuals, cryptococcal meningitis and toxoplasmosis predominate (Lucas *et al.*, 1988). An increasing number of neurological abnormalities are being documented in patients with HIV infection (Piot *et al.*, 1992). They may be the initial manifestation of HIV disease and are often atypical in presentation. About 30% of AIDS patients suffer from prolonged episodes of headache (Merrill, 1992). Sangster (1997) reported that a major segment of acquired Immunodeficiency syndrome (AIDS) patients suffer from neurological complications, including impairments in concentration and motor function. Chiodi *et al.*, (1996), also stated that a variety of neurological syndromes are observed in HIV-1 infected patients, of which the most frequent is a dementia-like syndrome. Neurological disorders that are probably caused by HIV include encephalitis with progressive pre-senile dementia, acute and chronic meningitis vacuolar myelopathy peripheral neuropathy, and polymyositis (Chiodi *et al.*, 1996). Other neurological

symptoms include AIDS encephalopathy (Price *et al.*, 1988), motor and sensory symptoms, (Chiodi *et al.*, 1996), focal cerebral lesions (Lucas *et al.*, 1991), meningeal signs (Mugerwa *et al.*, 1996) and autonomic nervous system abnormalities (Chiodi et *al.*, 1996).

Lymphadenopathy: Persistent generalized lymphadenopathy (PGL) is defined as enlargement of lymph nodes to more than 1 cm in diameter, involving one or more extra-inguinal sites, of at least 3 months duration and in the absence of any current illness or drug use known to cause lymphadenopathy (Tenner-Racz *et al.*, 1985). More than 50% of HIV-infected patients develop polyadenopathy. This may occur within months of acquisition of infection and lymph nodes may persist unchanged for several years. During the progression of HIV illness, lymphadenopathy may slowly regress and even disappear when the AIDS stage is reached (Racz *et al.*, 1986). Enlarged lymph nodes are detectable in 10-20% of patients within full-blown AIDS in Africa (Piot *et al.*, 1992). Lymph node biopsy is indicated only when the lymph nodes are abnormally large or have an unusual consistency when there is marked unilateral enlargement, or in the presence of fever of unknown origin (Tenner-Racz *et al.*, 1985; Racz *et al.*, 1986). The histology of lymph nodes from patients within PGL is characterized by hyperplasia of the germinal centres, with preserved structure of the lymph node. Regression or involution of the follicular structure and even marked lymphocyte depletion may be observed, particularly in patients whose clinical condition is progressing towards AIDS (Racz *et al*, 1986).

Oral manifestations: Oral manifestations include oral candidiasis (thrash), Herpesvirus ulceration, Kaposi's sarcoma lesions, hairy leukoplakia, rapidly progressive periodontal disease, gingivitis, persistent or recurrent Aphthous-types ulcer, trigerminal Zoster, and salivary gland enlargement, often with xerostomia and

warts caused by Papillomavirus (Piot *et al.*, 1992). Oral hairy leukoplakia presents as slightly raised, poorly demarcated lesions with a corrugated or "hairy" surface on the lateral borders of the tongue. The lesions may extend onto the central and dorsal surfaces of the tongue and usually produce no symptoms and may be associated with Epstein-Barr (E-B) virus infection (Schiodt *et al.*, 1990). While periodontal disease is characterized by gingival recession with marked gingival inflammation and rapid alveolar bone loss, symptoms of acute neurotizing gingival include painful erythematous swollen gingival, spontaneous or provoked gingival bleeding and necrosis of inter dental papillae with greyish necrotic slough (Sewankambo *et al.*, 1987).

Haematological manifestations: Anaemia is often present generally in advanced stages of HIV illness. Several conditions may contribute to anaemia in HIV-infected patients' concurrent infections: malignancies, malnutrition, and toxic drug treatments. However, in most HIV-infected patients, the cause of anaemia remains unclear. In AIDS patients, leukopenia and lymphopenia are also common and marrow hyperplasia with concomitant peripheral cytopenia is often found (Stanley *et al.*, 1992).

Renal manifestations: AIDS-associated nephropathy is a renal syndrome characterized by proteinuria and glomerulosclerosis (Wamukota and Lucas, 1986). The clinical presentation includes the nephritic syndrome with or without renal insufficiency as well as rapid deterioration of renal function, leading to end-stage renal disease within a few weeks.

Cardiac manifestations: Congestive cardiomyopathy may occur during HIV infection (Piot *et al*, 1992). The most common cardiac finding in Africa is pericardial effusion, often due to tuberculosis (TB). The contribution of HIV and HIV-related

viruses and cancers, as well as Toxoplasmosis to cardiac morbidity needs further evaluation (Mugerwa *et al*, 1996). Echocardiography features myocardial dysfunction simulating myocarditis, or dilated cardiomyopathy are common but endocarditis is rarely observed in HIV patients.

Hepatitis diseases: Liver disease occurs frequently in patients with AIDS (Siddiqui, 1983). Intra hepatic opportunistic infections or malignancies are found in 33 - 78% of post-mortem examinations. In general, hepatic involvement represents part of a disseminated disease process and there seems to be no pathognomonic lesion of the liver related to HIV infection.

Arthritis: A sub-acute oligoarthritic syndrome, vasculitis, polymyositis, Sjogren's syndrome, Reiter's syndrome and systemic lupus erythematosus (SLE) associated with HIV infection have been reported (Valeriano-Marcet *et al.*, 1990; Calabresse *et al.*, 1991). The cause of the arthritis is unknown, but HIV has been isolated from the synovial fluid.

Clinical presentation in children: Acquired immune deficiency syndrome (AIDS) was first described to occur in children in 1983 (Sharer *et al.*, 1985; Gutierrez-Ortega *et al.*, 1988). With experience of increasing number of cases of AIDS (Peterman *et al.*, 1993), pathologic lesions in various organs and tissues such as lungs, brain, gastrointestinal tract, heart, blood vessels, lymph nodes, spleen, bone marrow, etc., become evident in autopsy and biopsy specimens. These pathologic lesions were classified into four groups based on known or suspected pathogenesis namely: Primary lesions due to HIV infection itself (lymph nodes, brain, etc.), associated lesions related to direct or indirect sequelae of HIV infection (opportunistic infection,); lesions of undetermined pathogenesis (cardiomyopathy, arteriopathy, thrombocytopenia, nephropathy, etc.); and lesions of multifactorial pathogenesis

(villus atrophy of intestine, thymic lesions, etc.). More recent disorders are the reactive and neoplastic proliferative disorders. These disorders include nodal and extranodal lymphoproliferative lesions, smooth muscle tumours (SMTs), Kaposi's sarcoma, Human Papilloma virus-associated genital lesions and miscellaneous tumours (Joshi, 1996). In a recent study, it has been shown that Epstein-Barr virus (EBV) may be related to the pathogenesis of SMTs. The most recently recognized lymphoproliferative lesions include those of mucosa-associated lymphoid tissue of salivary glands, lungs and tonsils, which are of practical importance as distinctive lesions in paediatric AIDS (Joshi, 1996).Mortality among infants born to HIV infected mothers remains high in Africa due to lack of medical facilities for management of cases (Colebunders *et al.*, 1987). The WHO case definition for clinical AIDS, has been of less value in paediatric HIV infection. Infants with a persistent diarrhoea, prolonged fever, hepatosplenomegaly, malnutrition or TB may be incorrectly considered as infected (Akpede *et al.*, 1997) while many children who die with HIV-related illness do not meet the criteria (Tindyebwa *et al.*, 1993; 1994).

2.5.1 Immunopathology of HIV infection

Early after HIV infection, enlargement of the lymph nodes, described as lymphadenopathy, frequently develops and may persist for a long time. The enlarged lymph nodes show typical atrophy, with marked depletion of $CD4^+$ cells and destruction of follicular dendritic cells (FDCs) resulting either from direct virus infection (Racz *et al.*, 1986, Spiegel *et al.*, 1992, Fauci, 1993a), or from cytokine production by cells activated in response to increased virus replication (Emilie *et al*, 1990). At the late stage, lymphoid tissues therefore decrease in number and the architecture of the FDC network breaks down to leave an involuted and scarred lymph node (Mangkornkanok - Mark *et al.*, 1984). A loss of the FDC reduces efficient

antigen presentation and the ability to generate strong antiviral immune responses (Takahashi *et al.*, 1993), which results in the manifestation of many of the features of HIV- related immunosuppression.

Since HIV disturbs the balance of the immune system, it is not surprising that anti immune disorders (e.g. Reiter's syndrome (RS), systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), vasculitis, and polymyositis) are described as accompanying this viral infection (Ziegler and Stites, 1986; Valeriano-Marcet *et al.*, 1990; Calabrese *et al.*, 1991). In some early studies of AIDS patients, antibodies, often associated with clinical disorders, were detected against platelet, T cells and peripheral nerves (Morrow *et al.*, 1991). A few years back, auto antibodies to a large number of normal cellular proteins have been reported in HIV infected individuals (Bloom *et al.*, 1986; Weimer *et al.*, 1991; Muller *et al.*, 1993). The mechanisms of antibody production involve processes besides T-cell dysregulation, B cell activation and molecular mimicry as well as the roles of carrier-hapten and anti-idiotype antibodies.

As a result of immune responses of the host to the presence of HIV in the body system, whether shortly after infection or as the infection progresses to AIDS, a number of different classes of antibodies that are directed against HIV viral proteins were produced. These antibodies can be detected by a variety of techniques, which ushers a way out of many predicaments in the laboratory diagnosis of HIV infection.

2.6 LABORATORY DIAGNOSIS OF HIV INFECTION

Laboratory diagnosis of HIV infection is usually made by the detection of virus - specific antibody, and the technique remains central to the identification of virus-infected person. Sometimes, procedures for detecting circulating virus antigen

or genome and, rarely, virus isolation, may be used in conjunction with antibody detection. Before any laboratory diagnosis can take place, collection and storage of specimen are paramount in priority.

2.6.1 Collection and Storage of Specimens & Laboratory Safety

Blood samples (Pan *et al.*, 1993) routinely collected by veni-puncture, are generally used for viral detection and serological analysis. To isolate virus from peripheral blood mononuclear cells (PBMCs) (Levy, 1994), heparinized or Ethene - diamine tetracetic acid (EDTA) treated blood samples must be collected. HIV can also be detected in and isolated from other body fluids such as plasma (Spear, 1993), serum (Michealis and Levy, 1987), cerebrospinal fluid (Cheng-Mayer and Levy, 1988), saliva (Goto *et al.*, 1991), tears (Fujikawa *et al.*, 1985), milk (Ruff *et al.*, 1994), urine (Li *et al.*, 1990), genital secretions (Anderson *et al.*, 1991; Henin *et al*, 1993) and from biopsy specimens of infected tissues such as the bowel (Asmuth *et al.*, 1994; Batman *et al.*, 1994). Even though the amount of virus present in some biopsy may be low, precautions should be taken when handling any potentially HIV- infected clinical specimens. Gloves should be worn, and needles should be handled by safe procedures after collection of blood.

Plasma, serum or other body fluids to be tested serologically should be stored at -20^{o} or -70° C. For best results, virus isolation should be performed immediately after specimen collection. Nevertheless, PBMCs and other infected cells may be stored in liquid nitrogen in culture medium plus 10% dimethyl sulphoxide (DMS0) with reasonable recovery of virus from the PBMCs in most cases (Barker & Barnett, 1994).

Laboratories involved in HIV antibody testing should use biosafety level 2 standards. Clinical specimens from all individual should be considered infectious so as to avoid the confusion of having certain specimens handled differently. A class II type A biosafety cabinet with HEPA-filtered air should be employed for viral isolation work; gloves (two pairs) and a surgical gown with sleeve cuffs that can be tucked into gloves should be worn (Barker & Barnett, 1994). Because the levels of free virus in body fluids including blood are very low, transmission via aerosol from primary specimens is unlikely. However, when large quantities of virus are being grown, when concentrated viral preparations are being used, and when procedures that may produce droplets or aerosols are being performed, biosafety level 2 and 3 standards should be employed. In a laboratory, the following precaution should be taken:

1. All specimens should be stored in well-constructed containers with secure lids that prevent leakage during transport.
2. Persons processing, primary specimens or culturing virus should wear gloves, gowns (laboratory coats), surgical masks, and protective eyewear.
3. The generation of droplets, aerosols, and spills should be avoided, easily avoidable with the use of safety cabinet.
4. Mechanical pipetting devices should be used for manipulating fluids in the laboratory, and also for avoiding cross-contamination of specimen or even contamination of pipetting devices using cotton plugged pipettes.
5. The use of needles and glassware should be restricted to situations for which there are no alternatives, but needles and glass should be disposed of in closed puncture-proof containers.

6. Laboratory work surfaces and materials (including contaminated materials) should be decontaminated with an appropriate germicide after a spill and when work is completed.

7. Hand should be washed with soap and water immediately after infectious materials have been handled and after work is completed, even when gloves have been worn, should be a routine practice.

HIV can be readily inactivated under clinical laboratory conditions by several methods (Putkonem *et al.*, 1991). Although relatively stable in a dry or lyophilized state, the virus is very sensitive to many detergents (For example, Triton X and Nonidet P-40 but not Tween 20), including soap, and can be eliminated rapidly by bleach [0.5 % sodium hypochlorite ($NaOCl_3$)]. Alcohol and acetone - alcohol mixture (> 70%) can also inactivate virus effectively and efficiently at room temperature. HIV strains are also sensitive to iodophores such as organic iodine compounds, pH extreme (pH 2, pH12), ultraviolet (UV) and X irradiation, and heating in liquid solution at 56^{o}C. For inactivation of virus in serum prior to serological testing, specimens should be routinely heated at 56^{o}C for 30 minutes (Baker & Barnett, 1994).

There are several distinct situations in which laboratory diagnosis of HIV infection may be required: (1) Diagnosis of infection in an individual with or without symptoms; (2) monitoring disease progression, with or without antiviral therapy; (3) screening of blood and organ donors, voluntary screening of individuals considered to be at risk, and sero-epidemiological surveys; and (4) evaluation of new antivirals or vaccines. The preferred methodology differs, depending on the particular objective.

2.7 SEROLOGICAL ASSAYS TO DETECT HIV INFECTION

Detection of HIV antibodies is still the most efficient and most common way to determine whether an individual has been exposed to HIV and to screen blood and blood products for this infectious agent (Waldman & Calmann, 1986; Baker & Barnett, 1994). A test is considered positive when assays such as the enzyme - linked immunosorbent assay (ELISA) or Enzyme immuno assay (EIA), indirect immuno florescence assay (IFA), and Western blots (immunoblots) are considered reactive (Gurtler, 1996). A positive test result indicates exposure and, outside of the perinatal and neonatal periods, is presumed to indicate infection by the virus (i.e. HIV-1, HIV-2 or both).

2.7.1 Enzyme-linked Immunosorbent Assay (ELISA) or Enzyme Immunoassay (EIA)

The ELISA or EIA is the assay most commonly used to screen for HIV because of its relatively low cost, standardized procedure, high reliability, and ability to give rapid result. The sensitivity of the test is ranged from 93% to 100%. Under optimal laboratory conditions, the sensitivity and specificity in most cases are > 99% (Waldman & Calmann, 1986). Commercial ELISA kits are available for both routine diagnosis and research.

There are three types of ELISA: indirect ELISA, competitive ELISA & double antigen ELISA. There are also a number of generations of ELISA available with respect to trends in assays' development (Constantine, 1999). The first generation assay utilized purified HIV lysates but its disadvantage is that it often lacked sensitivity and specificity. The second generation ELISA kits rapidly became available to improve on the first based on recombinant proteins and/or synthetic

peptides, which also enabled the production of combined HIV-1/HIV-2 assays (Waldman and Calmann, 1986).

Another generation of sandwich ELISA, (so-called third generation), uses labelled antigen as conjugate, are extremely sensitive and has reduced the window period considerably (WHO, 2002). The latest generation of ELISA has produced the "combination assays" which combine p24 antigen ELISA with the traditional antibody ELISA, allowing for simultaneous detection of HIV antigen and antibodies using a single test (Feinberg, 1996; WHO, 1999). By and large, ELISA requires longer time (an average of 3 hours), equipment, adequate technical skill and constant supply of electricity, but these are responsible for its limitations (WHO, 2002; 2004).

2.7.1.1 Normal ELISA

Generally, the test procedure involves a non-competitive indirect staining procedure using immobilized HIV antigen to bind anti-HIV antibodies. Bound HIV antibodies are detected after being complexed with enzyme - labelled anti-human immunoglobulin G (IgG), which catalyzes a colourless substrate to a coloured product. The colour change, read using spectro-photometer for the optical density, is directly proportional to the concentration of HIV antibodies in the test sample. The result is evaluated on the basis of the cut off optical density values for positive and negative controls determined in each test (Waldman & Calmann, 1986).

Most assays use viral antigens obtained from HIV- infected T-lymphocyte cell (TLC) line. These preparations are usually rich in p24, p17, gp 160, and gp 41. False - positive results from this test can arise from contamination of the cell culture preparations with culture materials to which non-HIV human antibodies respond. The antigen that most commonly produces false-positive result is HLA - DR (Grant *et al.*,

1990). Because of this, many commercial ELISA kits use recombinant and synthetic peptides as antigens (e.g., Murex HIV 1 + 2 kit).

2.7.2 Simple, Rapid (S/R) Assays

This type of assay is very similar to the normal ELISA, but requires no special equipment for reading, washing or dispensing and also requires a very short duration (i.e., 5 minutes to 90 minutes) (Tamashiro *et al.*, 1993). Some of the methods employ the same basic principle of normal ELISA but derive an advantage of running single samples with the controls instead of waiting for a battery of samples, the result can also be read visually without use of any eye-aids or equipment (via identification of spots - grey, red, pink, blue, etc.) depending on the manufacturer's choice (e.g., Recombigen RTD kit gives blue spot).

There are a variety of simple, instrument-free screening tests such as agglutination (e.g., Capillus), immunofiltration (flow through tests e.g., HIV check), immunodot (flow through spot e.g., HIV spot), immunochromatographic (lateral flow tests e.g., Determine, Recombigen (RTD) and comb/dipstick tests (e.g., Immunocomb). Specimens and reagents are often added by means of a dropper to the test device. Some HIV test kits could use whole blood or saliva apart from the conventional serum (WHO, 2004).

A positive result is indicated by the appearance of a coloured dot or line, or shows an agglutination pattern. Most of these tests can be done in less than 10 minutes, and are therefore called simple/rapid (S/R) assays. On the other hand, some simple tests are less rapid and their procedures require between half an hour to 2 hours. The results are read visually. In general, these tests are most suitable for use in

settings with limited facilities where low numbers of specimens are processed daily (Kuun *et al.*, 1997; Koblavi-Deme *et al.*, 2001).

However, there could be the problem of false positive and negative results as well. False negative results can occur when the tests are done before sero-conversion, when the patient in immunosuppressed, and sometimes when the individual is late in the course of AIDS. In addition, some rapid kits used to detect HIV-1 may not detect antibodies to HIV-2. Other problematic clinical states that lead to false results include haemodialysis, anti-immune disorders, multiple myeloma, and haemophilia (Bylund *et al.*, 1992). Human error and variability in test kits may also contribute to some false results. Because of the possibility of false results, it is important to perform repetitive ELISA testing and to confirm ELISA results with Western blots and IFA as described earlier and more recently.

2.7.3 Western Blot method

The Western blot (WB) assay is the test most commonly used for confirming the presence of HIV-specific antibodies. However, WB is more expensive and more time consuming than ELISAs, and it requires more technical expertise owing to subjective interpretations. Sensitivity and specificity are high but are somewhat dependent on the interpretive criteria employed.

Western blots are prepared with partially purified whole virus, infected cell lysates, or recombinant viral proteins electrophoresed in denaturing gels (Kostrikis *et al.*, 1995). The advantage of using cell lysates rather than whole virus is the presence of relatively abundant viral protein not found in large amount in the virus. The viral proteins separated on the gel are transferred onto nitrocellulose paper. The nitrocellulose is treated with buffer containing non-specific proteins, and the antigen-

impregnated nitrocellulose is then exposed to test serum. Afterward, the nitrocellulose is exposed to an enzyme-linked secondary antibody. Adding substrate identifies the profile of antibodies bound to the various viral proteins. The colour change that occurs can be visualized as dark or purple bands on the nitrocellulose that correspond to the migrations of the different viral proteins. Positive and negative controls along with molecular weight markers are run simultaneously to allow identification of antibodies reactive with specific viral proteins.

Positive results are interpreted as the presence of two of three bands. Which bands should be present for the specimen to be considered positive depends on the health organization that sets the criteria. The Consortium of Retroviral Association considers any multiple bands (at least three) from any of the viral components as positive; the WHO considers any bands from at least two major components of the virus as positive; while the CDC considers any bands from at least three major components of the virus as positive. The absence of all bands on the nitrocellulose is interpreted as a negative result. If the number of positive bands does not meet the criteria for a positive result, such test is regarded as indeterminate. When indeterminate results are seen, the test subject's serum should be tested again, 6 months later to see whether the same band appears (Kostrikis *et al.*, 1995).

For reasons similar to those described above for ELISA, false - positive results can occur with WB assays. If they do, other tests such as viral culture or PCR should be used to confirm whether results are correct. Studies conducted in the 1980s showed that the Western blot assay has excellent sensitivity in confirming HIV-1 infection by using the CDC criteria (Hahn *et al.*, 1986; Kostrikis *et al.*, 1995).

2.7.4 Radio immunoprecipitation assay (RIPA)

Antibodies to HIV can also be detected by radio immunoprecipitation assay (RIPA), which combines immunoprecipitation of radioactively labelled HIV proteins with sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS - PAGE) (Baker & Barnett, 1994). Radio labelling can be performed either by incorporation of [^{35}S] cysteine (or [^{35}S] methionine), [^{3}H] leucine (or [^{3}H] isoleucine), or [^{14}C] glycosamine into culture or direct iodination of purified recombinant HIV proteins. After labelling, the sample cells are washed extensively or separated from excess label by gel filtration chromatography (proteins). Lysates or protein preparation are pre-absorbed with healthy serum and clarified with the addition of Staphylococcus protein A conjugated to sepharose beads. Subsequently, immunoprecipitation is performed by overnight incubation of the radio-labelled antigen preparation with the test serum. Antibody-antigen complexes are precipitated by the addition of protein A-sepharose, and precipitates are washed extensively with buffer. Precipitated proteins are eluted by boiling in a sample buffer, and after, analyzed by SDS - PAGE followed by auto-radiography. The main advantage of this method over WB is that antibodies can react with antigens in native conditions before undergoing electrophoresis. RIPA can also be employed to detect direct viral antigen with a known positive antiserum. The RIPA is useful in resolving the antibody status of individuals infected with HIV-1 and HIV-2.

2.7.5 Indirect Immunofluorescence Assay (IFA)

The indirect Immunofluorescence Assay (IFA), like Western blot is an excellent test for confirming results obtained in ELISA. However, IFA produces results much faster and at a fraction of the cost of Western blot, provided a fluorescence microscope is available (Tamashiro *et al.*, 1993).

This test uses cells from chronically infected T-cell lines that are fixed on a slide with acetone, air dried, and then incubated with patient serum. After incubation, the cells are washed and incubated with fluorescein-conjugated anti-human IgG. After the second incubation, the cells are washed again and stained with Evan's blue. Negative and positive control sera are tested simultaneously. The slides are evaluated microscopically for fluorescent staining patterns and intensity of fluorescence. Fluorescence of infected and un-infected cell lines is compared along with the negative and positive controls to eliminate background fluorescence and non-specific binding. Indeterminate results are usually followed up by Western blots. In some cases, false results arise as described for ELISA, and these results should be reconfirmed with other tests (Tamashiro *et al.*, 1993).

2.7.6 Recent Antibodies Detection Techniques

The recent antibody detection techniques include: serotyping of HIV by peptide immunoassays (Sherafa *et al.*, 1994); Enzymum-test HIV Combi (4th generation plus EIA for the simultaneous detection of HIV antigen, Immunoglobulin G (IgG) and Immunoglobulin M (IgM) antibodies to HIV-1 (including subtype O) and HIV-2 (Satten and Busch, 1997); Vidas HIV Duo as a 4th generation plus EIA for the simultaneous detection of p24 antigen and IgG antibodies against HIV-1 (including subtype O) and HIV-2 (Kasper *et al.*, 1996); and Reverse transcriptase inhibiting antibodies (RTb - Ab) assay as a 3rd generation Plus EIA for the detection of RTb Ab in HIV-1 infected patients (Neumuller *et al.*, 1991).

2.8 ISOLATION AND CULTIVATION OF HIV

While current serological tests determine whether an individual has been exposed to HIV a definitive test for an active infection is the recovery of HIV from

cells or cells free fluids of the infected individuals (Levy *et al.*, 1985; Evans *et al.*, 1988; Levy, 1994b).

In fact, the initial isolation of HIV was made by cultivating PBMCs from a person with lymphadenopathy syndrome (Barre-Sinoussi *et al.*, 1983). Patient specimens can be co-cultivated with phyto haemagglutinin-stimulated PBMCs obtained from seronegative individuals in a medium containing interleukin-2 (IL-2) (Clerici *et al.*, 1989; Shearer & Clerici, 1991).

Primary viral isolation from patient materials depends on the level of HIV being expressed. This level usually ranges from one Infected $CD4^+$ cell in one hundred to 1 in 10,000 (Brinchmann *et al.*, 1991). Thus, an adequate amount of heparinized blood is needed (not less than 2ml). For best results, the blood specimen should be cultured within few hours after venipuncture. Nevertheless, whole blood can be held up to 3 hours at room temperature without significant reduction of virus recovery from the cells. Any blood sample to be kept for more than 36 hours should be stored at room temperature for 24 hours and kept at 4^oC thereafter (Baker & Barnett, 1994). PBMCs should be recovered from the blood by Ficoll-hypaque gradient centrifugation (Ho *et al.*, 1995). Once purified, PBMCs can then be maintained in serum-containing medium with IL-2 for 7 to 10 days before being cultured for virus. The co-culture method is also applicable for detecting virus in body fluids; however, fluids should be stored at -70^oC within 3 hours following their removal from an individual (Barker & Barnett, 1994).

Usually 3×10^6 to 6×10^6 PBMCs from the infected individual are mixed with an equal number of un-infected PBMCs (which were previously stimulated with phytohaemagglutinin for 3 days and washed) to initiate the culture (Neate *et al.*, 1987;

Asjo *et al.*, 1988; 1990). In cases of low numbers of PBMCs (e.g., infants), as few as 10^6 cells can be used for cultivation if the culture vessel is kept small (Asjo *et al.*, 1988). Cultures are kept at 37°C in a 5% CO_2 atmosphere (WHO-NHIC, 1994). The cultures are monitored for the presence of virus in the medium, and then the medium is replaced every 3 to 4 days. For the detection of virus in culture fluid, the fluid is first filtered (0.45micron - pore-size filter) or centrifuged at 2,000 X g for 15 min to remove cell debris and then subjected to the RT assay and/or the p24 antigen ELISA (Barker & Barnett, 1994).

Culture supernatants possessing positive RT activity or p24 antigen ELISA reactivity can be saved and stored at -70°C for further studies. For most HIV- infected individuals, RT activity or detectable p24 levels become apparent within the first 2 weeks of culture; by 30 days, more than 90% of infected culture yield positive results (Barre-Sinoussi *et al.*, 1983). In some cases when levels of infectious virus are very low, these cultures may take longer, sometimes up to 60 days. In some instances, $CD8^+$ cells within the PBMC group should be removed to enhance the recovery of virus, since these cells suppress the ability of HIV to replicate in cells (WHO - NHIC, 1994). These methods can also be adapted and employed to detect and isolate HIV in other tissue specimens or cell free body fluids, provided the specimen is collected and used immediately or is stored at - 70°C within 3 hours (Rubsamen-Waigmann *et al.*, 1994; Ho, 1996; Hu *et al.*, 1996).

2.9 DETECTION OF VIRUS AND VIRUS - INFECTED CELLS

There are several methods employed for the detection of virus (i.e. viral particles) and virus-infected cells in specimens collected from patients. Some of these methods include:

1. Detection of HIV antigens in serum, plasma or cell culture supernatant capture technique, or "sandwich" ELISA with the aid of anti-HIV polyclonal or monoclonal antibodies coated on wells (Zsaaijer *et al.*, 1992; Des Roziers *et al.*, 1995).

2. The RT assay, although non-specific, can be used to monitor retrovirus replication during virus isolation procedures (von Briesen *et al.*, 1987).

3. Western blot assay, involving coated monoclonal antibodies to the specific compliments of the viral antigen, can be used for distinguishing between HIV-1 and HIV-2 (Burke *et al.*, 1987; Dodd & Fang, 1990).

4. Indirect IFA is also useful as a means of detecting virus in infected cells through the use of fluorescent-labelled anti-human IgG and monoclonal antibodies specific for HIV antigen (Gurtler, 1996).

5. Polymerase Chain Reaction (PCR) assay is the most sensitive method of detecting HIV infection. It can be used to detect the HIV particles (however minute) through the molecular target for integrated and/or free episomal HIV proviral DNA sequences (Gurtler, 1996). The PCR assay for HIV DNA can be performed using only 1μg of cellular DNA, which is equivalent to only 2μl of whole blood (Jackson *et al.*, 1990). This DNA amplification procedure uses two oligonucleotide primers that are complementary to the plus and minus strands of target DNA. In order to ensure detection of many different HIV strains, the sequence of the primers should correspond to regions of the HIV genome that are highly conserved among different HIV isolates. Such regions have been found in *gag, env, pol*, and the long terminal repeat. Repeated denaturation, renaturation, and elongation of the primers with a thermostable DNA polymerase are achieved by repeated temperature cycling. This process

results in an exponential increase in the number of copies of the DNA region that is flanked by the primers. This DNA is then denatured and hybridized in solution to a ^{32}P-labelled HIV probe that contains sequence complementary to those amplified. When HIV sequences are present, a target probe complex is formed that can be visualized by anti-radiography following PAGE. For some studies, the use of multiple primer pairs (and their respective probes) is recommended to further guarantee detection of HIV variants (Diamond *et al.*, 1998). Denaturation, renaturation, and elongation of the primers with a thermostable DNA polymerase are achieved by repeated temperature cycling. This process results in an exponential increase in the number of copies of the DNA region that is flanked by the primers. This DNA is then denatured and hybridized in solution to a ^{32}P-labelled HIV probe that contains sequence complementary to those amplified. When HIV sequences are present, a target probe complex is formed that can be visualized by anti-radiography following PAGE. For some studies, the use of multiple primer pairs (and their respective probes) is recommended to further guarantee detection of HIV variants (Diamond *et al.*, 1998).

The sensitivity of detection of HIV sequences by PCR with a *gag* primer pair and a *gag* probe has been reported to be 18 copies of viral genome in DNA from 150,000 cells (Grankvist *et al.*, 1992). In a study employing 57 infected individual, the virus could be recovered from the PBMCs in at least 89% of cases and from plasma in 75% of cases. In contrast, by PCR method, proviral HIV DNA was detected in many of the HIV - seronegative controls. Thus, PCR appears to be the most sensitive method of detecting virus in infected individuals (Jackson *et al.*, 1993).

While low levels of HIV can often be detected after *in vitro* propagation of infected cells in culture, the PCR method possesses the unique advantage of being able to detect non-replicating (latent) viral genomes. Another advantage of the PCR procedure is that it can be applied to DNA isolated directly from PBMCs of fresh blood, thus eliminating the need for cell culturing. The main disadvantage of the method is that owing to the extreme sensitivity of the assay (97 to 100%), it is highly prone to cross contamination with nucleic acid which causes many false - positive results. If measures are taken to minimize nucleic acid contamination (Jackson *et al.*, 1997), the specificity can be as high as 100%. Primer pairs should be carefully selected in order to avoid detection of non-infectious endogenous retroviruses (Loussert -Ajaka *et al.*, 1994).

6. Recent PCR methods includes: the use of PCR-DNA (single & nested) for qualification of cellular and plasma viral load (Simon *et al.*, 1993); detection of HIV - 1 RNA in plasma with quantitative PCR (Amplicor) (Jackson *et al.*, 1993); and nucleic acid sequence-based amplification (Amplicor monitor or NASBA) (Mulder *et al.*, 1994). Others are branched DNA signal amplification (Quantiplex) (Revets *et al.*, 1996; Mulder *et al.*, 1997; Lin *et al.*, 1998), amplification of HIV-RNA using combined reverse-transcription and amplification reaction (Nihjuis *et al.*, 1995); Oligonucleotide ligation assay using PCR for the detection of HIV-1 *pol* gene mutants (Gene chips technology) (Edelstein *et al.*, 1998); and Quantification of HIV-1 RNA from dried plasma spots collected on filter paper (Cassol *et al.*, 1997); as well as Line probe assay (LIPA) for detection of HIV-1 mutations conferring resistance to nucleoside inhibitors of reverse transcriptase (Stuyver *et al.*,

1997); the RNase A mismatch method (Galendez - Lopez *et al.*, 1991), and finally DNA hetero duplex mobility assay (HMA) for the determination of the genetic relationship among HIV-1 subtypes, HIV-2 subtypes, HIV, and between HIV and other viruses, or other microorganisms (Delwart *et al*; 1993 Bachmann *et al.*, 1994).

2.9.1 *In Situ* Hybridization

In situ hybridization is a histomolecular method which is used to detect HIV nucleic acids in different cells and tissues (Boom *et al.*, 1990). Slides containing fixed specimens are probed for the presence of intracellular HIV nucleic acids in infected cells (Asjo *et al.*, 1990). The technique involves fixation in para formaldehyde followed by rehydration, digestion with proteinase K, acetylation, and treatment with either RNase or DNase. Cells are then refixed with para formaldehyde and dehydrated. To detect both double - and single - stranded nucleic acids, samples are denatured in 95% formamide at 65^{o}C for 15 minutes, and the reaction is stopped on ice prior to dehydration.

Hybridization is performed by applying a denatured ^{35}S-labelled HIV DNA probe (approximate specific activity 2 x 10^{8} dpm/μg) to the cytological or histological sample. After a long incubation, slides are washed at high stringency, dehydrated, coated with Kodak NTB-2 photographic emulsion, and allowed to expose for several days at 4^{o}C. Slides are then developed, stained with Wright - Giemsa stain (or other cell type-specific reagent) and microscopically examined for autographical grains over cells, indicating intracellular HIV. Positive cells are defined in those with a number of grains five times that of background.

Several studies have shown that under optimal condition each grain above background represents about five genome equivalents of HIV RNA per cell (Neate *et al.*, 1987). One study reports that some infected cells are positive by *in situ* hybridization 1 to 2 days prior to the detection of cell associated viral antigens by immunocytochemistry (IFA or immunoprecipitation assay) and 2 to 5 days before RT activity. Hence, viral antigen could be measured in culture supernatants (Busch *et al.*, 1995). The *in situ* hybridization method has successfully identified HIV infections of various cell types, including those of lymphatic system, the CNS and brain, and the bowel (Eastman *et al.*, 1995). Thus, *in situ* hybridization is a very sensitive method that also allows direct correlation of a specific hybridization signal with the morphological and immuno-phenotypic properties of the cells and tissues studied.

2.10 PROGNOSTIC INDICATORS

Presently, there are no direct measures that determine the consequence of infection other than the use of surrogate markers. These markers are not only important in predicting the outcome of HIV infections but are also useful in determining when treatment should be administered. For example, surrogate markers can be used to monitor the course of treatment of an individual receiving anti-retroviral therapy or to determine the state of infection in an individual or risk of prenatal transmission of HIV (Thea *et al.*, 1997; Vigano *et al.*, 1996).

2.10.1 $CD4^+$-Cell Counts

One of the best predictors of disease progression thus far is the absolute $CD4^+$ count (CDC, 1985). An overt decline in the $CD4^+$ T cell count usually precedes clinical disease (Castro *et al.*, 1992). $CD4^+$ is a cell surface molecule found mostly on T lymphocytes that is important for the activation of the lymphocytes when they are

stimulated by antigens (Barker & Barnett, 1994). Since $CD4^+$ is a major receptor for viral surface glycoprotein gp 120. T cells expressing this surface marker are primary targets for infection. Cells expressing these markers are important in regulating the immune responses necessary for controlling pathogens and neoplasms. Thus, loss of these cells leads to an immunocompromised state that eventually leads to disease. Decline in response to certain stimuli by these cells leads to disease (Scott, 1993; Levy, 1994b).

Although in asymptomatic individuals, an average of 1/10,000 $CD4^+$ cells is actually infected, the number of $CD4^+$ cells lost greatly exceeds this amount (Brinchmann *et al.*, 1991). In addition; Groux *et al.* (1992) demonstrated that this loss of $CD4^+$ cells is partially attributable to programmed cell death, or apoptosis, which occurs in infected individuals but not in sero negative individuals.

In general, the lower the $CD4^+$ cell count, the greater the chance of entry to disease. Hence, the rate of decline of $CD4^+$ cells is also a good predictor of AIDS. The $CD4^+$ count can either be determined by direct or indirect immunofluorescence using monoclonal antibodies on a flow cytometry (Paxton *et al.*, 1995).

2.10.2 Measurement of Viral Load

Direct measurements of viral burdens can also be a good predictor of the outcome of infection and these include: plasma p24 antigen levels, titration of infectious virus in plasma or PBMCs and quantitation of proviral DNA and RNA by PCR (Lin *et al.*, 1998).

P24 antigen levels in plasma can be determined by ELISA and are therefore used to measure virus levels in the plasma. Although ELISA is very specific, its

sensitivity is usually too low for screening viral antigen in the plasma of asymptomatic individuals. Since the levels of p24 antigen in the plasma increase during the course of the disease, this method is a useful prognostic tool in monitoring individuals who are symptomtic or have AIDS (Vigano *et al.*, 1996; Mellors *et al.*, 1997; Thea *et al.*, 1997).

2.10.3 Titration of Infectious Virus in Plasma and Cells

Virus in plasma or cells is titrated by culturing various dilutions of cells or plasma with phytohaemagglutinin stimulated-PBMCs or other susceptible cells. The cultures should be set up within 3hrs. of acquiring the specimen from the test subject (Pan *et al.*, 1993). The level of viral infection is measured either by determining RT activity or by measuring p24 levels as described earlier in detection of virus and virus - infected cells.

2.10.4 Titration of Viral DNA or RNA by PCR

The PCR method as described earlier has enhanced our ability to detect one HIV-infected cell in 100,000. This technique has enabled researchers to demonstrate that the viral burden is substantially higher than what was believed, and that the burden changes with the variation in the clinical state of an individual. In this regard, viral culture isolation showed 1 provirus containing $CD4^+$ T cell per 4,000 to 150,000 $CD4^+$ T cells, while PCR demonstrated 1 per 2,500 to 26,000 cells (Brinchmann *et al.*, 1991). Hence, PCR has been used to quantify virus in the plasma by using a recent technique called quantitative competitive PCR (QC-PCR). This method was used to measure RNA in plasma by use of a competitive RNA template matched to the target sequence but differing from it slightly by virtue of an introduced internal deletion (Piatak *et al.*, 1993).

2.10.5 Immune Activation Markers

Infection by HIV induces activation of the immune system, which in turn increases the pool of HIV susceptible cells available and results in increased virus production. Thus, a measurement of immune activation markers is an indirect mean of measuring virus production in an individual. An indirect way to measure immune activation is to measure the amount of β_2 - microglobulin in plasma (Lifson *et al.*, 1992). This protein is part of class 1 MHC, and is present in almost all nucleated cells. This marker correlates with the degree of lymphocyte activation and also with progression to disease in HIV - infected individuals. Also recently employed is the use of adenosine deaminase (ADA) as a prognostic marker (Carrera *et al.*, 1995).

2.11 INTERPRETATION OF TEST RESULTS

Since the discovery of HIV as the causative agent of AIDS, the screening of individuals as well as blood supply for HIV infection has primarily relied on the use of anti-HIV antibody assays (Bylund *et al.*, 1992). Positive antibody status is considered equivalent to HIV infection, and the epidemiological evidence appears to support this assumption. However, the absence of antibodies to HIV is sometimes an unreliable indicator of the absence of infection, especially the early stages of infection and in certain population of high risk for infection (Blomberg and Schooley, 1985).

Since serological tests for HIV antibodies are inconclusive, virus detection methods may be desirable, especially in high risk individuals who, for treatment or management purposes, could benefit from early diagnosis. Although they are time-consuming and require highly technical training, virus cultivation and PCR assays are the most reliable tests for the determination of HIV infection in such high risk individuals.

After diagnosis (both clinical and laboratory), the next instantaneous step is to treat or manage the patients. In order to maximize the magnitude and durability of HIV RNA (provirus) suppression, therapeutic strategies that are effective against high levels of rapidly replicating virus (consisting of many genetic variants) must be implemented (Havlir and Richman, 1996).

2.12 HIV THERAPIES AND PREVENTION

After the use of early signs and symptoms, HIV p24 antigen, CD4 lymphocyte count, and plasma HIV-1 RNA and DNA as surrogate markers, immediate intervention of HIV with appropriate treatment at any stage of HIV/AIDS development should be employed. Hence, the need arises for appropriate therapy suitable for the patient. There are different types of HIV therapies. These therapies include monotherapy using antiretroviral drugs, combination therapy using only antiretroviral drugs or combining antiretroviral drugs with any other from of therapy; cytokines therapy, $CD8^+$ cell administration; post-infection immunization, passive immunotherapy and use of antibody-based approaches as well as gene therapy; antioxidants therapy; β-chemokines therapy; use of interferon and finally, herbal treatment. Detection of HIV antibodies in an infected individual has been the basis of laboratory diagnosis of HIV and treatments commonly employed are chemotherapy and the use of herbs. There is no viable and potent vaccine presently although research efforts are still on in this aspect.

2.12.1 Antiretroviral Therapy / Combination Therapy

Antiretroviral drugs are drugs that inhibit viral reverse transcriptase and therefore retrovirus (especially HIV) replication. A drug that had been previously synthesized for potential use against cancer, 3 – azido-3-deoxy thymidine or

zidovudine (AZT), was first noted to inhibit viral reverse transcriptase and HIV replication *in vitro* (Mitsuya *et al.*, 1985). As a nucleoside analogue, its mechanism involves both a termination of viral DNA production and a competition for nucleosides used by the viral polymerase in vitro. The use of AZT was immediately evaluated clinically and soon reported to be effective (Yarchoan *et al.*, 1986, Dournon *et al.*, 1988; Cardo *et al.*, 1997). At first, large doses were administered (1,500mg/day), with frequent side effects, especially toxicity to the bone marrow (Fischl *et al.*, 1987, Richman *et al.*, 1987). Subsequently, the amount of drug prescribed was reduced to 300 - 500mg/day with fewer harmful effects in infected individuals (Collier *et al.*, 1990). After a number of case studies, AZT was not found to prolong life, but it did maintain or improve the clinical state for about 18 months (Goudsmit *et al.*, 1997). This finding led to the advent of other antiretroviral drugs and other alternative therapies.

However, two major classes of antiretroviral drugs are now licensed: (1) ***nucleoside analogues*** (didanosine, lamivudine, stavudine, zalcitabine, and zidovudine), and (2) ***protease inhibitors*** (indinavir, ritonavir and saquinavir). As mentioned earlier, nucleosides analogues competitively inhibit viral reverse transcriptase for reverse transcription while protease inhibitors selectively inhibit HIV protease thereby causing the synthesis of deformed HIV particles which are less infectious or non-infectious. Also described recently are non-nucleoside antiviral drugs, such as nevirapine, and the thiobenzimidazolone (TIBO) derivation which inhibit reverse transcriptase. The TIBO compounds inactivate selectively, the RT of HIV-1 and not HIV-2 (De Clercq, 1991), most probably because of their specific predilection for the end chain of the HIV-1 polymerase (Shih *et al.*, 1991).

2.12.2 Other Chemotherapeutic Approaches

In addition, some investigators have considered immunosuppressive therapies such as use of cyclosporine because of the possible detrimental effects of hyperactive immune responses in HIV pathogenesis (Schwarz *et al.*, 1993) In contrast, some investigators have also considered using leucocyte-derived immunosupportive therapies as effective against HIV AIDS (Gottlieb *et al.*, 1996). Moreover, N-acetyl-L-cysteine, the antioxidant compound, has been proposed as a safe therapy in HIV infection (Roederer *et al.*, 1990; Lioy *et al.*, 1993; Muller, 1995). It likely inhibits intracellular factors and cytokines that enhance HIV replication. Others are use of pentoxifylline (Clerici *et al.*, 1997), use of human recombinant growth hormone and human recombinant insulin-like growth factor-I (Hirschfeld, 1996) and use of CD4-chemokine receptor pseudotypes (complex) (Endres *et al.*, 1997).

For perinatal HIV prevention and therapeutic intervention antiviral drugs (such as zidovudine) have been recommended (Wu *et al.*, 1995, Bryson, 1996; Doran, 1997; Mofenson, 1997). Recently, the effects of antiviral drug regimens for the management of HIV/AIDS have been proven through the mathematical modelling of various therapeutic interventions (Hraba and Dolezal, 1995). However, the ability to dramatically decrease vertical transmission of HIV from mother to child with antiretroviral therapy is encouraging. Thus, better understanding of HIV pathogenesis, disease progression antiviral therapy, and opportunistic infection prophylaxis are leading to more effective therapy and prevention (Carpenter *et al.*, 1996).

Globally, the control of HIV strategies have focused on the treatment of PLWHA all over the world as WHO (2004a) promised to treat 3 million of people in the developing countries with antiretroviral therapy by the year 2005 themed as "3 by 5 initiatives".

2.12.3 Gene Therapy

Gene therapy is based on the motion that transfer or incorporation of a therapeutic gene [such as Tat - antagonist; antisense oligonucleotides; genes expression modulator (GEM) 91; ribozyme, transdominant proteins], into target cells will render the body resistant to HIV replication (Bridges and Sarver, 1995). Infusion of protected cells to the patient may limit viral spread and delay disease progression. HIV gene therapy strategies can be broadly divided into three categories; those based on nucleic acid molecules that compete with viral RNA (vRNA) for binding essential HIV regulatory proteins or RNA molecules that cleave (or inactivate) HIV RNA, those based on intracellular protein molecules that compete or interferes with normal viral functions; and those based on inducible toxins or conditionally toxic molecules that kill host cells subsequent to infection by the virus.

