DETERMINATION OF EFFICIENCY OF INDIRECT IMMUNOFLUORESCENCE ASSAY TECHNIQUE IN THE DETECTION OF ANTIBODIES TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

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A thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science in Immunology of Kenyatta University.

December, 2002
DECLARATION

This is to certify that this thesis is my original work and has not been presented for a degree in another university or for another award.

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DEDICATION

I dedicate this thesis to my wife Janet, our daughter Memo, our parents and all Kenyans who are fighting for their lives with HIV/AIDS.
ACKNOWLEDGEMENT

I wish to express my gratitude to my supervisors, Dr. Z.W. Ng’ang’a of Kenyatta University, Department of Zoology and Dr. S. Mpoke of Kenya Medical Research Institute; for their guidance, encouragement and stimulating discussions during the course of the study and the preparation of this thesis.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction .............................................. 1

1.2 Literature review .................................................. 3

1.2.1 Discovery of acquired immune deficiency syndrome ............... 3

1.2.2 Identification of human immunodeficiency virus .................. 4

1.2.3 Transmission of human immunodeficiency virus .................. 6

1.2.4 Characteristics of human immunodeficiency virus type 1 ........... 7

1.2.4.1 Classification of human immunodeficiency virus .............. 7

1.2.4.2 The structure of human immunodeficiency virion ............. 8

1.2.4.3 Genomic organization of human immunodeficiency virus ... 10

1.2.4.4 Life cycle of human immunodeficiency virus................. 13
1.2.4.5 Viral phenotypes of human immunodeficiency virus..........15
1.2.4.6 Genetic variation of human immunodeficiency virus..........15
1.2.5 Pathogenesis of human immunodeficiency virus
infection.............................................................................16
1.2.6 Global epidemiology of HIV/AIDS............................................19
1.2.7 Clinical features and treatment of human
immunodeficiency virus infection..................................................21
1.2.8 Prevention of human immunodeficiency virus
infection......................................................................................25
1.2.8.1 Human immunodeficiency virus vaccines...............................28
1.2.9 Diagnosis of human immunodeficiency virus infection............30
1.2.9.1 Serological testing of human immunodeficiency virus...........31
1.2.9.1.1 Human immunodeficiency virus screening assays..............31
1.2.9.1.2 Human immunodeficiency virus type1/2 supplemental
(confirmatory) antibody tests.........................................................33
1.2.9.2 Viral identification assays......................................................33
1.2.10 Human immunodeficiency virus testing strategies...............35
1.2.11 Hypothesis..............................................................................36
1.2.12 Justification of the study........................................................36
1.2.13 Objectives of the study............................................................37
1.2.13.1 General objective.................................................................37
1.2.13.1 Specific objectives...............................................................38

CHAPTER 2: MATERIALS AND METHODS

2.1 Study population........................................................................39
2.2 Laboratory materials and reagents

2.2.1 Materials and equipment

2.2.2 Reagents and kits

2.3 Blood collection and separation

2.4 Cells for establishing human immunodeficiency virus infected cell lines

2.5 Determination of the optimum concentration of MOLT-4 Cells

2.6 Comparison of antigen production by MOLT-4/HIV MN and MOLT-4/HIV KS158 cell lines

2.6.1 Human immunodeficiency virus antigen preparation for SDS-PAGE

2.6.2 Sodium dodecyl sulphate poly-acrylamide gel electrophoresis and Western blotting

2.7 Particle agglutination assay

2.8 Enzyme-linked immunosorbent assay

2.9 Immunofluorescence assay

2.9.1 Preparation of immunofluorescence assay slides

2.9.2 Detection of antibodies in plasma

2.10 Growth curves of MOLT-4 cells, MOLT-4/HIV MN, MOLT-4/HIV KS158 cell lines and determination of antigen expression

2.11 Western blot assay

2.12 Detection of proviral deoxyribonucleic acid
### 2.12.1 Lymphocyte preparation

### 2.12.2 Deoxyribonucleic acid extraction

### 2.12.3 Polymerase chain reaction

### 2.12.4 Polymerase chain reaction product detection

### 2.13 Data management and statistical procedures

### CHAPTER 3: RESULTS

#### 3.1 Study population

#### 3.2 Growth curves of MOLT-4/HIV MN, MOLT-4/HIV KS158 and MOLT-4 cells

#### 3.3 Optimum concentration of MOLT-4 cells

#### 3.4 Comparison of HIV antigen expression and production by MOLT-4/HIV MN and MOLT-4/HIV KS158

#### 3.5 Antibodies detected by particle agglutination assay

#### 3.6 Antibodies detected by enzyme-linked immunosorbent assay

#### 3.7 Antibodies detected by immunofluorescence assay

#### 3.8 Antibodies detected by western blot

#### 3.9 Polymerase chain reaction testing for human Immunodeficiency virus type 1

#### 3.10 Determination of sensitivity and specificity of immunofluorescence assay using enzyme-linked immunosorbent assay, particle agglutination and western blot as the reference tests