In each case, the (i) antiviral gene chosen; (ii) intercellular expression and stability; (iii) delivery system; (iv) target cells and tissue and (v) localization of the gene product to the appropriate cellular compartment will determine therapeutic benefits. Importantly, the strategy should not be affected by HIV genetic variation "escape mutants" (Saver and Rossi, 1993; Yu *et al.*, 1994). HIV gene therapies require time and labour intensive *in vivo* manipulations that preclude their broad clinical use. Development of second generation delivery systems is likely to emphasize vehicles suitable for *in vivo* gene transfer.

2.12.4 Cytokine therapy

Cytokines - including IL-1, IL-2, IL-4, IL - 6, IL - 7, IL-10, IFN-γ, and TFN-α - have all shown the ability to enhance B cell proliferation, and can therefore be used for treating HIV/AIDS patients (Levy, 1994b). Recently discovered was

interleukin-12 (IL-12) (Romani *et al.*, 1997) which is a potent immunoregulatory cytokine while the role of IL-16 discovered by Cruickshank *et al.* (1994) has been described as one of the factors mediating anti-HIV activity of $CD8^+$ T cells by Mackewicz *et al.* (1996). The use of pentofylloxine against TFN-α is being evaluated for the potential of reducing HIV replication and thus disease progression (Fehsel *et al.*, 1991; Dezube *et al.*, 1993). Some studies have suggested that IL-2 in combination with IFN-α may transiently increase $CD4^+$ T cell numbers and reduce HIV replication *in vivo* (Schnittman *et al.*, 1994a). Finally, the administration of antiviral drug or cells along with cytokine therapy (Clapham *et al.*, 1991) should be evaluated, as is done in some anticancer protocols (Pardoll, 1992).

2.12.5 $CD8^+$Cell Administration

Based on the observation that $CD8^+$ cells can suppress HIV replication, some groups are evaluating whether autologous $CD8^+$ cells grown in large quantities in culture would be therapeutically effective if returned to patient. Preliminary studies on *ex vivo* cultured autologous $CD8^+$ cells have shown no obvious toxicity in patients (Ho *et al.*, 1993). In one small study, improvement in KS was noted in two patients (Klimas *et al.*, 1992). This cellular approach to immune modulation (in addition to cytokine, anti-cytokine antibody, and anti-cytokine drugs) should soon receive increased attention.

2.12.6 Post-infection Immunization

The suggestion that post infection therapy could involve immunization as preventive strategy (not vaccination) with a viral protein (Salk, 1987) has also been evaluated. It has been shown that immunizations with envelope-depleted virus still containing the HIV-1 *Gag* proteins (Levine *et al.*, 1993) for asymptomatic HIV-

infected individuals, significantly increases cell mediated antiviral immune cell responses, reduces rate of increase in viral load, and decreases rate of $CD4^+$ cell decline (Trauger *et al.*, 1994). Similar studies were also carried out by researchers (Redfield *et al.*, 1991; Kundu *et al.*, 1992). However, the potential value of post-infection immunization is controversial, and before any conclusions can be drawn, long term follow-up of the subjects will be required.

2.12.7 Passive Immunotherapy and Use of Antibody-Based Approach

Investigators have considered the possibility that passive immunotherapy, using plasma or purified immunoglobulins from HIV-seropositive individual who are symptomatic (Jackson *et al.*, 1988; Karpas *et al.*, 1990; Vittecoq *et al.*, 1992) would be helpful in therapy. The potential use of human anti-V_3 monoclonal antibodies with different epitopes has received attention (Emini *et al.*, 1992; Zolla - Pazner and Gorny, 1992). Protection of cats from feline immunodeficiency virus (FIV) infection by possible antibody administration has been reported (Hohdatsu *et al.*, 1993), as well as protection of chimpanzees (Prince *et al.*, 1991) and humanized-severe and combined immunodeficiency in mice (SCID-hu) (Baenziger *et al.*, 1993).

However, it is yet to be fully established in man, whether the immunoglobulin inoculated into a host will enhance rather than neutralize the HIV strain present in that infected individual, but Nord *et al.* (1990) have successfully used bovine hyperimmune colostrum to treat cryptosporidial diarrhoea in AIDS patients. It was also suggested by Musey *et al.* (1997), that induction of memory CTL, particularly those specific for *Env*, helps control viral replication and is associated with slower decline in $CD4^+$ cell counts in early HIV -1 infection.

2.12.8 Herbal Therapy

Early enough, the use of herbs in treating HIV/AIDS became pronounced with the detection of anti-HIV - active substances in some medicinal plants (Boyd, 1988, Ngan *et al.*, 1998; Tabba *et al.*, 1989). The search for selective antiviral agents, principally focused on anti-HIV agents, has been vigorous in recent years (De Clerq, 1988) but progress in the development of useful new antivirals has been painstakingly slow (Galasso, 1988). Although a combination of antibiotics and herbal medicine has been beneficial since the former limits the severity of the effects of the infection while the later supports the host's underlying constitutional (immunological) strength and ability to recover after the infection (i.e. immunomodulating effects) (Peterson, 1996).

With conservative estimates placing the number of patients trying unorthodox therapies at 70% - 80% of all HIV/ADS cases (Crawford, 1996), it has recently therefore become clearer that people with HIV can survive much longer than used to be thought. In addition to the recent news of a baby seroconverting from HIV + to HIV -, and the possibility that as many as a half of babies infected with the virus seroconvert through herbal treatment (Root-Bernstein, 1993), time will tell if nurturing and supporting a healthy and loving environment, will contribute to such a change. Of all the "immune herbs" (i.e. herbs with immunomodulating effects), *Uncaria tomentosa* (cat's claw or **Una de Gato**) either in capsule or tea form has been known as a unique herbal remedy for the treatment of HIV infection/AIDS (Steinberg, 1995).

2.12.9 Vaccine Development

All the observations made to date about the virus and the natural host immune responses against it provide valuable information for use in the eventual development

of therapy and an HIV vaccine (Cease and Berzofsky, 1994; Yang and Chen, 1997). Several features of HIV infection and transmission [such as its appreciation of viral heterogeneity, the need for local mucosal immunity (Forrest, 1991), the potential for auto immune responses, and virus transmission by infected cell (Levy, 1993b)] must be considered in developing vaccine approaches.

For the development of an effective vaccine, a major problem has been the inability to finding an appropriate animal model. From chimpanzee's SIV (Sharp *et al.*, 1994), magabey's SIV and rhesus monkey's SIV (Murphey-Corb *et al.*, 1991), vaccine models in pig-tailed macaques (*Macaca nemestrina*) with HIV-1 (Agy *et al.*, 1990) and HIV-2 (Mc Clure *et al.*, 1992; Jiang and Neurath, 1992) to the most recent model in mutant mouse strains which efficiently received human peripheral blood leucocyte (PBL) engraftment (hu - PBL - SCID mice) (Koyanagi *et al.*, 1997); all yielding no sufficient results, yet a reliable model is still hopeful. Also, for such development, eight different approaches are being explored (Levy, 1994c).

2.13 HIV TEST KITS

Two types of kits exist, those for diagnosis and those for research. Diagnostic kits are different from research kits in the following ways: diagnostic kits are usually standardized and evaluated whereas the research kits may or may not be standardized and evaluated, and they may undergo evaluation during research or other assessment studies. In addition, diagnostic kits assessment must focus on operational characteristics such as ease of performance, rapidity, suitability for use at referral and local centres, their sensitivity and specificity on panels of well-characterized sera of diverse geographical origins. A diagnostic kit is a kit that must have been tested and evaluated specifically for routine laboratory analysis of biological, chemical and physical parameters to ascertain human body system condition. Such a kit should be

free from any side effects on the users and applicators. Its accuracy must be close to 99.9% and it must be highly reliable (UNAIDS/WHO, 2000). Finally, diagnostic kits must be certified by the country's National Public Health Laboratory (NPHL), Associations on Laboratory Practice in Nigeria as well as National Agency for Food and Drug Administration and Control (NAFDAC) based on the standard laboratory methods and evaluation procedures.

2.13.1 The World Agenda on HIV Surveillance

Since the World Health Organization Global Programme on AIDS (GPA) initiated a programme to provide objective assessment of commercially available assays for detecting antibody to both types of HIV (HIV-1 and HIV-2) in 1988, assessment programmes at the local level with basic standards is yet to be done in Nigeria. There is need to assess, with the ultimate goal of regulating the circulation of commercially available kits, using the WHO biosafety guidelines of diagnostic research laboratories working with HIV, before they can be regarded as standard diagnostic kits. There is also the need to constantly and periodically update these diagnostic kits in the country so that they fit into the current needs. Such needs arise from recent discoveries of new methods and additional HIV properties enhancing the detection of HIV-1 & 2 antibodies/antigens in infected individuals (Garrette *et al.*, 1988).

Nigerian health policy makers, directors of blood banks and the Chief Executives of National AIDS Control Programme (NASCP), National Actions Committee on AIDS (NACA), NPHL and NAFDAC: ought to circulate the periodical assessments in the form of reports for use. These assessments may be used in conjunction with consideration of other factors, such as experience with a given test, availability, cost, service and trouble-shooting, provided locally by manufacturer, etc,

to help select HIV antibody assays as well as suitable HIV testing strategies appropriate to local needs (Garrett *et al.*, 1988).

2.13.2 HIV Testing Strategies

For any type of HIV testing algorithm, WHO and UNAIDS had recommended three testing strategies (Sato *et al.*, 1994). Criteria for choosing appropriate HIV testing strategies as given by WHO (2002) include:

1. Objective of the test (diagnosis, surveillance, blood safety, or research),
2. Sensitivity and specificity of the test(s) being used, and
3. HIV prevalence in the population being tested.

Strategy I involves only one test kit, sufficient enough for the final result of the assay. This is commonly used for transfusion/transplant safety, surveillance in a population with >10% prevalence and diagnosis in a population with 30% prevalence. Strategy II involves the use of two different kits (either in principle or type of coated HIV antigens) used either simultaneously (parallel algorithm) or consecutively (serial algorithm) in cases where the initial result is positive. This strategy is specific for surveillance and diagnosis of asymptomatic population with >10% prevalence and symptomatic population <30%. Strategy III involves the use of three different kits (either in principle or type of coated HIV antigens/antibodies) used either simultaneously or consecutively in cases where the initial result is positive. The strategy can only be used for diagnosis in a population with 10% prevalence.

Strategy I is a strategy involving only one ELISA or S/R assay used for testing all serum/plasma, strategy II is a strategy involving further testing of any serum/plasma found reactive on the first assay with a second ELISA or S/R assay based on a different antigen preparation and/or different test principle, while strategy

III is that improving on strategy II by a third ELISA or S/R assay to retest any reactive serum/plasma during testing with first, second or both first and second ELISA or S/R assays (Martin *et al.*, 1995; Henry, 1999).

2.13.3 Measures of Quality for HIV Test Devices

Sensitivity of a test is a measure of the probability of correctly identifying an HIV-infected person while specificity is a measure of the probability of correctly identifying an HIV-uninfected person (WHO, 1999). Reproducibility is a measure of the probability of obtaining the same result from a repeated test using the same technique. Rapidity is the measure of time taken by a particular test to finally examine a specimen. Positive predictive value is a probability that a reactive test indicates that the specimen actually contains HIV antibody, whereas the negative predictive value is a probability that a non-reactive test reveals a specimen without HIV antibody (WHO, 1999).

When a single screening assay is used for testing in a population with a very low prevalence of HIV infection, a very low positive predictive value means that the majority of people detected seropositive from such assay are not infected (WHO, 2004a). This problem still occurs even when a test with high specificity is used. The possible solution is the use of supplemental assays to retest all samples found positive by the first test and this can be described as the confirmatory test (WHO, 2002). However, those found negative by the first test are finally considered negative for HIV antibodies. All these virtues therefore serve as the key factors responsible for the quality of kits for HIV screening and/or confirmation. Hence, such tests therefore require the use of standardized kits and testing strategies, taking into consideration the peculiarity of the circulating viral types (WHO, 2004b).

2.13.4 Importance of HIV Serodiagnosis in Epidemiology

The need to know the spread and distribution of HIV cannot be underestimated. The UNAIDS and WHO's estimate of the number of PLWHAs at the end of the year 2004 was at 34-46 million, with 3 million deaths in 2003 alone (WHO, 2004). The challenges thrown up by HIV vary enormously from place to place, depending on how far and fast the virus is spreading and on whether those infected have started to fall ill or die in large numbers. In 2003, Africa was the home of two-thirds of PLWHA, although Africans represent 11% of the total world population (WHO, 2004). The pandemic has reversed decades of gradual gains in life expectancy in Sub-Saharan Africa (WHO, 2001). Transmission in these zones is basically by heterosexual means.

CHAPTER THREE
EVALUATION OF HIV TEST KIT (PHASE I)

3.0 EVALUATION OF HIV TEST KITS (PHASE I)

3.1 INTRODUCTION

The quality of human immunodeficiency virus (HIV) antibody testing is very significant in controlling the HIV/acquired immune deficiency syndrome (AIDS) epidemic in the country as well as serving as one of the major protectors of blood supply in the nation's blood banks. The major factors enhancing the quality of HIV antibody testing are the quality of an HIV testing method (strategy) and more importantly, the quality of test kits used. The quality of HIV test kits used depends on its sensitivity, specificity, ease of performance and storage conditions (suitability for use) (WHO, 2002). The more sensitive a test kit, the more readily it detects positive samples and vice versa. The more specific a test kit, the more selectively it detects positive samples and vice versa. The easier it is to perform a test using a test kit, the more efficient it is because errors due to staff proficiency are greatly reduced. The longer the shelf life of a test kit, the more durable and suitable it is. The less dependent a test kit is on cold chain system, the more suitable it is to our country's resources (WHO, 1998a).

The selection of test kits is based on the number of samples to be tested, the laboratory facilities available, the level of laboratory staff training, the objectives of the testing and finally, the testing strategy being followed (WHO, 2002; UNAIDS/WHO, 2004). The ELISA kits can be used to run pools of samples while the S/R test kits can be used to run single samples. The ELISA kits require constant electricity, water, and equipment while most S/R test kits do not require any of these facilities. The ELISA kits require skilled technical staff while most S/R test kits do not require skilled staff to run. Most ELISAs are appropriate for referral, conventional

and surveillance involving large batches of samples while most S/R test kits are appropriate for VCT, field and rural testing schemes.

Sub-Saharan Africa still remains the most affected with HIV epidemic (UNAIDS/WHO, 2002). Nigeria, being the most populous country in this sub-region, requires proper and constant attention with respect to the control of the spread of HIV/AIDS globally. Nigeria has however, improved on the control of the spread since recent sentinel seroprevalence surveillance has pegged down the seroprevalence to 5% (FMOH/NASCP, 2004), but this seemed to have caused efforts to be relented at improving on the quality of the HIV antibody testing in the country. Since control of epidemic is the critical entry point for both prevention and care efforts for HIV/AIDS, HIV antibody testing is therefore critical for such control.

Apart from the control of epidemics, the increasing supply of the Nigerian market with all sorts of HIV antibody test kits as commercially available kits without thorough accreditation through certification by both the National Agency for Food and Drug Administration and Control (NAFDAC) and the National Action Committee for AIDS (NACA), raises some questions on the quality of HIV antibody testing in the country. The Nigerian authority has not been able to classify test kits by their assessment into research and diagnostic kits; whereas the research kits serves the purpose of research which is for a short time, the diagnostic kits are routinely used for years (WHO, 1998a). Most Nigerian would rather go for the cheaper research kits and hence, stockpiling the market with kits of short shelf life.

With increasing challenges from the various NGOs at combating HIV/AIDS and stock of the Nigerian market with all sorts of HIV test kits as diagnostic kits across the country, there is an urgent need to evaluate and validate all HIV test kits found in circulation (WHO, 1998a). The absence of regulatory control of HIV test kits

use and standard evaluation programme for HIV testing kits stimulated the authors' curiosity to carry out both operational and experimental studies on the commercially available HIV test devices circulating in the Nigerian market. At the end of the evaluation, suitable diagnostic kits for determining HIV status of Nigerians would be identified.

3.2 OBJECTIVES

The main objective of this study is to evaluate three 2nd generation ELISA kits and six S/R test kits commonly circulating in the Nigerian market between 1996 and 1997.

3.2.1 Specific Objectives were to:

1. assess the evation parameters namely: sensitivity, specificity and reproducibility of three 2nd generation ELISA and six S/R test kits used in the study
2. determine the PPV and NPV of the three 2nd generation ELISA and six S/R test kits used in the study
3. evaluate the ease of performance of the three 2nd generation ELISA and six S/R test kits used in the study and,
4. evaluate the suitability of the three 2nd generation ELISA (for referral centres) and six S/R test kits (for rural centres/ VCT/ field) used in the study.

3.3 MATERIALS AND METHODS

3.3.1 SAMPLE POPULATION

3.3.1.1 Patients Population and Study Centres

Both College of Medicine of the University of Lagos (CMUL) and Central Public Health Laboratory (CPHL) served as the study centres. CPHL is the central and referral laboratory for diseases of public health importance (especially HIV/AIDS) in Nigeria and it serves other states and Federal hospitals in public health services in the country.

3.3.1.2 Sample size

A non-probability sampling method (as one of the random sampling method) was used to select the study population, based on the proportion of the parameter for the diagnostic kits involved in the study. All the patients recruited between July 1997 and December 1999 for the study, were criteria-free volunteers. Sample size was determined by the formula enunciated by Altman (1991).

$$N = \frac{(v-u)^2\{p_1 (100 - p_1)\, p_2 (100-p_2)}{(p_1 - p_2)^2}$$

where N = Required sample size

p_1 = Estimated minimum of sensitivity, specificity or reproducibility at 95% confidence interval = 100%

p_2 = Estimated minimum of sensitivity, specificity or reproducibility at 90% confident interval = 95%

v = Normal standard deviation for required confidence level of 95% = 1.96.

u = Normal standard deviation for required confidence level of 90% = 1.28.

So, sample size (N) = $\frac{10.5 \times .95 \times 5}{0.5^2}$ = 199.5 = 200

These samples were collected from all age groups and both sexes randomly. The sample size of 480 (280 HIV seropositive and 200 HIV seronegative) represented reference panels of sera with retrospectively known HIV serologic status for HIV kits' evaluation in the study.

3.4 LABORATORY METHODS

3.4.1 Evaluation of Common Commercially Available Kits

The sensitivities, specificities and reproducibility of six rapid tests and three 2nd ELISAs were determined on a reference panel of 480 sera with known HIV serologic status. All reference panels of sera used in this study were obtained from the CPHL serum bank in good storage conditions during the course of this study. This phase of the evaluation programme was done in August, 1998. The following commercially available ELISA and S/R test kits were used:

(a) Second Generation ELISA kits (Phase I)

1. Wellcozyme/Murex HIV-1+2 (Murex Diagnostics, Dartford, U.K.)
2. Genelavia Mixt (Sanofi Diagnostic Pasteur, Marnes la Coquette, France)
3. SUB-Recombigen HIV-1/HIV-2 EIA (Cambridge Biotech Diagnostics, Galway, Ireland)

(b) Simple Rapid assay kits (Phase I)

4. Gene II HIV-1/HIV-2 (Sanofi Diagnostic Pasteur, Marnes la Coquette, France)
5. Immunocomb II HIV-1 & 2 Bispot (Orgenics, Yavne, Israel)

6. Immunocomb II HIV-1 & 2 CombFirm (Orgenics, Yavne, Israel)
7. Recombigen HIV-1/HIV-2 RTD (Cambridge Biotech Diagnostics, Galway, Ireland)
8. Determine HIV-1/2 (Abbott Diagnostics, Pretoria, South Africa)
9. Omni-Sal HIV-1/2 & Subtype O - Saliva (Americare Biologicals, U.S.A.).

The properties of both normal ELISA and simple rapid test kits can be found in Appendix 1. All these kits were also sourced locally from laboratory kits suppliers and marketers. The assays for each kit to be evaluated and the supplementary assays (simple-rapid and ELISA using HIV Uni-Gold RTD, Camstrix HIV-1/HIV-2 and Murex HIV-1+2 kits as well as WB method using New Lav Blot 1 & 2 kits as CPHL's laboratory gold standards) were run concurrently based on the manufacturers' instruction and test principles (Appendices 2-9).

The criteria for ease of performance scoring (Tables 15 & 16) were adapted from C.D.C. (1998) and the suitability criteria for local and referral centres (Tables 17 & 18) were also designed based on UNAIDS/WHO's recommendations (WHO, 1998a, WHO, 2002).

3.4.2 STATISTICAL ANALYSIS

3.4.2.1 Data Collection and Statistical Analysis

The evaluation parameters (such as sensitivity, specificity and reproducibility) of all the HIV assays were calculated using the results of the approved supplement assays and Western blot method (serostatus of the sera used were also known). Statistical significance of comparative parameters was also determined. Exact binomial 95% confidence intervals (CIs) were also calculated for all proportions in

the study. The corresponding positive and negative predictive values were also determined. All data generated were analysed statistically using the computer software "Epi-Info 2002.

Table 15: Criteria for ease of performance of commercially available rapid assays to detect antibodies to HIV-1 & 2 in human specimen

Need to prepare

1.	Antigen	(Need = 0; No need = 1)
2.	Substrate	(Need = 0; No need = 1)
3.	Wash	(Need = 0; No need = 1)
4.	Conjugate	(Need = 0; No need = 1)
5.	Predilution of serum	(Need = 0; No need = 1)
6.	Dilution serum	(Need = 0; No need = 1)

Volume of serum needed

7. $\leq 25\mu l = 1; >25\mu l = 0$

Incubation temperature

8. Room = 1; other than room = 0

Stability after dilution (expiry = 1; less expiry = 0)

9. Antigen
10. Control
11. Sample diluted
12. Conjugate
13. Substrate
14. Buffer

Sufficiency of Reagent

15. Very sufficient = 1; slightly sufficient = 0.5; not sufficient = 0

Need for washing

16. Yes = 0; No = 1

Equipment needed but not provided in the test (provided = 1; not provided = 1)

17. Wash device
18. Incubator (water bath)
19. Spectrophotometer reader
20. Refrigerator (storage)
21. Agitator/shaker
22. Distilled water
23. Automatic pipette in microlitre
24. Aspiration device
25. Multichannel pipette
26. Dilution tubes rack/microlitres
27. Distilled water
28. Plate
29. Graduated pipette cylinder (ml)
30. Sulphur acid/sodium hydroxide

Total = **30**

(Adapted from WHO, 1999)

Table 16: Criteria for ease of performance of commercially available normal elisa assays to detect antibodies to HIV-1 & 2 in human specimen

	Need to prepare	
1.	Antigen	(Need = 0; No need = 1)
2.	Substrate	(Need = 0; No need = 1)
3.	Wash	(Need = 0; No need = 1)
4.	Conjugate	(Need = 0; No need = 1)
5.	Predilution of serum	(Need = 0; No need = 1)
6.	Dilution serum	(Need = 0; No need = 1)
	Volume of serum needed	
7.	$\leq 25\mu l = 1$; $>25\mu l = 0$	
	Incubation temperature	
8.	$<37^{\circ}C = 2$; $37^{\circ}C$ or $37^{\circ}C$ + humid condition = 1; $>37^{\circ}C = 0$	
	Stability after dilution (expiry = 1; less expiry = 0)	
9.	Antigen	
10.	Control	
11.	Sample diluted	
12.	Conjugate	
13.	Substrate	
14.	Buffer	
	Sufficiency of Reagent	
15.	Very sufficient = 1; slightly sufficient = 0.5; not sufficient = 0	
	Need for washing	
16.	Yes = 1; No = 0	
	Maximum number of sample run per test kit	
17.	>180 tests per kit = 4	
18.	90-180 test per kit = 3	
19.	<90 test per = 0	
	Equipment needed but not provided in the test (provided = 1; not provided = 0)	
20.	Plate	
21.	Tetraoxosulphate VI acid/sodium hydroxide	
22.	Washing buffer	
	Number of washing cycles per test (one cycle = 3; 2 cycles = 2; 3 cycles = 0)	
23.	Cycles of washing	
	Incubation Period	
24.	60-90mins. = 3; 100-120mins. = 2; 150mins. = 1; >150mins. = 0	
Total		= **30**

(Adapted from WHO, 1999)

Table 17: Suitability of rapid screening kits for use in the rural centres or districts

S/N	FACTORS	SCORES
1.	IR Sensitivity (0.5%) 99-100%	3
2.	IR Sensitivity (0.5%) 97.5-98.5%	2
3.	IR Sensitivity (0.5%) 96-97%	1
4.	IR Sensitivity (0.5%) 96-95%	0
5.	IR Sensitivity (0.5%) <95%	-1
6.	RR Specificity (0.5%) 99-100%	3
7.	RR Specificity (0.5%) 97.5-98.5%	2
8.	RR Specificity (0.5%) 96-97%	1
9.	RR Specificity (0.5%) 96-95%	0
10.	RR Specificity (0.5%) <95%	-1
11.	Incubation temperature (room)	3
12	Incubation temperature (other than room)	-1
13.	Shelf life (6 months at room)	2
14.	Shelf life (1 year at 2-8°C)	1
15.	Shelf life (<6 months at room/<1 year at 2-8°C)	0
16.	Storage (possible at room temperature)	3
17.	Storage (possible in cold boxes at ≤10°C)	2
18.	Storage (possible only at 2-8°C)	1
19.	Simplicity of procedure (very simple)	3
20.	Simplicity of procedure (simple)	2
21.	Simplicity of procedure (simple but too many steps)	1
22.	Pre-dilution requirement (none)	1
23.	Pre-dilution requirement (compulsory)	0
24.	Use of equipment (not requested at all)	2
25.	Use of equipment (not necessary)	1
26.	Use of equipment (automatic pipette compulsory)	0
27.	Running time per sample (≤5mins.)	5
28.	Running time per sample (6-10mins.)	4
29.	Running time per sample (11-15mins.)	3
30.	Running time per sample (16-25mins.)	2
31.	Running time per sample (26-45mins.)	1
32.	Running time per sample (>45mins.)	0
33.	Maximum samples per minimum running time (60-90)	2
34.	Maximum samples per minimum running time (30-60)	1
35.	Period for minimum running time/90 samples (90-120mins.)	3
36.	Period for minimum running time/90 samples (120-180mins.)	2
37.	Period for minimum running time/90 samples (>180mins.)	1
38.	Reading of test results (visual only)	3
39.	Reading of test results (visual or/and use of spectrophotometer)	2
40.	Reading of test results (use of spectrophotometer only)	1
	TOTAL	**33**

(Adapted from WHO, 1999)

Table 18: Suitability of normal elisa kits for use in the referral centres

S/N	FACTORS	SCORES
1.	IR Sensitivity (0.5%) 99-100%	3
2.	IR Sensitivity (0.5%) 97.5-98.5%	2
3.	IR Sensitivity (0.5%) 96-97%	1
4.	IR Sensitivity (0.5%) 96-95%	0
5.	IR Sensitivity (0.5%) <95%	-1
6.	RR Specificity (0.5%) 99-100%	3
7.	RR Specificity (0.5%) 97.5-98.5%	2
8.	RR Specificity (0.5%) 96-97%	1
9.	RR Specificity (0.5%) 96-95%	0
10.	RR Specificity (0.5%) <95%	-1
11.	Incubation temperature (room)	2
12	Incubation temperature (other than room)	1
13.	Shelf life (6 months at room)	2
14.	Shelf life (1 year at 2-8°C)	1
15.	Shelf life (<6 months at room/<1 year at 2-8°C)	0
16.	Storage (possible only at 2-8°C)	2
17.	Storage (possible in cold boxes at ≤10°C)	1
18.	Storage (possible at room temperature)	0
19.	Simplicity of procedure (Requires skill)	3
20.	Simplicity of procedure (very routine/may not require much skill)	2
21.	Simplicity of procedure (simple)	1
22.	Pre-dilution requirement (none)	1
23.	Pre-dilution requirement (compulsory)	0
24.	Use of equipment (automatic)	2
25.	Use of equipment (manual)	1
26.	Use of equipment (raw methods)	0
27.	Running time per 90 samples (60-90mins.)	5
28.	Running time per 90 samples (91-150mins.)	4
29.	Running time per 90 samples (151-180mins.)	3
30.	Running time per 90 samples (181-190mins.)	2
31.	Running time per 90 samples (191-210mins.)	1
32.	Running time per 90 samples (>210mins.)	0
33.	Maximum samples run (>180)	2
34.	Maximum samples run (90-180)	1
35.	Reading of test results (use of spectrophotometer only)	3
36.	Reading of test results (visual or/and use of spectrophotometer)	2
37.	Reading of test results (visual only)	1
38.	Cut-off determination (needed)	2
39.	Cut-off determination (not needed)	0
40.	Cut-off control (present)	1
41.	Cut-off control (not present)	0
	TOTAL	**31**

(Adapted from WHO, 1999)

3.5 RESULTS

A total of 9 HIV testing kits were evaluated in this phase of the study. Two of these kits namely: the SUB-Recombigen HIV-1/HIV-2 (ELISA kit), and the Determine HIV-1 & 2 (S/R kit) had sensitivity, specificity, reproducibility, positive predictive value (PPV), and negative predictive value (NPV) greater than 99.5% (>99.5%) for the evaluation process.

3.5.1 Evaluation of HIV testing kits (Phase I)

3.5.1.1 Second Generation ELISA kits

The outcome of the operational evaluation of second generation ELISA kits in phase I revealed that the SUB-Recombigen HIV-1/HIV-2 had the highest score (> 99.5%) in sensitivities, specificities, reproducibilities, PPV and NPV, while both Genelavia and Murex had scores less than 99.5% in three of these parameters (i.e, specificity, PPV and reproducibility), (Table 19). SUB-Recombigen also had a higher score of 28 (93.3%), above 27 (90%) of the other ELISA kits (Genelavia and Murex) used in the study with respect to ease of kit's performance (Table 20). SUB-Recombigen again had a higher score of 29 (93.6%), above 28 (90.3%) of the other ELISA kits (Genelavia and Murex) used in the study (Table 21) with reference to the suitability of the kits for referral centres.

3.5.1.2 Simple/Rapid test kits

Among the S/R test kits, Determine HIV-1/2 had the highest sensitivity (100%), specificity (100%), reproducibility (100%), PPV (100%) and NPV (100%) (Table 22) at the first phase of the assessment while Immunocomb II HIV-1 & 2 Bispot had the lowest sensitivity (97.9%) and NPV (97.09%). Other rapid kits (Immunocomb II HIV-1 & 2 CombFirm, Gene II, Omni-Sal HIV-1 & 2 and

Recombigen RTD) used in the study had <99.5% of sensitivity and NPV. With respect to ease of performance, Determine HIV-1/2 had the highest score of 29 (96.7%), followed by Omni-Sal HIV-1 & 2 [28 (93.3%)], Recombigen RTD [28 (93.3%)], Immunocomb II HIV-1 & 2 CombFirm [27 (90%)], Gene II [27 (90%)] and Immunocomb II HIV-1 & 2 Bispot [27 (90%)] in a decending order (Table 23). Similarly, Determine also had the highest score of 33 (100%), while Immunocomb II CombFirm HIV-1 & 2 had the lowest score of 28 (84.9%) with respect to suitability for rural settings (Table 24).

Table 19 : Quality assessment results for second generation ELISA kits used for HIV serodiagnosis in Nigeria (Phase 1)

Assay	Statistical Results of the Studies using a panel of 480 sera at CPHL									
	Sensitivity		Specificity		Predictive values				Reproducibility	
					Negative		Positive			
	n/N[b]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[b]	% (59% LCL/CI)	*n/N*[d]	% (95% LCL/CI)
SUB-Recombigen HIV-1/HIV-2 EIA	280/280	100 (99.96)	200/200	100 (99.96)	200/200	100 (99.96)	280/280	100 (99.96)	300/300	100 (99.96)
Genelavia Mixt	280/280	100 (99.96)	196/200	**98** (97.96-98.04)	196/196	100 (99.96)	280/284	**98.6** (98.56-98.63)	302/304	**99.3** (99.26-99.34)
Wellcozyme/Murex HIV-1+2	280/280	100 (99.96)	196/200	**98** (97.96-98.04)	196/196	100 (99.96)	280/284	**98.6** (98.56-98.63)	302/304	**99.3** (99.26-99.34)

[a]The sensitivities, specificities and reproducibilities of three ELISA assays were carried at CPHL, Lagos, Nigeria, using two laboratory gold standard ELISAs. Abbreviations: 95% LCL, 95% lower confidence limit; 95% CI, 95% confidence interval.

[b]n, number of samples positive by using rapid kits; N, number of samples positive using the two ELISAs

[c]n, number of samples negative by using rapid kits; N, number of samples negative using the two ELISAs

[d]n, number of samples with concordant results by using rapid kits; N, number of samples with concordant results when rerun using the same rapid kits by another laboratory scientists.

*Reproducibility test requires all positive samples and every 10^{th} negative.

Minimum acceptable sensitivity, specificity or reproducibility > or = 99.5%

Table 20: SCORES OF SECOND GENERATION HIV ELISA KITS BASED ON EASE OF PERFORMANCE (PHASE I) USING CRITERIA ADAPTED FROM WHO (1999)

S/N	Name of HIV ELISA Kits	Score (Total = 30)	(%)
1	SUB-Recombigen HIV-1/HIV-2 EIA	28	(93.3)
2	Genelavia Mixt	27	(90)
3	Wellcozyme/Murex HIV-1+2	27	(90)

Table 21: SCORES OF EVALUATED HIV KITS BASED ON SUITABILITY FOR REFERRAL CENTRES (PHASE I) USING CRITERIA ADAPTED FROM WHO (1999)

S/N	2nd Generation ELISA Kits	Score (Total = 31)	(%)
1	SUB-Recombigen HIV-1/HIV-2 EIA	29	(93.6)
2	Genelavia Mixt	28	(90.3)
3	Wellcozyme/Murex HIV-1+2	28	(90.3)

Table 22: Quality assessment results for simple rapid test kits used for HIV serodiagnosis in Nigeria (Phase 1)

Assay	Statistical Results of the Studies using a panel of 480 sera at CPHL									
	Sensitivity		Specificity		Predictive values				Reproducibility	
					Negative		Positive			
	n/N[b]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[b]	% (59% LCL/CI)	*n/N*[d]	% (95% LCL/CI
Determine HIV-1/2	280/280	100 (99.96)	200/200	100 (99.96)	200/200	100 (99.96)	280/280	100 (99.96)	300/300	100 (99.96)
Immunocomb II HIV-1 & 2 CombFirm	278/280	**99.3** (99.26-99.34)	200/200	100 (99.96)	200/202	**99** (98.96-99.04)	278/278	100 (99.96)	300/300	100 (99.96)
Gene II HIV-1/HIV-2	277/280	**98.9** (98.86-98.94)	200/200	100 (99.96)	200/204	**98.04** (98-98.08)	276/276	100 (99.96)	297/297	100 (99.96)
Omni-Sal HIV-1/2 & Subtype O (Saliva test)	276/280	**98.6** (98.56-98.64)	200/200	100 (99.96)	200/204	**98.04** (98-98.08)	276/276	100 (99.96)	297/297	100 (99.96)
Recombigen HIV-1/HIV-2 RTD	275/280	**98.2** (98.16-98.24)	200/200	100 (99.96)	200/204	**98.04** (98-98.08)	276/276	100 (99.96)	297/297	100 (99.96)
Immunocomb II HIV-1 & 2 Bispot	274/280	**97.9** (97.86-98.94)	200/200	100 (99.96)	200/206	**97.09** (97.05-97.13)	274/274	100 (99.96)	295/295	100 (99.96)

[a]The sensitivities, specificities and reproducibilities of six simple/rapid assays were carried at CPHL, Lagos, Nigeria, using two laboratory gold standard ELISAs. Abbreviations: 95% LCL, 95% lower confidence limit; 95% CI, 95% confidence interval.

[b]n, number of samples positive by using rapid kits; N, number of samples positive using the two ELISAs

[c]n, number of samples negative by using rapid kits; N, number of samples negative using the two ELISAs

[d]n, number of samples with concordant results by using rapid kits; N, number of samples with concordant results when rerun using the same rapid kits by another laboratory scientists.

*Reproducibility test requires all positive samples and every 10th negative.

Minimum acceptable sensitivity, specificity or reproducibility > or = 99.5%

Table 23: Scores of simple rapid HIV test kits based on ease of performance (Phase I) using criteria adapted from WHO (1999)

S/N	Name of Simple/Rapid HIV Test Kits	Score (Total = 30)	(%)
1	Determine HIV-1/2	29	(96.7)
2	Recombigen HIV-1/HIV-2 RTD	28	(93.3)
3	Omni-Sal HIV-1/2 & Subtype O (Saliva test)	28	(93.3)
4	Gene II HIV-1/HIV-2	27	(90)
5	Immunocomb II HIV-1 & 2 Bispot	27	(90)
6	Immunocomb II HIV-1 & 2 CombFirm	27	(90)

Table 24: Scores of evaluated HIV kits based on suitability for rural/district centres (Phase I) using criteria adapted from WHO (1999)

S/N	Simple/Rapid HIV Test Kits	Score (Total = 33)	(%)
1	Determine HIV-1/2	33	(100)
2	Recombigen HIV-1/HIV-2 RTD	32	(97)
3	Gene II HIV-1/HIV-2	31	(93.9)
4	Immunocomb II HIV-1 & 2 Bispot	30	(90.9)
5	Omni-Sal HIV-1/2 & Subtype O (Saliva test)	29	(87.9)
6	Immunocomb II HIV-1 & 2 CombFirm	28	(84.9)

3.6 DISCUSSION

In this study, three 2nd generation ELISA and six S/R test kits were separately compared using 7 criteria: sensitivity, specificity, reproducibility, PPV, NPV, ease of performance and suitability for use in both rural/field and urban/referral centres. These are the major factors determining the quality of HIV antibody testing and are therefore referred to as operational characteristics of test kits (UNAIDS/WHO, 2001). The quality of HIV antibody testing is the backbone of controlling the HIV/AIDS epidemic in the country as well as serving as one of the major protectors of blood supply in the nation's blood banks. The value of HIV antibody testing requires constant evaluation of the quality of an HIV testing method (strategy) and more importantly, the efficacy of testing kits used. The assessment of these key qualities would strengthen the serodiagnosis of HIV and standardize the circulating kits being used for this purpose.

Although Sub-Saharan Africa had a slight drop in prevalence as against years 2000 – 2003 since it had maintained the lead, the sub-region still remains the most affected with HIV pandemic (WHO, 2004; UNAIDS/WHO, 2004). Nigeria, being the most populous country in this sub-region and even the third most affected country in the world after South Africa and India, require proper and constant attention with respect to the control of the spread of HIV/AIDS globally (WHO, 2004). Nigeria, with her health education and public awareness campaign, had tried to improve on the control of the spread since recent sentinel seroprevalence surveillance has pegged down the seroprevalence to 5% (FMOH/NASCP, 2004) from 5.4% in 2000 (FMOH/NASCP, 2001). The country, however, should not relent in its efforts to improve on the quality of the HIV antibody testing in the country.

Since control of epidemics is the critical entry point for both prevention and care efforts for HIV/AIDS, HIV antibody testing is therefore critical for such control. The need for HIV prevention and management care for people living with HIV/AIDS (PLWHA) to reduce mortality rate and control spread of HIV/AIDS cannot be over-emphasized. For such need, HIV serodiagnosis is inevitable, especially in the implementation of prevention of mother-to-child transmission (PMCTC) of HIV (Wiktor, *et al.*, 1999; Marseille, *et al.*, 1999; Guay *et al.*, 1999). A gold standard kit/method for HIV serodiagnosis in any country in the world must be established for quality HIV testing and voluntary counselling and testing (VCT) (CDC, 1998; WHO, 1998; 1999). This gold standard involves standard testing strategies (algorithms), standard diagnostic kits and equipment, standard proficiency system and continuous assessment programmes.

Now that VCT has become the order of the day and with large groups (both governmental (GO) and non-governmental organizations (NGOs)), it is crucial to set standards in order to control the use of just any kits for serodiagnosis in the country. This study therefore demonstrated the capacity of 9 common commercially available kits to appropriately suit the Nigerian environmental conditions for serodiagnosis of HIV. With the limited supplemental and confirmatory resources (vis-à-vis, ELISA using HIV Uni-Gold RTD, Camstrix HIV-1/HIV-2 and Murex HIV-1+2 kits as well as WB method using New Lav Blot 1 & 2 kits as CPHL's laboratory gold standards), the outcome of the study was strengthened with statistical analysis.

From various criteria used in this phase of the study, SUB-Recombigen HIV-1/HIV-2 ELISA kit clearly showed that it was an outstanding kit out of other (Murex/Welcozyme and Genelavia) ELISA kits as at the period of the study while Determine HIV-1/2 appeared to be an exceptional kit out of other (Immunocomb II

HIV CombFirm, Gene II, Omni-Sal, Recombigen RTD and Immunocomb II HIV Bispot) rapid kits as at the period of the study.

SUB-Recombigen HIV-1/HIV-2 is a second generation ELISA or EIA kit of high sensitivity and specificity for the detection of anti-HIV antibodies in human serum. SUB-Recombigen had 100% sensitivity, specificity, PPV, NPV and reproducibility in concordance with all supplementary assays and Western Blot used in the study. Like the outcome of the sensitivity and specificity of the multi-centre European evaluation exercise reported by Francois – Gerrard *et al.*, (1996), this study's results on SUB-Recombigen were therefore similar, although the European strain of HIV is different from the Nigerian type. The results of this study on SUB-Recombigen were however contrary to that of Andersson *et al.*, (1997) with respect to the specificity of 89.6% while comparing ELISA kits with S/R test kits in the study of alternative strategies in HIV screening. With respect to ease of performance and suitability of SUB-Recombigen for use at the referral level, the kit is still suitable for use since its scores were above 90% in both and better than others.

Among other normal selected ELISA-based commercially available kits evaluated are Genelavia Mixt and Wellcozyme/Murex HIV-1+2. Both had less specificity (98.0%), PPV (98.6%) and reproducibility (99.2%) than SUB-Recombigen HIV-1/HIV-2 kit's (100%). Apparently, they cannot be chosen as standard diagnostic kits since they both could not meet up with the minimum 99.55% serving as cut-off value. Although Murex HIV-1+2 kit has been extensively used in evaluation studies, especially in the evaluation of rapid test kits using oral fliud (Granade *et al.*, 1995; King *et al.*, 1995) where it was used as one of the supplemental kits. Constantine *et al.*, (1997) found Murex to be very sensitive in support of and less reproducible as against the results of this study while evaluating it among other ELISA kits on

European and Asian specimens. Contrarily, Murex and Genelavia Mixt among others also had the best performance for detection of early seroconversion samples (Ly *et al.*, 2001). Combination of Murex and Innotest were rated higher than Genelavia/3rd Generation Vironostika by Chishawa *et al.*, (2001) with respect to efficiency, sensitivity and specificity. Although both Murex and Genelavia had ease of performance and suitability values equal to or above 90%, SUB-Recombigen was still the best in this respect.

Determine HIV-1/2 is a very simple, rapid immunochromatographic test for the detection of antibodies to HIV-1 and HIV-2 in human serum, plasma, and whole blood. Since it does not require any specific instrumentation or any skill or expertise for reading of the results, it is therefore suitable for use as an anti-HIV antibody detection device in remote areas without electricity. Determine HIV-1/2 showed 100% sensitivity, specificity NPV, PPV and reproducibility for the detection of HIV-1 and HIV-2 antibodies, and the results completely agreed with all the supplemental assays as well as Western Blot method employed in the study. Its results were similar to the findings of Aria *et al.*, (1999), in their hospital-based and field evaluation of rapid kits in India and South East Asia, and Koblavi-Deme *et al.*, (2001) in their evaluation studies on the rapid-based serial serologic testing algorithm in Cote d'Ivoire.

Determine HIV-1/2 was recommended by Koblavi-Deme *et al.*, (2001) and Lien *et al.*, (2000), as one of the rapid kits used for HIV serologic testing algorithms in researches carried out in Cote d'Ivoire and Vietnam respectively. Koblavi-Deme *et al.*, (2001) reported that Determine HIV-1/2 detected all 11 samples from patients recently infected with HIV-1 non–B subtypes and had best results in the evaluation with the seroconversion panel in their studies. Determine HIV-1/2 was selected with

respect to the criteria on ease of performance and suitability for use at the local/district centre because of its simplicity, easy reading of results and tolerability of room temperature over long period of months before its expiry date (having the longest shelf life than any other kit used in the study). As a standard commercially available rapid kit for local/district HIV serodiagnosis, Determine therefore demonstrated its capabilities that surpassed other S/R test kits used in this study.

Among other selected commercially available rapid kits used, the next most efficient test kit found in this study was Immunocomb II HIV–1/2 CombFirm with 99.3% sensitivity, 99% NPV, 100% PPV, specificity and reproducibility respectively. Immunocomb II HIV CombFirm therefore had values shortfall of the minimum 99.5% in both sensitivity and NPV and cannot be considered, as a diagnostic kit in the study. It had a shortfall based on the criteria for ease of performance (90%) and suitability for use at the district/local level (84.9%) against a minimum of 90% in the study. Immunocomb II CombFirm was used in studies carried out by Wisnom *et al.*, (1997), and Abrao-Ferreira *et al.*, (1999) during comparative assessment of serum and oral S/R test kits where it had values of specificity and sensitivity between 99.5% and 100%. Their findings were similar to the results of this study but cannot substantiate the effectiveness or efficacy of the kit in respect of ease of performance and suitability for use in rural communities that lack storage facilities.

Just following Immunocomb II CombFirm in the trends is Genie II with sensitivity of 98.9% and NPV of 98% that made it less sensitive than Determine HIV–1/2 and Immunocomb II HIV-1/2 CombFirm mentioned earlier and not meeting up with the minimum standard. Genie II HIV – 1/HIV-2 rapid kit has also been extensively used successfully in many studies and considered very efficient (Constantine *et al.*, 1997; Koblavi-Deme *et al.*, 2001).

Genie II however lacks some abilities in support of this study because Koblavi-Deme *et al.*, (2001) reported that Genie II can only detect 3 out of 11 samples from patients recently infected with HIV-1 non-B subtypes while it also yielded discordant results of 0.7% & 4.5% with Determine HIV–1/2 in the parallel and serial testing algorithm respectively. Genie II HIV–1/HIV-2 rapid kit as a very good rapid test device, contrary to the above position, was still recommended by Constantine *et al.*, (1997) as the rapid test kit of choice among others for HIV screening test in developing countries. Apart from a lower ease of performance and suitability values than Determine, it also lacked the ability to detect some HIV–1 subtypes in spite of this ability to detect HIV-I subtype O which circulates in Cameroon and some parts of Nigeria.

The detection of Immunoglobulin A (IgA) HIV antibodies using ELISA is an effective method for early diagnosis of HIV-infected infants in comparison with conventional Immunoglobulin G (IgG) HIV antibody tests. It is a simple and inexpensive method that could be used in both developed and developing countries. Statistical analysis was assessed comparing IgA results with HIV infection status as the gold standard. Sensitivity (95%) and specificity (100%), positive predictive value (100%), and negative predictive value (94%) of IgA antibody determination were analyzed taking into account only one sample per child and only children older than 6 months (King *et al.*, 1995). Of these saliva test devices is Omni-Sal HIV-1/2 and Subtype O RTD for saliva samples. Omni-Sal HIV–1/2 and Subtype O saliva test device had similar results with the Determine HIV–1/2 except for the sensitivity of 98.6%, ease of performance of 93.3% and suitability for rural/district centre of 87.9% as against the 100%, 96.7% and 97% for Determine HIV-1/2 respectively.

Omni-Sal HIV –1/2 results was similar to the works of Granade *et al.*, (1995), Grant *et al.*, (1990), and Chohan *et al.*, (2001), as a simple saliva-based rapid test device that can accurately be utilized in a variety of clinical settings, providing great promise for further applications. Its specificity value is supported by the fact that its performance varies with HIV-1 group O and HIV–2 infected individual (Saville *et al.*, 1997), and that its reliability depends on the sensitivity of normal ELISA test (Francois-Gerrard *et al.*, 1996). Omni-Sal HIV-1/2 is also an efficient rapid testing device of minor limitations and therefore was only recommended for surveillance purposes for HIV-1 Subtype O (Liberatore *et al.*, 1996).

Recombigen HIV-1/HIV-2 rapid test device had one of the least sensitivity (98.2%) and NPV (98%) following Omni-Sal HIV-1/2, Subtype O salvia test device, an accurately specific and reproducible rapid kit as confirmed by other researchers (De Souza *et al.*, 2002) who successfully used it in their HIV surveillance studies in Caracas, Venezuela, but there is need to improve on its sensitivity to some HIV-1 Sub types and variants. Recombigen RTD was ranked second to the Determine with respect to ease of performance and suitability for use. The kits would have been as well recommended for further evaluation as diagnostic kits if not for its sensitivity and NPV that could not meet up the minimum of 99.5% standard.

It is not surprising to find the Immunocomb II HIV-1 & 2 Bispot being the least sensitive (98%) among the evaluated rapid test devices since it has been reported by Constantine *et al.*, (1997), that its sensitivity varied between 83% and 100% among other six rapid test kits evaluated using testing sera from individuals infected with HIV-1 group O and HIV–2. Kamat *et al.*, (2000), however, successfully used Immunocomb II HIV-1 and 2 Bispot for differentially identifying HIV-1 and /or HIV-2 infections among replacement blood donors in Mumbai (Bombay). Although

Immunocomb II HIV-1 and 2 Bispot did not qualify based on this study's criteria, it was still a recommended rapid test device for HIV surveillance and diagnosis.

3.7 CONCLUSION

In the first phase of the evaluation of common commercially available kits in Nigeria, SUB-Recombigen (a 2nd generation ELISA kit) clearly emerged as the only diagnostic kit of choice among two other ELISA kits based on the evaluation criteria used in the study. Similarly, Determine (a simple, rapid immunochromatographic kit) clearly appeared as the only diagnostic kit of choice among five other S/R test kits based on the evaluation criteria used in the study. This comparative evaluation of several kits was the first of its kind conducted in Nigeria.

CHAPTER FOUR
EVALUATION OF HIV TEST KIT (PHASE II)

4.0 EVALUATION OF HIV TEST KITS (PHASE II)

4.1 INTRODUCTION

Transmission of human immunodeficiency virus through blood transfusion and diagnosis in hospitals and public health settings has become a great concern. Detection of HIV antigen (Ag) and antibodies (Ab) during early viremic window period of HIV infection and seroconversion continues to be a challenge (Ly *et al.*, 2001). In this respect, a careful choice of ELISA and S/R test kits must be made by any laboratory to minimize possible errors.

Simple-rapid test kits and ELISA kits are specific for different risk populations, prevalence and purpose. The kits meant for research cannot be used for diagnosis because it cannot be error-proved since such kits are subject to experiments. If a diagnosis kit has been standardized and a test of inferior quality is chosen for testing a high-risk population, following up on false results will consume time, money (Nishanian *et al.*, 1987) and even life (in cases of blood transfusion). False positive results could cause considerable unnecessary anxiety to patients when used diagnostically and could result in unnecessary waste of valuable blood in blood banks, or even infection of victims through transfusion of such blood. The damage caused by false negative results is also serious. A person who is erroneously informed that he or she is negative and not properly followed up, could readily spread the virus through sexual contacts in the following months.

The discovery of HIV was rapidly followed by the development of assays to detect the antibodies to the HIV. Since the discovery, a number and variety of commercial assays have been available to screen blood, diagnose infection and monitor disease progression in individuals infected with HIV-1 & 2. These assays are

categorised into four main classes, including tests that detect HIV antibody, detect p24 antigen, detect or quantify viral nucleic acid, and estimate T-lymphocytes number (cell phenotyping) (Constantine, 1999). The ELISA is the most common immunoassay used for the detection of HIV antibody and antigen. This method has evolved from the first-generation viral lysate-based IgG tests, to the second-generation tests incorporating recombinant and/or synthetic peptide antigen, to the third-generation tests which detect IgG and IgM (antigen sandwich techniques) and finally to the third-generation-plus assays which also detect HIV-1 group O (Constantine, 1999).

Specific antibody to HIV is synthetized soon after infection, although the precise time may depend on several factors, including both host and viral characteristics. The antibody is detected in majority of individuals within 6 to 12 weeks after infection with the earlier generation of assays, but such antibody can be detected within 3 to 4 weeks when newer third-generation antigen sandwich assays are used (Constanine *et al.*, 1994). This window period can be shortened to 2 weeks using p24 antigen assays or to 1 week by nucleic acid detection assays (Feinberg, 1996). In addition to increased sensitivity and specificity with incorporation of recombinant proteins and synthetic peptide antigens, the ELISA offers several advantages over other types of assays in that it is inexpensive, relatively simple, suitable for testing sizeable numbers of samples and easily adapted to automated platforms. Although nucleic acid testing and viral culture are highly sensitive and specific methods to identify infections, respectively, these procedures are time-consuming, laborious and expensive (Constantine, 1999).

The detection of p24 antigen by ELISA is a simple and cost-effective method to demonstrate viral components in blood, thereby verifying infection and/or

identifying early infection, and offer the same performance advantages as the ELISA for antibody detection (Constantine, 1999a). The combination of this new antigen detection assay with antibody detection leads to advent of a fourth generation ELISA kit. The 4th generation ELISA kit is the existence of an assay that provides combination ELISA for simultaneous antigen and antibody detection in order to reduce the diagnostic window period of infection as well as decrease the time, personnel and cost necessary to perform both assays (Gurtler *et al.*, 1998; Weber *et al.*, 1998).

Similarly, the advent of a variety of simple, instrument-free screening tests such as agglutination (e.g., Capillus), immunofiltration (flow through tests e.g., HIV Check), immunodot (flow through spot e.g., HIV Spot), immunochromatographic (lateral flow tests e.g., Determine, Recombigen (RTD) and comb/dipstick tests (e.g., Immunocomb) led to the development and circulation of various types of S/R test kits in the markets (WHO, 2002; 2004). Both the new generation (4th Generation) ELISA and new S/R test kits need to be assessed to determine whether they can fit in as diagnostic kits suitable for our environment and can detect the circulating virus strains and antibodies in infected individuals. There is also need to update the old kits to determine their recent capacity and efficiency and compare there with the new kits in circulation. Such needs would enable the country to achieve her objectives of controlling and preventing the spread of HIV/AIDS by improving on the quality of HIV antibody testing methods.

Apart from the control of epidemics, the increasing flooding of Nigerian markets with all sorts of HIV antibody testing kits as commercially available kits without thorough accreditation through certification by both National Agency for Food and Drug Administration and Control (NAFDAC) and National Action

Committee for AIDS (NACA), raises more questions on the quality of HIV antibody testing in the country. The absence of regular assessment of HIV test kits use and continuous operational evaluation programme for HIV test kits stimulated the authors' curiosity to carry out periodic, operational and experimental studies on the commercially available HIV test devices circulating in the Nigerian market. At the end of the evaluation, update of current diagnostic kits for determining HIV status of Nigerians would have been implemented.

4.2 OBJECTIVES

The main objective of this study was to evaluate two 4th generation ELISA kits and three new S/R test kits commonly circulating Nigerian market between 1999 and 2000.

4.2.1 Specific Objectives are to:

1. assess the evation parameters namely: sensitivity, specificity and reproducibility of two 4th generation ELISA and three S/R test kits used in the study
2. determine the PPV and NPV of the two 4th generation ELISA and three S/R test kits used in the study
3. evaluate the ease of performance of the two 4th generation ELISA and three S/R test kits used in the study and,
4. evaluate the suitability of the two 4th generation ELISA (for referral centres) and three S/R test kits (for rural centres/ VCT/ field) used in the study.

4.3 MATERIALS AND METHODS

4.3.1 SAMPLE POPULATION

4.3.1.1 Patients Population and Study Centres

See texts 3.3.1.1

4.3.1.2 Sample size

See texts 3.3.1.2.

4.4 LABORATORY METHODS

4.4.1 Evaluation of Common Commercial Available Kits

The sensitivities, specificities and reproducibility of nine rapid tests and five ELISAs were determined on a reference panel of 480 sera with known HIV serologic status. All reference panels of sera used in this study were obtained from the CPHL serum bank in good storage conditions during the course of this study. This phase of the evaluation programme was done in July, 2000. The following commercially available ELISA and S/R test kits were used:

(a) Fourth Generation ELISA kits (Phase II)

1. Vironostika HIV Uni-Form II Ag/Ab (Organon Teknika, Portugal)
2. Genscreen Plus HIV-Ag (Biorad, Marnes la Coquette, France)

(b) Simple Rapid assay kits (Phase II)

3. Capillus HIV-1/HIV-2 (Cambridge Biotech Diagnostics, Galway, Ireland)
4. EFOORA HIV RTD (Efoora Inc., Buffalo Grove, IL, U.S.A.) and
5. HIV-TRI-DOT (J. Mitra & Co., New Delhi, India).

The properties of both normal ELISA and simple rapid test kits can be found in Appendix 1. All these kits were also sourced locally from laboratory kits

suppliers and marketers. The assays for each kit to be evaluated and the supplementary assays (simple-rapid and ELISA using HIV Uni-Gold RTD, Genscreen Plus HIV-Ag and Vironostika HIV Uni-Form II ag/Ab kits as well as WB method using New Lav Blot 1 & 2 kits as CPHL's laboratory gold standards) were run concurrently based on the manufacturers' instruction and test principles (Appendices 2-9).

The criteria for ease of performance scoring (Tables 15 & 16) were adapted from C.D.C. (1998) and the suitability criteria for local and referral centres (Tables 17 & 18) were also designed based on UNAIDS/WHO's recommendations (WHO, 1998a, WHO, 2002).

4.4.2 STATISTICAL ANALYSIS

4.4.2.1 Data Collection and Statistical Analysis

See text 3.4.2.1

4.5 RESULTS

A total of 5 HIV testing kits were evaluated in this phase of the study. Three of these kits namely: the Genscreen Plus HIV-Ag, the Vironostika HIV Uni-Form II ag/Ab (ELISA kit), and the Capillus HIV-1/HIV-2 (S/R kit) had sensitivity, specificity, reproducibility, positive predictive value (PPV), and negative predictive value (NPV) greater than 99.5% (>99.5%) for the evaluation process.

4.5.1 Evaluation of HIV testing kits (Phase II)

4.5.1.1 Fourth Generation ELISA kits

The outcome of the operational evaluation of fourth generation ELISA kits in phase II revealed that both Genscreen Plus HIV-Ag and Vironostika HIV Uni-Form II ag/Ab had the score > 99.5% in sensitivities, specificities, reproducibilities, PPV and NPV (Table 25), but Vironostika had higher score of 29 (96.7%) above 28 (93.3%) of the other ELISA kits (Genscreen Plus) used in the study with respect to kit's ease of performance. Vironostika again had higher score of 30 (96.8%) above 28 (90.3%) of the other ELISA kits (Genscreen Plus) used in the study with reference to the suitability of the kits for referral centres.

4.5.1.2 Simple/Rapid test kits

Among the S/R test kits, Capillus HIV-1/2 + 0 had the highest sensitivity (100%), specificity (100%), reproducibility (100%), PPV (100%) and NPV (100%) (Table 26) at the second phase of the assessment while HIV TRI DOT had the lowest sensitivity (97.1%) and NPV (96.15%). Other rapid kit (EFOORA HIV RTD) used in the study had <99.5% of sensitivity and NPV. With respect to ease of performance, Capillus HIV-1/2 + 0 had the highest score of 29 (96.7%), followed by both EFOORA HIV [28 (93.3%)], and HIV TRI DOT [28 (93.3%)]. Similarly, Capillus HIV-1/2 + 0

also had highest score of 33 (100%) while EFOORA HIV had the lowest score of 29 (87.9%) with respect to suitability for rural settings (Table 27).

Table 25: Quality assessment results for fourth generation HIV ELISA kits used for HIV serodiagnosis in Nigeria (Phase II)

Assay	Statistical Results of the Studies using a panel of 480 sera at CPHL									
	Sensitivity		Specificity		Predictive values				Reproducibility	
					Negative		Positive			
	n/N[b]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[b]	% (59% LCL/CI)	*n/N*[d]	% (95% LCL/CI)
Vironostika HIV Uni-Form II Ag/Ab	280/280	100 (99.96)	200/200	100 (99.96)	200/200	100 (99.96)	280/280	100 (99.96)	300/300	100 (99.96)
Genscreen Plus HIV-Ag	280/280	100 (99.96)	200/200	100 (99.96)	200/200	100 (99.96)	280/280	100 (99.96)	300/300	100 (99.96)

[a]The sensitivities, specificities and reproducibilities of two ELISA assays were carried at CPHL, Lagos, Nigeria, using two laboratory gold standard ELISAs.
Abbreviations: 95% LCL, 95% lower confidence limit; 95% CI, 95% confidence interval.
[b]n, number of samples positive by using rapid kits; N, number of samples positive using the two ELISAs
[c]n, number of samples negative by using rapid kits; N, number of samples negative using the two ELISAs
[d]n, number of samples with concordant results by using rapid kits; N, number of samples with concordant results when rerun using the same rapid kits by another laboratory scientists.
*Reproducibility test requires all positive samples and every 10th negative.
Minimum acceptable sensitivity, specificity or reproducibility > or = 99.5%.

Table 26: Quality assessment results for simple rapid HIV test kits used for HIV serodiagnosis in Nigeria (Phase II)

Assay	Statistical Results of the Studies using a panel of 480 sera at CPHL									
	Sensitivity		Specificity		Predictive values				Reproducibility	
					Negative		Positive			
	n/N[b]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[b]	% (59% LCL/CI)	*n/N*[d]	% (95% LCL/CI
Capillus HIV-1/HIV-2	280/280	100 (99.96)	200/200	100 (99.96)	200/200	100 (99.96)	280/280	100 (99.96)	300/300	100 (99.96)
EFOORA HIV RTD	274/280	**97.9** (97.86-97.94)	200/200	100 (99.96)	200/206	**97.09** (97.05-97.13)	274/274	100 (99.96)	280/295	**94.5** (94.46-94.54
HIV-TRI-DOT	272/280	**97.1** (97.06-97.14)	200/200	100 (99.96)	200/208	**96.15** (96.11-96.19)	272/272	100 (99.96)	293/293	100 (99.96)

[a]The sensitivities, specificities and reproducibilities of three simple/rapid assays were carried at CPHL, Lagos, Nigeria, using two laboratory gold standard ELISAs. Abbreviations: 95% LCL, 95% lower confidence limit; 95% CI, 95% confidence interval.

[b]n, number of samples positive by using rapid kits; N, number of samples positive using the two ELISAs

[c]n, number of samples negative by using rapid kits; N, number of samples negative using the two ELISAs

[d]n, number of samples with concordant results by using rapid kits; N, number of samples with concordant results when rerun using the same rapid kits by another laboratory scientists.

*Reproducibility test requires all positive samples and every 10th negative.

Minimum acceptable sensitivity, specificity or reproducibility > or = 99.5%.

Table 27: Scores of evaluated HIV kits based on suitability for rural/district centres (Phase II) using criteria adapted from WHO (1999)

S/N	Simplr/Rapid HIV Test Kits	Score (Total = 33)	(%)
1	Capillus HIV-1/HIV-2	33	(100)
2	HIV-TRI-DOT	30	(90.9)
3	EFOORA HIV RTD	29	(87.9)

4.6 DISCUSSION

Kits assessed in Phase I were carried out earlier than the second phase kits because the ELISA kits of the first phase were clearly different from those of the second phase based on 2nd phase kits' additional detection capacity. Hence, the importance of second phase kits emphasized the need for dynamism in HIV testing technology. Weber *et al.*, (1998) concluded that fourth generation assays permit an earlier diagnosis of HIV infection than third generation antibody screening assays through the detection of p24 antigen, which may be present in serum samples from individuals with recent HIV infection prior to seroconversion. In addition, Courouce (1999) in his update review concluded that the constant improvement of anti-HIV screening tests which leads one to shorten the 'window' period permits an earlier diagnosis of HIV infection and progressive decrease of transfusion risk. The fourth generation ELISA kits were introduced into the market in order to reduce the diagnostic window experienced by the other generations of HIV ELISA devices in the HIV serodiagnosis (Weber *et al.*, 1998; Constantine, 1999; Weber *et al.*, 2002)

In the second phase, Vironostika HIV Uni-Form II Ag/Ab ELISA was shown to be better than Genscreen plus HIV-Ag as a diagnostic kit of choice of the commercially available fourth generation ELISA kits based on the evaluation parameter while Capillus HIV-1/HIV-2 latex agglutination test kit also emerged as a diagnostic kit of choice for S/R HIV antibody test kit in this study.

Of the two normal ELISA kits assessed in the second phase, Vironostika Uni Form II Ag/Ab was better than Genscreen Plus HIV Ag-Ab based on ease of performance and suitability for referral settings. The two fourth generation ELISA kits however proved to be very effective for HIV serodiagnosis with 100% in all their evaluation parameters, namely; sensitivity, specificity, reproducibility, NPV and PPV.

However, Ly *et al.*, (2001) found that Vironostika Uniform II Ag-Ab was better in sensitivity than Genscreen Plus Ag-Ab in France during their study while Chishawa *et al.*, (2001) reported the suitability of Vironostika as Gold standard in their referral centre in Zimbabwe.

Vironostika HIV Uni-Form II Ag/Ab has been extensively used as a normal ELISA-based control for standardizing the evaluation of a number of commercially available kits (Kuun *et al.*, 1997, Wisnom *et al.*, 1997, Ettiegne-Traore *et al.*, 1998). Its outcome as having 100% sensitivity, specificity, NPV, PPV and reproducibility on all tested panels of sera was evocative of its ability and suitability for detecting HIV-1, HIV-2, HIV-1 group O and HIV-Ag (HIV antigen) as the basic qualities it possessed in the studies carried out by the aforementioned researchers. However, Vironostika HIV Uni-Form II Ag/Ab was more efficient and more suitable by certain qualities than Genscreen Plus Ag due to the outcomes of ease of performance (96.7% against 93.3%) and suitability for use (96.8% against 90.3%).

The study of Ly *et al.*, (2001) was in favour of Genscreen and Murex as against Vironostika. Genscreen and Murex HIV Duo Ag/Ab were found to be more specific than Vironostika over pooled samples from Asia, Europe and East Africa used in their study. According to the findings of Shima *et al.*, (2001), specificity and sensitivity of Genscreen HIV Ag-Ab was 100 and 99.7% involving 90 positive and 760 negative serum panels and this is in support of the findings of this study. Genscreen Plus Ag was used as a reference test by De Souza *et al.* (2002). Although Genscreen Plus was reliably used in the study carried out by both Shima *et al.* (2001) and Chohan *et al.* (2001), its ease of performance (93.3%) and suitability for use (90.3%) made it inferior to Vironostika HIV Uni-Form II Ag/Ab kit with 96.7% and 96.8% respectively. Apart from Ly *et al.*, (2001), Weber *et al.*, (2002) also rated

Genscreen among the best ELISA kits. Genscreen was also considered very effective and efficient but less preferred than Vironostika by selection criteria.

Of the 3 selected commercially available fourth generation rapid test devices, Capillus HIV–1/HIV-2, a capillary latex agglutination assay, was identified as the S/R diagnostic kit of choice, based on sensitivity (100%), specificity (100%), NPV (100%), PPV (100%), and reproducibility (100%); as well as ease of performance (96.7%) and suitability for use (100%). These findings were similar to those reported by Kuun *et al.*, (1997), and Koblavi-Deme *et al.*, (2001). Supporting the results further, Capillus HIV-1/2 has been extensively used in HIV seroepidemiological studies and found to be very reliable in Nigeria and some countries (Omotade *et al.*, 2001, Ramalingam *et al.*, 2001).

Other workers however slightly disagreed with these findings claiming that Capillus failed to detect certain subtypes of HIV-1 in specimens (Philip *et al.*, 1995) although Capillus HIV-1/HIV-2 rapid test device is widely known to detect anti-HIV-1, anti-HIV-2 and anti-HIV-1 subtype O antibodies in plasma, serum or whole blood specimens (Koblavi-Deme *et al.*, (2001). Capillus was however reported by Urassa *et al.*, (2002) in Dar Es Salaam, Tanzania to have 100% sensitivity but 98.7% specificity in the 1,412 fresh serum samples evaluated. Andersson *et al.*, (1997), recommended in Sweden that Capillus is a suitable rapid screening test (RST) for Africa, with sensitivity of 100% and specificities of initial and repeated testing of 99.8 and 99.9% respectively.

HIV TRI-DOT rapid test device followed Capillus HIV-1/HIV-2 in order of capability and suitability with a lower sensitivity of 97.1% and NPV of 96.15% than Capillus (100%), ease of performance of 93.3% and suitability for rural/district centres of (90.9%) as against 96.7% and 100% for Capillus respectively. Although it

did not achieve the minimum 99.5% in all the evaluation parameters, its results however reflected an hospital–based evaluation study carried out by Kannagai *et al.*, (2000) and Shah *et al.*, (2002), that it is a suitable rapid test device for use where facilities and laboratory expertise are limited.

Nonetheless, Efoora HIV RTD had higher sensitivity (97.9%) and NPV (97.1%) than HIVTRI-DOT (97.1%) and (96.15%) but was much less reproducible (94.5%) than HIV TRI-DOT (100). Hence, Efoora could not meet up with the minimum standard of 99.5% of the evaluation parameters used in the study, and was even the least effective rapid test devices in this study because it was also scored less suitable for rural/district centre due to its demand for storage facilities requiring electricity. Results of the study done by Engelbrecht *et al.*, (1994), identified Efoora as a less suitable commercially available kit for serodiagnosis but good enough for HIV surveillance studies.

The need for HIV prevention and management care for people living with HIV/AIDS (PLWHA) to reduce mortality rate and control spread of HIV/AIDS cannot be over-emphasized. For such, HIV serodiagnosis is inevitable, especially in the implementation of prevention of mother-to-child transmission (PMCTC) of HIV (Guay *et al.*, 1999). A gold standard for HIV serodiagnosis in any country in the world must be established for quality HIV antibody testing and voluntary counselling and testing (VCT) (Giles *et al.*, 1997; Henry, 1999). This gold standard involves standard testing strategies (algorithms), standard diagnostic kits and equipment, standard proficiency system and continuous assessment programmes. Now that VCT has become the order of the day and with large groups [both governmental (GO) and non-governmental organizations (NGOs)] showing interest, it is imperative to set

standards in order to control use of just any kits or methods for serodiagnosis in the country.

4.7 CONCLUSION

In the second phase of the evaluation of common commercially available kits in Nigeria, Vironostika HIV Uni-Form II Ag/Ab clearly emerged as a diagnostic kit of choice representing the 4th generation ELISA kit based on the evaluation criteria used in the study. Similarly, Capillus HIV-1/HIV-2 (a simple, rapid latex agglutination test kit) clearly appeared as a diagnostic kit of choice representing the S/R test kits based on the evaluation criteria used in the study. This comparative evaluation of another generation of HIV test kits was the first of its kind conducted in Nigeria.

CHAPTER FIVE
A RAPID-BASED HIV TESTING ALGORITHM

5.0 A RAPID-BASED HIV TESTING ALGORITHM

5.1 INTRODUCTION

With the advent of "3 by 5 initiatives" by the WHO, HIV infected persons need to benefit from the antiretroviral drug treatments in Africa. And with the existence of safe blood transfusion practices, all blood and its product must be certified fit for use. Hence, there is need for appropriate diagnosis anywhere for safe blood supply and its products as well as immediate determination of the status of such HIV-infected persons (CDC/WHO/APHL, 2002). Thus, accurate and cost-effective testing is of great importance in combating the spread of HIV, considering the serious ethical, legal and social issues that accompany HIV infection. Conventionally, an ELISA or S/R assay is carried out for the first step screening and subsequently any positive sample is further subjected to most commonly-used confirmatory tests (WB) or any other confirmatory test such as line immuno-assay (LIA), immunofluorescence assay (IFA) and PCR method (Sato *et al.*, 1994). A rapid evolution in diagnostic technology since the advent of the first HIV antibody tests became commercially available, has led to possibilities for alternative strategies or algorithms.

For the development of HIV testing algorithms, it is noteworthy that algorithms are designed based on the objectives of the HIV antibody testing. There are three main objectives for which HIV antibody testing is performed. These are, screening of donated blood for blood safety; epidemiological surveillance of HIV prevalence; and diagnosis of infection in individuals (WHO, 1998a). UNAIDS and WHO therefore recommended three testing strategies (algorithms) based on the objectives of the tests and prevalence of HIV in the population. The algorithms have been recently updated, to maximize accuracy and minimize cost (Henry, 1999).

These algorithms are: strategy I; involving use of one ELISA or S/R assay for testing all sera/plasma (mostly applicable for transfusion and transplant safety, most prevalence studies, especially in >10% general population and for diagnosis of >30% symptomatic PLWHA); strategy II involving first testing all sera/plasma with one ELISA or S/R assay and further re-testing of any serum found reactive with a second ELISA or S/R assay based on a different antigen preparation and/or different test principle (mostly applicable for prevalence studies of ≤ 10% general population, for diagnosis of ≤ 30% symptomatic and > 10% asymptomatic PLWHA); and strategy III; involving another test for any serum/plasma that is reactive in the first and non-reactive in the second assay or vice versa, in addition to strategy II (mostly applicable for the diagnosis of ≤ 10% asymptomatic PLWHA) (UNAIDS, 1999; WHO, 2002; Henry, 1995).

Use of these strategies (algorithms) for diagnosis depends on the nature and prevalence among the sample population. For instance, diagnosis among tuberculosis (TB) patients (of about 30% prevalence) would require strategy I, but should be repeated with another fresh blood from the tested individual to eliminate any technical errors. In lower prevalence population (of about 10% prevalence), strategy II would be very suitable to determine the HIV status. Strategy III is only relevant where there is very low prevalence in the population (of about 1% prevalence) to determine the final HIV status of individuals. In the selection of HIV antibody tests for use in strategies II and III, the first test should have the highest sensitivity, whereas the second and third tests should have a higher specificity than the first (UNAIDS/WHO, 1999). The number of initial discordant, indeterminate results should not exceed 5%. If it does, quality assurance procedures should be checked and/or a new test combination should be adopted.

Strategy II has been adopted by many countries, especially in Sub-Saharan Africa (Kassler *et al.*, 1998). Such algorithm could be run concurrently and referred to as parallel algorithm while the algorithm could also be run subsequently and referred to as serial algorithm. Some algorithms may involve rapid assay only and are therefore called rapid-based, some may involve both rapid and ELISA assays, referred to as ELISA/rapid-based, while others may involve ELISA assays only and are hence called ELISA-based. The classic algorithm for the serodiagnosis of HIV infection include screening sera using ELISA or R/S assay and confirming screened positive samples using WB, LIA, IFA, PCR, etc. (CDC, 1989).

For this purpose, the use of standard ELISAs, designed for batch testing, followed by confirmatory WB tests, if necessary, is now considered time- and money-consuming (CDC, 1990). Sophisticated equipment (such as automatic pipettes, incubators, and readers) must be available, and these are costly to purchase and maintain, and must be located near clean water and a reliable supply of electricity. The validity of the results obtained by these techniques strongly depends on the skills and proficiency of the technicians, and their interpretation requires skilled training and supervision (UNAIDS/WHO, 1998). These conditions are often lacking in most rural areas and many urban centres in the country.

With the report of sentinel seroprevalence study of the country in 2004 pegging down the prevalence to 4.4% from 5.0% in 2003 (FMOH/NASCP, 2006), there is an urgent need to design methods of determining HIV status of Nigerians anywhere in the country. Recently, the government adopted an interim HIV testing strategy, after many years of sentinel seroprevalence studies without evaluating the suggested methods or strategy (The Guardian Newspaper, 2006). The strategies were just suggested to be carried out in parallel rather than serial.

For the rapid-based HIV serological testing algorithms, the S/R assays which may use flow-through or immunochromatographic membranes and are presented in kit form, do not require either equipment or refrigeration. Their procedures are easy to perform, and their formats allow persons with minimal instruction and training to perform them correctly (Giles *et al.*, 1999). At the end of each procedure, a result can be read visually within few minutes. Even if the cost of these diagnostic procedures is higher (at rate of cost per test) than ELISA, their cost effectiveness is better than those of ELISAs in situations in which small numbers of test are carried out at one time (Speilberg and Kassler, 1996).

Since most clinics and VCT (in the rural centres and some in the urban areas) lack the laboratory infrastructure suitable for diagnostic tests; do not have access to a reference laboratory; lack equipment necessary to perform the test and maintain cold chain; and lack skilled technical staff but require urgent feedbacks of test results for further decisions and actions; (UNAIDS/WHO, 1999), it is imperative to design an algorithm that is capable of overcoming these prevailing inadequacies. In addition, such an algorithm would get to the people at the grassroots (i.e., various local government areas of the country, both rural and urban) at affordable prices to determine their HIV status and hasten treatment of cases at various clinics, especially those awaiting surgical operation, dialysis and blood transfusion. In fact, HIV status determination is a pre-requisite for admission into many hospitals before treatment of patients commences (WHO, 2004b).

Most communities in both rural and urban centres either have epileptic electric power supply or none. The problem of electricity contributes to the disadvantage of normal ELISA in such settings, apart from availability of equipment and trained personnel (CDC, 1989). Since rapid ELISA is very similar to the normal ELISA, but

requires no special equipment for reading, washing or dispensing and also requires a very short duration (i.e., 5 minutes to 90 minutes) (Tamashiro *et al.*, 1993; Spielberg and Kassler, 1996), it is suitable in rural/field settings while the normal ELISA still fits in to referral and urban settings where there are some equipment, trained personnel and electricity supply.

Although most of the published evaluations, especially those performed in Africa (Andersson *et al.*, 1997; Arai *et al.*, 1999; Urassa *et al.*, 2002), were done retrospectively in reference laboratories with stored plasma and/or with limited numbers of samples taken prospectively, the present study is carried out prospectively in a reference laboratory with fresh samples collected within two years of the evaluation. In addition, the demographic and other risk factors of the patients whose blood samples were collected during the evaluation study were analysed.

5.2 OBJECTIVES

The main objectives of this study are to develop and evaluate a rapid-based serial HIV serological testing algorithm.

5.2.1 Specific objectives are to:

1. develop a rapid-based serial HIV serological testing algorithm
2. evaluate the rapid-based serial HIV serological testing algorithm using the evaluation parameters (such as sensitivity, specificity, reproducibility, NPV, and PPV), for the S/R test kits used in the algorithm
3. use the algorithm to study prospectively the infection rate in CPHL patients between 1997 and 1999
4. analyse the demographic pattern among the CPHL patients and determine the risk groups with respect to occupation and other exposures

5.3 MATERIALS AND METHODS

5.3.1 SAMPLE POPULATION

5.3.1.1 Patients Population and Study Centres

See texts 3.3.1.1.

5.3.1.2 Sample size

In addition to 3.3.1.2 for the study design and sample size, another sample size use in the study is stated as follows: A non-probability sampling method known as convenience sampling was used to select the study population. All the patients recruited between July 1997 and December 1999 for the study, were criteria-free volunteers. Sample size was determined by the formula enunciated by Oyejide (1992)

$$N = \frac{Z^2 P(1 - P)}{d^2}$$

where N = Required sample size

P = Estimated prevalence of the condition which is about 5% in this study.

Z = Normal standard deviation for required confidence level of 95% = 1.96.

d = Required level of precision which is 0.05.

$$\text{So, sample size (N)} = \frac{1.96^2 \times .05 \times 0.95}{0.05^2} = 73$$

Two major sample sizes were designed for the study. The first sample size of 480 represented a panel of sera with retrospectively known HIV serologic status for HIV S/R test kits' evaluation. The second sample size of 2,500 was designated as baseline data for the determination of HIV infection rate at the study site. These samples were collected from all age groups and both sexes randomly.

5.3.2 LABORATORY METHODS

5.3.2.1 Sample Collection and Processing

Venous blood samples were collected in 10 ml plain bottles from the referred patients by veni-puncture. All serum samples were processed within 3 hours of collection by separating the serum from coagulated blood (by centrifugation for 10 minutes at 800 x g at ambient temperature). The separated serum was removed and centrifuged again at 800 x g for an additional 10 minutes. The serum samples obtained were stored at –70°C in 1ml aliquots until assayed in a batch. All aliquots preserved between 1997 and 1999 were pooled for serological assays to evaluate the HIV testing algorithms and other aspects of the study.

The serum samples were inactivated in a water bath at 56 °C for 30 minutes prior to assays. Serum inactivation reduces anti-compliment factors and inactivates proteins (including those of all infectious agents) in the serum (Server, 1962).

5.3.2.2 Evaluation of two simple/rapid test kits used for the algorithm

The sensitivities, specificities and reproducibility of two rapid tests were determined on a reference panel of 480 sera with known HIV serologic status. All reference panels of sera used in this study were obtained from the CPHL serum bank in good storage conditions during the course of this study. This phase of the evaluation programme was done in August, 2000. The following commercially available S/R test kits were used:

1. Determine HIV-1/2 (Abbott Diagnostics, Pretoria, South Africa)
2. Capillus HIV-1/HIV-2 (Cambridge Biotech Diagnostics, Galway, Ireland)

See last two paragraph of texts 4.4.1

5.3.2.3 Development of HIV Testing Algorithms

Based on the results of the above evaluation, two rapid kits (Determine and Capillus) were selected to prospectively evaluate serial and parallel HIV testing algorithms (WHO, 1998a; 2002; 2004b).

(a) Rapid-based serial HIV testing algorithm

Determine and Capillus as candidate kits were set in serial order to perform rapid-based HIV testing algorithm on 2,500 sera obtained from prospective patients/individuals referred to CPHL consecutively.

(b) Parallel HIV testing algorithm

The rapid-based HIV testing algorithm was carried out using Determine and Capillus. The two candidate kits were run for 40 samples simultaneously. The parallel HIV testing algorithms were discontinued for cost ineffectiveness as they yielded similar results with other algorithms.

5.3.2.4 Supplemental serological assays for the HIV testing algorithms

All seropositive concordant and discordant samples were subjected to serological assays at the CPHL. HIV-1 & 2 antibodies were detected by screening with Vironostika HIV Uni-Form II Ag/Ab (Organon Teknika, Portugal) as Enzyme-linked Immunosorbent Assay (ELISA) method and Capillus HIV-1/HIV-2 (Trinity Biotech Diagnostics, Galway, Ireland) as latex agglutination. All screened positive samples were subjected to confirmatory test using New Lav Blot 1 & 2 (Pasteur Diagnostic Laboratories, Marnes la Coquette, France) as Western Blot method.

5.4 ETHICAL CONCERNS

Individuals were educated on the research and on the importance of solving problems of HIV serodiagnosis in Nigeria. They were told that their blood samples would be used for research on evaluation of HIV test kits and the development of algorithms that would standardize diagnostic kits locally and at referral levels. They were informed that their samples might be used for further research if there was such need but that it was not mandatory to participate and that their identity and facial picture would not be recorded in any part of the research (Appendix 10). After these explanations, they freely and readily gave their consent (Appendix 11). The study was approved by the Central Public Health Laboratory, FMOH. For confidentiality, only the consent document had the names of the patients. Study reference number was assigned to each patient/individual involved in the study.

5.5 STATISTICAL ANALYSIS

5.5.1 Data Collection and Statistical Analysis

See text 3.4.2.1

5.6 RESULTS

Two S/R test kits were evaluated for rapid-based HIV serologic testing algorithms used in the study. The Determine HIV-1/2 and the Capillus HIV-1/HIV-2 + O (S/R kits) had sensitivity, specificity, reproducibility, positive predictive value (PPV), and negative predictive value (NPV) greater than 99.5% (>99.5%) for the evaluation process. Determine HIV-1/2 and Capillus HIV-1/HIV-2 were the candidate kits used for serial HIV testing algorithms as rapid-based. The rapid-based serial HIV testing algorithm had a total of 512 (20.5%) samples confirmed by concordance as HIV seropositive individuals during the study.

5.6.1 Evaluation of HIV S/R test kits

The outcome of the operational evaluation of the two rapid test kits revealed that the Determine HIV-1/2 and the Capillus HIV-1/HIV-2 + O had score > 99.5% in sensitivities, specificities, reproducibilities, PPV and NPV (Table 28).

5.6.2 Rapid-based serial HIV testing algorithm

Determine HIV-1/2 and Capillus HIV-1/HIV-2 were the candidate kits used for serial HIV testing algorithms as rapid-based. They had a total of 512 (20.5%) samples confirmed by concordance as HIV seropositive individuals during the study (Figure 9). With initial screening using the Determine kit in the algorithm, 1,988 samples (79.5%) were found seronegative while 512 samples (20.5%) were found seropositive. Of the 512 screened positive samples by Determine kit, only 509 (20.4%) were concordantly positive while 3 (0.1%) were discordant negative using Capillus HIV-1/HIV-2. Subjecting the outcome to further evaluation with reference laboratory standard confirmed 512 samples as HIV seropositive with known HIV-1(17.7%), HIV-2 (0.5%) and both HIV-1 & 2 (2.3%) serostatus (Figure 9).

5.6.3 OBSERVATION

5.6.3.1 Rapid-based parallel HIV testing algorithm

Rapid-based parallel HIV testing algorithm run (using Determine and Capillus as candidate kits) for 40 samples gave exactly the same results with its serial counterparts. It was observed that only concordant positives and discordant negatives were repeated with rapid-based serial algorithm whereas all samples were repeated in rapid-based parallel algorithm. The rapid-based parallel HIV testing algorithm was therefore discontinued for cost ineffectiveness as it yielded similar results with its serial counterpart.

5.6.4 Demographics

Two thousand five hundred patients were referred to CPHL during the study period (1997 and 1999). Of these patients, 1,607 were male (64.3%) and 893 were female (35.7%). The patients' ages ranged from 6 months to 77 years, with mean age of 28 ± 4.56. Nine hundred and ninety-eight (40%) were single (female and male were 331 (33.2%) and 667 (66.8%) respectively), while 1,468 (58.7%) were married (female and male were 548 (37.3%) and 920 (62.7%) respectively). Fifty-eight (2.32%) claimed to have acquired HIV through blood transfusion, 438 (17.5%) through their spouses (heterosexually), 54 (2.2%) through contaminated sharp objects and 28 (1.1%) through the child's mother (perinatally) while none claimed to have HIV transmission through homosexual or surgical operation. Six hundred and fifty-six (26.2%) business people and traders enrolled as the largest occupational group while 70 (2.8%) military personnels enrolled as the smallest occupational group for the study (Table 29).

5.6.5 Outcomes of rapid-based serial HIV testing algorithm

Three hundred and fourteen males (61.3%) were confirmed seropositive while 198 females (38.7%) were seropositive. The rate of HIV infection within the females was 22.2% while that of the male group was 19.6%. In addition, 16 (27.6%, CI: 20.89-34.31) of those who claimed they were infected through blood transfusion were HIV seropositive out of those transfused with blood in the study (Figure 10). The highest infection rate was in the Drivers/Other transporters group (37.7%) while the lowest infection rate was in the Bankers group (14.3%) (Table 29).

Table 28: Quality assessment results for simple rapid HIV test kits used for rapid-based serial HIV testing algorithm in Nigeria

Assay	Statistical Results of the Studies using a panel of 480 sera at CPHL									
	Sensitivity		Specificity		Predictive values				Reproducibility	
					Negative		Positive			
	n/N[b]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[b]	% (59% LCL/CI)	*n/N*[b]	% (95% LCL/CI
Capillus HIV-1/HIV-2	280/280	100 (99.96)	200/200	100 (99.96)	200/200	100 (99.96)	280/280	100 (99.96)	300/300	100 (99.96)
Determine HIV-1/2	280/280	100 (99.96)	200/200	100 (99.96)	200/200	100 (99.96)	280/280	100 (99.96)	300/300	100 (99.96)

[a]The sensitivities, specificities and reproducibilities of two simple/rapid assays were carried at CPHL, Lagos, Nigeria, using two laboratory gold standard ELISAs.
Abbreviations: 95% LCL, 95% lower confidence limit; 95% CI, 95% confidence interval.
[b]n, number of samples positive by using rapid kits; N, number of samples positive using the two ELISAs
[c]n, number of samples negative by using rapid kits; N, number of samples negative using the two ELISAs
[d]n, number of samples with concordant results by using rapid kits; N, number of samples with concordant results when rerun using the same rapid kits by another laboratory scientists.
*Reproducibility test requires all positive samples and every 10^{th} negative.
Minimum acceptable sensitivity, specificity or reproducibility > or = 99.5%.

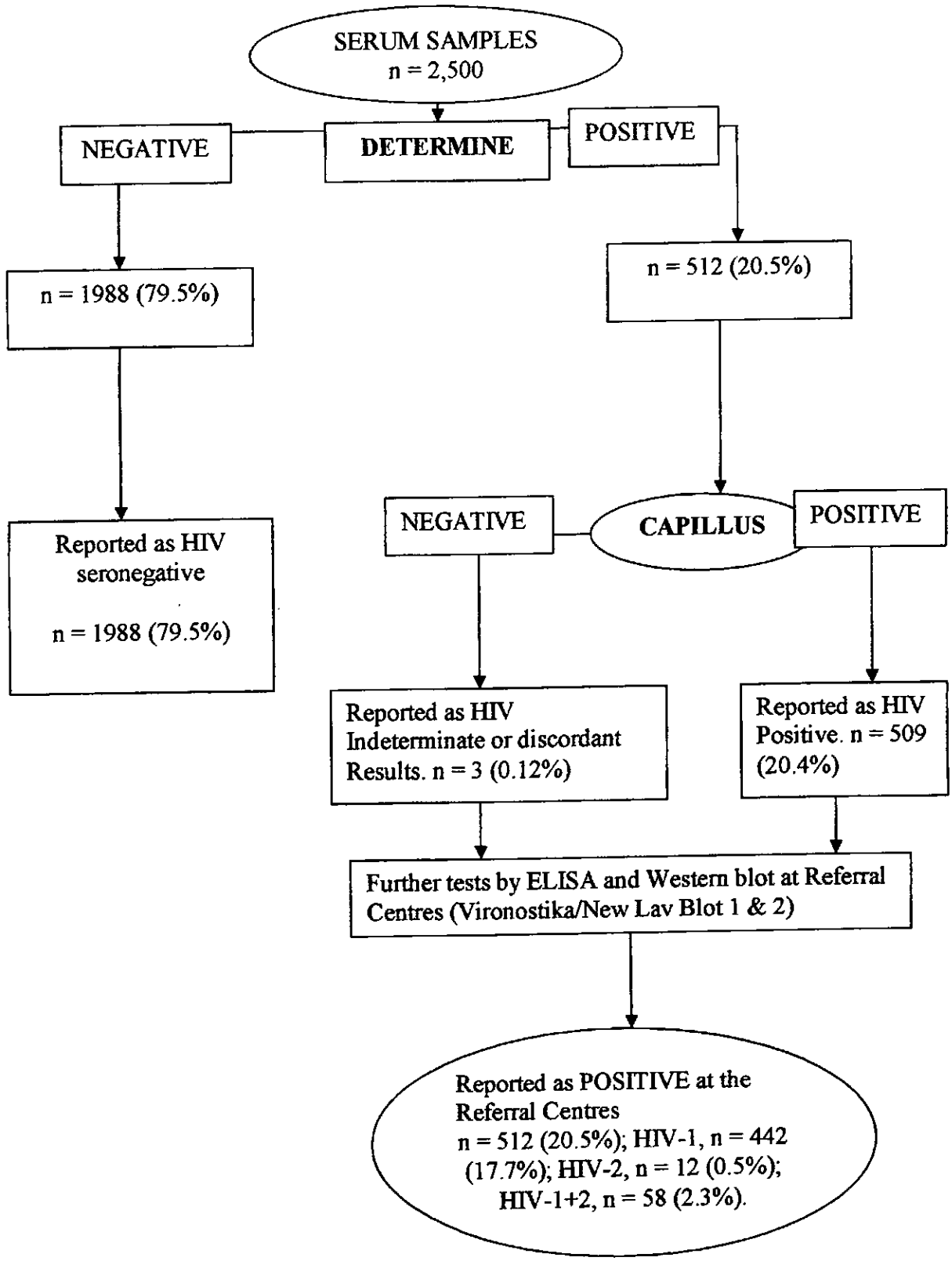


Figure 9: Rapid HIV testing results obtained in the study using serial algorithm (as rapid-based algorithm for rural/district centres) for 2,500 serum samples collected from CPHL between June 1997 and December 1999

* Majority of seropositive patients in the transfused group were children and teenagers aged between 5-19yrs

Figure 10: HIV Transfusion Pattern at CPHL – Lagos, Nigeria (1997-1999)

Table 29: Distribution of HIV patients attending CPHL based on notable occupational group between 1997 and 1999

Occupation Group	Total No. of Patients	Total No. of Male Patients	Total No. of Female Patients	Total No. of Seropositive	No. of Male Seropositive	No. of Female Seropositive	% Infectivity	Confident Interval (CI)
Bankers	133	106	27	19	12	7	14.3	(8.26-20%)
Business/Traders	656	416	240	142	88	54	**21.6**	(18-25%)
Civil Service	288	209	79	53	37	16	18.4	(14-23%)
Fashion designers/Tailors	75	18	57	17	3	14	**22.7**	(13-32%)
Medical Service	76	24	52	12	6	6	15.8	(7.4-24%)
Military	70	67	3	16	16	0	**22.9**	(13-33%)
Students	334	186	148	57	29	28	17.1	(13-21%)
Teachers	113	66	47	22	13	9	19.5	(12-27%)
Transporters/Drivers	77	77	0	29	29	0	**37.7**	(27-49%)
Other Occupations	678	437	241	145	81	64	**21.3**	(13-29%)
TOTAL	**2500**	**1606**	**894**	**512**	**314**	**198**	**20.5**	

5.7 DISCUSSION

For diagnosis, surveillance and blood transfusion in the rural areas and some urban centres with HIV screening-limited-resources, the HIV status of Nigerians and blood/blood products can be determined as quickly as possible anywhere in the country with the advent of a novel strategy known as rapid-based serial HIV serologic testing algorithm. This study has demonstrated the ability of serial algorithm using HIV S/R test kits to function accurately and conveniently in spite of several HIV testing constraints in the rural/resource-limited areas of the country, as an alternative strategy to final determination of the HIV status of a person. Similar algorithms have been implemented in some parts of West Africa (except Nigeria) (Koblavi-Deme *et al.*, 2001; Rouet *et al.*, 2004) and some other African countries (Kassler *et al.*, 1998) based on the circulating HIV strains and needs in such countries.

Considering the problems encountered by the VCT centres and clinics in the rural and suburban centres in determining the final HIV status of their clients, in order to commence treatment and/or surgical operation on patients (especially during emergencies) that constraints encountered by local health centres at determining the HIV status of the patients in any urgent health related matter, it is now high time a solution emerged as a result of the development of a rapid-based serial HIV testing algorithm in this study. The algorithm has been implemented successfully in many developing countries (Henry, 1999) and had contributed immensely to the 3 by 5 initiatives of the WHO on the antiretroviral treatment of 3 million PLWHA by the year 2005 target across the world (WHO, 2004).

Although parallel and serial algorithms gave the same results with the few samples (40 samples) tested, the rapid-based parallel algorithm was discontinued due to the cost and time inefficiency, since the cost of the S/R test kits was one of the

limitations to this study. However, Koblavi-Deme *et al.*, (2001), extensively carried out prospective evaluation of the cost and turn around time of rapid-based parallel and serial testing algorithms and found that parallel testing had 2.5 fold-higher cost and 2 fold-higher turnaround time than serial testing. Hence, a rapid-based parallel HIV testing algorithm is not suitable for rural/field and clinical settings in Nigeria considering the cost of the kits to be used simultaneously per test.

Although both the Determine and Capillus were equally efficient as an S/R test during the evaluation exercise, Capillus HIV-1/HIV-2 + O kit showed its limitations as a second-line discriminatory rapid test by its poor performance for the identification of dual HIV-1 and HIV-2 infections in some individual in the study. Koblavi-Deme *et al.*, (2001) showed that Determine had better detection of seroconversion panels than Capillus. Similar observation on Genie II was made by Rouet *et al.*, (2004), in the evaluation of serial algorithm for the treatment of pregnant women in West African with antiretroviral regimen.

This study undoubtedly revealed that the candidate kits (Determine and Capillus) used as a rapid-based serial HIV testing algorithm have been evaluated to be suitable for not only in rural or field settings (where sophisticated equipment, electricity, etc, are not available) but also in clinics or hospitals where urgent HIV diagnosis is required for treatment/surgical operation during emergencies. Similar algorithm constituting Determine and Genie II was developed for field/rural settings involving pregnant women in West Africa (Rouet *et al.*, 2004). In this algorithm all concordant positive and negative results were reported as confirmed positive and negative without further tests, while discordant positive or negative results were subjected to further conventional HIV antibody tests in referral laboratories. The

algorithm reduces the burden of samples sent for, and duration of time spent on conventional confirmatory tests.

To arouse consciousness for the need to carry out studies for baseline data entry for HIV surveillance in the country, the ELISA/rapid -based serial HIV testing algorithm was applied in a form of prospective study of samples collected at CPHL. A 20.5% infection rate was shown by patients, unlike sentinel seroprevalence surveillance (5.5%) reported in Nigeria in the year 2000 involving only the antenatal group as at the time of this study (NASCP/FMOH, 2001). Use of Determine and Capillus (as rapid-based for rural centres) as a gold standard is therefore recommended for sentinel seroprevalence studies so that quality surveillance and database for HIV is sustained.

Comparing the HIV infection rate of individuals who claimed to have acquired the infection by blood transfusion 6.4%, [i.e., (27.6%, CI: 20.89-34.31) of (2.32%, n = 2, 500)] with that of blood donors (0.3%, 2%, and 4%) as reported by Akinsete *et al.*, (1989 & 1991), it was shown that trends in transmission of HIV through blood transfusion from blood donors is steadily increasing, although the difference between the three variables was still significant ($0.0001 \leq p \leq 0.01$). Hence, there has not been effective improvement in measures reducing the HIV transmission via blood or its products in spite of low prevalence of this group in the population.

The study suggested further research on other occupational groups apart from the established groups (such as the commercial sex workers (CSW) and drivers) (Orubuloye *et al.*, 1991; Dada *et al.*, 1993) as high risk workers for HIV/AIDS because it showed that military, tailors/fashion designers, and traders/business people as new groups with high infection rate. The poverty level and the unemployment rate might also be the possible cause of drive towards promiscuity among the teachers and

students. As for the business personalities and traders, the group falls within the same risk as the transporters and drivers by virtue of occupation. The group is prone to the HIV susceptibility through extra-marital sex, except for the few of them that are well-mannered. Surprisingly, the bankers had the least infection rate among the group. The rate was still very low in this group simply because of their pre-employment screening test and the annual medical check ups to ensure that the staff members are in a state of good health. The military were expected to have a high infection rate, according to some reports (Ekong *et al.*, 2002) due to the nature of their job (i.e., involving urgent movement from one station to another without their entire family).

5.8 CONCLUSION

In this study, a rapid-based serial HIV testing algorithm has been proven to be suitable for rural/field and clinical settings. Of 2,500 of patients attending CPHL between 1997 and 1999, 512 (20.5%) were HIV seropositive [of which 442 (17.7%) were HIV-1, 12 (0.5%) were HIV-2 and 58 (2.3%) were both HIV-1 & 2. Military personnel, tailors and business people appeared as groups to which attention should be drawn as high risk, apart from the CSW and drivers/transporters, while the rate of transmission through blood transfusion remained the same as from the past. All the data were obtained from the outcome of the rapid-based serial HIV testing algorithm.

CHAPTER SIX
AN ELISA/RAPID-BASED HIV TESTING ALGORITHM

6.0 AN ELISA/RAPID-BASED SERIAL HIV TESTING ALGORITHM

6.1 INTRODUCTION

Testing for HIV status of an individual and/or blood product at a referral centre is one of the basic components in the diagnosis and treatment of persons infected with the virus, by screening of blood and its products for transfusion, in surveillance and in HIV/AIDS related research (WHO, 2002a). Transmission of HIV through blood transfusion and diagnosis of infection in hospitals and public health settings continue to be of worldwide concern. Detection of HIV antigen and antibody during the early vireamic window period between HIV infection and seroconversion also continues to be a challenge. With the progress in the development of several generations of testing methods and kits, new assays were developed to detect HIV-1 antigen using anti-p24 antibodies (either polyclonal or monoclonal) on the solid phase and p24 specific antibodies conjugated to an enzyme for the detection of HIV antigen bound to the solid phase (Kessler, *et al.*, 1987; Busch *et al.*, 1990).

As an alternative to ELISA or S/R assay being the first step for screening and subsequent confirmatory test (WB) or any other confirmatory test such as line immuno-assay (LIA), immunofluorescence assay (IFA) and PCR method, a number of HIV testing strategies have been and would still be developed based on new HIV testing technology (Henry, 1999). A rapid evolution in diagnostic technology since the advent of the first HIV antibody tests became commercially available, has led to possibilities for alternative strategies or algorithms. The antibody is detected in majority of individuals within 6 to 12 weeks after infection with the earlier generation of assays, but such antibody can be detected within 3 to 4 weeks when newer third-generation antigen sandwich assays are used (Constanine *et al.*, 1994). This window

period can be shortened to 2 weeks using p24 antigen assays or to 1 week by nucleic acid detection assays (Feinberg, 1996).

Although nucleic acid testing and viral culture are highly sensitive and specific methods to identify HIV and other infections, these procedures are time-consuming, laborious and expensive (Constantine, 1999). The detection of p24 antigen by ELISA is a simple and cost-effective method to demonstrate viral components in blood, thereby verifying infection and/or identifying early infection, and offer the same performance advantages as the ELISA for antibody detection (Constantine, 1999a). The combination of this new antigen detection assay with antibody detection led to the advent of a fourth generation ELISA kit. The 4th generation ELISA kit is the existence of an assay that provides combination ELISA for simultaneous antigen and antibody detection in order to reduce the diagnostic window period of infection as well as decrease the time, personnel and costs necessary to perform both assays (Gurtler *et al.*, 1998; Weber *et al.*, 1998).

With the recent modification of type of HIV test termed as detuned assay or standardized testing algorithm for recent HIV seroconversion (STARHS) as a research tool for measuring incidence (rate of new HIV infection), the evolution of fourth generation ELISA kits would facilitate STARHS use as surveillance tool at the national level in any referral laboratory. This tool allows for determining whether an HIV-infected person has been recently infected i.e., whether the person has sero-converted within an average of 130 days prior to being tested (Janssen *et al.*, 1998). Hence, a testing system involving the new generation ELISA can adopt and implement the STARHS for the country's baseline data with ease.

The use of standard ELISAs, designed for batch testing, followed by confirmatory WB tests has been the traditional role of most reference laboratories

(UNAIDS/WHO, 2002). In Nigeria, clean water and a reliable supply of electricity are major problems encountered by these laboratories. In spite of the presence of sophisticated equipment (such as automatic pipettes, incubators, and readers) skilled/trained technicians and good supervision, the cost of fuelling and maintaining an alternative electric power generating set as well as that of purchasing WB test kits is too overwhelming for the reference laboratory to perform its HIV testing role. An alternative HIV testing strategy or algorithm needs to be developed and evaluated in order to determine its suitability and reliability in the country's electricity-constrained reference laboratories. It would ensure cost-effective determination of HIV status of samples pool in referral centres.

The algorithm, adopted by many countries [especially in Sub-Saharan Africa (Kassler *et al.*, 1998)], applies an initial test involving a more sensitive test kit followed by a more specific test kit. Such algorithm could be run simultaneously and referred to as parallel algorithm while the algorithm could also be run successively and referred to as serial algorithm. The more suitable algorithms may involve both rapid and ELISA assays and are therefore called ELISA/rapid-based or ELISA assays only and so called ELISA-based (Henry, 1999), since batches or pools of samples are involved. The 4th generation ELISA kit is cheaper and more sensitive than the S/R assays and can be used to run 90 or more samples, unlike the S/R that can take a maximum of 10 tests at once (Courouce, 1999).

An ELISA-based or ELISA/rapid-based algorithm would be most appropriate for blood banks where electricity and cost of WB kits constitute the major constraints. As for early detection and reduction of diagnostic window designate additional advantages (Constantine, 1999a), of an algorithm using 4th generation ELISA kit as one of the candidate kits, it would be cost effective to involve such algorithm in the

determination of the final HIV status of blood or blood products for safe, effective and timely blood transfusion, especially in emergency cases and erratic electric power supply.

HIV seroprevalence information among antenatal clinic attendees has been available since the mid-1980s from Nigeria. However, reports from more than one or two sites per year did not begin until 1991-92. By 1993-94 ten major urban sites were reporting HIV prevalence among women attending antenatal clinic. HIV prevalence remained low for many years in Nigeria. But, by 1988-90, one percent of women attending antenatal clinic in the major urban areas tested positive for HIV. By 1993-94, a median of nearly 4% were tested positive and in 1999, nearly 5% tested positive. Among the 10 major urban sites in 1999, HIV prevalence ranged from 3 % to 8% of this group of women tested.

There was a rise between 1999 and 2001 in seroprevalence from 5% to 5.4% (FMOH/NASCP, 2003) and this rise motivated NASCP to strengthen campaigns with health education and public awareness. However, with the report of sentinel seroprevalence study of the country in 2005 pegging down the prevalence to 4.4% from 5.0% in 2003 (FMOH/NASCP, 2006), there is an urgent need to design methods of determining an evidence-based HIV status of Nigerians anywhere in the country. Recently, the government has adopted an interim HIV testing strategy after many years of sentinel seroprevalence studies without evaluating the suggested method or strategy (The Guardian Newspaper, 2006). The strategy was just suggested to carry out HIV testing in parallel rather than in serial, for standard HIV serodiagnosis in the country.

None of the published evaluations, especially those performed in Africa (Anderson *et al.*, 1997; Arai *et al.*, 1999; Urassa *et al.*, 2002), ever ventured into the

ELISA/rapid-based HIV testing algorithm although ELISA-based type had been evaluated by Ly *et al.*, (2001). The present study was however carried out prospectively in a reference laboratory with fresh samples collected within two years of the evaluation. In addition, some demographic qualities, the state of origin of the patients whose blood samples were collected and the trend/rate of infection during the evaluation study were analysed.

6.2 OBJECTIVES

The main objectives of this study are to develop and evaluate an ELISA/rapid-based serial HIV serological testing algorithm.

5.2.1 Specific objectives are to:

1. develop an ELISA/rapid-based serial HIV serological testing algorithm
2. evaluate the ELISA/rapid-based serial HIV serological testing algorithm using the evaluation parameters (such as sensitivity, specificity, reproducibility, NPV, and PPV), for the S/R test kits used in the algorithm
3. use the algorithm to study prospectively the trend and rate of infection in CPHL patients between 1997 and 1999
4. analyse some demographic qualities among the CPHL patients and determine the distribution of seropositive cases based on their states of origin.

6.3 MATERIALS AND METHODS

6.3.1 SAMPLE POPULATION

6.3.1.1 Patients Population and Study Centres

See texts 3.3.1.1.

6.3.1.2 Sample size

See text 5.3.1.2

6.3.2 LABORATORY METHODS

6.3.2.1 See text 5.3.2.1

6.3.2.2 Evaluation of two simple/rapid test kits used for the algorithm

The sensitivities, specificities and reproducibility of one S/R tests and two ELISAs were determined on a reference panel of 480 sera with known HIV serologic status. All reference panels of sera used in this study were obtained from the CPHL serum bank in good storage conditions during the course of this study. This phase of the evaluation programme was done in October, 2000. The following commercially available test kits were used:

1. Vironostika HIV Uni-Form II Ag/Ab (Organon Teknika, Portugal)
2. Determine HIV-1/2 (Abbott Diagnostics, Pretoria, South Africa)
3. SUB-Recombigen HIV-1/HIV-2 EIA (Cambridge Biotech Diagnostics, Galway, Ireland)

See the last two paragraphs of texts 4.4.1

6.3.2.3 Development of HIV Testing Algorithms

Based on the results of the above evaluation, three kits (SUB-Recombigen HIV-1/HIV-2, Vironostika and Determine) were selected to prospectively ELISA/rapid-based and ELISA-based serial HIV testing algorithms (WHO, 1998; 2002; 2004).

(a) ELISA/rapid-based serial HIV testing algorithm

Vironostika and Determine as candidate kits were set in serial order to perform an ELISA/rapid-based HIV testing algorithm on 2,500 sera obtained from prospective patients/individuals referred to CPHL consecutively.

(b) ELISA-based serial HIV testing algorithm

The ELISA-based HIV testing algorithm was carried out using Vironostika and SUB-Recombigen HIV-1/HIV-2. The two candidate kits were run for 90 samples simultaneously. The ELISA-based HIV testing algorithm was discontinued for low efficiency as they yielded more concordant false positive than the ELISA/rapid-based algorithm.

6.3.2.4 Supplemental serological assays for the HIV testing algorithms

See text 5.3.2.4

6.4 ETHICAL CONCERNS

See text 5.4

6.5 STATISTICAL ANALYSIS

6.5.1 Data Collection and Statistical Analysis

See text 3.4.2.1

6.6 RESULTS

Two ELISA and one rapid test kits were evaluated for the development of ELISA-based and ELISA/rapid-based HIV testing serologic algorithms in this study. The SUB-Recombigen HIV-1/HIV-2, Vironostika HIV Uni-Form II Ag/Ab and Determine HIV-1/2 had sensitivity, specificity, reproducibility, positive predictive value (PPV), and negative predictive value (NPV) greater than 99.5% (>99.5%) for the evaluation process. Vironostika and Determine were the candidate kits used for serial HIV testing algorithms as ELISA/rapid-based tests. The ELISA/rapid-based serial HIV testing algorithm had a total of 512 (20.5%) samples confirmed by concordance as HIV seropositive individuals during the study.

6.6.1 Evaluation of HIV test kits

The outcome of the operational evaluation of the three test kits revealed that SUB-Recombigen HIV-1/HIV-2, Vironostika HIV Uni-Form II Ag/Ab and the Determine HIV-1/2 had the score > 99.5% in sensitivities, specificities, reproducibilities, PPV and NPV (Table 30).

5.6.2 ELISA/rapid-based serial HIV testing algorithm

Vironostika HIV Uni-Form II Ag/Ab and Determine HIV-1/2 were the candidate kits used for serial HIV testing algorithms as ELISA/rapid-based. They had a total of 512 (20.5%) samples confirmed by concordance as HIV seropositive individuals during the study (Figure 11). With initial screening using the Vironostika HIV Uni-Form II Ag/Ab kit in the algorithm, 1,976 samples (79%) were found seronegative while 524 samples (21 %) were found seropositive. Of the 524 screened positive samples by Determine kit, only 512 (20.5%) were concordantly positive while 12 (0.5%) were discordant negative using Determine HIV-1/2. Subjecting the

outcome to further evaluation with reference laboratory standard, 512 samples were confirmed as HIV seropositive with known HIV-1(17.7%), HIV-2 (0.5%) and both HIV-1 & 2 (2.3%) serostatus (Figure 11).

6.6.3 OBSERVATION

6.6.3.1 ELISA-based serial HIV testing algorithm

ELISA-based HIV testing algorithm (using Vironostika and SUB-Recombigen HIV-1/HIV-2 as candidate kits) was run for 90 samples alongside an ELISA/rapid-based algorithm (using Vironostika and Determine as candidate kits) with 90 samples, it was observed that the algorithm had the same sensitivity (100%) but the ELISA-based algorithm was less specific (96.7%) than ELISA/rapid-based type (100%). The ELISA-based HIV testing algorithm was therefore discontinued for cost ineffectiveness and less specificity than its ELISA/rapid-based counterpart.

6.6.4 Outcomes of ELISA/rapid-based serial HIV testing algorithm

Three hundred and fourteen males (61.3%) were confirmed seropositive while 198 females (38.7%) were seropositive. The rate of HIV infection within the females was 22.2% while that of the male group was 19.6%. Individuals between 20 and 49 years had the highest infection rate (Table 31) with the modal infection rate in the age group 30-34. One hundred and twenty-four females (62.6%) were HIV seropositive in age group 20-34 years, while 191 males (60.8%) were also HIV seropositive in age group 30-44 years.

On marital status, single females had 23% infection rate as compared with married females, 21%. Infection rate for married males was 18.6% while that of single males was 20%. Generally, there was no significant difference in infection rates ($p <$

0.0001) between single and married couples but there was a statistical significant difference ($0.001 \leq p \leq 0.01$) between single males (18.6%) and single females (23%).

An average of 20.5% (n = 2,500; Figure 12) HIV infection rate was recorded between June, 1997 and December, 1999 in this study. An average of 20.3% (n = 2,482; Figure 12) were HIV seropositive among Nigerians between June, 1997 and December, 1999 while 38.9% (n = 18; Figure 12) were HIV seropositive among other countries (such as Burkina Faso, Cameroon, Gambia, Ghana, Guinea, India, Japan, Sierra Leone, South Korea, Togo and United Kingdom) citizens resident in Nigeria during this study.

The HIV infection rates and outcome pattern in all 36 states and FCT of the Federation of Nigeria ranged between 0% (n = 2; Nassarawa State) and 66.7% (n = 9; Borno and n = 3; Kebbi States) as well as marginally between 0 (n = 2; Nasarawa State) and 49 (n = 276; Imo State) respectively (Table 32). Out of 36 States and FCT in Nigeria, Benue State (59%, n = 78) had the highest seropositivity (Figure 13) while Nasarawa State (0%, n = 2) had the least (Figure 13; Table 32) in the study. Patients turnout at CPHL continually increases over the years between 1997 and 1999, but worthy of note is a drop in the patients' rate of infection between 1998 and 1999 (Figure 15) that is from 30.7% to 15.4%.

Table 30: Quality assessment results for simple rapid HIV test kits used for ELISA/rapid-based serial HIV testing algorithm in Nigeria

Assay	Statistical Results of the Studies using a panel of 480 sera at CPHL									
	Sensitivity		Specificity		Predictive values				Reproducibility	
					Negative		Positive			
	n/N[b]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[c]	% (95% LCL/CI)	*n/N*[b]	% (59% LCL/CI)	*n/N*[b]	% (95% LCL/CI
SUB-Recombigen HIV-1/HIV-2 EIA	280/280	100 (99.96)	200/200	100 (99.96)	200/200	100 (99.96)	280/280	100 (99.96)	300/300	100 (99.96)
Vironostika HIV Uni-Form II Ag/Ab	280/280	100 (99.96)	200/200	100 (99.96)	200/200	100 (99.96)	280/280	100 (99.96)	300/300	100 (99.96)
Determine HIV-1/2	280/280	100 (99.96)	200/200	100 (99.96)	200/200	100 (99.96)	280/280	100 (99.96)	300/300	100 (99.96)

[a]The sensitivities, specificities and reproducibilities of two ELISA and one S/R assays were carried at CPHL, Lagos, Nigeria, using two laboratory gold standard ELISAs. Abbreviations: 95% LCL, 95% lower confidence limit; 95% CI, 95% confidence interval.

[b]n, number of samples positive by using rapid kits; N, number of samples positive using the two ELISAs

[c]n, number of samples negative by using rapid kits; N, number of samples negative using the two ELISAs

[d]n, number of samples with concordant results by using rapid kits; N, number of samples with concordant results when rerun using the same rapid kits by another laboratory scientists.

*Reproducibility test requires all positive samples and every 10^{th} negative.

Minimum acceptable sensitivity, specificity or reproducibility > or = 99.5%.

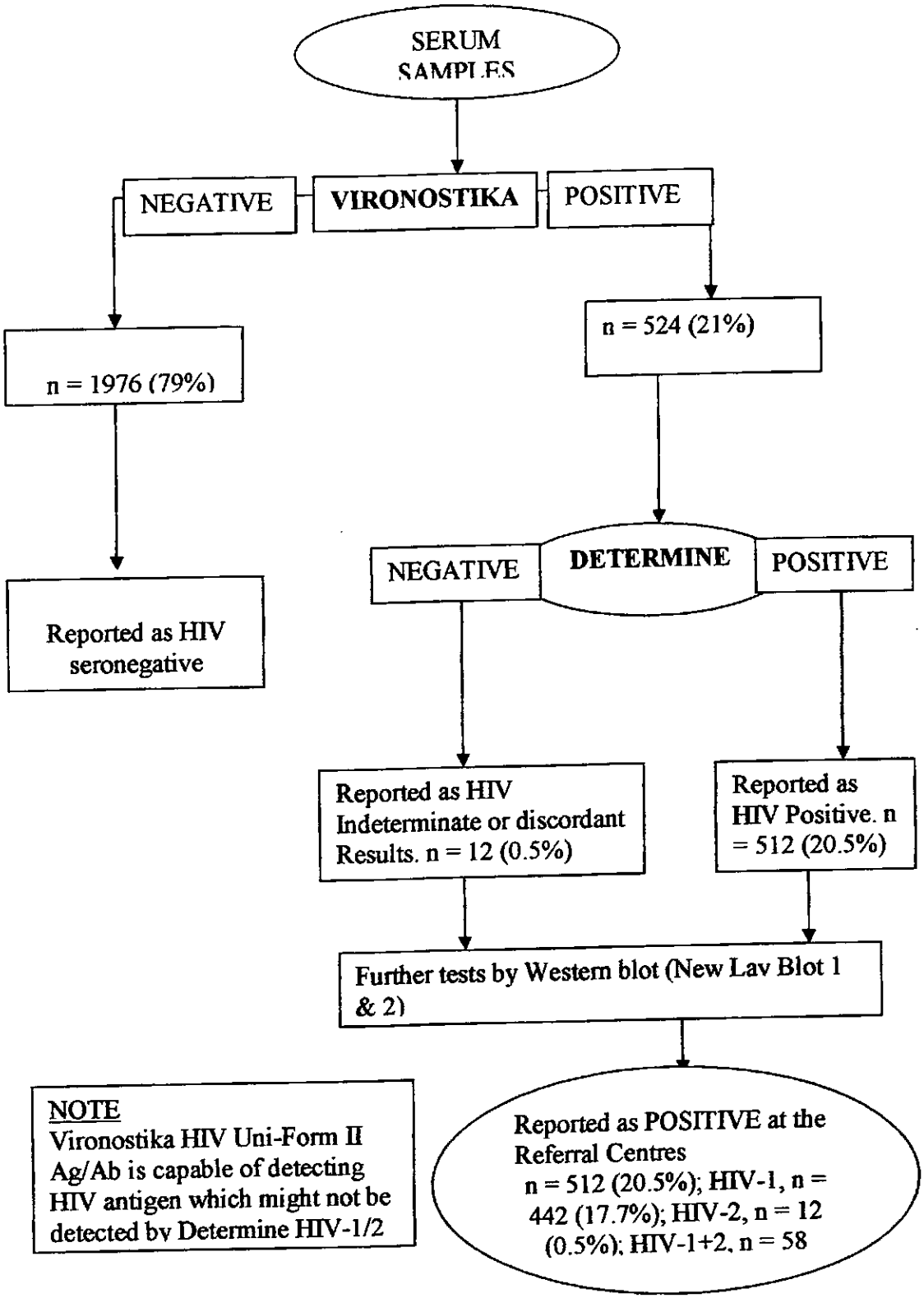
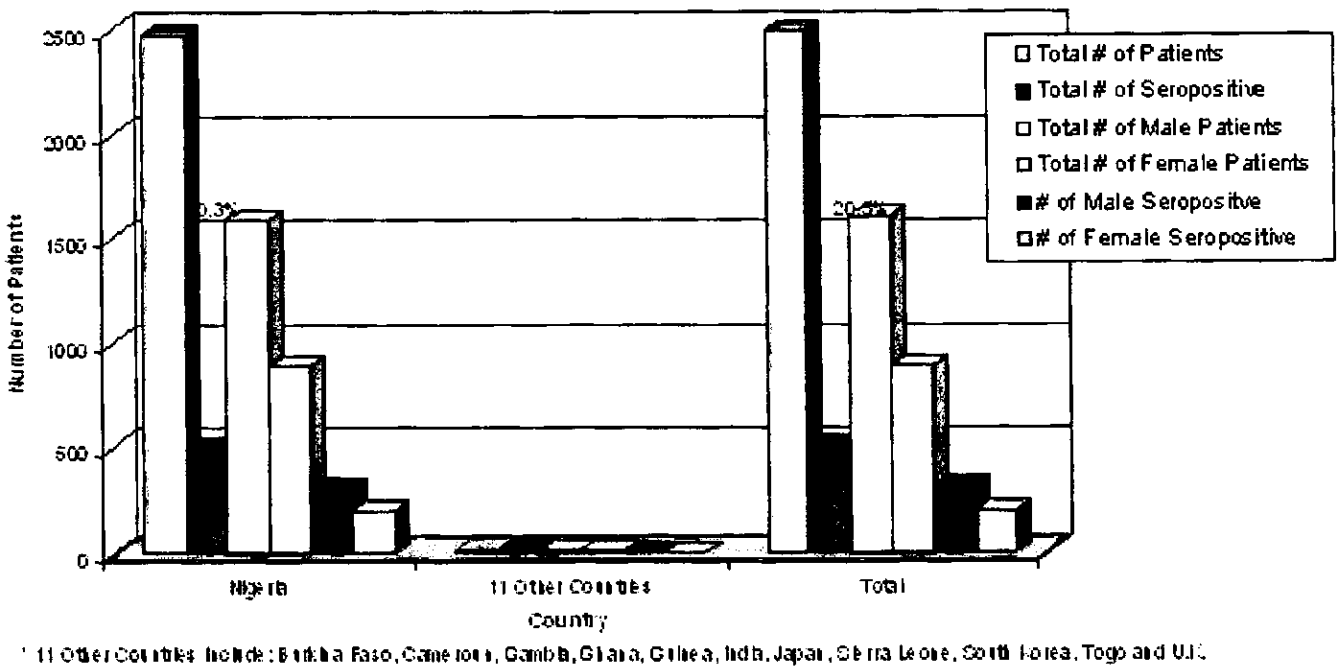


Figure 11: HIV testing results obtained in the study using serial algorithm (as rapid/ELISA-based algorithm for referral centres) for 2,500 serum samples collected from CPHL between June 1997 and December 1999

Table 31: Distribution Pattern of cases of HIV infection at CPHL by age and sex

Age Group (yrs.)	Total No. of Patients	Total No. of Male Patients	Total No. of Female Patients	Total No. of Seropositive	No. of Male Seropositive	No. of Female Seropositive	Infection Rate %	Confident Interval (CI)
0 – 4	32	18	14	12	6	6	**37.5**	(26-34%)
5 – 9	10	5	5	2	1	1	**20**	35%
10 - 14	10	3	7	3	2	1	**30**	20%
15 - 19	61	27	34	10	3	7	16.6	(13-27%)
20 - 24	280	122	158	53	22	31	18.9	(14-24%)
25 - 29	533	296	237	112	54	58	**21**	(18-24%)
30 - 34	643	452	191	118	83	35	18.4	(15-21%)
35 - 39	440	325	115	93	65	28	**21.1**	(17-25%)
40 - 44	246	187	59	59	43	16	**24**	(19-29%)
45 - 49	102	76	26	26	22	4	**25.5**	(17-34%)
50 - 54	72	51	21	14	10	4	19.4	(11-23%)
55 - 59	29	19	10	3	1	2	10.3	17%
60+	42	25	17	7	2	5	16.7	(17-30%)
TOTAL	**2500**	**1606**	**894**	**512**	**314**	**198**	**20.5**	



* 11 Other Countries include: Burkina Faso, Cameroon, Gambia, Ghana, Guinea, India, Japan, Sierra Leone, South Korea, Togo and U.K.

Figure 12: HIV Profile of patients collected at CPHL – Lagos, Nigeria, between June, 1997 and December, 1999

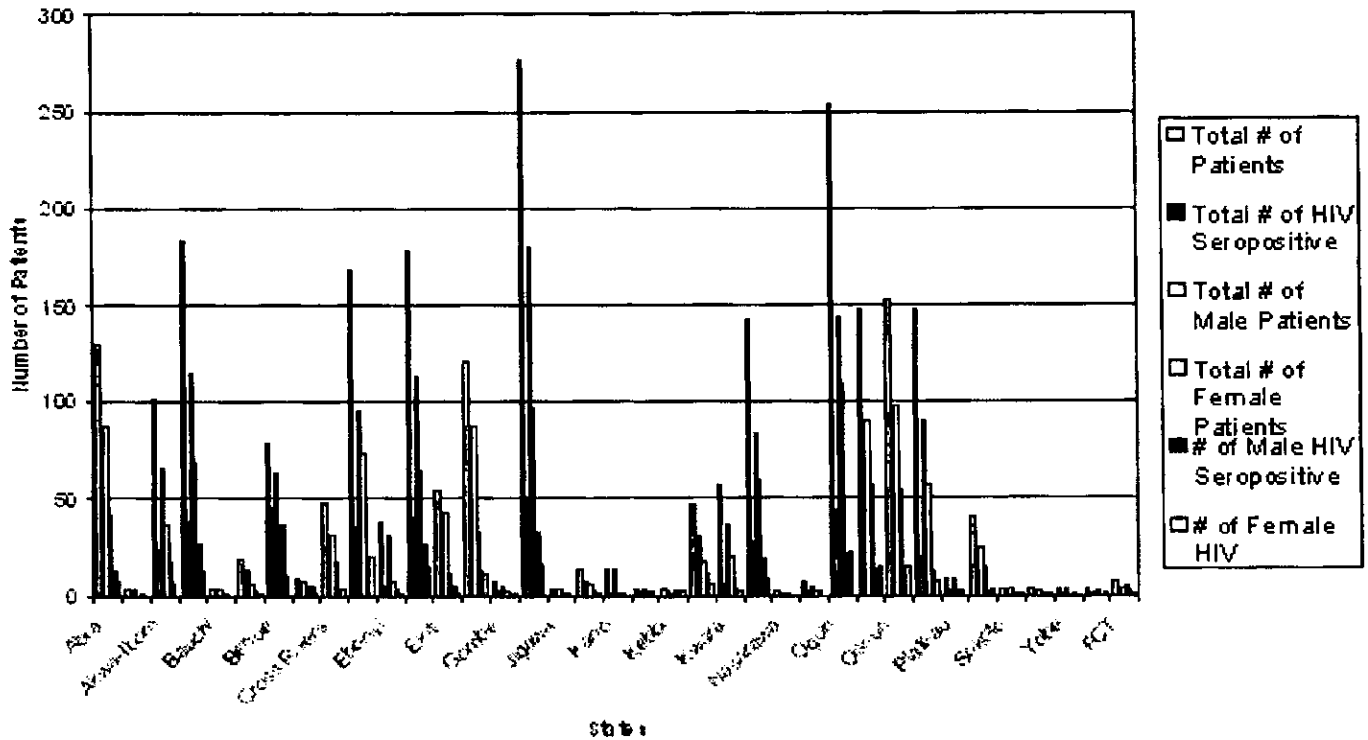


Figure 13: HIV Profile based on patient's state of origin in CPHL – Lagos, Nigeria, between June, 1997 and December, 1999

Table 32: Reported cases of HIV infection at CPHL from various states in Nigeria between 1997 & 1999 during the study

STATE	Total # of Patients	Total # of Seropositive	Total # of Male Patients	Total # of Female Patients	# of Male Seropositive	# of Female Seropositive	% Proportion	Confident Interval (CI)
Abia	129	20	87	42	12	8	15.5	14.2 – 16.8%
Adamawa	4	1	4	0	1	0	25	-
Akwa-Ibom	102	24	66	36	18	6	23.5	22 – 25%
Anambra	183	38	114	69	26	12	20.8	19.9 – 21.7%
Bauchi	4	1	3	1	1	0	25	-
Bayelsa	19	3	13	6	2	1	15.8	7.1 – 24.5%
Benue	**78**	**46**	63	15	36	10	**59**	**58 – 60%**
Borno	9	6	7	2	5	1	**66.7**	-
Cross Rivers	48	6	31	17	2	4	12.5	8.9 – 16.1%
Delta	168	35	95	73	15	20	20.8	19.9 – 21.7%
Ebonyi	38	5	31	7	4	1	13.2	8.7 – 17.7%
Edo	178	41	113	65	26	15	23	22.1 – 23.9%
Ekiti	54	6	43	11	5	1	11.1	7.9 – 14.3%
Enugu	121	23	88	33	12	11	19	17.7 – 20.3%
Gombe	7	2	5	2	1	1	28.6	-
Imo	**276**	**49**	179	97	33	16	17.8	17.2 – 18.4%
Jigawa	4	1	3	1	1	0	25	-
Kaduna	13	3	7	6	2	1	23.1	11.5 – 34.7%
Kano	14	1	13	1	1	0	7.1	-
Katsina	4	2	4	0	2	0	**50**	-
Kebbi	3	2	1	2	0	2	**66.7**	-
Kogi	47	17	30	17	11	6	36.2	33.5 – 38.9%
Kwara	57	6	37	20	4	2	10.5	7.4 – 136%
Lagos	161	35	96	65	24	11	21.7	20.7 – 22.7%
Nasarawa	**2**	**0**	1	1	0	0	0	-
Niger	7	2	5	2	2	0	28.6	8.6 – 48.6%
Ogun	253	44	144	109	21	23	17.4	16.8 – 18%
Ondo	147	29	90	57	14	15	19.7	18.6 – 20.8%
Oshun	153	28	98	55	13	15	18.3	17.3 – 19.3%
Oyo	147	20	90	57	12	8	13.6	12.5 – 14.7%
Plateau	9	2	9	0	2	0	22.2	5.2 – 39.2%
Rivers	40	6	25	15	2	4	15	10.8 – 19.2%
Sokoto	4	2	3	1	1	1	**50**	-
Taraba	3	1	2	1	0	1	33.3	-
Yobe	3	1	3	0	1	0	25	-
Zamfara	3	1	1	2	0	1	33.3	-
FCT	8	3	3	5	2	1	37.5	22.2 – 52.8%
Nigeria	**2500**	**512**	**1607**	**893**	**314**	**198**	**20.5**	

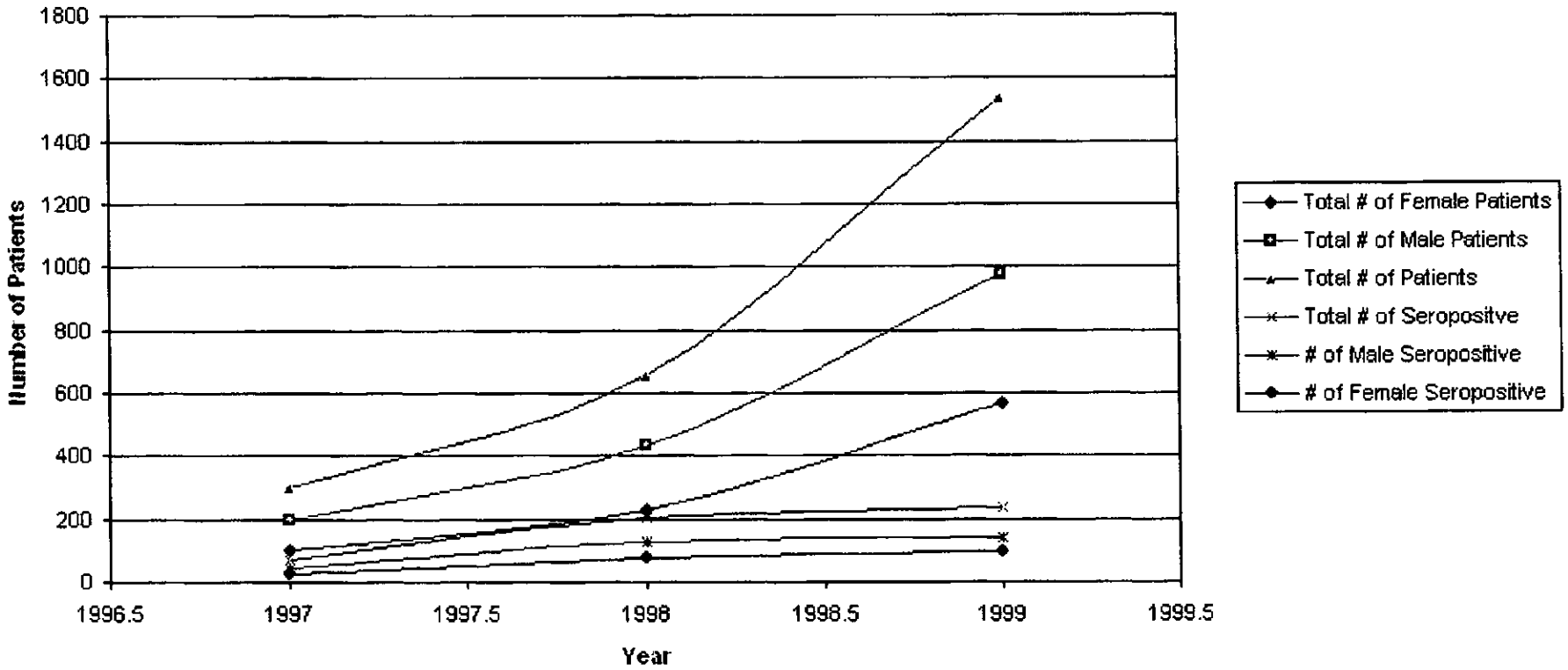


Figure 14: The trend of HIV outcome of reported cases at CPHL between June 1997 and December 1999

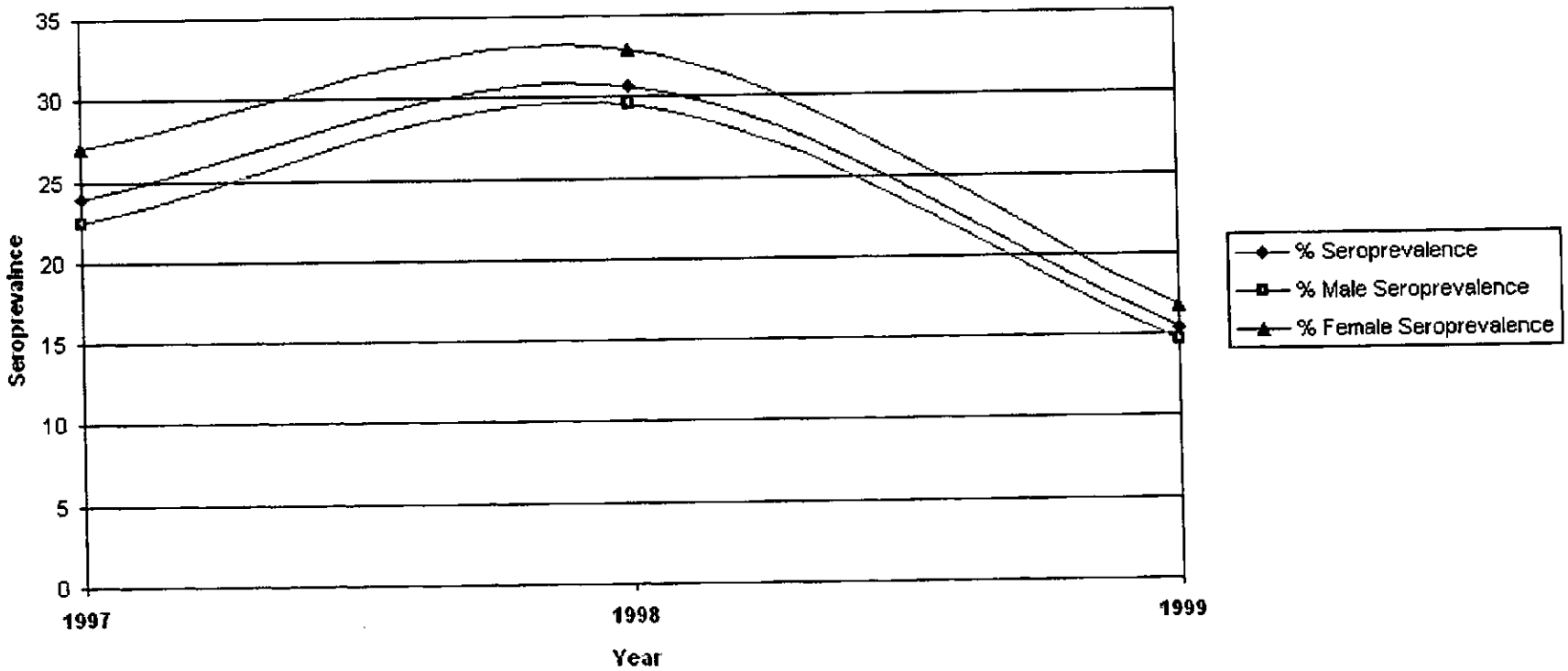


Figure 15: The trend of HIV seropositive cases in CPHL between June 1997 and December 1999

6.7 DISCUSSION

The importance of ELISA/rapid-based serial HIV testing serologic algorithm, for referral centres and blood banks, was emphasized by the need for dynamism in HIV testing technology. Weber *et al.* (1998) concluded that fourth generation assays permit an earlier diagnosis of HIV infection than third generation antibody screening assays, through the detection of p24 antigen which may be present in serum samples from individuals with recent HIV infection prior to seroconversion. In addition, Courouce (1999) in his update review concluded that the constant improvement of anti-HIV screening tests which leads one to shorten the 'window' period permits an earlier diagnosis of HIV infection and progressive decrease of transfusion risk. This study has demonstrated the ability of serial algorithm using 4th generation HIV ELISA and S/R test kits to function accurately and conveniently, in spite of HIV testing constraints due to electricity power supply and cost of purchasing WB test kits in various referral laboratories of the country, as an alternative strategy to final determination of HIV status of large samples and blood/blood products.

Considering the problems encountered with determining the final HIV status of referred samples or patients by the referral centres in the urban centres, those encountered by the blood banks at determining the HIV status of blood and blood products for transfusion of patients (especially during emergencies) as well as constraints encountered by reference laboratories at determining the HIV status of samples obtained from surveillance and specific diagnoses, it is high time that a solution to these problems and constraints emerged as a result of the development of an ELISA/rapid-based serial HIV testing algorithm in this study. This is a novel algorithm which has just been developed and evaluated successfully in this study. With the new algorithm in place, the delay in blood supply at the blood banks, due to

electric power failure and turn around time for WB test, would become a thing of the past.

With 90 samples used in the experimental trial, ELISA/rapid-based serial algorithm clearly showed better performance than its ELISA-based counterpart because of its higher specificity of 100% as against 97% of the other. However, Ly *et al.* (2001), extensively carried out retrospective evaluation of the ELISA-based testing algorithm and found that their combination assays yielded appropriate sensitivity. They did not comment on the specificity of these combination assays and did not evaluate the ELISA/rapid-based alternative. The ELISA-based HIV testing algorithm is not suitable for referral settings and blood banks in Nigeria considering the specificity of the kits.

Although all test kits used namely; SUB-Recombigen HIV-1/HIV-2, Vironostika HIV Uni-Form II Ag/Ab and Determine HIV-1/2 were equally efficient as HIV test kits during the evaluation exercise, SUB-Recombigen HIV-1/HIV-2 kit showed its limitations as a second-line discriminatory ELISA test by its poor performance for the wrong identification of 2 seronegative individuals as HIV-1&2 infections in those individuals in the study. Francois-Gerrard *et al.*, (1996), at a multicentre evaluation of European HIV diagnostic kits, described SUB-Recombigen as one of the best among all second generation ELISA kits of its time, with sensitivity of 100% and specificity of 99.6% - 99.8%. Similar observations were made by Leon de Gonzalez *et al.*, (1994) in their review of European diagnostic kits in France.

The study undoubtedly revealed that the candidate kits (Vironostika and Determine) used as an ELISA/rapid-based serial HIV testing algorithm was able to function perfectly, not only in referral settings (where electricity supply and kit cost constitute major problems) but also in blood banks where urgent HIV diagnosis is

required for blood transfusion. This algorithm was the first of its kind to be developed and evaluated, but Ly *et al.*, (2001) carried out combination assays for several 4th generation ELISA that can be referred to as ELISA-based HIV testing algorithm. In this algorithm all concordant positive and negative results were reported as confirmed positive and negative without further tests while discordant positive or negative results were subjected to further conventional HIV antibody tests in the reference laboratory (CPHL). The algorithm reduces the burden of samples sent for, and duration of time spent on conventional confirmatory tests.

To awaken perception for the call for studies on baseline data entry for HIV surveillance in the country, the ELISA/rapid -based serial HIV testing algorithm was applied in a form of prospective study of samples collected at CPHL. A 20.5% infection rate was shown by patients, unlike sentinel seroprevalence surveillance (5.5%) reported in Nigeria in the year 2000 involving only the antenatal group as at the time of this study (NASCP/FMOH, 2001). Use of Vironostika and Determine (as ELISA/rapid-based for urban/referral centres) as a gold standard is therefore recommended for sentinel seroprevalence studies so that quality surveillance and database for HIV can be sustained.

The country's health system is yet to establish standard operating procedure (SOP) based on a genuine HIV testing algorithm, and are yet to identify other high risk groups and States in the entire nation. Although a good number of HIV seroprevalence studies (Williams *et al.*, 1989; Olaleye *et al.*, 1993; Osotimehin *et al.*, 1994; Awofeso, 1995; FMOH/NASCP, 2006) have been reported in the country, each has just been able to address sentinels based on selected and expected high risk groups as well as pockets of surveys carried in some locations in the country.

The trends in age groups further supported the views in the study by Orubuloye *et al.*, (1992), where younger females and elderly males are more vulnerable to HIV infection. The socio-cultural aspects and economic situation of the country create more avenues for promiscuity among the young ladies and older (married) adults whereby the ladies exchange sex for money to survive while the men preferred young vulnerable ladies to the more matured older ones.

The study revealed that the single female group had the highest infection rate under marital status, although the overall married group had statistically significant ($p > 0.01$) HIV infection rate greater than that of the single group. Orubuloye *et al.*, (1992), had given adequate explanation in view of the fact that most married men and some single young women (spinsters) transmit HIV to their respective partners, with more spinsters falling victims.

The overall infection rate of 20.5% in the study could not represent the true seroprevalence of HIV infection in the country; although Lagos is an epicentre representing many communities in Nigeria since most people from every part of the country settle in Lagos for economic reasons. The CPHL, being a national reference laboratory, does not really serve the entire country since it was functioning far below its capacity during the period of the study. Sample size used in the study was not true representation of the whole country's population. The message expressed by this study is that, there is need to conduct a true sentinel seroprevalence study using a uniform and standard algorithm throughout the Federation; others carried out in the past were bias based on the testing methods, testing kits and sampling methods used.

The results from all 36 States of origin and FCT in the Federation were statistically significant in 15 States of origin namely, Benuè, Imo, Enugu, Ogun, Lagos, Edo, Anambra, to mention but a few. However, other States of origins' results

were statistically insignificant for scientific judgment. Some of these States of origin were Nasarawa, Zamfara, Bomo, Kebbi, Kastina, Jigawa, Adamawa, Sokoto, Yobe and Taraba States. The sample sizes were too small for any statistical analysis. The state profile revealed that nearly all the States in the Federation had significant value of HIV seroprevalence, therefore indicating a generalized epidemic. In addition, some states appeared to be approaching thresholds level (20%) of the epidemic (Benue, Adamawa, and Kogi). Benue State had the modal HIV infection rate among others. This finding confirms all the previous sentinel seroprevalence survey by FMOH/NASCP (1995, 1997 & 2001). Although Borno state came second, similar to that of FMOH/NASCP (2004), the sample size was however too small.

Across the country, Benue State still emerged the state with highest seroprevalence of HIV in the country (Asagba *et al.*, 1992; FMOH/NASCAP, 1995; 1997 & 2001). The states that recently featured in the year 2005 sentinel studies in the country are Ebonyi, Bauchi, Kebbi, Katsina, Nasarawa, Niger, Ekiti, Kogi, Akwa-Ibom, Jigawa and Sokoto States. All these states had significant seroprevalence of HIV except Nasarawa State. Similar to FMOH/NASCP's reports on sentinel HIV seroprevalence in Nigeria (2001), the magnitude and intensity of the HIV epidemic vary in size and prevalence according to the states and geographical zones of the country. The trends in both HIV seropositive pattern and infection rate between 1997 and 1999 served as an indication of public awareness on HIV/AIDS and possibilities of interventional strategies in action. These agree with the findings of FMOH/NASCP, (2006), and Badaru *et al.*, (2002).

6.8 CONCLUSION

In this study, an ELISA/rapid-based serial HIV testing algorithm (using the Vironostika HIV Uni-Form II Ag/Ab and Determine HIV-1/2 as candidate kits) has been proved to be suitable for referral settings and blood banks. The HIV infection rates and outcome pattern in all 36 states and FCT of the Federation of Nigeria ranged between 0% and 66.7% infection rates as well as marginally between 0 and 49 seropositive people respectively in the study. Benue State was the State with highest infection rate. Individuals between 20 and 49 years had the highest infection rate with the modal infection rate in the age group 30-34, while single females had the highest risk based on the marital status of individuals enrolled in the study. All the data were obtained from the outcome of the novel ELISA/rapid-based serial HIV testing algorithm that was accurate, cost-effective, and of great importance in combating the spread of HIV, considering the serious ethical, legal and social issues that accompany this infection.

CHAPTER SEVEN
DETECTION & EVALUATION OF HIV CF ANTIBODY TITRES AS A PROGNOSTIC MARKER FOR HIV/AIDS

7.0 DETECTION AND EVALUATION OF HIV COMPLEMENT FIXING ANTIBODY TITRES AS A PROGNOSTIC MARKER FOR HIV/AIDS

7.1 INTRODUCTION

Clinical manifestations of Human Immunodeficiency Virus (HIV) infection may not be only very diverse, but may also vary in different populations according to the relative frequencies of other endemic, potential opportunistic infections. Acquired immunodeficiency syndrome (AIDS), recognized as a distinct syndrome in 1981, represents the late clinical stage of HIV infection which results in progressive damage to the immune and other systems, especially the central nervous system (CNS) (Levy, 1994). The damage of the immune system leads to immune deficiency while that of CNS leads to neurological diseases and disorders. With the millennium goals and the "3 by 5 initiatives" by WHO (2004b), there is need to identify the (people living with HIV/AIDS) PLWHA to commence anti-retroviral drugs (ARV) treatment. The laboratory requirement for safe and effective use of ARV drugs and other forms of HIV/AIDS management is inevitable for sustainable testing, monitoring and management of PLWHA (Marseille *et al.*, 1999).

Identification of laboratory tests that can help predict progression to AIDS in people infected with HIV is important for clinical management and counselling (Lifson *et al.*, 1992). The identified laboratory tests are CD4+ T cell count, viral load titre measurement, reverse transcrpitase (RT) activity, p 24/25 antigen assay, anti-p24/25 antibodies assay, serum beta-2 microglobulin assay, anti-p18 antibodies assay, complement fixation test as well as a host of other similar assay tests.

One of the best predictors of disease progression thus far is the absolute $CD4^+$ count (CDC, 1985). An overt decline in the $CD4^+$ T cell count usually precedes

clinical disease (Castro *et al.*, 1992). $CD4^+$ is a cell surface molecule found mostly on T lymphocytes, which is important for the activation of the lymphocytes when they are stimulated by antigens (Barker & Barnett, 1994). Since $CD4^+$ is a major receptor for viral surface glycoprotein gp 120. T cells expressing this surface marker are primary targets for infection. Cells expressing these markers are important in regulating the immune responses necessary for controlling pathogens and neoplasms. Thus, loss or depletion of these cells leads to an immunocompromised state that eventually leads to disease and decline in response to certain stimuli by these cells leads to disease (Scott, 1993; Levy, 1994).

Although in asymptomatic individuals, an average of one out of 10,000 $CD4^+$ cells is actually infected, the number of $CD4^+$ cells lost greatly exceeds this amount (Brinchmann *et al.*, 1991). In addition; Groux *et al.*, (1992), demonstrated that this loss of $CD4^+$ cells is partially attributable to programmed cell death, or apoptosis, which occurs in infected individuals but not in sero-negative individuals.

In general, the lower the $CD4^+$ cell count, the greater the chance of entry of disease. Hence, the rate of decline of $CD4^+$ cells is also a good predictor of AIDS. The $CD4^+$ count can either be determined by direct or indirect immunofluorescence using monoclonal antibodies on a flow cytometer. Direct measurement of viral burdens is now considered the best predictor of the outcome of infection and this includes: plasma p24 antigen levels, titration of infectious virus in plasma or PBMCs and quantitation of proviral DNA and RNA by PCR (Lin *et al.*, 1998).

p24 Antigen levels in plasma can be determined by the ELISA and are therefore used to measure virus levels in the plasma. Although ELISA is very

specific, its sensitivity is usually too low for screening viral antigens in the plasma of asymptomatic individuals. Since the levels of p24 antigen in the plasma increase during the course of the disease, this method is a useful prognostic tool in monitoring individuals who are symptomatic or have AIDS (Vigano *et al.*, 1996; Mellors *et al.*, 1997; Thea *et al.*, 1997).

Virus in plasma or cells is titrated by culturing various dilutions of cells or plasma with phytohaemagglutinin stimulated-PBMCs or other susceptible cells. The cultures should be set up within 3hrs. of acquiring the specimen from the test subject (Pan *et al.*, 1993). The level of viral infection is measured either by determining RT activity or by measuring p24 levels as described earlier in detection of virus and virus-infected cells.

The polymerase chain reaction (PCR), a method involving thermal amplification of specific genetic sequences of a target organism or DNA using a pair of primers and thermocycler, has enhanced our ability to detect one HIV-infected cell in 100,000 cells or more. This technique has not only been known to be very sensitive at detecting most recent infections and viral load but has also enabled researchers to demonstrate that the viral burden was substantially higher than was believed and that the burden changed with a change in the clinical state of the individual. In this regard, viral culture by isolation showed 1 provirus containing $CD4^+$ T cell per 4,000 to 150,000 $CD4^+$ T cells, while PCR demonstrated 1 per 2,500 to 26,000 cells and above (Brinchmann *et al.*, 1991). It is therefore clear that PCR is more effective at detecting the aetiological agents of viruses, especially the fastidious ones like HIV, than viral isolation. Hence, PCR has been used to quantify virus in the plasma by using a recent technique called quantitative competitive PCR (QC-PCR). This method was used to measure RNA in plasma by use of a competitive RNA template matched to the target

sequence but differing from it slightly by virtue of an introduced internal deletion (Piatak *et al.*, 1993).

Infection by HIV induces activation of the immune system, which in turn increases the pool of HIV susceptible cells available and results in increased virus production. Thus, a measurement of immune activation markers is an indirect mean of measuring virus production in an individual. An indirect way to measure immune activation is to measure the amount of β_2 - microglobulin in plasma (Lifson *et al.*, 1992). This protein is part of class 1 MHC, and is present in almost all nucleated cells. This marker correlates with the degree of lymphocyte activation and also with progression to disease in HIV - infected individuals. Also recently employed is the use of adenosine deaminase (ADA) as a prognostic marker (Carrera *et al.*, 1995).

Some studies have indicated that some neutralizing and non-neutralizing antibodies can lyse HIV via complement fixation (Spear *et al.*, 1990, 1991). With the neutralizing antibodies, the antiviral titre can sometimes be increased 10-fold by the addition of high levels of complement to the assay (Spear *et al.*, 1992). A number of viral aetiologic agents have been recently diagnosed in the laboratory using complement fixation test (CFT) to detect the complement fixing (CF) antibodies (Oni *et al.*, 1994; De Petris *et al.*, 2002; Travassos da Rosa *et al.*, 2002) and especially in HIV seropositive individuals (Lord *et al.*, 2000; Sykora *et al.*, 1992; Ragnaud *et al.*, 1994).

Presently, there are no direct measures that determine the consequence of infection other than the use of surrogate markers. These markers are not only important in predicting the outcome of HIV infections but are also useful in determining when treatment should be administered. For example, surrogate markers can be used to monitor the course of treatment of an individual receiving anti-retroviral therapy, to determine the state of infection in an individual or risk of

prenatal transmission of HIV (Vigano *et al.*, 1996; Thea *et al.*, 1997). Therefore, the complement fixing antibodies can also be used as a surrogate or prognostic marker for HIV/AIDS especially in cases of HIV/AIDS management and treatment.

Nigeria, presently known for its poor resource setting, is challenged with possible means of adopting any of the above methods for detecting HIV surrogate/prognostic markers in order to monitor and follow-up the management and treatment of HIV patients. With the reports on the efficacy of various surrogate and prognostic markers, CD4 cell count and viral load have been generally accepted (Lillo *et al.*, 1997; Morris *et al.*, 1998; Mofenson *et al.*, 1999) while beta microglobulin, p24 antigen and anti-p24 antibody assays were recommended by others with some disadvantages (Goldschmidt *et al.*, 1998; Morand-Joubert, 1998; Castillo *et al.*, 2000). Any of these prognostic and surrogate markers could therefore be applied for the assessment of the HIV/AIDS patients' states of health and response to treatments.

Finally, the cost and technicalities involved in the standard prognostic markers for the disease trend of PLWHA, namely – CD4 counts and viral load, largely account for difficulties in monitoring the disease trend of PLWHA in poor resource settings. Nigeria, being one of such countries, needs alternative prognostic markers. Such markers must meet up with the standards established by CD4 counts [Cyflow (Paxton *et al.*, 1995)] and viral load [RNA/DNA Quantiplex (Revets *et al.*, 1996; Mulder *et al.*, 1997) – Roche]. For easy HIV/AIDS clinical diagnosis in this part of the tropical world, common HIV/AIDS symptoms need to be identified and compared with other parts of the world. This study is therefore focused on these key areas of needs so as to intensify efforts in the control of spread of HIV/AIDS as well as to conduct the follow up of HIV/AIDS disease trends in PLWHA.

Such needs, stimulated the quest to experiment with the suitability and possibility of using anti-p 25 HIV antibody and CF antibodies for HIV as prognostic markers in place of HIV viral load determination and CD4 T cell counts. An alternative method to the expensive and highly electricity-dependent methods for monitoring the progression of PLWHA from asymptomatic condition to full blown AIDS needs to be developed and experimented on, considering the economic situations and low per capital income of Nigerians. Most patients cannot afford the cost of determining their CD4 counts and viral load titres, and individual organisations and NGOs find it difficult to establish their own testing centre because of the cost of equipment and reagents for these two standard prognostic markers for HIV/AIDS. Although CD4 T cells counts and viral load determination have generally been accepted worldwide (Morris *et al.*, 1998; Mofenson *et al.*, 1999), cost per test and cost of running the assays contribute to the need for such alternative methods as anti-p25 antibody assay and CFT. Hence, the clinical symptoms, viral load titres and CD4 T cells counts are analysed along with the anti-p25 assay and CFT.

7.2 OBJECTIVES

The main objective of this study is to determine the possibility of using anti-p 25 antibody levels, CF antibodies titres or both as prognostic marker(s) for monitoring the progression of PLWHA from asymptomatic to symptomatic conditions.

7.2.1 Specific objectives are to:

1. determine whether the presence of anti-p 25 antibody alone can serve as a marker to distinguish asymptomatic from symptomatic PLWHA

2. determine whether the presence CF antibody alone can serve as a marker to distinguish asymptomatic from symptomatic PLWHA
3. determine whether the CF antibody titres can serve as markers to distinguish asymptomatic from symptomatic PLWHA
4. compare the ability of either CF antibodies, presence of anti-p 25 antibody or both with the standards (i.e., viral load titres and CD4 T cells counts) to measure the degree of progression from asymptomatic to symptomatic in PLWHA
5. determine the distribution of HIV serotypes among HIV seropositive Nigerians.

7.3 MATERIALS AND METHODS

7.3.1 SAMPLE POPULATION

7.3.1.1 Patients Population and Study Centres

See texts 3.3.1.1.

7.3.1.2 Sample size

A non-probability sampling method known as convenience sampling was used to select the study population. All the patients recruited between July 1997 and December 1999 for the study, were criteria-free volunteers. Sample size was determined by the formula enunciated by Oyejide (1992)

$$N = \frac{Z^2 P(1-P)}{d^2}$$

where N = Required sample size

P = Estimated prevalence of the condition which is about 5% in this study.

Z = Normal standard deviation for required confidence level of 95% = 1.96.

d = Required level of precision which is 0.05.

$$\text{So, sample size (N)} = \frac{1.96^2 \times .05 \times 0.95}{0.05^2} = 73$$

These samples were collected from all age groups (mostly adults) and both sexes randomly. The sample size of 250 HIV seropositive volunteer patients consisting of both asymptomatic and symptomatic PLWHA was designed for the study.

7.3.2 ASSESSMENT OF HIV PROGNOSTIC MARKERS

7.3.2.1 Patients Clinical Assessment Record

A questionnaire (Appendix 12) for 250 HIV seropositive patients assessing their state of health and symptoms experienced was also prepared to analyze associations between presence of symptoms and lack of it. Patients' symptoms were evaluated (Plate 1 & 2) and ascertained using CD4+ counts as characterized and classified by CDC (1993) before enrolment.

7.3.3 LABORATORY METHODS

7.3.3.1 Sample Collection and Processing

See text 5.3.2.1

7.3.3.2 Elavia Anti-p 24 Assay

Ten millilitre of blood per sample was collected from 250 HIV seropositive patients by veni-puncture. All serum samples were processed within 3 hours of collection and stored as earlier mentioned. All 250 seropositive samples were subjected to Elavia Anti-p24 (Sanofi Diagnostics Pasteur, S.A., France) kit for the detection of HIV-1 p24 antibodies and the prognosis of HIV-1 infections by enzyme immunoassay. Ninety-six-well flat-bottomed microtitre plates coated with HIV p24 antigen were used for the assay. The plates (Plate 3) were read using a spectrophotometer after adding 50µl of (1N HCl) stop solution.

6.3.3.3 Complement Fixation Test

All 250 processed sera were inactivated at 56°C for 30 minutes while shaking at 10 minute intervals in a water bath. Thereafter, HIV – complement fixing antibodies were screened for and titrated using HIV-1 antigen (Sanofi Diagnostic

Pasteur, Marnes la Coquette, France) commercially sourced. The micro-titre technique employed was adapted from the standard procedure described by Server (1962). See one of the plates read at the end of the assay (Plate 4).

6.3.3.4 CD4+ T Cells Count

Of the 250 HIV seropositive volunteers enrolled for the study, 2ml each of 150 EDTA-treated blood samples were randomly collected from 75 symptomatic (test) and 75 asymptomatic (control) individuals respectively. A minimum of 200µl of monocytes depletion-treated blood samples were used to determine the number of CD4+ T cells present per ml of blood using Dynabeads technique (Sanofi Diagnostic Pasteur, Marnes la Coquette, France).

6.3.3.5 HIV Viral Load Determination

A total of 150 EDTA-treated plasma samples from 150 HIV seropositive volunteers enrolled for the study was randomly collected in a similar manner as that of CD4+ counts, and was stored frozen at -70°C until ready for use. Plasma RNA was extracted from 50µl of plasma by Qiagen technique (Roche Diagnostics GmbH, Mannheim, Germany), which was based on the lysing and nuclease-inactivating properties (with diatoms' nucleic acid – binding properties of guanidinium isothiocyanate). The plasma viral load of the samples was then determined by reverse transcription and polymerase chain reaction (PCR) amplification using Amplicor HIV-1 Monitor, version 1.5 technique (Roche Diagnostics GmbH, Mannheim, Germany).

6.3.3.6 HIV Serotyping Assay

Two hundred and fifty-three (253) seropositive samples collected from volunteer patients among all the seropositive patients were subjected to Enzyme

Immunoassay (EIA) screening with V3 synthetic peptides specific to different HIV-1 & 2 subtypes (namely HIV-1 subtypes A, B, C, D, & E, and HIV-2 subtypes A & B) for the detection of HIV-1 & 2 serotypes' antibodies [reagents were kindly supplied by Professor O.D. Olaleye, Virology Department, University College Hospital (UCH), Ibadan]. The assay was carried out in a similar manner to the principle of Enzyme-linked immunosorbent assay (ELISA).

6.4 ETHICAL CONCERNS

See text 5.4

6.5 STATISTICAL ANALYSIS

6.5.1 Data Collection and Statistical Analysis

See text 3.4.2.1

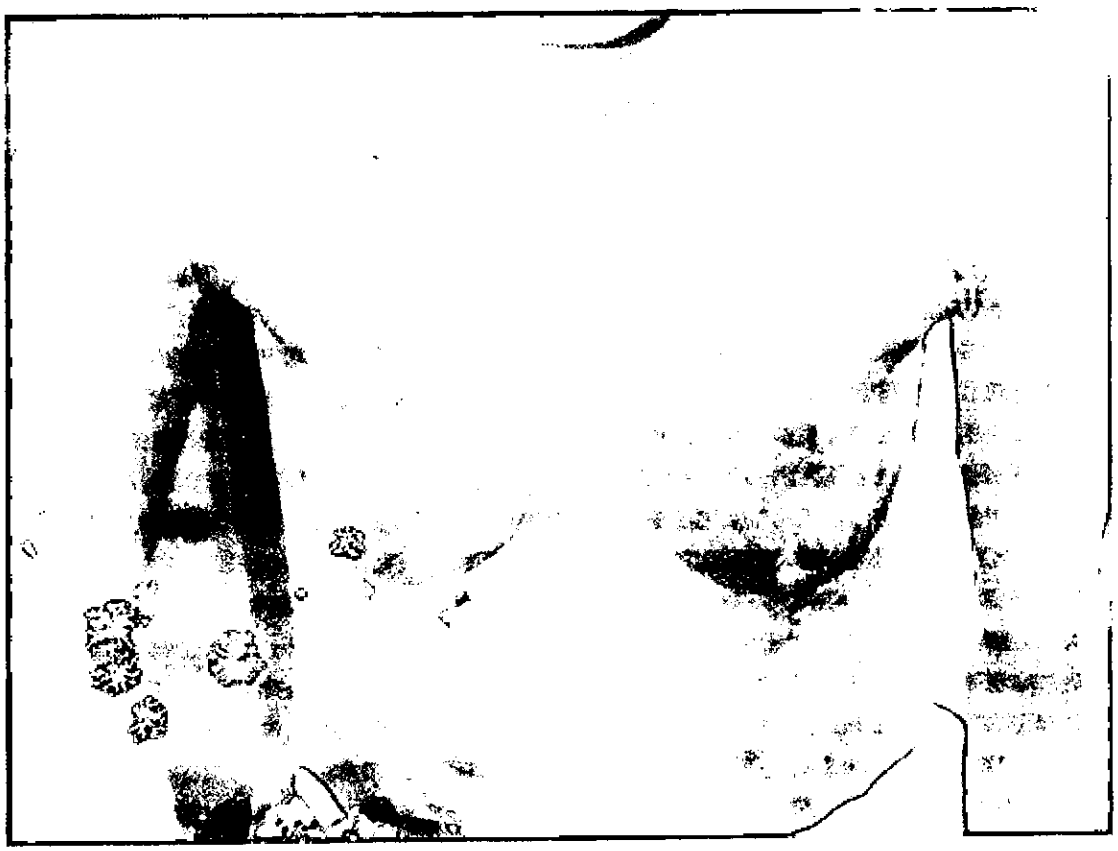


Plate 1: A patient with wasting syndrome and Kaposi sarcoma

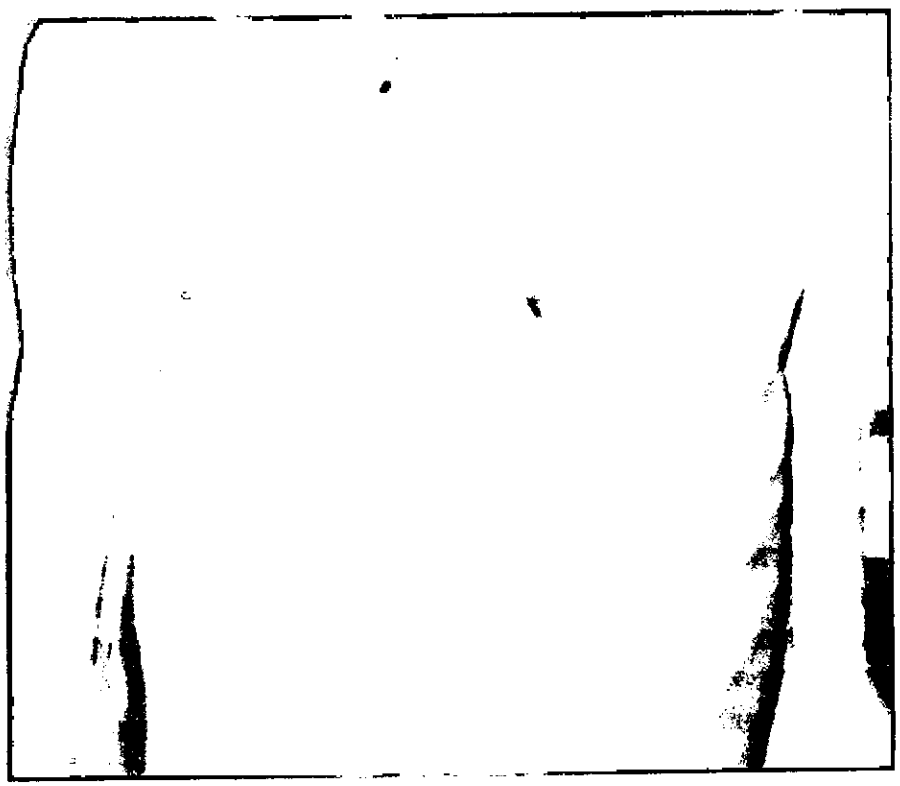


Plate 2: A patient with extreme loss of weight (wasting syndrome)

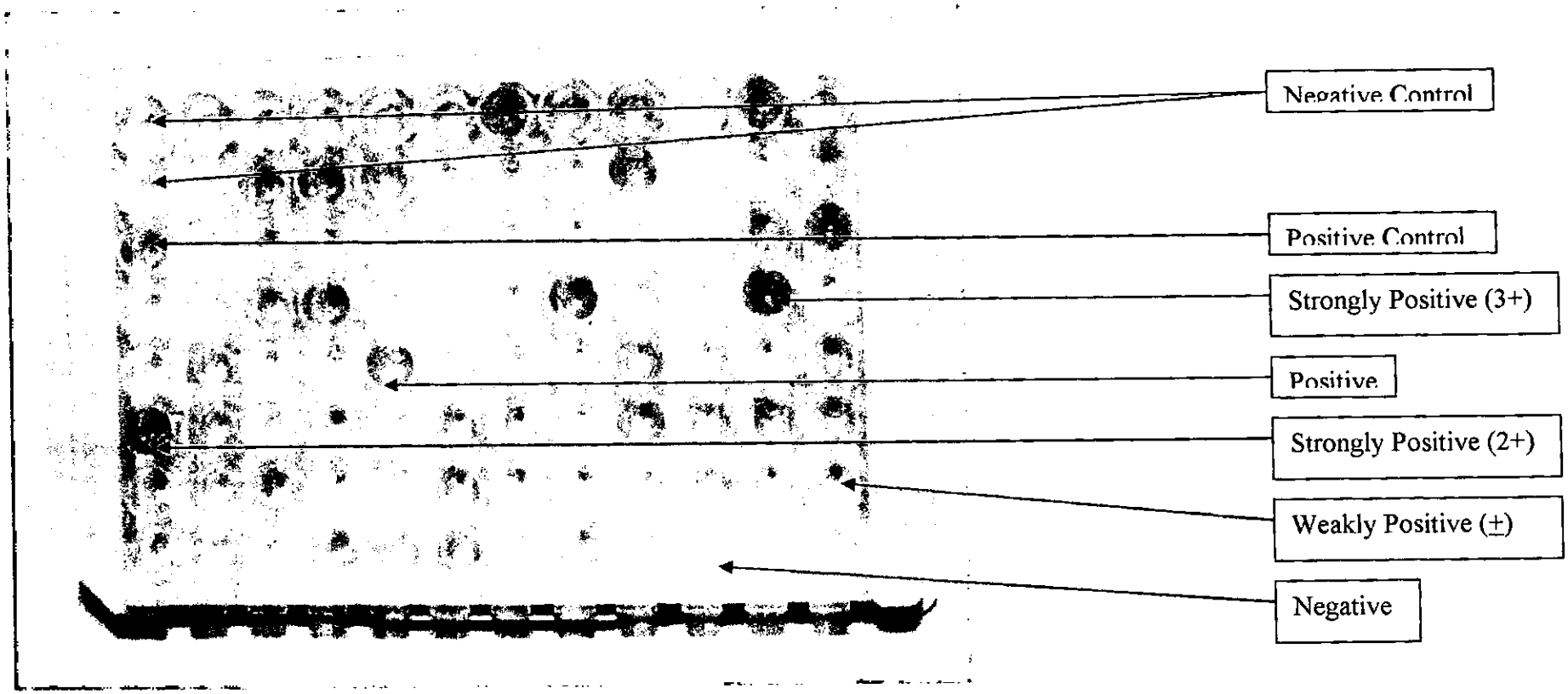


Plate 3: The Plate of anti-p 25 assay (at the end of the assay)

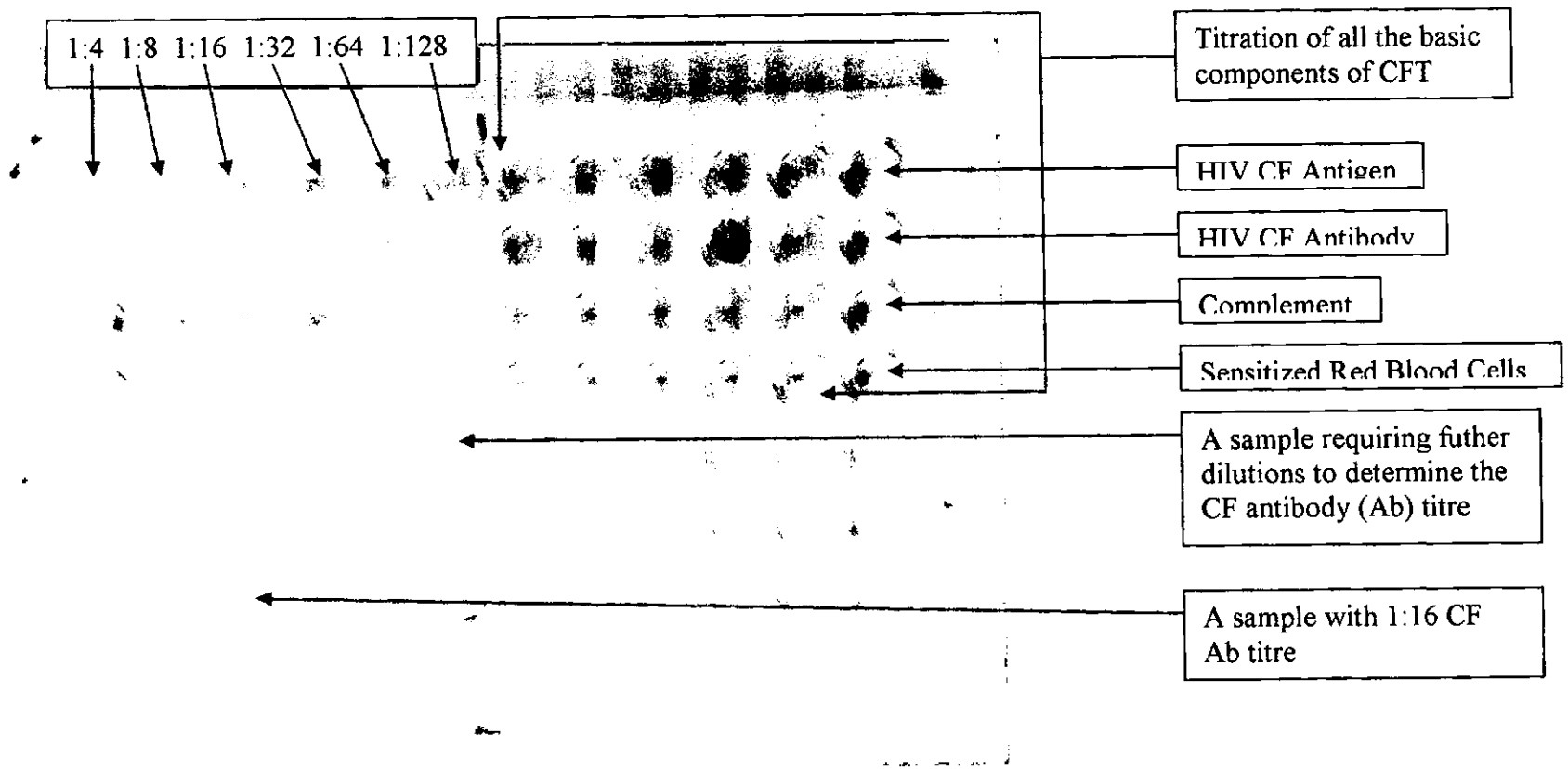


Plate 4: The Plate of complement fixation test (at the end of the test)

7.6 RESULTS

One hundred and sixty (64%) of the volunteered patients were symptomatic while the rest (36%) were asymptomatic. Of these, 36.3%, 22.4% and 19.2% had wasting syndrome, diarrhoea and tuberculosis respectively. One hundred and forty-six (58.4%) were reactive to both anti-p24 and complement fixing (CF) antibodies with CF antibody titre ranging between 1:8 and 1:512 with a modal titre of 1:256. The presence of anti-p 25 antibody and CF antibody were not significant enough to distinguish between asymptomatic and symptomatic patients. However, the frequency curve of asymptomatic and symptomatic individuals with the CF antibody titre revealed that the asymptomatic group had its peak in the frequency curve at 1: 32 while the symptomatic group had its peak at 1: 256. Of 20 serotypes of HIV and many unknown others identified, HIV-1 serotype A (27%) slightly emerged the highest from HIV-1 serotype C (26%) among all HIV-1& 2 serotypes.

7.6.1 Clinical assessment of patients

Of the 250 individuals, 160 (64%) had one or more symptoms while 90 (36%) were asymptomatic (Table 33). Ten different symptoms or groups of symptoms were recorded with wasting syndrome (36.3%) topping the list, followed by diarrhoea (22.4%) and tuberculosis (19.2%) respectively (Table 33). After HIV screening and confirmatory test by ELISA, rapid and Western blot methods (Figure 16 & 17), HIV-1 was highest with 87.2%, followed by HIV-2 (8.4%) and lastly, HIV-1 & 2 mixed infections (4.4%). The female HIV seropositive volunteers (53.2%) were slightly higher than the males (46.8%) in the study. Among the participants 93.5% were Nigerians while the rest (6.5%) were foreigners.

7.6.2 Presence of anti-p 25 antibody and clinical conditions

One hundred and forty-six (58.4%) of 250 samples tested for anti-p24 antibody were positives (Figure 18). Seventy-two (61.5%) male were positive while 74 (55.6%) female were also positive. However, more female were negative than male although the proportion of male positive to negative was more than that of female. The higher female value was due to the fact that more females were seropositive to HIV-1&2 tests than males (133 versus 117). Of the age groups, 20-29 group had the highest value while 0-9 group had the lowest among those positive to the anti-p25 antibody. The most predominant group for anti-p25 antibody positivity was the 20-29 year-old females (Figure 19). Anti-p25 antibodies assay (Table 34) revealed that 59 of 90 asymptomatic individuals (65.6%) were positive while 87 of 160 symptomatic individuals (54%) were positive, there was no significant difference between them ($p = 0.08$).

7.6.3 Presence of complement fixing (CF) antibody and clinical conditions

Similarly, 146 (58.4%) of 250 samples tested for CF antibody were positive (Figure 20). Seventy-one (60.7%) male were positive while 75 (56.4%) females were also positive. However, more females were negative than males although the proportion of male positive to negative is more than that of female. The higher female value was due to the fact that more females were seropositive to HIV-1&2 tests than male (133 versus 117). Of the age group, 20-29 group had the highest value while 10-19 group had the lowest among those positive to the CF antibody. In the age groups, the most predominant group for CF antibody positivity was the 20-29 year-old females (Figure 21). Complement fixing (CF) antibodies pattern (Table 35) also

revealed that 56 of 90 asymptomatic individuals (62.2%) were positive while 90 of 161 symptomatic individuals (55.9%) were positive, but there was no significant difference between them (p = 0.33).

7.6.4 Frequency of complement fixing (CF) antibody and clinical conditions

The frequency curve of asymptomatic and symptomatic individuals with the CF antibody titre (ranging between 1:8 and 1: 512 with a modal titre of 1: 256) revealed that the asymptomatic group had its peak in the frequency curve at 1: 32, while the symptomatic group had its peak at 1: 256 (Figure 22), and there were significant differences in their curves (p = 0.03). Similarly, the frequency curve of asymptomatic and symptomatic individuals with viral load titre (Figure 23) showed that most peaks produced by symptomatic individuals in the frequency curve were greater than those of the asymptomatic (p = 0.05). Likewise, the frequency curve of asymptomatic and symptomatic individuals with CD4 counts (Figure 24) showed a sharp peak at 200 cells/μl in the frequency curve for the symptomatic individuals but a blunt peak at 840 cells/μl for the asymptomatic. There were significant differences in these curves (0.0001).

7.6.5 Distribution of HIV Serotypes by ELISA Methods in Lagos, Nigeria

By serotyping with the ELISA method (an old but only available method) of detecting circulating HIV subtypes antibodies among the seropositive patients in the population, 20 serotypes of HIV and many unknown others were identified. HIV-1 serotype A (27%) slightly emerged the highest from HIV-1 serotype C (26%) among all HIV-1& 2 subtypes (Figure 25; Table 36). There was a substantial amount of mixed infections of various serotypes of HIV-1. There was however no mixed infection within HIV-2 serotypes.

Table 33: The frequency of major types of symptoms in HIV-infected individuals recruited in the study

Major Types of Symptoms	Frequency	Percent	HIV Type
Prolonged Fever	28	17.5	HIV-1
Chronic Diarrhoea	20	12.5	HIV-1
Tuberculosis	31	19.4	Both HIV-1 & 2
Generalized adenopathy	5	1.9	Both HIV-1 & 2
Typhoid fever	3	1.9	HIV-1
Wasting syndrome (Loss of Body Weight)	43	26.9	Both HIV-1 & 2
Hepatitis	2	1.3	HIV-1
Persistent and Prolonged Pneumonia	6	3.7	HIV-1
Gonorrhoea	4	2.5	HIV-1
Kaposi sarcoma/Skin rashes	18	11.2	Both HIV-1 & 2

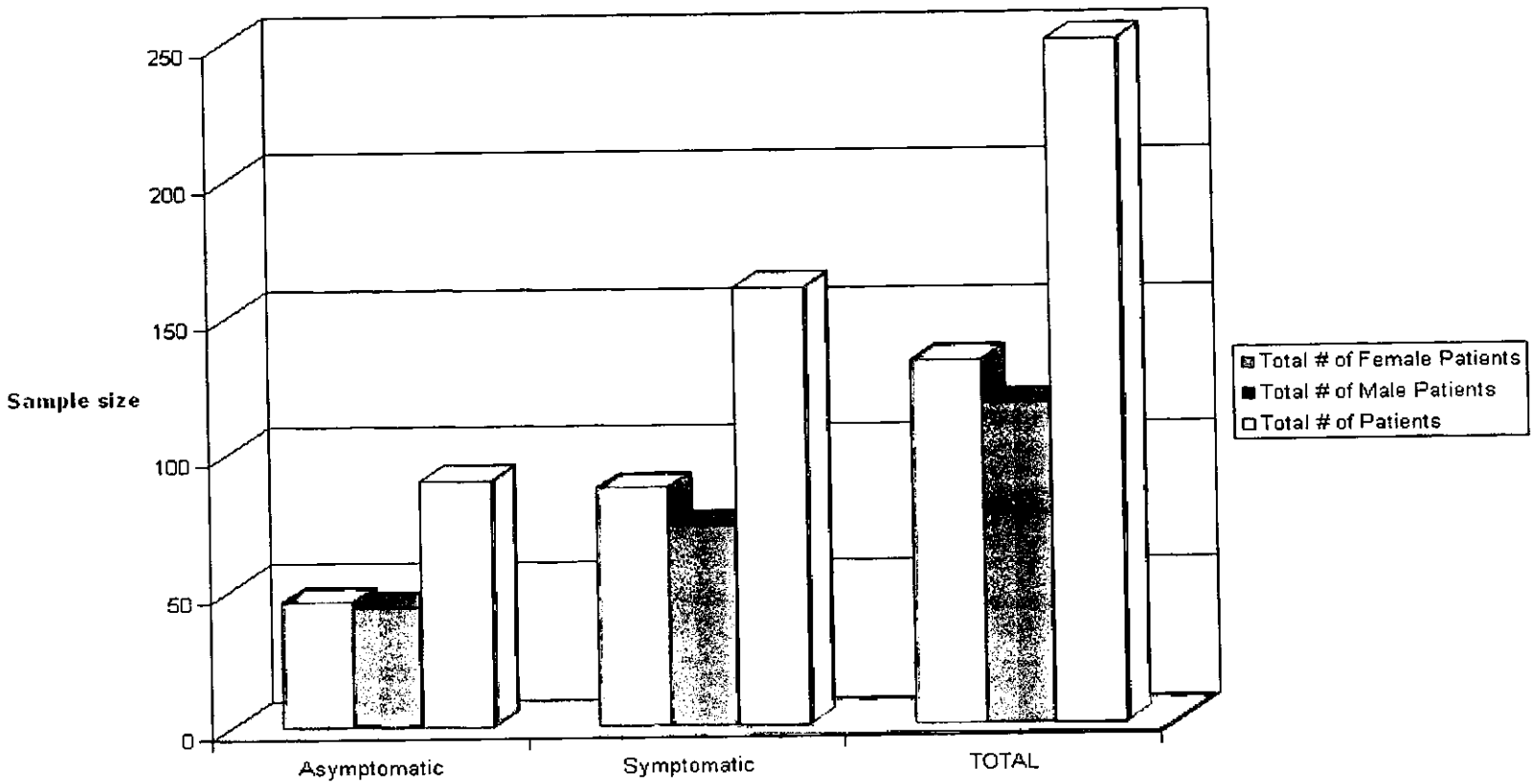


Figure 16: Proportion of asymptomatic and symptomatic patients in the study

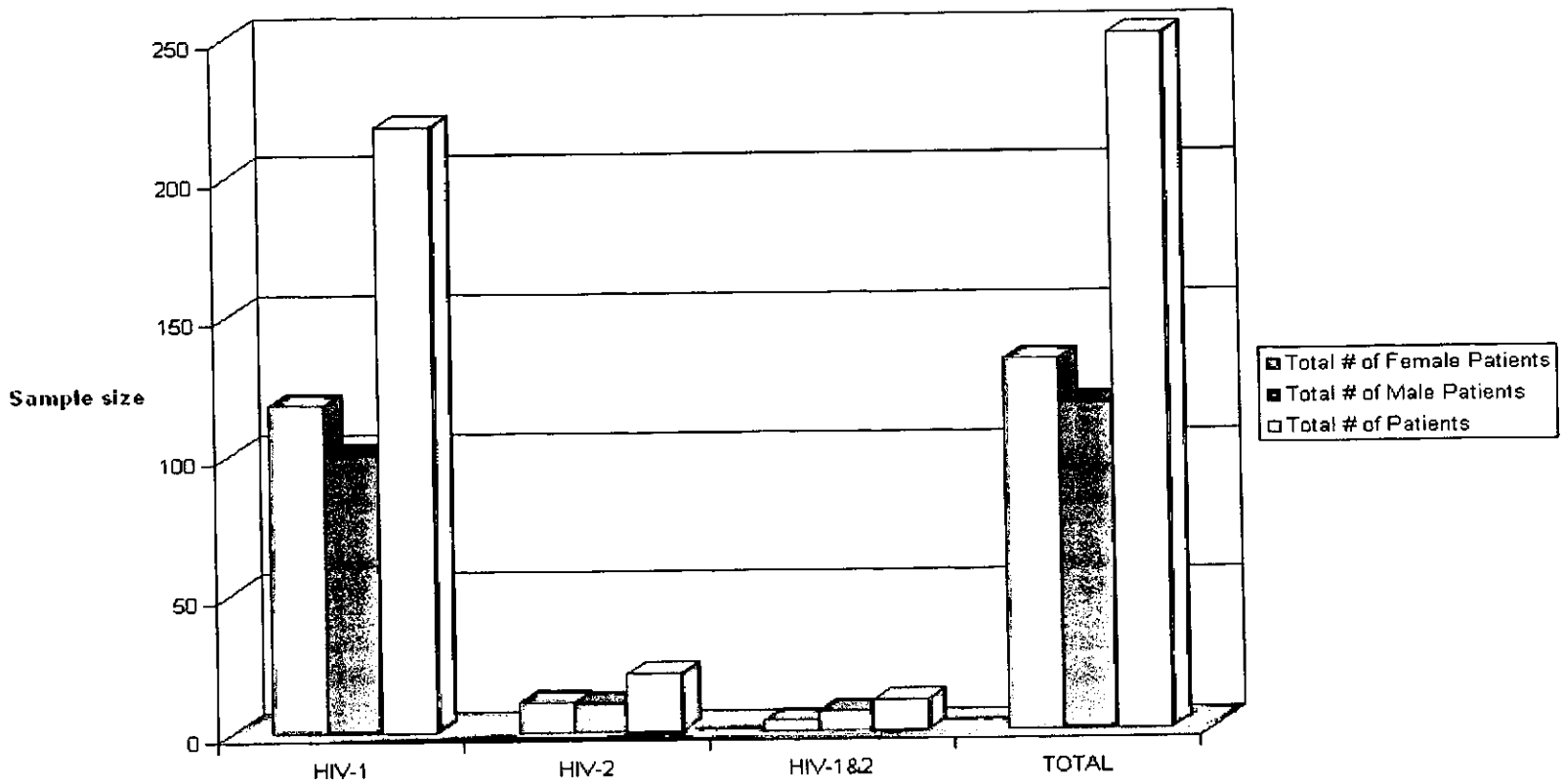


Figure 17: Distribution of HIV Types among the patients for clinical assessment

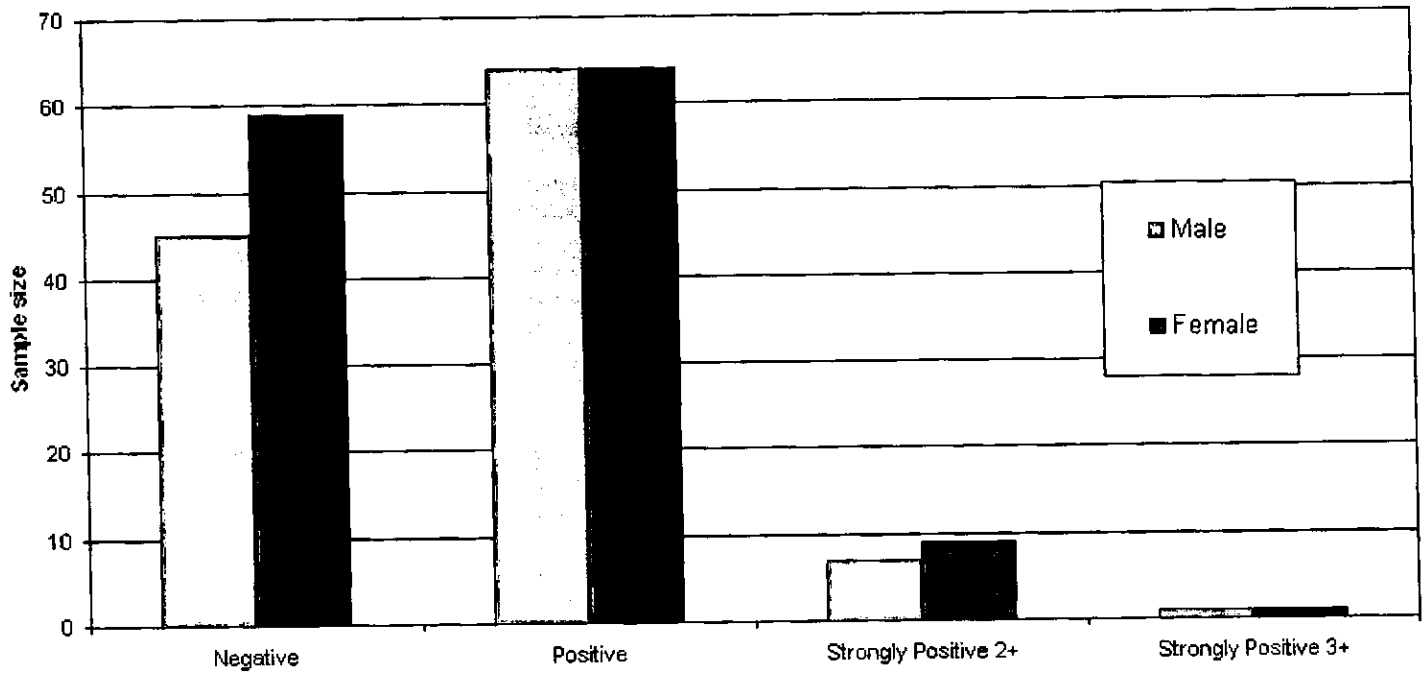


Figure 18: Proportion of anti-p 25 antibody levels by sex

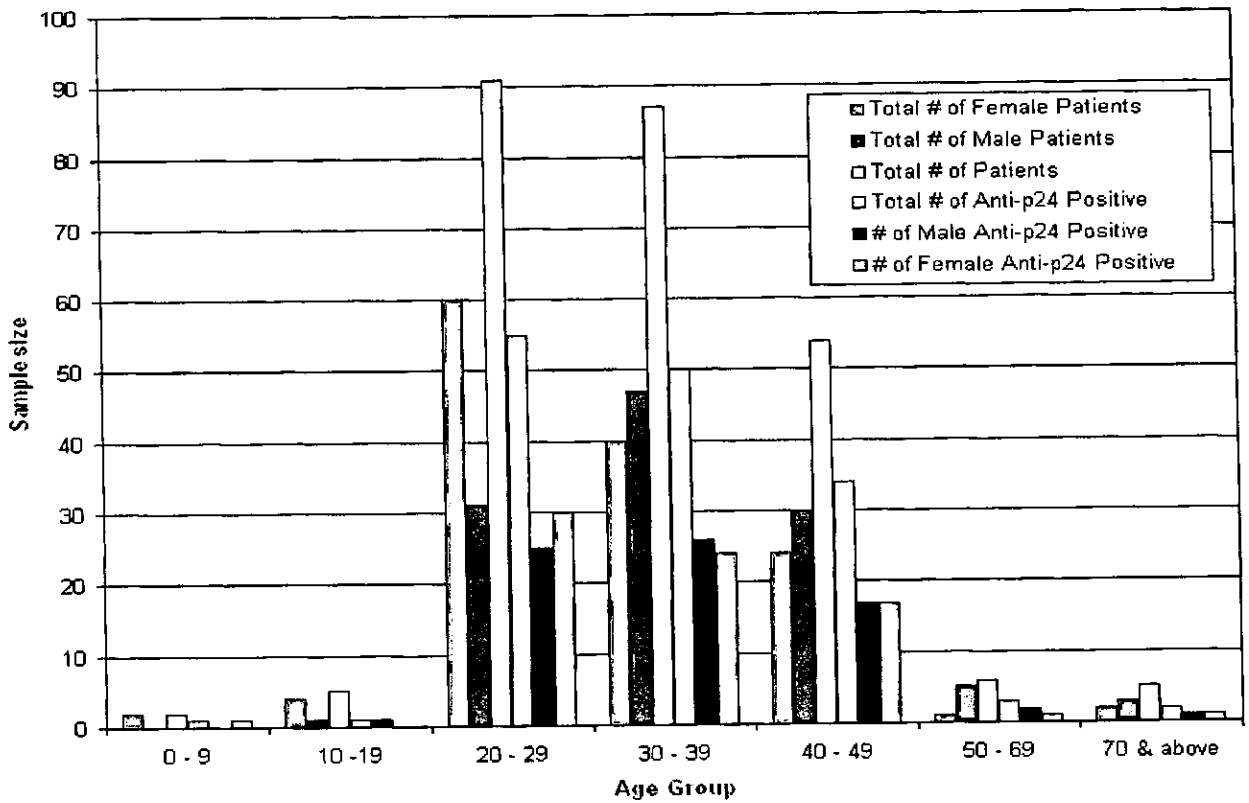


Figure 19: Distribution of anti-p 25 antibody by age and sex

Table 34: Clinical stages and anti-p24 antibodies detection

STAGE OF INFECTION	ANTI-P24 ANTIBODY ASSAY RESULT		TOTAL
	Negative	**Positive**	
Asymptomatic (-)	31	59	90
Symptomatic (+)	74	87	161
Total	**105**	**146**	**251**

Chi-Square Tests

	VALUE	DF	P VALUE
Pearson Chi-Square	3.148	1	0.076

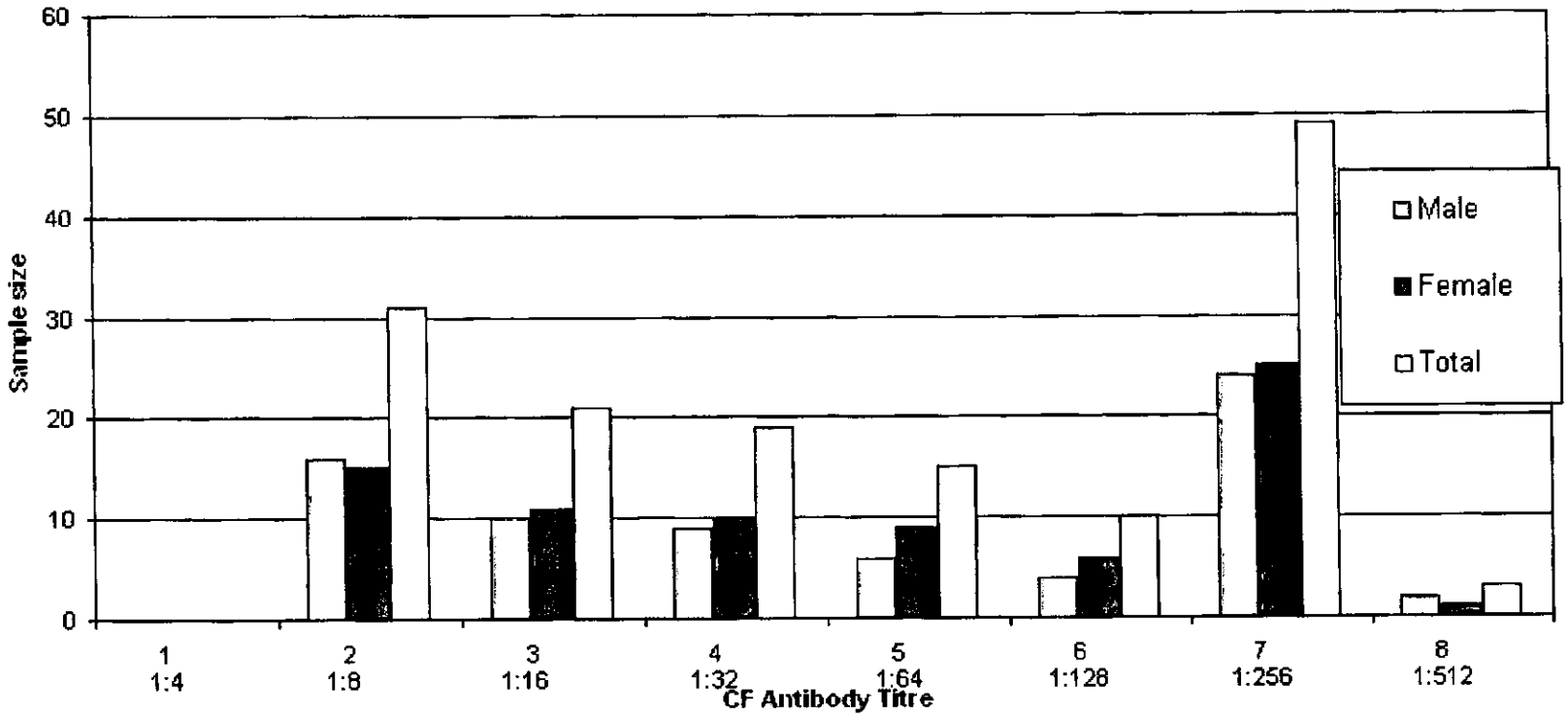


Figure 20: Distribution of HIV CF antibody titre by sex

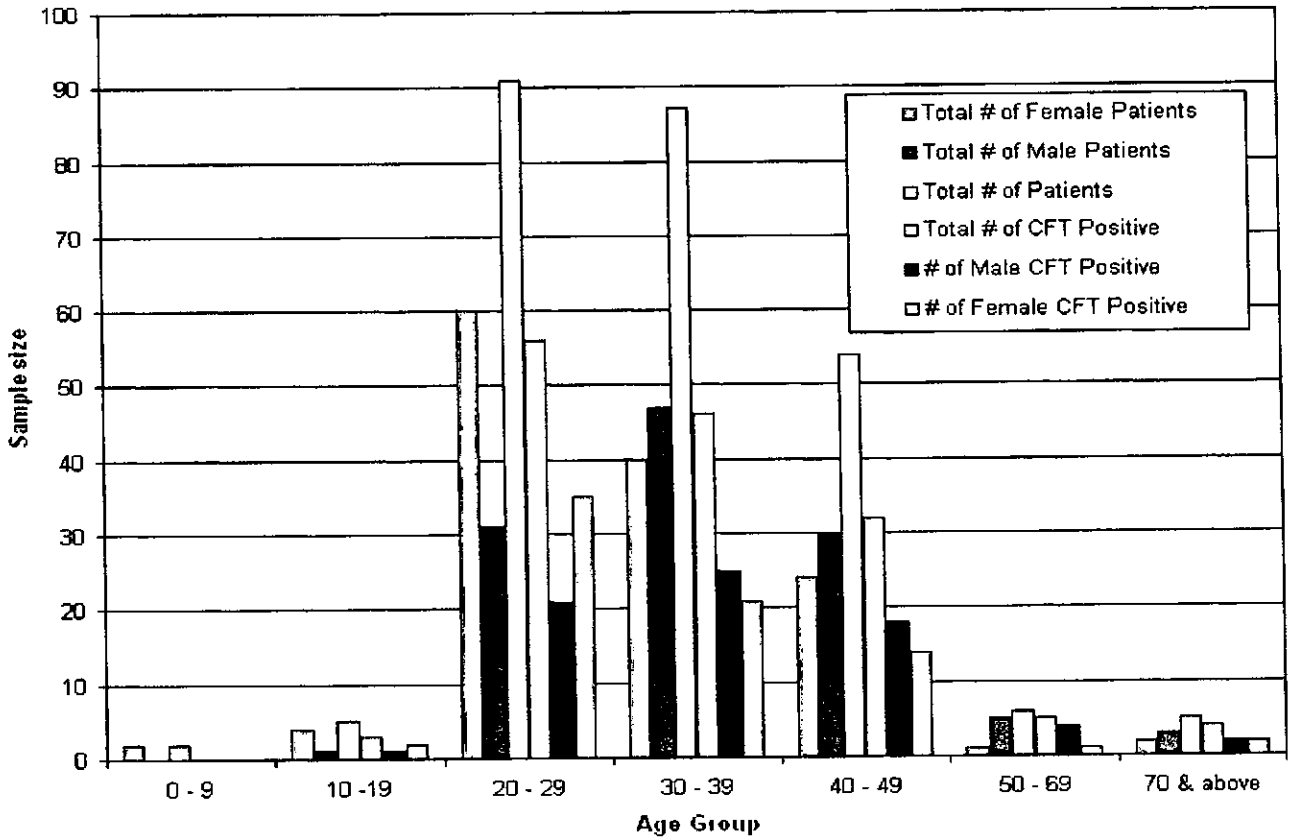


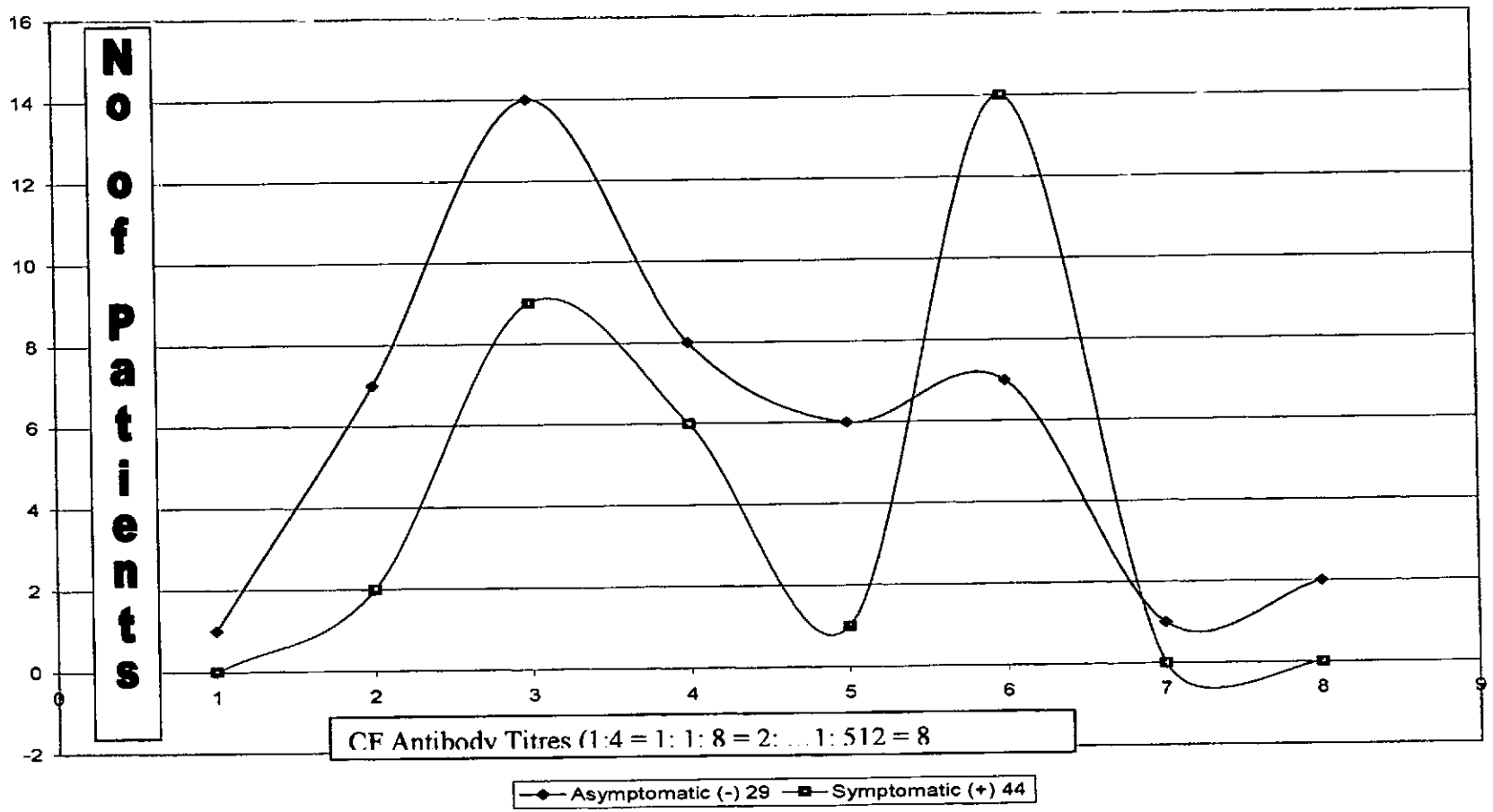
Figure 21: Distribution of HIV CF antibody titres by age and sex

Table 35: Clinical stages and HIV CF antibodies detection

STAGE OF INFECTION	HIV CF ANTIBODY ASSAY RESULT		TOTAL
	Negative	Positive	
Asymptomatic (-)	34	56	90
Symptomatic (+)	71	90	161
Total	**105**	**146**	**251**

Chi-Square Tests

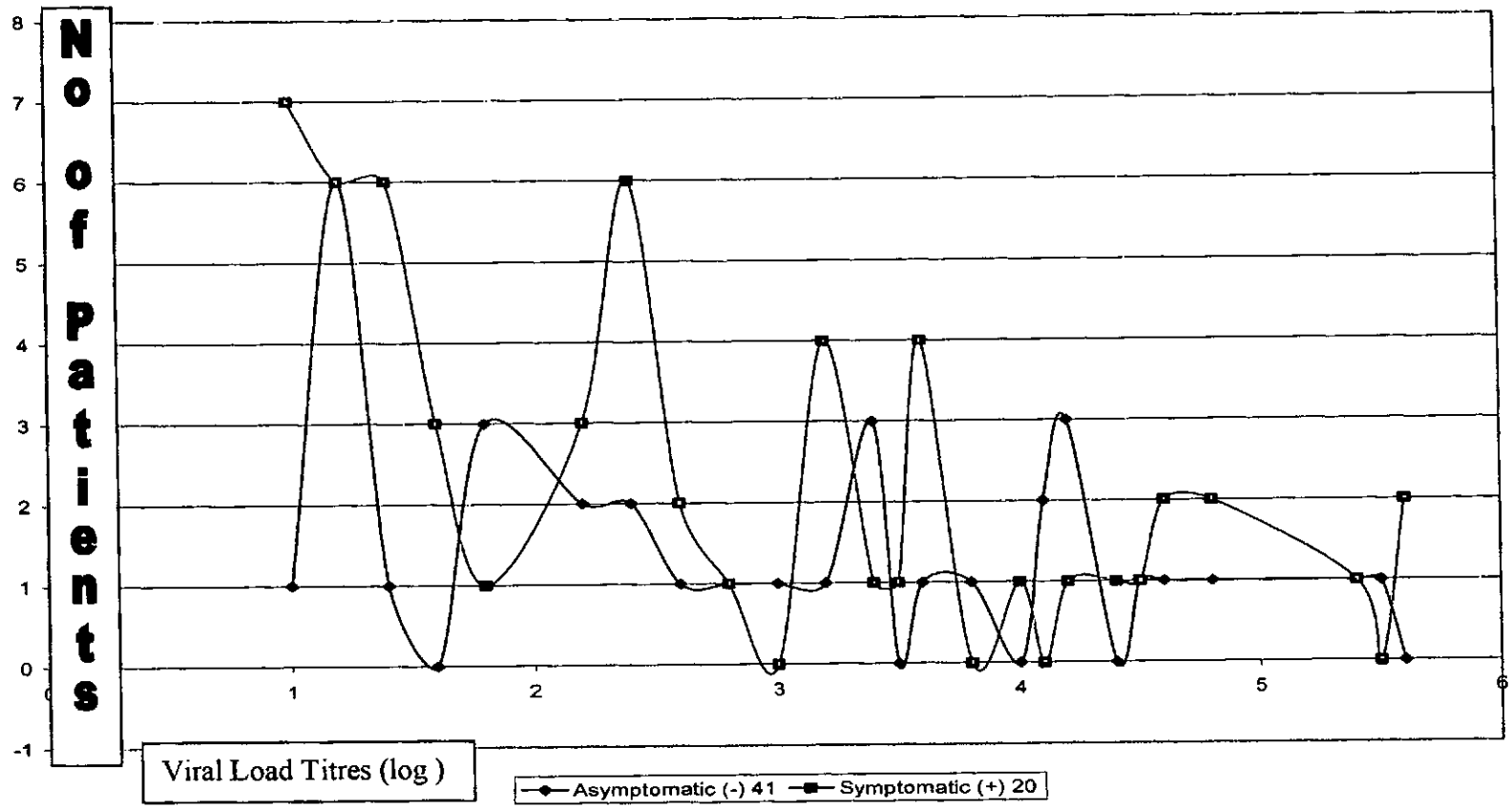
	VALUE	DF	P VALUE
Pearson Chi-Square	0.948	1	0.330



Chi-Square Tests

	VALUE	DF	ASYMP. SIG. (2-SIDED)
Pearson Chi-Square	17.132	8	0.029

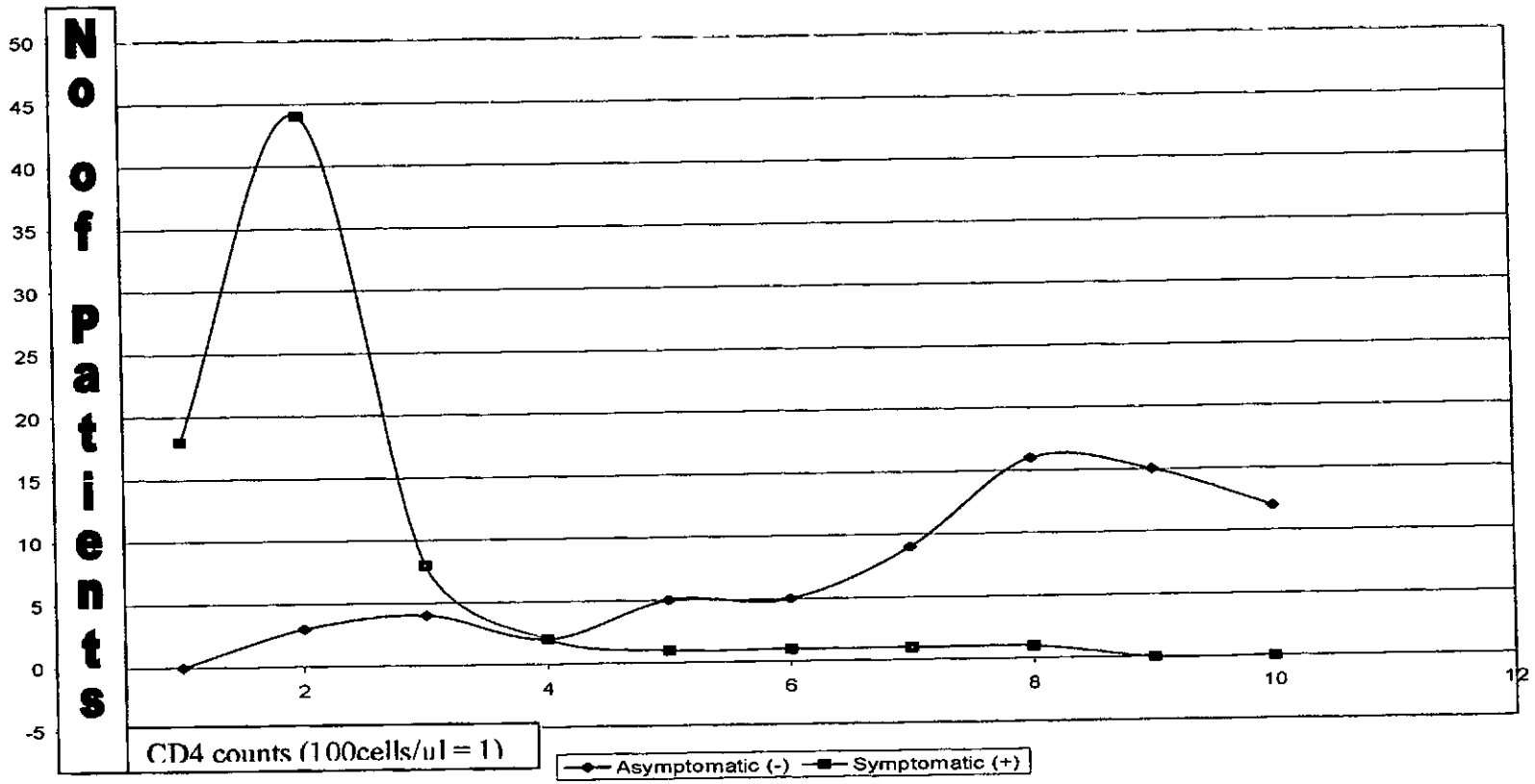
Figure 22: Frequency of asymptomatic and symptomatic individuals with the level of HIV CF antibody titre



Chi-Square Tests

	VALUE	DF	ASYMP. SIG. (2-SIDED)
Pearson Chi-Square	38.096	25	0.045

Figure 23: Frequency of asymptomatic and symptomatic individuals with HIV viral load titre



Chi-Square

	VALUE	DF	ASYMP. SIG. (2-SIDED)
Pearson Chi-Square	128.018	47	0.0001

Figure 24: Frequency of asymptomatic and symptomatic individuals with CD4 counts

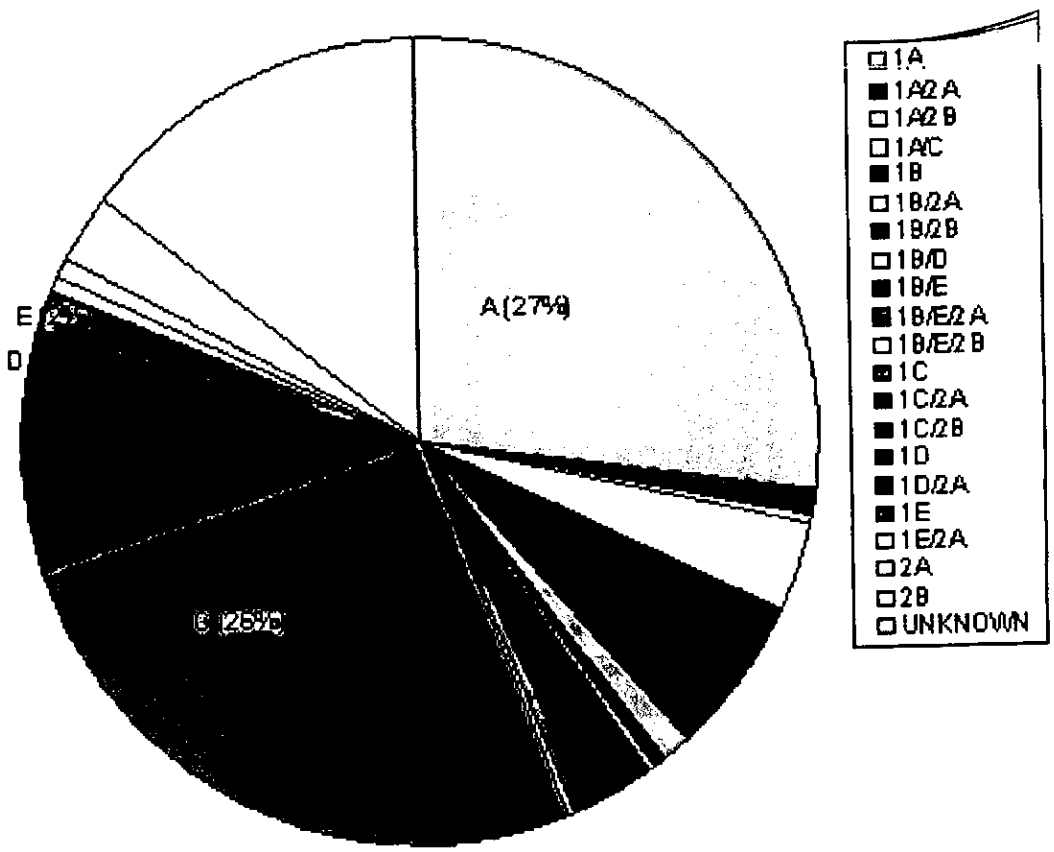


Figure 25: Proportion of HIV-1 & 2 Serotypes in Lagos, Nigeria

Table 36: Distribution of HIV serotypes among the recruited cases in the study

HIV serotype	No. of Male with HIV serotype	No. of Female with HIV serotype	Total No. with HIV serotype	% HIV serotype
HIV-1 serotype A	27	41	68	27.1
HIV-1 serotype A/ HIV-2 serotype A	1	2	3	1.2
HIV-1 serotype A/ HIV-2 serotype B	0	1	1	0.4
HIV-1 serotype A & C	4	5	9	3.6
HIV-1 serotype B	8	8	16	6.3
HIV-1 serotype B/ HIV-2 serotype A	2	1	3	1.2
HIV-1 serotype B/ HIV-2 serotype B	0	1	1	0.4
HIV-1 serotype B & D	0	1	1	0.4
HIV-1 serotype B & E	5	2	7	2.8
HIV-1 serotype B & E/ HIV-2 serotype A	0	1	1	0.4
HIV-1 serotype B & E/ HIV-2 serotype B	1	0	1	0.4
HIV-1 serotype C	35	30	65	25.9
HIV-1 serotype C/ HIV-2 serotype A	7	3	10	4.0
HIV-1 serotype C/ HIV-2 serotype B	4	6	10	4.0
HIV-1 serotype D	1	2	3	1.2
HIV-1 serotype D/ HIV-2 serotype A	0	1	1	0.4
HIV-1 serotype E	2	2	4	1.6
HIV-1 serotype E/ HIV-2 serotype A	0	2	2	0.8
HIV-2 serotype A	2	0	2	0.8
HIV-2 serotype B	4	3	7	2.8
Unknown	15	21	36	14.3
Total	**118**	**133**	**251**	**100**

7.7 DISCUSSION

Of the 64% symptomatic volunteers, 36.3%, 22.4% and 19.2% had wasting syndrome, diarrhoea and tuberculosis respectively. Titres ranging between 1:8 and 1:512 with a modal titre of 1:256 were obtained from 146 (58.4%) reactive volunteers to complement fixing (CF) antibody as well as anti-p24 antibody. The presence of anti-p 25 and CF antibodies were not significant enough to distinguish between asymptomatic and symptomatic patients. However, the frequency curve of asymptomatic and symptomatic individuals with the CF antibody titre exceptionally revealed that the asymptomatic group had its peak in the frequency curve at 1: 32 while the symptomatic group had its peak at 1: 256. The CF antibody titres have therefore proved to be suitable for use in a resource-limited country like Nigeria. Of 20 serotypes of HIV obtained from the study, HIV-1 serotype A (27%) slightly emerged the highest from HIV-1 serotype C (26%).

The proportion of individuals having one or more symptoms (64%) as compared with asymptomatic individuals (36%) in this study had no significant value other than the stage of HIV infection represented by these individuals during and after their enrolment for the study. The advantages of this proportion are detecting the incidence of AIDS among the HIV infected individuals and enlarging the sample size for symptoms types for better analysis. Of the full blown (symptomatic) AIDS patients, 43 patients (26.9%) had wasting syndrome, 31 (19.4%) had tuberculosis, 28 (17.5%) had prolonged fever and 20 (12.5%) had persistent diarrhoea. The reports of this study are therefore synonymous with others with respect to prevailing symptoms exhibited by HIV/AIDS patients in various clinics across the Globe (Fauci, 1988; Benenson, 1990; Piot *et al.*, 1992).

On symptoms or clinical stages of HIV infection, much work had been done (CDC, 1993, Pan *et al.*, 1993) with similar results to those of this study. Since only HIV/AIDS cases reporting at any health institutions were enrolled in these studies, the actual rate of HIV-infected individuals developing symptoms in the general population would require application of many epidemiological tools. Hence, the fraction/percentage of symptomatic patients among the PLWHA in this study should be accepted with caution.

HIV- 1 has been generally reported as the more common of the two types of HIV (Chiengsong-Popov *et al.*, 1991; 1993; Barre-Sinoussi, 1996) so it was convincing to have HIV-1 recording 87.2%. The percentage of HIV-1 & 2 mixed infections was surprisingly low compared with the reports of various mixed HIV-1 & 2 subtypes occurring in the samples studied previously. The significance of HIV-1 having the highest percentage among others was due to higher virulence of HIV- 1 virus than HIV-2 (Ho *et al.*, 1995) and the predominance of HIV-1 than HIV-2 (WHO/NHIC, 1994). There was no significant difference between males and females seropositive for HIV- 1 & 2 in the study, although the female population is still more vulnerable to HIV infection than the male (WHO, 2004). Since the study was carried out in Nigeria and based on random selection, most of the subjects were therefore expected to be Nigerians. The 93.5% Nigerian in the study was then appropriate.

More than half (58.4%) of all HIV seropositive individual participants were anti-p24 antibody positive. Since this percentage was close to that of the symptoms, anti-p24 antibody levels and titres could therefore be used as a reliable prognostic marker for HIV progression (Forster *et al.*, 1987; Goldschmidt *et al.*, 1998). However, anti-p24 antibodies

pattern in the study revealed that 59 of 90 asymptomatic individuals (65.6%) were positive while 87 of 161 symptomatic individuals (54%) were positive, there was no significant difference between them ($p = 0.08$). Hence, presence of anti-p 24 antibody in the blood of the PLWHA could not be used to distinguish asymptomatic from symptomatic patients.

Complement components, particularly C3, are known to be involved in the pathogenesis of AIDS and macrophages may serve as a source of C3 at sites of infection (Bajtay *et al.*, 1998). Monocytes cultured with either the monocytotropic or the T lymphocytotropic HIV-1 strains produce C3 in large amounts. The effect of both HIV-1 & 2 viruses is dose dependent and the amount of C3 induced by HIV was up to 20-fold higher than in the control samples (Bajtay *et al.*, 1998). C3 production was also enhanced by *gp*160, the envelope protein of the virus. Secretion of IL-6 by the cells was also measured and found to be elevated up to threefold as a consequence of the interaction with the virus.

HIV-1-activated monocyte-derived macrophages acquired the capacity to cleave exogenous C3 and to fix generated C3 fragments on their cell membrane. Hence, CF antibodies are being released during this process (Spear *et al.*, 1991) and could therefore be detected and quantified (Spear *et al.*, 1992). Complement fixing (CF) antibodies' presence alone also revealed that 56 of 90 asymptomatic individuals (62.2%) were positive while 90 of 161 symptomatic individuals (55.9%) were positive, but there was no significant difference between them ($p = 0.33$). Hence, presence of HIV CF antibody alone could not be used to distinguish asymptomatic from symptomatic patients.

Although there were similar results shown by the frequency of asymptomatic and symptomatic individuals with CF antibody titres when compared with viral load titres and CD4 counts, the use of CF antibody titre for the monitoring of HIV/AIDS progression in PLWHA is yet to be reported. However, symptomatic and asymptomatic frequencies with qualitative complement fixing antibody test and anti-p24 antibody assays similarly showed no differences with 0.33 and 0.08 respective p values. It was therefore shown that the qualitative CF test and anti-p24 antibody assay could not distinguish between symptomatic and asymptomatic stages of HIV infection. Anti-p24 antibody had been known through a number of findings (Foster *et al.*, 1987, Goudsmit *et al.*, 1988) to decline as HIV-infected individuals progress to manifestation of HIV/AIDS symptoms. In addition, quantitative analysis for anti-p24 antibody assay was not carried out in this study, and hence the quantitative parameter needs to be examined. Such study would reveal specific relationship between anti-p24 antibody, CF antibody, CD4 counts and viral load. This study has therefore clearly shown that it is possible to use CF antibody titres to monitor the trends of clinical stages of HIV in PLWHA from asymptomatic conditions to full blown AIDS.

The distribution of HIV-1 & 2 based on serotyping therefore revealed a quassi-species of HIV-1 & 2 involving 20 serotypes and unknown others, and this confirms the high degree of HIV-1 and HIV-2 diversity as discussed by Cheingsong-Popov *et al.* (1998). The study is however inadequate without thorough analysis of the various subtypes in circulation in Nigeria, using molecular genetic methods (such as V3 loop, real time PCR, reverse transcriptase and ribonuclease genotypmg, gene sequencing and heteroduplex mobility assay) in order to actualize specific subtypes' distribution of HIV-

1, HIV-2 and their hybrids circulating among the HIV infected individuals in Nigeria. The detection of various specific subtypes would pave way for designing a suitable vaccine for protecting Nigerians (and the whole world) against HIV/AIDS in the near future.

The need for management of HIV/AIDS patients in order to reduce mortality rate and further spread of HIV/AIDS cannot be over-emphasized. For such need to arise there should be in place, assessment of HIV/AIDS cases and monitoring of stages of clinical conditions. Nigeria, being a resource-limited country, needs alternative methods to serve as prognostic markers for HIV/AIDS. Complement fixation test could therefore be used as a surrogate or prognostic marker of HIV progression to AIDS. Reports were yet to be obtained from around the Globe with respect to the possibility, efficacy and effectiveness of using CFT as a prognostic tool for HIV/AIDS management although there were reports of CF antibody in HIV infected individuals (Spear *et al.*, 1990 & 1992, Bajtay *et al.*, 1998).

7.8 CONCLUSION

In this study, complement fixing HIV antibody titres have been developed and evaluated to be a possible and an efficient tool as one of the prognostic markers for monitoring the progression of HIV infection in PLWHA from asymptomatic status to full blown AIDS. One hundred and sixty (64%) of the confirmed HIV seropositive patients were symptomatic with 43 patients (26.9%) having wasting syndrome, 31 (19.4%) having tuberculosis, 28 (17.5%) having prolonged fever and 20 (12.5%) having persistent diarrhoea. Both the presence of anti-p 24 antibody and HIV CF antibody alone could not

be used to distinguish asymptomatic from symptomatic patients. Twenty serotypes of HIV and many unknown others were identified with HIV-1 serotype A (27%) slightly emerging the highest from HIV-1 serotype C (26%) among all HIV-1& 2 subtypes (including some HIV-1 mixed infection). All serotypes reported in the study still need further identification and confirmation. The CF antibody titre is therefore significant for monitoring HIV/AIDS trends in PLWHA.

CHAPTER EIGHT
GENERAL DISCUSSION

8.0 GENERAL DISCUSSION

8.1 DISCUSSION

The need for any country's evaluation/standardization of commercially available kits, which is one of the objectives of this study, cannot be overemphasized, as differences in sensitivity, specificity, reproducibility, and suitability of some kits have been severally reported in different countries, due to the compatibility of the kit's antigen with the country's circulating virus (es). The outcome of the first phase showed that SUB-Recombigen and Determine HIV-1 & 2 were the diagnostic kits of choice for ELISA and S/R assays respectively while that of the second phase demonstrated that Vironostika and Capillus HIV-1/HIV-2 were also respective ELISA and S/R assays' diagnostic kits of choice. They all had sensitivities, specificities, reproducibilities, PPV and NPV of over 99.5%. Both the ease of performance and suitability for use of these kits of choice were better than any of the other kits assessed.

A rapid-based serial HIV testing algorithm (using Determine and Capillus as candidate kits) was developed for 2,500 patients of which 20.5% were seropositive with 442 HIV-1(17.7%), 12 HIV-2 (0.5%) and 58 HIV-1 & 2 (2.3%) confirmed serostatus. Of the 2,500 samples used in the algorithm, concordant negatives were 1,988 (79.5%), concordant positives were 509 (20.4%) while discordant negatives were 3 (0.12%). The algorithm was suitable for rural/field settings, [most especially for the voluntary counselling and testing (VCT) programme and emergencies in the remote localities], hospitals and clinics.

An ELISA/rapid-based serial HIV testing algorithm (using Vironostika and Determine as candidate kits) was developed for 2,500 patients, of which 20.5% were

seropositive with 442 HIV-1(17.7%), 12 HIV-2 (0.5%) and 58 HIV-1 & 2 (2.3%) confirmed serostatus. Of the 2,500 samples used in the algorithm, concordant negatives were 1,976 (79%), concordant positives were 524 (21%) while discordant negatives were 12 (0.5%). The algorithm was suitable for early turnout of HIV confirmatory results in blood banks and referral centres with electricity-constraint. It would also be suitable for HIV surveillance study.

Use of CF antibody titres for measuring the progression from healthy carriers to symptomatic HIV/AIDS patients was demonstrated successfully in the study. There was a significantly higher frequency of the symptomatic patients with CF antibody titre > 1:64 in the study. The complement fixation test (CFT) can therefore be used to monitor the commencement of treatment for the people living with HIV/AIDS (PLWHA) that cannot afford the expensive HIV RNA viral load determination and CD4 T cells count methods.

Nigeria, being a country rated 3rd among others harbouring between 3.2 and 3.6 million of PLWHA worldwide (WHO, 2004), still has its recent seroprevalence at 4.4% after the 2005 sentinel seroprevalence study across the country (FMOH/NASCP, 2006). If the sentinel study was anything to go by, it means that 5.6 million out of 127 million Nigerians (WHO, 2005) were infected by the virus as against the WHO's estimate based on the outcome of the surveillance study on women's antenatal clinic survey (the main HIV indicator). The sentinel report contradicts the WHO estimate and its figure is alarming. Hence, the sentinel study needs to be assessed technically.

Considering the activities of both NASCP and NAFDAC on authenticating commercial HIV diagnostic kits, there are many flaws in the accreditation and evaluation exercise. The two governmental organizations send out incoming HIV kits to the

Nigerian market to any referral laboratory known for HIV confirmatory tests giving no guidelines on how to carry out the evaluation exercise on the kits. Most of these referral laboratories have no standard operating procedure (SOP) based on WHO's (2002) standard. In fact, most do not have specific sample size, sampling method, evaluation parameters and laboratories' gold standard diagnostic supplementary kits.

Again, there is no distinction between diagnostic and research kits whereby some of the laboratories (including referral laboratories) use the research kits as diagnostic or even as their gold standard. The influx of these kits into the Nigerian market without regulation by the authorities constitutes one of the problems encountered in the determination of HIV status in individuals and blood, for blood transfusion. Even if there were diagnostic kits in the country, are these kits efficient enough to recognise the local HIV strains circulating among Nigerians? Most HIV kits are manufactured with their country's local antigens; and these kits might not recognise the local strains in Nigeria due to the diversity and mutation rate of HIV-1 and HIV-2 (WHO/NHIC, 2004).

The dynamism in HIV testing technology has not been put into consideration by the Nigerian regulatory authority to update already certified commercial HIV kits in order to reflect the recent changes in the development of ELISA (Constantine, 1999) and rapid test (Henry, 1999) assays. In addition, there was no suitable testing strategy (algorithm) for the hard-to-reach and rural settlers, and none for emergency blood transfusion and patient's clinical treatment. Also, there were no new designs to resolve the delay at the blood banks and referral laboratories on the final determination of HIV status by confirmatory methods in spite of the cost of the WB kit and the erratic power supply from the national grid.

Surveillance system in the country is yet to have a reliable HIV database since the establishment of NASCP in 1989 and NACA in 1999, with about 6 sentinel seroprevalence surveillance studies implemented. While many countries in Sub-Saharan Africa have known HIV prevalence, strains distribution and database (Peters *et al.*, 1999; UNAIDS, 2002, UNAIDS/WHO, 2004), Nigeria is still struggling to have a national standard format for determining HIV status. In addition, Nigeria is yet to have an alternative to the major prognostic markers (viral load and CD4 T cell count) to monitor the trend among the symptomatic and asymptomatic PLWHA, many of whom cannot afford the cost of these two markers.

All these underlying difficulties stimulated the path-finding approach to resolve them. The need for HIV prevention and management for people living with HIV/AIDS (PLWHA) to reduce mortality rate and control the spread of HIV/AIDS cannot be over-emphasized, therefore, HIV serodiagnosis is indispensable, especially in the implementation of prevention of mother-to-child transmission (PMTCT) of HIV (Andersson *et al.*, 1997). A gold standard for HIV serodiagnosis in any country in the world must be established for quality HIV antibody testing and voluntary counselling and testing (VCT) (Ziyambi *et al.*, 2002). This gold standard involves standard testing strategies (algorithms), standard diagnostic kits and equipment, standard proficiency system and continuous assessment. Now that VCT has become the order of the day and with large groups (both governmental (GO) and non-governmental organizations (NGOs)), it is imperative to set standards in order to control the use of just any kit or method for serodiagnosis in the country.

The SUB-Recombigen HIV-1/HIV-2, a 2nd generation normal ELISA kit, was demonstrated as the best among 3 other selected commercially available HIV ELISA kits in the study. The Recombigen HIV-1/HIV-2 test kit had been assessed by both Leon de Gonzalez *et al.*, (1994) and Francois-Gerrard *et al.*, (1996) in their review of European diagnostic kits at a multicentre evaluation of Europeans HIV diagnostic kits (France), as one of the best among all second generation ELISA kits of its time, with sensitivity of 100% and specificity of 99.6% - 99.8%.

Determine HIV-1/2, an immunochromatographic rapid kit, was the best among 5 other selected commercially available HIV rapid kits in the first phase of the study. Aria *et al.*, (1999) revealed in Bangkok (Thailand) that Determine had 100% sensitivity, specificity and reproducibility, and recommended it to be very suitable for field and local HIV screening. Similarly, Rouet *et al.* (2004), reported in Ivory Coast on the field evaluation of a rapid HIV serial serologic testing algorithm using 10,135 samples of pregnant West African women, that the Determine had better sensitivity (100%) than Genie II (99.5%). Determine was the most suitable kit for rural/field settings in the study by virtue of its ability to retain its shelf life at room temperature and above, therefore being independent of a cold chain system for its storage.

Of the two normal ELISA kits assessed in the second phase, Vironostika Uni Form II Ag/Ab was better than Genscreen Plus HIV Ag-Ab based on ease of performance and suitability for referral settings. The two fourth generation ELISA kits however proved to be very effective for HIV serodiagnosis with 100% in all their evaluation parameters, namely; sensitivity, specificity, reproducibility, NPV and PPV. But Ly *et al.* (2001) found that Vironostika Uniform II Ag-Ab was better in sensitivity than Genscreen

Plus Ag-Ab in France during their study while Chishawa *et al.* (2001) reported the suitability of Vironostika as the gold standard in their referral centre in Zimbabwe. Therefore, Vironostika Uni-Form II Ag/Ab could also serve as a gold standard in Nigeria.

In the second phase, the Capillus HIV-1/HIV-2, a capillary latex agglutination rapid kit, demonstrated to be the best among two other S/R commercially available HIV kits based on its sensitivity (100%), specificity (100%), reproducibility (100%), NPV (100%) and PPV (100%). Capillus was however reported by Urassa *et al.* (2002) in Dar Es Salaam, Tanzania to have 100% sensitivity but 98.7% specificity in the 1,412 fresh serum samples evaluated. Andersson *et al.* (1997), recommended in Sweden that Capillus is a suitable rapid screening test (RST) for Africa, with sensitivity of 100% and specificities of initial and repeated testing of 99.8 and 99.9% respectively.

Phase I kits were not assessed comparatively with the second phase kits but ELISA kits of the first phase are clearly different from those of second phase based on 2nd phase kits' additional detection capacity. Hence, the importance of second phase emphasizes the need for dynamism in HIV testing technology as Weber *et al.* (1998) concluded that fourth generation assays permit an earlier diagnosis of HIV infection than third generation antibody screening assays through the detection of p24 antigen, which may be present in serum samples from individuals with recent HIV infection prior to seroconversion. In addition, Courouce (1999) in his update review concluded that the constant improvement of anti-HIV screening tests which leads one to shorten the 'window' period permits an earlier diagnosis of HIV infection and progressive decrease of transfusion risk.

The candidate kits (Determine and Capillus) used as a rapid-based serial HIV testing algorithm was able to function perfectly well, not only in rural or field settings (where sophisticated equipment, electricity, etc, are not available) but also in clinics or hospitals where urgent HIV diagnosis is required for treatment/surgical operation during emergencies. Similar algorithm constituting Determine and Genie II was developed for field/rural settings involving pregnant women in West Africa (Rouet *et al.*, 2004). In this algorithm all concordant positive and negative results are reported as confirmed positive and negative without further tests while discordant positive or negative results were subjected to further conventional HIV antibody tests in referral laboratories. The algorithm reduces the burden of samples sent for and duration of time spent on conventional confirmatory tests. Apart from these advantages, anybody anywhere in the country can determine his or her HIV status, whether in Sahel savannah/arid desert or the Delta riverine areas, if this algorithm were adopted for the standard testing for rural/field settings. In addition, accident victims would no longer die due to loss of blood once the HIV status of the blood to be transfused is known at that local road side with this algorithm being implemented for emergency at various clinics in the country.

Another set of candidate kits (Vironostika and Determine) used as an ELISA/rapid-based serial HIV testing algorithm has been demonstrated to function perfectly well for blood bank settings (where emergency blood transfusion is predominant) or referral settings (with electricity outage problem). The algorithm was developed to reduce the time factor affecting conventional confirmatory tests and sample size for such tests. The algorithm is very suitable for early diagnosis of HIV infection (even before seroconversion) because of the role of Vironostika as a 4th generation

ELISA kit (capable of detecting HIV p24 antigen in blood and serum). The WHO (2002) has proposed and recommended this algorithm (strategy) for Africa. The algorithm can hasten release of confirmatory test results at the referral levels for immediate action on suspected blood samples as well as enhance the safe and faster delivery of blood and blood products in blood banks across the country.

To arouse consciousness for the need to carry out studies for baseline data entry for HIV surveillance in the country, the ELISA/rapid -based serial HIV testing algorithm was applied in a form of prospective study of samples collected at CPHL. A 20.5% infection rate was shown by patients, unlike the sentinel seroprevalence surveillance (5.5%) reported in Nigeria in the year 2000 involving only the antenatal group as at the time of that study (NASCP/FMOH, 2001). The trends in both HIV seropositive pattern and infection rate between 1997 and 1999 served as an indication of public awareness on HIV/AIDS and possibilities of interventional strategies in action. These agree with the findings of NASCP/FMOH, (2002) and Badaru *et al.*, (2002). Use of both Vironostika and Determine (as ELISA/rapid-based for urban centres) and Determine and Capillus (as rapid-based kits for rural centres) as a gold standard therefore recommended for sentinel seroprevalence studies so that quality surveillance and database for HIV is sustained.

Comparing the HIV infection rate of individuals who claimed to have acquired the infection by blood transfusion 6.4%, [i.e., (27.6%, CI: 20.89-34.31) of (2.32%, n = 2, 500)] with that of blood donors (0.3%, 2%, and 4%) as reported by Akinsete *et al.*, (1989 & 1991), it was shown that trends in transmission of HIV through blood transfusion from blood donors is steadily increasing, although the difference between the three variables was still significant ($0.0001 \leq p \leq 0.01$). Hence, there has not been effective improvement

in measures reducing the HIV transmission via blood or its products in spite of low prevalence of this group in the population.

The study revealed that the single female group had the highest infection rate under marital status, although the overall married group had statistically significant ($p > 0.01$) HIV infection rate greater than that of the single group. Orubuloye *et al.*, (1992), had given adequate explanation in view of the fact that most married men and some single young women (spinsters) transmit HIV to their respective partners, with more spinsters falling victims. The study suggested intensive research on other occupational groups apart from the established groups (such as the commercial sex workers and drivers) (Orubuloye *et al.*, 1991; Dada *et al.*, 1993) as high risk workers for HIV/AIDS because it showed that military, tailors/fashion designers, and traders/business people as new groups with high infection rates. The trends in age groups further supported the views in the study by Orubuloye *et al.*, (1992), where younger females and elderly males were more vulnerable to HIV infection.

On symptoms or clinical stages of HIV infection, much work has been done (CDC, 1993, Pan *et al.*, 1993) with similar results to those of this study. Since only HIV/AIDS cases reporting at any health institution were enrolled in these studies, the actual rate of HIV-infected individuals developing symptoms in the general population would require application of many epidemiological tools. Hence, the fraction/percentage of symptomatic patients among the PLWHA in this study should be accepted with caution.

Although there were similar results shown by the frequency of asymptomatic and symptomatic individuals with CF antibody titres when compared with viral load titres and

CD4 counts, the use of CF antibody titre for the monitoring of HIV/AIDS progression in PLWHA is yet to be reported. However, symptomatic and asymptomatic frequency with qualitative complement fixing antibody test and anti-p24 antibody assays similarly showed no differences with 0.33 and 0.08 respective p values. It was therefore shown that the qualitative CF test and anti-p24 antibody assay could not distinguish between symptomatic and asymptomatic stages of HIV infection, although anti-p24 antibody had been known through a number of findings (Foster *et al.*, 1987, Goudsmit *et al.*, 1988) to decline as HIV-infected individuals progress to manifestation of HIV/AIDS symptoms. In addition, quantitative analysis for anti-p24 antibody assay was not carried out in this study, and hence the quantitative parameter needs to be examined. Such study would reveal specific relationships between anti-p24 antibody, CF antibody, CD4 counts and viral load.

The study experienced some limitations. The results of HIV subtyping require Real-Time PCR to clearly differentiate the serotypes (Figure 29; Table 34). There is need to develop normal ELISA and WB kits using local strains of HIV circulating in the country as the target antigens. These pressing issues could be thoroughly addressed if there were enough funds and resources to continue.

CHAPTER NINE
GENERAL CONCLUSION AND RECOMMENDATIONS

9.0 GENERAL CONCLUSIONS

9.1 CONCLUSIONS

The study has been able to develop, demonstrate and evaluate successfully, a novel rapid-based HIV serologic testing algorithm using Nigerian subjects resident in Lagos, Nigeria although an interim algorithm (not tested nor evaluated) has just been recently recommended to the authorities of the health sectors in the country. The algorithm involves the use of Determine HIV-1 & 2 and Capillus HIV-1/HIV-2 kits as candidate kits used serially, and has been proven to be very suitable for determining HIV status of any Nigerian anywhere (whether rural or urban) as urgently as possible (especially in cases of emergencies, blood transfusion and clinical treatment/management). The most fascinating development is that the algorithm can be run on the spot, especially during field activities.

A novel ELISA/rapid-based HIV serologic testing algorithm ever reported was developed, demonstrated and evaluated with full accomplishment in this study. The algorithm entails the use of Vironostika Uni-Form II HIV Ag/Ab and Determine HIV-1 & 2 kits as applicant kits used serially, and has been proven to be very suitable as an alternative mean for final determination of HIV status in developing countries embattled with resources constraint (like electricity and expensive equipment/kits). The algorithm served as a watershed to the delay commonly encountered at referral centres and blood banks for the final outcome of HIV serologic tests.

After 25 years of the discovery of the acquired immune deficiency syndrome (AIDS), this is the first kind of evaluation study in Nigeria, involving commercially available HIV test kits of different methods and principles. In this study, Determine HIV-

1 & 2 is the rapid diagnostic kit of choice of all S/R kits used in the first phase of the evaluation for the serodiagnosis of HIV. Although SUB-Recombigen is the best ELISA kit of all ELISA kits in this first phase, the improvement in the quality of testing among all generations of ELISA due to the dynamism in testing technology made SUB-Recombigen extraneous in recent times, being a second generation ELISA kit.

The second phase of the evaluation of the commercially available ELISA kits identified Vironostika Uni-Form HIV Ag/Ab (4^{th} generation ELISA) kit as the ELISA diagnostic kit of choice for the serodiagnosis of HIV among others evaluated. In addition, the evaluation exercise for the commercially available rapid test kits in the second phase recognised the Capillus HIV-1/HIV-2 kit as the rapid diagnostic kit of choice for the serodiagnosis of HIV among others assessed in the study. Although the studied kits in the first phase were not compared with those of the second phase, most (especially the 4^{th} generation ELISA) have been documented to have better performance than those of the first phase (Weber *et al.*, 1998; Courouce, 1999; 1999a).

More than 20% of the entire patient population enrolled for this study were confirmed HIV-infected. This finding illustrated a generalized epidemic among the patients referred to Central Public Health Laboratory (CPHL) during the study. Since the group belonged to the hospital-referred, it is difficult to compare with the actual country's situations. This study however served as a pointer for thorough investigations on HIV incidence and prevalence using the gold standard methods and HIV test kits, as well as an appropriate sentinel seroprevalence surveillance study for the country.

Although the need to study other high-risk groups for HIV infection has been severally recommended in many studies (Brew-Graves (1994; Dada *et al.*, 1993; 1995),

this study also emphasized such need so that intervention strategies would be focused on these groups. The occupational high-risk groups identified in this study were the military force, tailor/fashion designers and business people. There is need to investigate other groups because this study may still miss out some other high-risk groups due to limited sample size and coverage of this study.

Another novel study reported is the detection and use of complement fixing (CF) antibody titres to monitor the progression of HIV/AIDS among the people living with HIV/AIDS (PLWHA). This study clearly demonstrated the possibility of using CF antibody titres as a prognostic marker for HIV/AIDS by comparing the novel method with HIV RNA viral load determination and CD4 T cells count as standards. The method would serve as an alternative to the standard for those who cannot afford the cost of the standard markers among the PLWHA, especially in a resource-constrained developing country like Nigeria.

Conclusively, the Determine HIV-1/2, Capillus HIV-1/HIV-2 (as rapid test kits) and Vironostika Uni-Form HIV Ag/Ab (4^{th} generation ELISA) kits have been assessed and ratified to be the national gold standard kits among others evaluated in this study. An ELISA/rapid-based serial HIV testing algorithm using Vironostika and Determine has been proven suitable for blood banks and referral centres with electricity-constrain while a rapid-based serial HIV testing algorithm has been proven to be suitable for rural/field and clinical settings.

9.2 RECOMMENDATIONS

Based on the outcome of this study and if the Government and people of the Federal Republic of Nigeria were ready to combat the spread of HIV and minimize the viral load of PLWHA beyond transmissible level in order to control and eliminate the virus; it is pertinent to recommend the following:

9.2.1 Since this study is a litmus test for accessing the level or gravity of HIV/AIDS surveillance and intervention course prevailing in Nigeria, major outcomes of this study should be adopted or subjected to further evaluation studies using larger sample sizes (or other investigations)

9.2.2 Operational evaluation of all commercially available HIV test kits in the country should be re-evaluated using the WHO standards before certification as diagnostic kits

9.2.3 Updating of the circulating diagnostic HIV test kits should be conducted biennially in order to ascertain the effectiveness and efficiency of the test kit, as well as to compare their performance with recently developed test kits

9.2.4 All the country's identified evaluation centres should have standard operating procedures (SOPs), gold standard HIV test kits for ELISA and rapid assays (like Determine, Capillus and Vironostika), WB kits and its facilities, and at least 400 positive and negative panels of sera

9.2.5 That the authority in the health sector should uphold publishing of accredited diagnostic kits and adopt the Determine HIV-1 & 2, Capillus HIV-1/HIV-2 and Vironostika Uni-Form II Ag/Ab as the national gold standard HIV test kits for a start

9.2.6 That the authorities should advocate for the use of serial testing algorithm in preference to the parallel testing for final determination of HIV status of Nigerians and their blood/blood product at any level of HIV serodiagnosis, surveillance and blood transfusion

9.2.7 That the authority should adopt the use of the rapid-based serial HIV testing algorithm using Determine and Capillus as candidate kits at the grassroots, VCT, mobile clinics and hospitals for final determination of HIV status of individuals and blood for a start

9.2.8 That the authority should approve the use of the ELISA/rapid-based serial HIV testing algorithm using Vironostika and Determine as candidate kits as an alternative confirmatory strategy at the referral centres and blood banks for the final determination of HIV status of individuals and blood whenever there were constraints due to electricity or/and cost of Western blot (WB) test kits

9.2.9 Since studies have revealed a generalized epidemic of HIV/AIDS in Nigeria based on the outcome of the seropositivity of individuals by state of origin, HIV/AIDS surveillance should be carried out at the grassroots' levels

9.2.10 That a standard HIV testing algorithm (strategy) should be adopted nationwide for screening and confirmatory test of individuals and blood/blood products if the above evaluated algorithms were not accepted

9.2.11 That further studies should be carried out on the quality assurance of various blood banks in providing safe blood for transfusion, apart from adhering to the alternative

confirmatory strategy since there are other highly infectious agents that need effective laboratory diagnosis for their detection in blood/blood products

9.2.12 That further assessment of all the qualities (various units) of the health sector (including structure, personnel and materials) involved in the diagnosis and management of HIV/AIDS should also be implemented

9.2.12 That the authorities should provide fund and logistics for capacity building of various health units involved in care and management of HIV/AIDS

9.2.13 Mass campaign and education on knowledge and prevention of HIV/AIDS should fully be funded by both governmental and non-governmental organizations

9.2.14 If sentinel studies were to be organized, it should be extended to not only the six geopolitical zones in the country but also the entire 36 states and Federal Capital Territory of the Federation, with sentinel sites drawn from probability-dependent random sampling method to avoid biased outcomes

9.2.15 A programme for vaccine development should be designed, funded and organized based on the knowledge of circulating HIV strains in the country, in order to obtain potent vaccines that can completely neutralize the wild strains in the body system and protect individuals from wild HIV infections

9.2.16 Surveillance, focused on hospital patients and patients found in other health care delivery centres, should be carried out using the gold standards as Vironostika and Determine for reliable database on HIV/AIDS

9.2.17 That local HIV screening and confirmatory kits using the local HIV circulating strains as its basic antigens should be developed for the detection of those false negative cases arising from the foreign kits

9.2.18 An incumbent priority for authorities in health sector of the country is to employ experts on HIV/AIDS to survey its spread throughout the country with more emphasis on its molecular epidemiology

9.2.19 That the Government should encourage the setting up of more voluntary counselling and testing (VCT) centres across the country (especially in the rural areas) to enable more Nigerians to have access to free HIV tests

9.2.20 The Government and other stakeholders for control and prevention of HIV/AIDS should channel more resources at strengthening materials, logistics, equipment, staff and infrastructural capacity for HIV/AIDS management system in Nigeria

9.2.21 With the WHO 3 by 5 initiatives still on, procurement of affordable antiretroviral drugs and/or medicinal herbs, if any, should be improved and ARV treatment centres should be increased

9.2.22 That the CF antibody titres should be adopted as an alternative prognostic marker to viral load determination and CD4 T cells counts for monitoring the progression of people living with HIV/AIDS (PLWHA)from asymptomatic condition to full blown AIDS as a cheap and suitable surrogate marker

9.2.23 That the health care providers should intensify efforts on thorough laboratory research on new strains, especially the ARV-resistant strains, and follow up of cases of PLWHA that do not respond to treatment and those that have contraindications

9.2.24 Finally, the Government and all stakeholders should improve on the techniques of HIV/AIDS surveillance using recent technological devices, which are adaptable to our poor-resource settings.

CHAPTER TEN
REFERENCES

10.0 REFERENCES

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APPENDICES

Appendix 1: Properties of Commercially Available kits used in the study

S/N	Name of ELISA Kit	Manufacturer	Lot No	Expiry Date	Test Run Date	Running Incubation Time	Running Incubation Temperature	Storage Requirement	Specimen Predilution Requirement	Manufacturer's Instruction	Auxillary Equipment	Method
1	Wellcozyme/Murex HIV-1+2	Murex Diagnostics, Dartford, U.K	F776910	28/09/99	12/08/98	2-3hrs	37oC, humid condition + 18-25oC Room Temp.)	2-8oC	None	Clear + Technical skill	Auto. Microplate Reader & Washer	2nd Generation ELISA
			F776810	15/08/99								
			F776710	22/07/99								
			F776800	05/08/99								
			F776610	18/06/99								
			F776910	21/09/99								
2	Genelavia Mixt	Sanofi Diagnostics Pasteur, Marnes la Coquette, France	8F213-R	28/01/99	14/08/98	2-3hrs	37oC, humid condition + 18-25oC Room Temp.)	2-8oC	None	Clear + Technical skill	Auto. Microplate Reader & Washer	2nd Generation ELISA
			8F214-R	15/02/99								
			8F215-R	09/03/99								
			8F218-R	15/04/99								
			8F217-R	9/4/1999								
			8F219-R	25/04/99								
3	SUB-Recombigen HIV-1/HIV-2 EIA	Cambridge Biotech Diagnostics, Galway, Ireland	D44509/1 (5)	26/4/99	17/08/98	2-3hrs	37oC, humid condition + 18-25oC Room Temp.)	2-8oC	None	Clear + Technical skill	Auto. Microplate Reader & Washer	2nd Generation ELISA
4	Immunocomb II HIV-1 & 2 Bispot	Orgenics, Yavne, Israel	980821 (5)	21/08/99	19/08/98	38-40mins	18-25oC (Room Temp.)	2-8oC	None	Clear + Simple	None	Rapid EIA
			980823 (5)	23/08/99								
			980825 (4)	25/08/99								
5	Immunocomb II HIV-1 & 2 CombFirm	Orgenics, Yavne, Israel	980822 (10)	22/08/99	21/08/98	38-40mins	18-25oC (Room Temp.)	2-8oC	None	Clear + Simple	None	Rapid EIA
			980820 (10)	20/08/99								
			980819 (10)	19/08/99								
6	Recombigen HIV-1/HIV-2 RTD	Cambridge Biotech Diagnostics, Galway, Ireland	N33904/1 (3)	26/03/98	24/08/98	5-10mins	18-25oC (Room Temp.)	2-8oC	None	Clear + Simple	None	Rapid EIA
			N33904/3 (2)	14/03/98								
7	Gene II HIV-1/HIV-2	Sanofi Diagnostics Pasteur, Marnes la Coquette, France	980408	08/04/99	26/08/98	5-10mins	18-25oC (Room Temp.)	2-8oC	Present	Clear + Simple	None	Rapid EIA
			980406	29/03/99								
			980404	16/03/99								
			980402	27/02/99								
			980400	12/02/99								
8	Determine HIV-1/2	Abbott Diagnostics, Pretoria, South Africa	43100U100 (5)	16/12/99	28/08/98	3-5mins	18-25oC (Room Temp.)	2-8oC	None	Clear + Simple	None	Rapid EIA
9	Omni-Sal HIV-1/2 & Subtype O (Saliva test)	Americare Biologicals, U.S.A	AB-201 (10)	12/10/1999	31/08/98	3-5mins	18-25oC (Room Temp.)	2-8oC	None	Clear + Simple	None	Rapid EIA
10	Genscreen Plus HIV-Ag	Biorad, Marnes La Coquette, France	99123003 (3)	14/01/02	19/07/00	2-2.5hrs	37oC, humid condition + 18-25oC Room	2-8oC	None	Clear + Simple	None	4th Gen. ELISA
			99123005 (2)	30/01/02								
11	Vironostika HIV Uni-Form II Ag/Ab	Organon Teknika, Portugal	84138(00031402) (4)	2/4/2002	21/07/00	1-2hrs	37oC, humid condition + 18-	2-8oC	None	Clear + Simple	None	4th Gen. ELISA
12	EFOORA HIV RTD	Efoora, Buffalo Grove, IL, U.S.A.	22621	15/04/02	24/07/00	5-10mins	18-25oC (Room Temp.)	2-8oC	None	Clear + Simple	None	Rapid EIA
			22618	21/03/02								
			22623	3/4/2002								
			22628	10/5/2002								
			22634	25/07/02								
			22630	9/7/2002								
13	Capillus HIV-1/HIV-2	Cambridge Biotech Diagnostics, Galway, Ireland	HO4210	12/12/2001	26/07/00	3-5mins	18-25oC (Room Temp.)	2-8oC	None	Clear + Simple	None	Rapid EIA
			HO4205	20/11/01								
			HO3820	14/05/01								
			HO3819	17/03/01								
			HO3818	13/03/01								
14	HIV-TRI-DOT	J.Mitra & Co., New Delhi, India	HT22300 (3)	24/09/2001	28/07/00	5-10mins	18-25oC (Room Temp.)	2-8oC	None	Clear + Simple	None	Rapid EIA
			HT22400 (2)	12/11/2001								

APPENDIX 2: A SECOND GENERATION ELISA METHOD WITH AN EXAMPLE OF ITS TECHNIQUE

PRINCIPLE OF THE GENSCREEN HIV-1/2 version 2 KIT as an example of 2nd ELISA

GENSCREEN HIVI/2 version 2 is an enzyme immunoassay based on the principle of the two-step sandwich technique for the detection of the various antibodies associated with HIV1 and/or HIV2 virus in human serum or plasma.

GENSCREEN® HIV1/2 version 2 is based upon the use of a solid phase coated with purified antigens (gpl20 and p25 recombinant proteins of HIV1 and a peptide mimicking the immunodominant epitope of the HIV2 envelope protein) and of an antigens - peroxidase conjugate (peptides mimicking the immunodominant epitopes of the HIV1 and HIV2 envelope glycoproteins, and nucleocopsid recombinant protein).

STORAGE CONDITIONS - SHELF LIFE

The kit should be stored at ÷2-8°C. When stored at this temperature, each reagent contained in the GENSCREEN® HIV /2 version 2 kit can be used until the expiry date mentioned on the package except specific instruction:

Ri: After the vacuum-sealed bag has been opened, the microwell strips stored at +2-8°C in the carefully reclosed bag remain stable for 4 weeks.

R2: The diluted washing solution stored at +2-8°C remains stable for 15 days.

R7a + b : The reagents stored at +2-8°C remain stable for 4 weeks after the vials have been opened and reconstituted.

R8 + R9 : After the reconstitution, the reagent stored in the dark remain stable for 6 hours at room temperature (1 8-30°C).

MATERIAL REQUIRED BUT NOT PROVIDED

- Distilled water.
- Sodium hypochlorite (household bleach) and sodium bicarbonate.
- Automatic or semiautomatic, adjustable or preset pipettes or multipipettes to measure and dispense 25 pJ, 75 pI, 80 pl and 200 pl.
- Graduated cylinders of 25 ml; 100 ml; 1000 ml capacity.
- Container for biohazardous waste.
- Water-bath or equiv. microplate incubator, thermostatically set at 37°C± 1
- Manual, semiautomatic or automatic microplate washer (*)•
- Microplate reader equipped with 450nm and 620nm filters (*)•

(Contact Sanofi Diagnostics Pasteur for monochromatic procedure).

- Absorbent paper.

(*) Consult us for detailed information about the equipment recommended by our technical department.

Autoclaving for at east one hour at 121 °C, is the best method to inactivate the HIV viruses and the HB virus.

DO NOT PLACE SOLUTiONS CONTAINING SODIUM HYPOCHLORITE IN THE AUTOCLAVE.

The Safety Data Sheet is available upon request.

The treatment of contaminated solutions and equipment with sodium hypochlorite at the final concentration of 5% for 30 minutes also allows the inactivation of the HIV viruses and of the HB virus.
Avoid any contact of the substrate buffer, the chromogen and the stopping solution with the skin and mucosa (toxicity, irritation or burn hazard).
Chemicals should be handled and disposed of in accordance with Good Laboratory Practice.
Some reagents contain sodium azide as a preservative. Sodium azide may react with laboratory plumbing to form copper or lead azides. Such azides are explosive. To prevent ozide build-up, flush the pipes with a large quantity of water if solutions containing ozide are disposed of in the sink after inactivation.

RECONSTITUTION OF THE REAGENTS

Note: Before use, allow reagents to reach room temperature (18-30°C). Reagent 1 (Ri) Microp
12 strips of 8 wells coated with the purified antigens. Each tray containing 12 strips is wrapped in a sealed toil-lined bag. Cut the bag with scissors or a scalpel 0.5 - 1 cm above the line. Open the bag and remove the tray. Return unused strips in the bag. Re-seal the bag and return to +2-8°C.
Reagent 2 (R2) : Washing solution (lOx concentrate)
Tris NaCl buffer pH 7.4 containg 1% Tween® 20 and maximum 0.01% sodium merthiolate. Dilute 1:10 in distilled water to obtain the ready-for-use washing solution. Prepare 800 ml for one plate of 12 strips.
Reagent 3 (R3) Negative control serum (ready-for-use)
Human serum, negative For HIV1 and HIV2 antibodies, HCV antibodies and HBs Ag. Contains 0.1 % sodium azide as a preservative.
Reagent 4 (R4) : Cut-off serum (ready-for-use)
Heat inactivated human serum, negative for HBs Ag and HCV antibodies and containing HIV antibodies. Contains 0.1 % sodium azide as a preservative.
Reagent 5 (R5): Positive control serum (ready-for-use)
Heat inactivated human serum, negative for HBs Ag and HCV antibodies and containing HIV antibodies. Contains 0.1% sodium ozide as a preservative.
Reagent 6 (R6) : Sample diluent (ready-for-use)
Calf serum solution (Tris buffer with 0.1% chloroform and Proclin, and coloured indicator).
Reagent 7a (R7a) : Lyophilized conjugate
Purified HIV1 and HIV2 antigens labeled with peroxidase. Contains BSA and 0.1% Proclin.
Gently tap the vial on the workbench to remove any substance from the rubber cap.
Carefully remove the cap and pour the contents of a Con Diluent vial in the Lyophilized Conjugate vial.
Put the cap on and let stand for 10 minutes while gently shaking and inverting from time to time to ease dissolution.
Reagent lb (R7b) : Conjugate diluent
Skimmed milk solution (Tris buffer with 0.1% chloroform and ProClin). Reagent 8 (R8) Substrate Buffer

Ready-for-use solution of citric acid and sodium acetate pH 4.0 containing 0.015% hydrogen peroxide, and 4% dimethyl sulfoxide
(DMSO).
Reagent 9 (R9) : Concentrate Chromogen Solution
Solution containing tetramethylbenzidine (TMB). Dilute 1:11 the
solution in the Substrate Buffer (ex: 1 ml reagent R9+ 10 ml reagent
R8). Stability for 6 hours in the dark once prepared. Reagent 10 (R10) Stopping solution
Ready-for-use 1 N sulphuric acid solution.

ASSAY PROCEDURE
Strictly follow the proposed procedure.
Use the negative, positive and cut-off Controls for each series of
determinations to validate the test results.
Follow the following Good Laboratory Practice
• Carefully establish the sample distribution and identification plan
2. Prepare the dilute washing solution,
3. Take the carrier tray and the strips (R 1) out of the protective pouch,
4. Apply directly, without prior washing of the plate and in succession
4.1 25 µl of diluent in each well
4.2 75 µI of negative control serum (R3) in well A1
75 µl of cut-off control serum (R4) in wells B1, C1 and D1.
75 µl of positive control serum (R5) in well El,
75 µl of specimen 1 in well Fl,
75 µl of specimen 2 in well Gl, etc.
Depending on the utilized system, it is possible to modify the position of the controls.
Homogeneize the mixture by a minimum of 3 aspirations with 75 pµl pipette or by shaking the microplate after the pipeting step.
It is also possible to apply 100 µl of a sample previously diluted 3:4 (ex: 150 µl serum + 50 µl diluent).
NB : The sample distribution can be visually controlled at this step of the manipulation : after adding the sample, the diluent turns from purple to blue. Frefer to section 12 for automatic verification
- SPECTROPHOTOMETRIC VERIFICATION OF SAMPLE AND CONJUGATE PIPETING)
5.Cover the microplate with adhesive film. Press firmly all over the plate to ensure adequate tightness.
6. Incubate the microplate in a thermostat-controlled water-bath or microplate incubator at 37°C ± 1°C for 30 ± 5 minutes.
7. Remove the adhesive film. Aspirate the contents of all wells into a
container for biohazardous waste (containing sodium hypochlorite).
Add into each well a minimum of 0.370 ml of washing solution.
Respect a soak time of a minimum of 30 seconds. Aspirate again.
Repeat this procedure at least twice (i.e. a total of a minimum of
3 washes). The residual volume must be lower than 10 1 (if necessary, dry the plate by turning it upside down on absorbent paper). If an automatic washer is used, follow the same procedure (refer to section 10: recommendations)

8. Distribute 100 µl of the conjugate solution into all wells. The conjugate must be shaken gently before use.
NB : The distribution of the conjugate which is coloured green can be visually controlled at this step of the manipulation (refer to section 12 for automatic verification - SPECTROPHOTOMETRIC VERIFICATION OF SAMPLE AND CONJUGATE PIPETING)
9. Cover with new adhesive film and incubate for 30 ± 5 minutes at Room temperature (18 - 30°C).
10.Remove the adhesive film, empty all wells by aspiration and wash a minimum of 5 times as described above. The residual volume must be lower than 10 pl (if necessary, dry the strips by turning them upside down on absorbent paper.)
11 .Quickly dispense into each well 80 pl enzyme reaction development solution (R8+R9) freshly prepared before use. Allow the reaction to develop in the dark for 30 ± 5 minutes at room temperature (18 - 30°C). Do not use adhesive film during this incubation.
1 2. Add 100 pµ stopping solution (Ri 0) by using the same sequence and rate of distribution as for the development solution.
13.Carefully wipe the plate bottom. Read the optical density at 450/62Onm using a plate reader within 30 minutes of stopping the reactions (the strips must always be kept away from light before reading).
14. Check all results for agreement between the reading and the plate and sample distribution and identification plan.

10- RECOMMENDATIONS

CAUTION: AVOID CONTAMINATION WHILST PERFORMING ASSAY
* IN CASE OF CONTAMINATION (spills, etc)
• Non-acid spills should be wiped up thoroughly with a 5% (minimum) sodium hypochlorite solution.
• Acid spills should be wiped dry. The spilled area should then be cleaned with a 5% (minimum) sodium hypochlorite solution.

The material used for cleaning must be thrown assay in the biohazardous waste container and disposed of in accordance with the relevant safety rules (see precautions, §7).
• NEVER USE THE SAME CONTAINER TO DISTRIBUTE THE CONJUGATE AND THE ENZYMATIC DEVELOPMENT SOLUTION.
• RESPECT THE PRESCRIBED NUMBER OF WASHING CYCLES.
• CHECK THE ENZYMATIC DEVELOPMENT SOLUTION (BUFFER SUBSTRATE AND CHROMOGEN)
BEFORE DISTRIBUTION. It should be colourless.
The appearence of a blue colour within a few minutes after reconstitution indicates that the reagent may be contamined by metal ions cannot be used and must be replaced.
We recommend the use of plastic containers and distribution equipment, or alternatively glassware previously washed with 1 N hydrochloric acid, carefully rinsed with distilled water and dried. WASHING Carefully follow the washing procedures described to obtain maximum test performance.
LP 35 washing procedures.

After switching on LP35, validate the washing parameter. Choose a test number (between 1 and 20) and validate. Choose base procedure number 82 and plate mode. Enter minimum of 04 as contact time and 05 as bottom time. Once odjustements have been made, the washer returns to washing mode.
Contact Sanafi Diagnostics Pasteur for the adaptations and special procedures.

11 CALCULATION AND INTERPRETATION OF THE RESULTS

The presence or absence of antibodies to HIV1 and/or l-IIV2 is determined by comparing the absorbance measured for each sample to that of the calculated cut-off value.
1) Cakulate the mean absorbance of the cut-off control serum (ODR4)
— OD(B1)÷OD(C1)+OD(D1 ODR4
2) Calculate cut-off value
The cut-off value is given by the ratio:
3) Assay validation
The absorbance of the negative control serum should be less than 70% of the cut-off value
ODR3 <0.7 x C.O
The mean absorbance of cut-off control serum should be greater than 0.80:
ODR4 > 0.80
Optional The ratio : ODR5/OD R4 should be greater than or equal to 1 .3 (This optional norm will be applied when the linearity of the utilized reader is over 3.000).
4) Interpretation of the results
Samples with absorbance values less than the cut-off value are considered to be negative by the GENSCREEN® HIV1/2 version 2 test.
Results just below the cut-off value (C. 0 -10% < OD c C0) should however, be interpreted with caution (it is advise to retest in duplicate the corresponding samples when the utilized systems and laboratory procedures allow it).
Samples with absorbance values equal to or greater than the cut-off value are initially considered to be positive by the GENSCREEN® HIV1/2 version 2 test. They should be retested in duplicate before final interpretation.
If after retesting of a sample, the absorbance values of the 2 duplicates are less than the cut-off value, the initial result is non repeatable and the sample is declared to be negative with the GENSCREEN® HIV1/2 version 2 test.
Non repeatable reactions are often caused by:
- inadequate microplate washing,
- contamination of negative samples by serum with a high antibody titre,
- contamination of the development solution by oxidizing agents (bleach, metal ions, etc.)
- contamination of the stopping solution.

APPENDIX 3: AN EXAMPLE OF RAPID IMMONOBLOT/CONCENRATION METHODS (HIV Sav, MedMira etc)

For the quantitative detection of antibodies to Human Immunodeficiency Virus (HIV) 1&2 in serum or plasma in serum or plasma

Kit Content

1.	Cassettes.	50 units
2.	Buffer Solution in dropper bottle (blue cap), 18ml	1 dropper bottle
3.	Wash Solution in dropper bottle (red cap), 20ml	1 dropper bottle
4.	Lyophilized Gold Conjugate in dropper bottle (white cap)	2 dropper bottles
5.	Reconstitution Solution in dropper bottle (white cap), 4ml	2 dropper bottles
6.	Positive Control, 0.5ml	1 vial
7.	Pipettes	50 units
8.	Instruction Manual	1

Precautions

1. Do not mix reagents from different lots.
2. Do not smoke, eat or drink in areas in which samples are handled.
3. Wear disposable gloves when handling samples and thoroughly wash hands afterwards.
4. Use a separate disposable pipette for each sample.
5. Dispose of all samples and used materials as if they contained infectious agents.

Storage Instructions

1. Store all kit reagents in a refrigerator (2-8° C).
2. Return the reagents to the refrigerator after use.

Reconstitution of Lyophilized Gold Conjugate

1. Remove dropper cap from Lyophilized Gold Conjugate bottle.
2. Add TOTAL AMOUNT of the vial of Reconstitution Solution by gently squeezing the bottle without removing its dropper cap.
3. Return dropper cap of conjugate bottle into position and mix gently by inversion and/or agitation for at least 5 minutes, or until the content is completely dissolved.

Note: Lyophilized Gold Conjugate may scatter throughout the inner sides of the bottle. Make sure lyophilized material is completely dissolved prior to test.

4. Reconstituted Gold Conjugate is stable for 3 months at 4°C, or for 1 month at room temperature (20-25° C).

Sample Preparation

1. The Recombigen HIV-1&2 Rapid Sero Test Device TM should be used with fresh serum or plasma samples which have not been frozen. When possible, clear samples should be used. Viscous or particulate samples giving inconsistent or invalid results may be centrifuged and retested.
2. Store samples in a refrigerator (2-8°).
3. Frozen samples should be prepared by the following procedure:

• Allow the sample to thaw completely, and shake gently. Then put the sample in a vertical position without further shaking. This allows particles to settle at the bottom. It is recommended to centrifuge the serum sample.

• Insert the pipette just below the surface of the sample and withdraw one drop of sample. Add this drop as instructed in "Test Procedure" (see below, step 2) and follow procedure steps.

4. If samples yield a high background, or if blocking of the membrane occurs, It is recommended to centrifuge the serum sample.

Procedure notes

1. Reagents should be allowed to reach room temperature (20-25°C) prior to running the assay.
2. When adding solutions and sample to the cassette well, always allow liquids to soak into the membrane before proceeding to the next step.
3. A Positive Control should be run with each assay and a red dot should be observed.
4. The reagents supplied in this kit are intended for use as an integral unit Do not mix identical reagents from kits with different lot numbers.

Test Procedure

1. Add 2 drops of Buffer Solution to the cassette.
2. AId 1 drop of sample or control using the pipette provided.
3. Add 2 drops of Buffer Solution.
4. Add 2 drops of Wash Solution.
5. Add 2 drops of Gold Conjugate (see instructions for reconstitution).
6. Add 3 drops of Wash Solution.
7. Read results

APPENDIX 4: AN EXAMPLE OF RAPID IMMUNOCOMB ELISA METHOD (Bispot & CombFirm

Principle of the Test

The ImmunoComb II HIV I & 2 BiSpot test is an indirect solid-phase enzyme immunoassay (EIA). The solid phase is a comb with 12 projections ("teeth"). Each tooth is sensitized at three spots:

upper spot — goat antibodies to human immunoglobulin (Internal Control)
middle spot — HIV-2 synthetic peptides.
lower spot — HIV-1 synthetic peptides.

The Developing Plate has 6 rows (A-F) of 12 wells, each row containing a reagent solution ready for use at a different step in the assay. The test is performed stepwise, by moving the Comb from row to row, with incubation at each step.

To start the test, serum or plasma specimens are added to the diluent in the wells of row A of the Developing Plate. The Comb is then inserted in the wells of row A. Anti-HIV antiborbes, if present in the specimens, will specifically bind to the synthetic peptides on the lower and/or middle spots on the teeth of the Comb (Figure 1). Simultaneously, immunoglobulins present in the specimens will be captured by the anti-human immunogbbulin antibodies on the upper spot (internal Control). Unbound components are washed away in row B. In row C, the lgG captured on the teeth will react with anti-human lgG antibodies labeled with alkaline phosphalase (AP). In the next two rows, unbound components are removed by washing. In row F, the bound alkaline phospliatase will react with chrornogenic components. The results are visible as grey-blue spots on the surface of the teeth of the Comb.

Contents Combs

The kit contains 3 plastic Combs. Each Comb has 12 teeth, one tooth for each test (Figure 2). Each tooth is sensitized with three reactive areas:

upper spot — goat antibodies to human immunoglobulin (Internal Control)
middle spot — HIV-2 synthetic peptide (derived from the env glycoprotein gp36)
lower spot — HIV-1 synthetic peptides (derived from the env glycoproteins gp4l and gpl2O)

The Combs are provided in aluminum pouches containing a desiccant bag.

Developing Plates

The kit contains 3 Developing Plates, covered by aluminum foil. Each Developing Plate (Figure 3) contains all reagents needed for the test. The Developing Plate consists of 6 rows (A—F) of 12 wells each. The contents of each row are as follows:

Row A	specimen diluent
Row B	washing solution
Row C	alkaline phosphatase-labeled goat anti-human lgG antibodies
Row D	washing solution
Row E	washing solution
Row F	chromogenic substrate solution containing 5-bromo-4-chloro- 3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT)

Storage of the Kit

Store the kit in its original box at 2 8CC. Under these conditions, the kit will remain stable until the expiry date on the label. Do not freeze the kit.

Test Procedure

Equipment Need

- Precision pipette with disposable tips for dispensing 50 pl
- Scissors
- Laboratory timer or watch

Preparing the Test

Bring all components, developing plates, combs, reagents and specimens to room temperature and perform the test at room temperature (22°-26°C).

Preparing the Developing Plate

1 Incubate the Developing Plate in an incubator at 37CC for 20 minutes; or leave at room temperature (22 for 3 hours.

2. Cover the work table with absorbent tissue to be discarded as biohazardous waste at the end of the test.

3. Mix the reagents by shaking the Developing Plate.

Note: Do not remove the foil cover of the Developing Plate. Break the foil cover by using the disposable tip of the pipette or the perforator, only when instructed to do so by the Test Instructions.

Preparing the Comb

Caution: To ensure proper functioning of the test, do not touch the teeth of the Comb.

1. Tear the aluminum pouch of the Comb at the notched edge. Remove the Comb.
2. You may use the entire Comb and Developing Plate or only a part. To use part of the Comb:

a. Determine how many teeth you need for testing the specimens and controls. You need one tooth for each test. Each tooth displays the code number "32 of the kit, to enable identification of detached teeth.

b. Bend and break the Comb vertically or cut with scissors (see Figure 4) to detach the required number of teeth (No. of tests including 2 controls).

c. Return the unused portion of the Comb to the aluminum pouch (with desiccant bag). Close pouch tightly, e.g. with a paper clip, to maintain dryness. Store the Comb in the original kit box at 2 for later use.

Tests Results

Validation

In order to confirm that the test functions properly and to demonstrate that the result a are valid, the following three conditions must be fulfilled (see Figure 5):

1. The Positive Control must produce three spots on the Comb tooth.
2. The Negative Control must produce an upper spot (Internal Control) and no other spots.
3. Each specimen tested must produce an upper spot (Internal Control D. This will also confirm that the specimen was added.

If any of the three conditions are not fulfilled, the results are invalid, and the specimens and controls should be retested.

Limitations

The ImmunoComb® HIV 1 & 2 BiSpot kit is a screening test. Reactivity for antibodies to HIV-1IHIV-2 must not be considered a diagnosis of Acquired Immunodeficiency Syndrome (AIDS) or of infection with HIV. Since the production of antibodies to HIV may be delayed following initial exposure, non-reactivity with this test must not be considered conclusive evidence that the patient has not been exposed to or infected by HIV.

APPENDIX 5: AN EXAMPLE OF RAPID IMMONOCHROMATOGRAPHIC METHODS (Determine, Efoora, Triline, etc)

BIOLOGICAL PRINCIPLES, SUMMARY, AND EXPLANATION OF THE TEST

Determine HIV Rapid Test is a membrane based immunochromatographic test for the qualitative detection of antibodies to HIV-1, HIV-2, and HIV-1 group O.
A measured sample of whole blood, serum or plasma is added to the sample pad accessible in the circular area of the cassette. Diluent buffer is then added to the sample pad. The sample pad is adjoined to a conjugate pad that contains dried colloidal gold-antibody binding protein. As the sample migrates through the conjugate pad, it reconstitutes and mixes with this conjugate. This mixture continues to migrate through the solid phase to the area of the membrane (T) containing immobilized I-IIV antigens. These antigens represent the immunoconserved domains ofgp4l and gp 120 of 1 and gp36 of H1V.2. If antibodies to HIV-I, HIV-2, and/or H1V-I group O are present in the sample, the antibodies bind to the antigens, forming a visible tine at the area designated T. If no detectable levels of antibodies to HIV-1, HIV-2 and/or HIV-I group O are present, no test line is formed. A separate procedural control zone is included on each test, where an antibody binding protein is immobilized on the membrane (designated by the "C" printed on the plastic test cassette). Antibody-colloidal gold complexes are captured in the control zone, forming a line that confirms the reactivity of the reagents and the satisfactory performance of the test. The control line must be visible for the test to be considered valid.

KIT CONTENTS

Determine HIV Rapid Test (100 test cartridges)
Buffer (2 ml)

MATERIALS REQUIRED BUT NOT SUPPLIED AS PART OF THE KIT

Timer
Devices capable of delivering 5μ of specimen to the Efoorã HIV Rapid test sample well
Lancets
Note that sample loops' and lancets may be purchased separately.

PROCEDURE (See diagram on the next page)

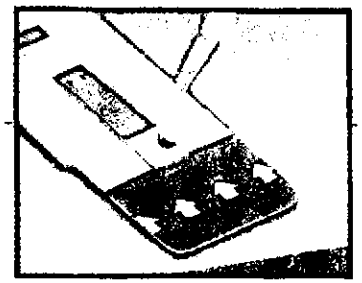
Read the test at 20 minutes. Results are void after 30 minutes.

5. **Interpretation:**

1) 2 lines present: If two lines, one in the designated test "T" area and one in the designated control ("C") area are seen after 20 minutes the test should be considered reactive.
2) One line present: If only a control line is seen in the control area of the device the test is non-reactive.
3) If only the test line appears, the test is considered invalid and should be repeated.

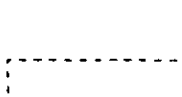
DETERMINE HIV-1/2 TEST PROCEDURE IN DIAGRAM

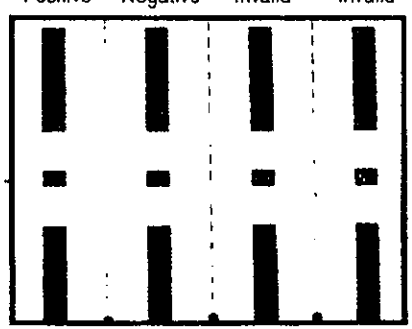
SERUM / PLASMA PROCEDURE



1 - Add sample, (50µl) to sample pad

2 - Wait 15 minutes

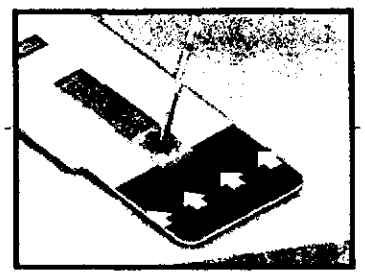




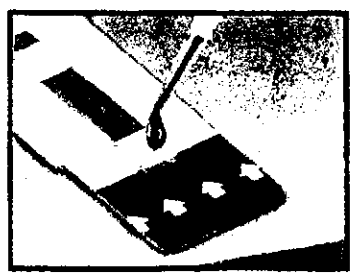
3 - Read Results

WHOLE BLOOD PROCEDURE

1 - Fingerstick sample collection



2 - Add sample, (50µL) to sample pad (Fingerstick method)



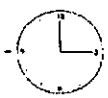
2 - Add sample, (50µL) to sample pad (Venipuncture method)

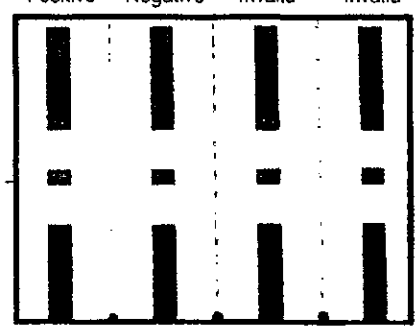
WHOLE BLOOD PROCEDURE

1 minute

3 - Add chase buffer - one drop

4 - Wait 15 minutes





5 - Read Results

APPENDIX 6: AN EXAMPLE OF 4TH GENERATION ELISA METHOD

(Vironostika, Genscreen Plus, Murex Vidos, etc)

PRINCIPLE OF THE GENSCREEN® PLUS HIV Ag-Ab

GENSCREEN® PLUS HIV Ag-Ab is an enzyme immunoassay based on the principle of the sandwich technique for the detection of HIV antigen and of the various antibodies associated with HIV and/or HIV2 virus in human serum or plasma.

The GENSCREEN® PLUS HIV Ag-Ab solid phase is coated with

- monoclonol antibodies against p24 HIC\V 1- antigen
- purified antigens : gp160 recombinant protein with on artificial functional consensus polypeptide which is composed of variable sequences of the virus inserted into well-preserved sequences of HP group 0 strains and a peptide mimicking the immunodominant epitope of the HIV2 envelope protein

The GENSCREEN® PIUS HIV Ag-Ab conjugates are based upon the use of

- biotinylated polyclonal antibodies to HIV Ag (conjugate 1)
- Avidin and l-HIV antigens - peroxidase conjugate (gp4i and gp3o peptides mimicking the immunodominant epitopes of the HIVI and HIV2 envelope glycoproteins, and the some functional consensus polypeptide of HIV1 used for the solid phase) conjugate 2)

The assay procedure includes the following reaction steps

1. Conjugate 1 (biotinylated polyclonal antibody to p HIV1 Ag) is added into the microplate wells
2. Serum samples to be assayed and controls are p into the wells.
   - If present, HIV antigens bind with the monoclonol antibody bound to the solid phase and the conjugate 1.
   - HIVI and/or HIV2 antibodies, if any, bind to the antigens immobilised on the solid phase.
   - Deposition of conjugate 1 and sample is validated through a colour change, from yellow-green to blue.
3. After incubation at 37°C then washing, conjugate 2 is added:
   - Avidin reacts with biotinylated Ab-Ag-Ab complexes.
   - Peroxidase labelled, purified HIV1 and HIV2 antigens bind in turn to the IgG, IgM or IgA antibodies captured on the solid phase.
4. After incubation at 18-30°C the unbound conjugate 2 fraction is removed by washing. After incubation in presence of the substrate at room temperature) I 8-30°C) the presence of the complexed conjugate is shown by a change of colour.
5. The reaction is stopped and absorbance's are read using a spectrophotometer at 450/620-700 nm. The absorbance measured on a sample determines the presence or absence of HIV Ag or HIV1 and/or HIV2 antibodies.

CONTENTS OF THE GENSCREEN® PLUS HIV Ag-Ab KIT

All reagents are exclusively for in vitro diagnostic use

LABEL	NATURE OF THE REAGENTS	PRESENTATION 72375	72376
R1	Microplate 12 strips of 8 wells coated with nonoclonal antibodies to p24 HIV1 (mouse) and purified HIV 1 and HIV 2 antigen	1 plate	5 plates
R2	Concentrated washing solution 10 x Tris NaCl buffer pH 7.4 Preservative: Thimerosal 0.01%	1 vial 100 ml	2 vials 2 x 250 ml
R3	Negative control Heat inactivated human plasma negative for HBs antigen, HIV agent, anti-HIV1 and anto-HCV antibodies Preservative: sodium azide < 0.1%	1 vial 1 ml	1 vial 1 ml
R4	Cut-off control Heat inactivated human plasma for anti-HIV antibodies, negative for HBs antigens and anti-HCV antibodies Preservative: Sodium azide < 0.1%	1 vial 25 ml	1 vial 25 ml
R5	HIV Ag Positive Control Purified HIV 1 antigen inactivated with a choatropic agent, in synthetic diluent Preservative: ProClin ™300 0.5%	1 vial 1ml	1 vial 1 ml
R6	Conjugate 1 Biotinylated polyclonal antibodies to p24 HIV 1 (sheep) colour yellow – green Preservation: ProClin™ 300. 0.5%	1 vial 1ml	1 vial 1 ml
R7a	Conjugate 2 Lyophilised peroxidase labeled avidin and purified HIV 1 and HIV 2 antigens	1 vial sqf 12.5 ml	2 vials 2 x 10 ml
R7b	Conjugate 2 Diluent Skimmed milk solution coloured red Preservation: ProClin™ 300 0.5%	1 vial 12.5 ml	2 vials 2 x 30 ml
R8	Peroxidase substrate buffer Citric and Sodium acetate solution pH 4.0 containing H2O2 [0.015%] and DMSO [4%]	1 vial 60 ml	2 vials 2 x 60 ml
R9	Chromogen Solution containing tetramethyl benzidine (TMB)	1 vial 5 ml	2 vials 2 x 60 ml
R10	Stopping solution 1N sulphuric acid solution	1 vial 28 ml	3 vials 3 x 28 ml
	Adhesive film for microplates	4	12

MATERIAL REQUIRED BUT NOT PROVIDED

- Distilled water
- Sodium hypochlorite (household bleach) and sodium bicarbonate.
- Automatic or semiautomatic, adjustable or preset pipettes or multipipettes to measure and dispense 25µl, , 75µl 80µl and 700µl.
- Graduated cylinders of 25 ml; 100 ml; 1000 ml capacity.
- Container for biohazardous waste.
- Water-bath or equivalent microplate incubator, thermostatically set at 37°C ± 1 °C (*).
- Manual, semiautomatic or automatic microplate washer (*),
- Microplate reader equipped with 450nm and 620-700 nm filters (*).
- Absorbent paper.

(*) Consult us for detailed information about the equipment recommended by our

PREPARATION FOR REAGENTS.

Note: Before use, allow reagents to reach room temperature [18-30°C]

1) Ready for use reagents

Reagent I (RI) : Microplate

Each tray containing 12 strips is wrapped in a sealed foil-lined bag. Cut the bag with scissors or a scalpel 0.5

1 cm above the line. Open the bag and remove the tray. Return unused strips in the bag. Re-seal the bag and return to +2-8°C,

Reagent 3 (R3) Negative control

Reagent 4 (R4) : Cut-off control

Reagent 5 (R5) : HIV Ag positive control

Reagent 6 (R6) : Conjugate I

Reagent 10 (R10) : Stopping solution

2) Reagents to reconstitute

Washing solution (10 x concentrate) : Reagent 2 (R2)

Dilute 1:10 in distilled water to obtain the ready-for-use washing solution. Prepare 800 ml for one plate of 1 2 strips.

Conjugate 2 working solution : Reagent 7a (R7a) + Reagent 7b (R7b)

Gently tap the vial of the lyophilised conjugate 2 (R7a) on the work-bench to remove any substance from the rubber cap. Carefully remove the cap and pour the contents of Conjugate Diluent vial (R7b) into the Lyophilised Conjugate vial)R7a). Replace the cap and let stand for 10 minutes, whilst gently shaking and inverting from time to time to ease dissolution.

Enzyme development solution : Reagent 8 (R8) + Reagent 9 (R9)

Dilute 1:11 the clirornogen (R9) in the Substrate Buffer (R8) (ex : 1 ml reagent R9+ 10 ml reagent R8). Stability is for 6 hours in the dark once prepared.

STORAGE CONDITIONS - SHELF LIFE

The kit should be stored at +2-8°C. When stored at this temperature, each reagent contained in the GENSCREEN® PWS HIV Ag-Ab kit can be used until the expiry date mentioned on the package (except for specific instructions).

R1: After the sealed bag has been opened, the microwell strips stored at +2-8°C in the carefully resealed bag remain stable for 1 month.
R2: The diluted washing solution stored at +2-8°C remains stable for 2 weeks. The concentrated washing solution (P2) can be stored at +2-25°C.
R7a + b The reagents stored at +2-8°C remain stable for 1 month after the vials have been reconstituted.
R8 + R9: After the reconstitution, the reagent stored in the dark remains stable for 6 hours at room temperature

1. **ASSAY PROCEDURE**

Strictly Follow the proposed procedure.
Use the negative (R3), cut-off (R4) and HIV Ag positive (R5) controls for each series of determinations to validate the test results.
Follow the following Good Laboratory Practice:
Carefully establish the sample distribution and identification plan.
2. Prepare the diluted washing solution (refer to chapter 8).
3. Prepare the con 2 working solution (refer to chapter 8).
4. Take the carrier tray and the strips (P1) out of the protective pouch,
5. Apply directly, without prior washing of the plate and in succession (suggested plate distribution)

5.1 25µl of con 1 in each well

5.2 75µl of negative control (P3) in well Al
75µl of cut-off control (P4) in wells B), Cl and Dl.
75µl of HIV Ag positive control (P5) in well El,
75µl of specimen I in well Fl
75µl of specimen 2 in well Gl, etc...

Homogenise the mixture by a minimum of 3 aspirations with 75µl pipette or by shaking the microplate after the pipetting step.
NB.: The sample and conjugate I distribution can be visually controlled at this step of the monipulotion:
after adding the sample, the conjugate I turns from yellow - green to blue (refer to section 14 for automatic verification: SPECTROPHOTOMETRIC VERIFICATION OF SAMPLE AND CONJUGATE PIPETING).

6. When possible, cover the microplate with adhesive film. Press firmly all over the plate to ensure a tight seal.
7. Incubate the microplate in a thermostat-controlled water-both or microplate incubator at 37°C ± 1 °C for 1 hour ± 5 minutes.

CALCULATION AND INTERPRETATION OF THE RESULTS

The presence of absence of detectable HIV Antigen of antibodies to HIB 1 and /or HIV 2 is determined by comparing the absorbance measured for each sample to the calculated cut-off value.

1. Calculate the mean absorbance of the cut-off control serum (ODR4)

$$\overline{ODR4} = \frac{OD\,[B1] + OD\,[C1] + OD\,[D1]}{3}$$

2. Calculate cut-off value
The cut-off value is given by the ratio:

$$C.O. = \frac{\overline{ODR4}}{5}$$

3. Assay validation
The absorbance of the negative control [R3] should be less than 70% of the cut-off value:
OD R3 < 0.7 X C.O

The mean absorbance of cut-off control (R4) should be greeter than 0.35

$$\overline{OD\ R4} > 0.35$$

The OD of the HIV Ag positive control (R5) must be greeter than 0.50
OD R5 > 0.50

4) **Interpretation of the results**

Samples with absorbance values less than the cut-off value are considered to be negative by the GENSCREEN® PLUS HIV Ag-Ab test.
Results just below the cut-off value C.0 -10% c 00 c C.0) should however be interpreted with caution (it is advisable to retest in duplicate the corresponding samples when lie systems and laboratory procedures permit) Samples with absorbance values equal to or greater hen the cur-off value are initially considered to be positive by the GENSCREEN® PLUS HIV Ag-Ab test. They should be retested in duplicote before final interpretation. If after retesting of a sample, the ob,orbonce value, of the 2 duplicates are l than he cut.off value, he ini result is non repeatable and the sample is declared to be negative with the GENSCREEN HIV A test Non repeatable reactions are ohen caused by

- inadequate rnicroplote washing,
- contamination of negative samples by serum or plasma with a high antibody titre
- contamination of the substrate solution by oxidising agents (b metal cons, etc
- contamination of the stopping solution.

if after retesting the obsorbance of one of the duplicates is equal 0 or greeter than the cut-off value, the initial result is repeatable and the sample is declared to be po5ifive s*h he GENSCRE&N® PLUS HIVAg test sub(ect to the limitotions of the procedure, described below.

APPENDIX 7: AN EXAMPLE OF RAPID IMMUNODOT METHOD (HIV TRI-DOT, Uni-Gold, etc.)

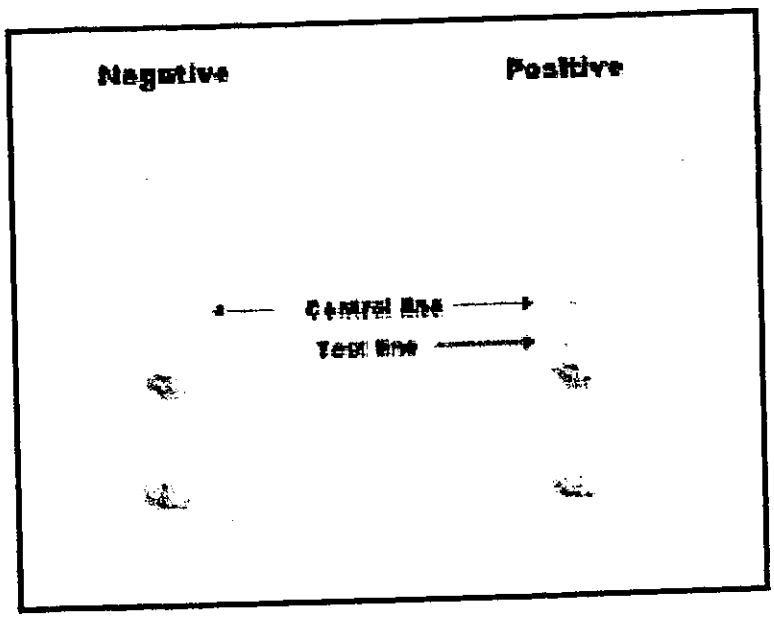
TEST PROCEDURE FOR HIV TRI-DOT RAPID TEST

1. Add 3 drops of Buffer Solution to the centre of the device.
2. Add 1 drop of patients sample (serum or plasma) using the sample dropper provided (use a separate sample dropper for each specimen to be tested).
3. Add 5 drops of Butter Solution.
4. Add 2 drops of Liquid Conjugate directly from the conjugate vial.
5. Add 5 drops of Buffer Solution and read results,

IMPORTANT. IT IS IMPORTANT TO ALLOW EACH SOLUTION TO SOAK IN THE TESTDE VICE BEFOREADDINO THE NEXT SOLUTION,
Read results immediately and discard the device considering it to be potentially infectious.

CONTENTS	**HIV TRI DOT**
Store at 4-8°C	
HIV TRIP-DOT Test Device (Individual packed)	10 Units
Buffer Solution	1 Vial (6.5 ml)
Conjugate (ready to use)	1 Vial 91.5 ml)
Disposable Sample Droppers	10 Units

J. Mitra & Co Ltd.
A-180, Okhia Ind. Area Phase 1, new Deihi-110 020 India.



APPENDIX 8: AN EXAMPLE OF A RAPID LATEX AGGLUTINATION (Recombigen HIV RTD, Capillus, etc.)

Intended use: The Cambridge Diagnostice Ireland Limited (DIL) Capillus HIV-1/HIV-2 is a rapid quantitative assay for the detection of antibodies to HIV-1 and HIV-2 in human serum or plasma. The Capillus HIV-1/HIV-2 is intended as an initial screening test in low volume testing facilities, in emergency situations and in supplemental assay in test algorithms.

Summary and Explanation of Test: Available data indicate that the AIDS is caused by a virus transmitted by sexual contact, by an infected mother to her foetus or child during the perinatal period, by exposure to blood including sharing of contaminated needles and syringes and by certain blood products or other body fluids.

Human immunodeficiency virus Type 1 (HIV-1) has been isolated from patients with AIDS and AIDS-realted complex (ARC) and from healthy persons at high risk for AIDS. The incidence of antibodies specific for HIV-1 in AIDS and ARC patients is universal. In persons at increased risk for AIDS the incidence is high. Human immunodeficiency virus Type 2 (HIV-2) is responsible for AIDS in West Africa with isolated cases reported in Europe, Central Africa, Western United States, Canada and Brazil.

The major antigens from the envelope proteins of HIV-1 and HIV-2 have been identified and cloned using recombinant DNA technology. These HIV-1 and HIV-2 polypeptides have been expressed and purified. The CDIL Capillus HIV-1/HIV-2 uses these two proteins bound to polystyrene latex beads to form the basis of a direct latex aggregation assay for the detection of antibodies to HIV-1 and HIV-2 in serum and plasma. The assay is performed on a patented capillary slide. (The word "Slide" will be used from here forward when reference is made to CDIL's patented capillary).

The slide consists of a well area for mixing of latex reagent and sample. At one end of the mixing well there is a capillary flow channel which leads to a view window. The latex reagent and test sample are mixed in the mixing well on the slide. The mixed reagents are drawn to the flow channel and the reagents begin to flow by capillary action towards the viewing window. Samples positive for HIV-1 or HIV-2 will cause the antigen coated latex to aggregate. The capillary flow enhances the binding of specific antibodies to the latex and hence promotes aggregation. The reaction is read visually when the latex solution reaches the viewing window. Aggregation in the viewing window should be considered as initially reactive. A smooth milky white appearance is considered non-reactive. The test may also be performed on the CDIL Capillus Digital Reader which gives a positive, negative or threshold readout result.

Specimen collection and preparation: The CDIL Capillus HIV-1/HIV-2 is designed to be used with freshly collected serum and plasma samples. Plasma specimens must be collected properly into blood collection containers containing anticoagulant. Serum and plasma samples should not be contaminated woth fibrin or other cellular debris.

Store serum or plasma specimens frozen at -20 oC, or alternatively store refrigerated (2-8oC) for no more than 5 days. Avoid multiple freeze-thaws. The adverse effects of long term freezer storage on sample reactivity have not been characterized. Do nt use a freezer with a frost free cycle. Shipped specimen s should be packed in compliance woth country regulations coering the transportation of etiologic agents.

Do not use "heat inactivated" (e.g., 56oC, 30 minutes) serum or plasma.

Visual interpretation assay procedure: Allow reagent sto reach room temperature (18 to 25oC) before use.

Record patient sample identification number.

Place up to 10 slides on the interpretation station.

Remove calibrated dropper from latex and eject any latex in the dropper back into the bottle. Replace the now empty dropper back into the latex bottle.

Mix the latex reagent well to ensure that the latex suspension is homogenous. Avoid foming of the latex reagent. Also draw latex up and down a few times woth the calibrated dropper to ensure good mixing before latex is dispensed onto the slide.

Draw the latex reagent to the calibated mark (120ul volume). Avoid drawing up air bubbles. Dispense the reagent onto the slide at the edge of the mixong well which is furthest away from the capillary channel.

Note: If the latex begins travelling down the slide before sample addition, discard slide and start again.

Using the precalibrated pipette tip provided in the kit and retrieve test sample or control (10 µl volume).

Dispense sample directly into latex solution. Using the pipette mix the sample in the latex by pumping the mixture in and out of the tip three times and stir in a circular motion at least five (5) times.

Note: Effective mixing of the sample in the latex is critical to ensure reproducible and accurate test results.

Continue to use the pipette tip to move the well mixed sample and latex solution to the opening of the channel until the capillary flow begins.

Allow the latex mixture to flow through the entire capillary channel and into the viewing window before interpreting result. This will require approximately 3-7 minutes.

Observe viewing window for aggregation. Samples demonstrating any latex aggregation should be considered initially reactive. Samples showing non aggregation should be interpreted as non-reactive.

Record results on a data sheet.

Any reactive sample or questionable samples should be released in duplicate with the test or with a supplemental test e.g., CDIL Recombigen HIV-1/HIV-2 RTD test. This membrane based rapid test has distinct reactive areas for HIV-1 and HIV-2, hence this test can be used as an indicator of HIV-1 or HIV-2 antibody reactivity. Western blot can be used for confirmatory testing for repeatedly reactive samples.

Limitations:

a. The Capillus HIV-1/HIV-2 detects antibodies to both HIV-1 and HIV-2. The test will not discriminate between antibodies to HIV-1 and HIV-2.

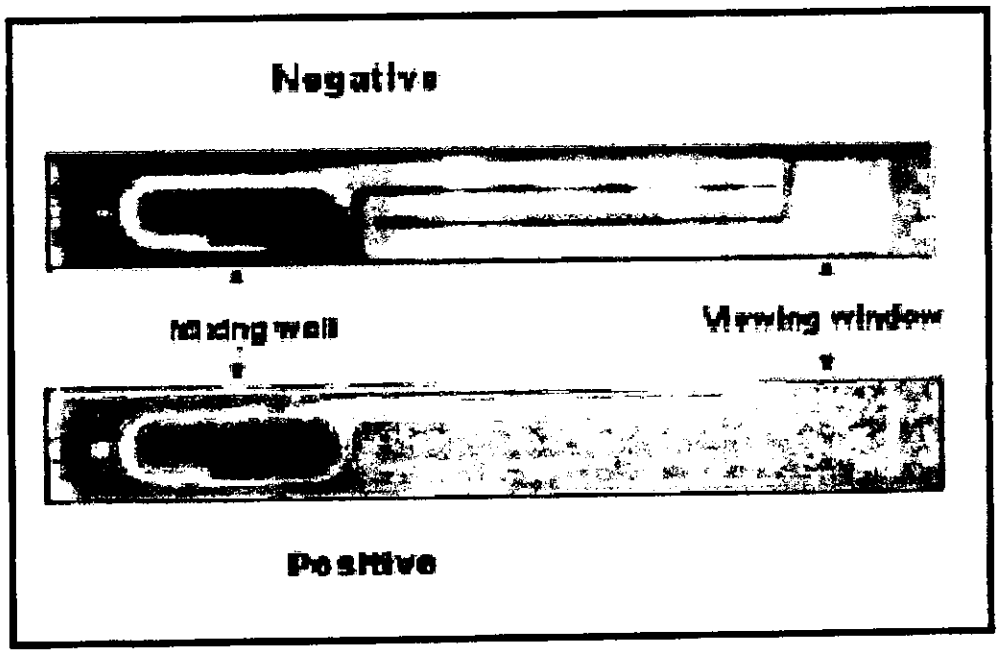
b. Insufficient data are available to interpret tests performed on other body specimens, pooled blood or processed plasma, and products made from such pools; testing of these specimens is not recommended.

c. Immunosuppressed or immunocompromised individuals may not produce antibodies after infection with HIV-1 or HIV-2 ; thus negative results with any antibody detection kit may not be reliable for these patients.

d. Infants may not produce antibodies or they may passively receive them from an infected mother, therefore, great care is necessary to interpret their positive or negative test results in relation to HIV-1 and/or HIV-2 infection. Infant samples should not be tested on Capillus HIV-1/HIV-2.

e. Capillus HIV-1/HIV-2 detects circulating antibodies to HIV-1 and HIV-2, thus, is useful in screening blood and plasma donated for transfusion and further manufacture, in evaluating patients with signs or symptoms of AIDS, and in establishing infection with HIV-1 or HIV-2 in an asymptomatic carrier. For most uses it is recommended that repeatedly reactive specimens be investigated by an alternative assay or supplemental test. A person who has antibodies to HIV-1 and/or HIV-2 is presumed to be infected with the virus and appropriate counselling and medical evaluation should be offered. Such an evaluation should be considered an important part of HIV antibody testing and should include test result confirmation on a freshly drawn sample.

f. AIDS and AIDS-related conditions are clinical syndromes and their diagnosis can only be established clinically. Capillus testing alone cannot be used to diagnose AIDS, even if the recommended investigation of reactive specimens suggests a high probability that the antibody to HIV-1 or HIV-2 is present. A negative result at any point in the investigation of individual subjects does not preclude the possibility of exposure to or infection with HIV-1 or HIV-2.


Negative
Viewing window
Positive

APPENDIX 9: AN EXAMPLE OF IMMUNOBLOTING (WESTERN BLOT) METHOD (New LAV Blot)

BY IMMUNOBLOTTING.
RINCIPLE OF THE METHOD
The test uses the indirect ELISA technique on a nitrocellulose strip containing all the constitutive proteins of the HIV1 virus and an anti-IgG internal control. The band corresponding to the internal control is located towards the strip end without any number, before the p] 8 reaction, and it is used to validate the addition of the sample and reagents as well as the correct execution of the test protocol.

Inactivated HIV 1 viral proteins are separated according to their molecular weights by polyacrylamide gel electrophoresis in dissociating and reducing medium and subsequently transferred onto a nitrocellulose membrane sheet.
The procedure comprises the following steps 1. Strip rehydration.
2. Incubation of the samples to be confirmed or the control serums. If anti-HIV 1 antibodies are present, they will bind to the viral proteins recognized, present on the strip.
3. After washing, the alkaline phosphatase-labeled anti-human IgG conjugate is added and incubated to allow the binding of the conjugate to the anti-HIV 1 antibodies captured on the solid support.
4.After washing and removing the excess conjugate, the colour development solution allows the enzymatic activity of the complexed compounds bound to nitrocellulose to be revealed.
5.The appearance of specific coloured bands allows the presence of anti-HIV 1 antibodies in the serum to be confirmed.

8 strips
in 3 trays
(6 cells each)
1 vial, 0.2 ml
• The samples can be inactivated by heating (30 minutes at +56°C).
• Remove by centrifugation the Fibrin particles or aggregates, if need be.
The samples should be stored at +2 + 8°C if the test is to be carried out within 24 hours or frozen at -20°C for several months. Avoid successive freezing/defreezing. If necessary, centrifuge after defreezing.
If the samples are to be shipped, they must be packed in accordance with the regulations concerning the transfer of pathogenic agents.
- EQUIPMENT AND MATERIALS REQUIRED
• Distilled water.
• 100 ml, 250 ml and 500 ml graduated cylinders.
• 2 ml graduated pipettes.
• Automatic or semi-automatic pipetfes, adjustable or fixed, allowing the measuring or dispensing of 20 pl.
• 1, 2 or 3 dimensional shaker (slow shaking).
• Disposable gloves.
• Liquid jet vacuum pump with safety bottle.
• Sodium hypochiorite (Bleach).
• Absorbent paper.
• Tweezers.
eqs - PRESENTATION - RECONSTITUTION
AND STORAGE OF THE REAGENTS
All reagents contained in the kit are intended for in vifro diagnostic use only. Each kit contains enough reagents For 1 8 determinations. The determinations may be carried out separately.
A) REAGENTS READY FOR USE
Ri: 1 8 nitrocellulose strips activated by the transfer of HIV 1 viral proteins and anti-IgG internal control The strips are placed in disposable trays
R3: Negative control serum Human serum negative for HBs Ag,anti-HIV1 and HIV2 and anti-HCV antibodies
Preservative agent: 0.1 % sodium azide
R4: Positive control serum I vial, 0.2 ml Human serum positive for anti-HIVI
antibodies, negative For anti-HCV
antibodies and HBs Ag, inactivated by heat Preservative agent : 0.1 % sodium azide
This serum must be used to validate the test and to evaluate the sfrips correctly. It must be used for each test run.
R5: Conjugate (lx) Alkaline phosphatase-labeled 1 vial, 40 ml anti-human IgG goat antibodies.
Preservative agent: 0.1 % sodium azide.
R6: Colour development solution 1 vial, 40 ml 5 Bromo-4-Chloro-3-Indolyl Phosphate)BCIP)
and NitroBlue Tetrazolium (NBT) as developing buffer.

B) REAGENTS TO BE RECONSTITUTED

R2: Washing solution/diluent5x concentrated 1 vial, 100 ml

Preparation: shake the vial before using.

Dilute the washing solution diluent to 1:5 in distilled water (e.g. for a complete tray: 30 ml of washing solution + 120 ml of distilled water).

C) STORAGE

Store the kit at +2 + 8°C beFore use. All reagents are stable at this temperature until the expiry date printed on the box.

Avoid any microbial contamination of the reagents.

The reconstituted washing solution/diluent (R2) is stable for 1 month at + 2 + 8°C.

1 PROCEDURE

Remove the transparent cover of the tray being used.

Make sure that the strip side with the reference mark and the number is visible, so that the viral proteins on this side are covered by the various reaction media throughout the test.

Strips should be handled with plastic tweezers.

2. Add 2 ml of the reconstituted washing solution/diluent in each cell. Incubate for 5 minutes under slow shaking.

3. Add 20 il of each sample or control serum in the corresponding cell. Incubate for 2 hours at room-temperature under slow shaking.

4. Completely drain the contents of each cell using a vacuum pump with a trap containing a disinfectant (sodium hypochlorite at the concentration of 25 %).

Take care not to remove the strip during aspiration. Rinse under the tap the suction tip which is in contact with the samples between each aspiration to avoid sample cross-contamination.

Wash each strip with 2 ml of the reconstituted washing solution/diluent and immediately remove it by aspiration, using the same precautions. Wash each strip twice for 5 minutes, under slow agitation, with 2 ml of the reconstituted washing solution/diluent (i.e. a total of 3 washings). Remove the solution used for the last washing.

5. Dispense 2 ml of conjugate in each cell. The conjugate solution should be previously stabilized at room-temperature. Incubate for 1 hour at room-temperature under slow shaking.

6. Washing : proceed as described in Paragraph 4

7. Dispense 2 ml of development solution in each cell.

If suspended particles are present in the development solution, leave to decant in the vial before pipetting. (These particles do not interfere with the test.)

Incubate under slow shaking and monitor the appearance of the colouring. All the bonds corresponding to the viral proteins must be displayed with the positive control serum.

(Development time : about 5 minutes).

8. Stop the reaction by removing the development solution and by rinsing the strips 3 times with distilled water.

9.Dry the strips between 2 sheets of absorbent paper at room- temperature.

Class the strips, position them perfectly using the reference mark and evaluate.

CAUTION : do not stick adhesive plastic on the strip side corresponding to the viral proteins.

VALIDATION AND INTERPRETATION OF THE RESULTS

Validation of the results

The anti-IgG internal control band must be present with a strong colour. It is used to validate the addition of the sample and reagents as well as the correct execution of the test protocol. The absence or a low colour intensity of the anti-IgG internal control band indicates either that the sample or reagents were not dispensed or that the test protocol was not followed.

Interpretation of the results

A)PROTEINS CONSTITUTIVE OF THE HIV-1 VIRUS

The presence of anti-HIV 1 constitutive proteins antibodies in the samples examined is shown by the appearance 0 specific coloured bands (blue-violet).

APPENDIX 10: CONSENT FORM FOR HIV TESTING

Statement

In addition to providing a blood sample, we would like your permission to do a blood test on you to find out if you have human immunodeficiency virus (HIV) infection. The HIV is a type of germ that can cause AIDS (acquired immunodeficiency syndrome), which is usually a serious health problem and can be deadly. Someone can look and feel perfectly healthy and still be infected. The only way to know is by doing a blood test. Over time HIV infection decreases the body's fighting power and increases a person's risk of catching other diseases, including tuberculosis. In order to help you decide whether or not you wish to be tested for HIV infection, the services of a counsellor are available. You will be counselled before testing for HIV infection. The reason why we would like to test if you have HIV or not, is that the tests we normally confirm any result brought to this centre as a referral to be certainly sure for, the result of a wrong diagnosis may be serious. Therefore it is important for us to have HIV results on the blood samples.

The benefit of HIV testing is that, if you agree, you will be given the results of these tests and if positive, you will be referred to the standard HIV medical care services at the Lagos University Teaching Hospital (LUTH) or the Nigerian Institute of Medical Research (NIMR), Yaba. This research project will not pay for any form of HIV treatment.

There may be emotional discomfort or stress associated with knowledge of the results of this test.

The risks of doing a blood test are minimal. They include the discomfort of drawing a sample of blood, rare bruising and infection at the site of needle stick, and very rarely, fainting. New needles will be used for each patient so there is no risk for transmitting diseases.

All information that you provide will be considered confidential, and no mention of your name or any other identifying information will appear on the samples or in any publication in connection with this study. The blood samples will be coded so that your name will not be revealed. No persons other than the research staff and the health care workers overseeing your care will have access to any information that identifies you individually.

You may also choose not to participate in this study. Your participation in this important study is voluntary. You may refuse to participate at any time without penalty or loss of benefits to which you would otherwise be entitled. You do not have to explain why you do not wish to participate. If you have any questions now you may ask me, or if you have questions later, you may contact

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Muhammed Way
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APPENDIX 11: CONSENT FOR HIV TESTING (DOCUMENT)

Purpose

The purpose of HIV testing is to be able to determine if the blood samples you provide are infected with HIV.

Procedures

If I agree to testing, a small amount of blood will be collected from my arm using a sterile needle and syringe. The result of the test will be available to me in 3-7 days. I will need to come back for results. To be sure no one else knows my test result, no names will be attached to the blood sample, only a number code; therefore, only study personnel will be able to trace the test back to me and these records will be stored in locked files. The result of the test will be told to me only in person. A positive HIV test means that my blood sample tested positive for HIV. Laboratory tests are not perfect and the consequences of having a positive HIV test are serious. Therefore, it is standard procedure to repeat HIV testing to confirm the first test if it is positive. A negative HIV test means that at this time, no evidence of HIV was found in my blood sample. The social worker/ nurse is available to counsel as to the risks for transmitting HIV to others, risks for developing AIDS, and the available treatments for HIV infection.

Risks:

The risks of participating are minimal. They include the discomfort of drawing a sample of blood, rare bruising and infection at the site of needle stick, and very rarely, fainting. If I choose to collect the test results, there may be emotional discomfort or stress associated with this knowledge.

Benefits:

The benefit of undergoing HIV testing is that I will be given the results of these tests and referred for available HIV medical care, if needed and desired.

Compensation:

There will be no compensation to me if I decide to take part in this study.

Confidentiality

All information that I provide will be considered confidential, and no mention of my name or any other identifying information will appear in any publication in connection with this study. No persons other than the research staff and the health care workers overseeing my care will have access to any information that identifies me individually.

Right to refuse:

I do not have to take part in this testing. Counselling is available to me before I make the decision to participate in this testing. If I refuse HIV testing, it will not change the medical care I will receive at this hospital/clinic. I am free to ask any questions about the test, about HIV infection or AIDS, or in general about my health.

Participant's Statement

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a subject in this study and understand that I have the right to withdraw from the study at any time without in any way affecting my further medical care.

If participant is illiterate

_____
Name of Participant

_____
Name of Literate Witness

_____
Thumb print/Signature of Participant

_____
Signature of Literate Witness

_____
Date

_____
Date

APPENDIX 12: QUESTIONNAIRE ON HIV ASSOCIATION WITH STATE OF HEALTH OF SEROPOSITIVE INDIVIDUALS IN NIGERIA: HIV CASE MANAGEMENT STUDY

Name of Patients ..... Age ..... Sex .....

State of Origin ..... Nationality .....

Religion ..... Address .....

Occupation ..... No. of Children .....

Marital Status ..... No. of sexual partners .....

Which of the following symptoms do you have including those indicated by your physician? Tick those applicable.

Headache ..... Fever ..... Weight loss .....

Skin infection ..... Chronic diarrhoea ... Syphilis .....

Gonorrhoea ..... Herpes ..... T.B. .....

Pregnancy ..... Chlamydia ..... Hepatitis .....

Cough ..... Cold ..... Boil .....

Lymphadenopathy ... Allergy ..... Fibroid .....

Jaundice ..... Candidiasis ..... Vomiting .....

Hypertension..... Arthritis ..... Rashes .....

Typhoid ..... Pneumonia ..... Diabetes .....

Others ..... Please, specify.

S/N	Name of ELISA Kit	Positive Serum Panel	Negative Serum Panel	No. Positive with Kit	No. Negative with Kit	No Positive by 3 Different Kits	No. Negative by 3 Different Kits	Western blot Confirmed Positive	Western blot Confirmed Negative	# of Samples Rerun	# of Concordant Results
1	Wellcozyme/Murex HIV-1+2	90	40	90	39	90	40	90	40	92	91
		60	30	61	29	60	30	60	30	90	90
		90	40	91	39	90	40	90	40	90	89
		60	30	60	30	60	30	60	30	92	92
		90	40	91	39	90	40	90	40	90	90
		10	20	10	20	10	20	10	20	90	90
2	Genelavia Mixt	90	40	90	40	90	40	90	40	92	91
		60	30	61	29	60	30	60	30	90	90
		90	40	91	39	90	40	90	40	90	90
		60	30	61	29	60	30	60	30	92	91
		90	40	91	39	90	40	90	40	90	90
		10	20	10	20	10	20	10	20	90	90
3	SUB-Recombigen HIV-1/HIV-2 EIA	90	40	90	40	90	40	90	40	90	90
		60	30	60	30	90	30	60	30	90	90
		90	40	90	40	90	40	90	40	60	90
		60	30	60	30	60	30	60	30	90	90
		90	40	90	40	90	40	90	40	90	90
		10	20	10	20	10	20	10	20	90	90
4	Immunocomb II HIV-1 & 2 Bispot	20	14	20	14	20	14	20	14	30	30
		20	14	19	15	20	14	20	14	20	20
		20	14	19	15	20	14	20	14	20	20
		20	14	20	14	20	14	20	14	20	20
		20	14	20	14	20	14	20	14	20	20
		20	14	19	15	20	14	20	14	20	20
		20	14	20	14	20	14	20	14	20	20
		20	14	19	15	20	14	20	14	20	20
		20	14	20	14	20	14	20	14	20	20
		20	14	19	15	20	14	20	14	20	20
		20	15	19	16	20	15	20	15	20	20
		20	15	20	15	20	15	20	15	20	20
		20	15	20	15	20	15	20	15	20	20
		20	15	20	15	20	15	20	15	25	25
5	Immunocomb II HIV-1 & 2 CombFirm	20	14	20	14	20	14	20	14	30	30
		20	14	20	14	20	14	20	14	20	20
		20	14	20	14	20	14	20	14	20	20
		20	14	19	15	20	14	20	14	20	20
		20	14	20	14	20	14	20	14	20	20
		20	14	20	14	20	14	20	14	20	20
		20	14	20	14	20	14	20	14	20	20
		20	14	20	14	20	14	20	14	20	20
		20	14	20	14	20	14	20	14	20	20
		20	15	20	15	20	15	20	15	20	20
		20	15	19	16	20	15	20	15	20	20
		20	15	20	15	20	15	20	15	20	20
		20	15	20	15	20	15	20	15	30	30
6	Recombigen HIV-1/HIV-2 RTD	98	40	97	41	98	40	98	40	60	60
		98	40	97	41	98	40	98	40	60	60
		98	40	97	41	98	40	98	40	60	60
		98	40	97	41	98	40	98	40	60	60
		48	40	47	41	48	40	48	40	57	57
7	Gene II HIV-1/HIV-2	98	40	97	41	98	40	98	40	60	60
		98	40	98	40	98	40	98	40	60	60
		98	40	97	41	98	40	98	40	60	60
		98	40	97	41	98	40	98	40	60	60
		48	40	48	40	48	40	48	40	57	57
8	Determine HIV-1/2	98	40	98	40	98	40	98	40	60	60
		98	40	98	40	98	40	98	40	60	60
		98	40	98	40	98	40	98	40	60	60
		98	40	98	40	98	40	98	40	60	60
		48	40	48	40	48	40	48	40	60	60
9	Omni-Sal HIV-1/2 & Subtype O (Saliva test)	30	18	30	18	30	18	30	18	30	30
		30	18	29	19	30	18	30	18	30	30
		30	18	30	18	30	18	30	18	30	30
		30	18	29	19	30	18	30	18	30	30
		30	18	29	19	30	18	30	18	30	30
		30	18	30	18	30	18	30	18	30	30
		30	18	29	19	30	18	30	18	30	30
		30	18	30	18	30	18	30	18	30	30
		30	18	30	18	30	18	30	18	30	30
		10	30	10	30	10	30	10	30	27	27
10	Genscreen Plus HIV-Ag	90	40	90	40	90	40	90	40	90	90
		60	30	60	30	60	30	60	30	90	90
		90	40	90	40	90	40	90	40	90	90
		60	30	60	30	60	30	60	30	90	90
		90	40	90	40	90	40	90	40	90	90
		10	20	10	20	10	20	10	20	90	90
11	Vironostika HIV Uni-Form II Ag/Ab	90	40	90	40	90	40	90	40	90	90
		60	30	60	30	60	30	60	30	90	90
		90	40	90	40	90	40	90	40	90	90
		60	30	60	30	60	30	60	30	90	90
		90	40	90	40	90	40	90	40	90	90
		10	20	10	20	10	20	10	20	90	90
12	EFOORA HIV RTD	98	38	99	39	90	38	90	38	60	56
		90	38	98	40	90	38	90	38	60	54
		60	38	99	39	90	38	60	38	60	54
		60	38	99	39	60	38	60	38	60	56
		40	48	39	49	40	48	40	48	55	53
13	Cusabs HIV-1/HIV-2	60	38	60	38	60	38	60	38	60	60
		60	38	60	38	60	38	60	38	60	60
		60	38	60	38	60	38	60	38	60	60
		60	38	60	38	60	38	60	38	60	60
		40	48	40	48	40	48	40	48	60	60
14	HIV-TRUST	60	38	59	39	60	38	60	38	60	60
		60	38	58	40	60	38	60	38	60	60
		60	38	58	40	60	38	60	38	60	60
		60	38	58	40	60	38	60	38	60	60
		40	48	39	49	40	48	40	48	53	53