#### 3.10.1 Comparison of immunofluorescence assay and particle
agglutination.................................................................69

3.10.2 Comparison of immunofluorescence assay and enzyme-linked immunosorbent assay .............................................69

3.10.3 Comparison of immunofluorescence assay and western blot.................................................................72

3.11 Overall sensitivity, specificity, positive and negative predictive values of immunofluorescence assay.................72

CHAPTER 4: DISCUSSION

4.1 Characteristics of the study population.................................75

4.2 Kinetics of HIV antigen expression and production.............75

4.3 Comparison of anti-HIV-1 detection by immunofluorescence assay and particle agglutination............76

4.4 Comparison of anti-HIV-1 detection by immunofluorescence assay, particle agglutination, enzyme-linked immunosorbent assay, western blot and polymerase chain reaction..............................................77

4.5 Use of polymerase chain reaction as a gold standard test.....79

CHAPTER 5: CONCLUSIONS......................................................81

CHAPTER 6: RECOMMENDATIONS FOR FUTURE WORK........82

REFERENCES....................................................................83
LIST OF FIGURES

Figure 1. 20-Year History of HIV/AIDS..........................................................2

Figure 2. Phylogenetic tree of Lentiviruses.....................................................8

Figure 3. The human immunodeficiency virus..............................................9

Figure 4. Genomic organization of human immunodeficiency virus type 1..........................................................11

Figure 5. The life cycle of human immunodeficiency virus..........................14

Figure 6. Distribution of human immunodeficiency virus subtypes in
Africa............................................................................................................17

Figure 7. Viral dynamics over the course of human immunodeficiency virus type 1 infection..................................................24

Figure 8. Procedure for IFA using MOLT-4 cells.......................................48

Figure 9. Growth curves of HIV infected and non-infected MOLT-4 cells..........................................................57

Figure 10. Kinetics of HIV antigen expression...........................................61
LIST OF TABLES

Table 1. Proteins coded for by human immunodeficiency virus genes and their functions ................................................................. 12

Table 2. Conditions defining acquired immune deficiency syndrome ........................................................................................................ 22

Table 3. Human immunodeficiency virus prevalence within the age groups ........................................................................................................ 55

Table 4. Average number of cells within the photographic field ................................................................................................................ 58

Table 5. Sensitivity and specificity of IFA using PA as the reference test ................................................................................................. 70

Table 6. Sensitivity and specificity of IFA using ELISA as the reference test ............................................................................................. 71

Table 7. Sensitivity and specificity of IFA using WB as the reference test ................................................................................................. 73

Table 8. Sensitivity and specificity of IFA using the determined HIV status .............................................................................................. 74
LIST OF PLATES

Plate 1. Distribution of MOLT-4 cells..................................................59
Plate 2. SDS-PAGE and Western blotting.............................................62
Plate 3. Particle agglutination results..................................................63
Plate 4. Enzyme-linked immunosorbent assay results..........................64
Plate 5. Immunofluorescence assay staining of HIV-1 infected
          MOLT-4 Cells...........................................................................66
Plate 6. Western blot strips.................................................................67
Plate 7. Electrophoretic patterns of PCR products...............................68
LIST OF APPENDICES

Appendix 1. STC study form.................................................................101

Appendix 2. PA test worksheet...........................................................102

Appendix 3. Enzyme-linked immunosorbent assay worksheet............103
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS:</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>ARV:</td>
<td>AIDS-related virus</td>
</tr>
<tr>
<td>BIV:</td>
<td>Bovine immunodeficiency virus</td>
</tr>
<tr>
<td>CA:</td>
<td>Capsid</td>
</tr>
<tr>
<td>CAEV:</td>
<td>Caprine arthritis-encephalitis virus</td>
</tr>
<tr>
<td>CCB:</td>
<td>Coomassie Brilliant Blue</td>
</tr>
<tr>
<td>CCR5:</td>
<td>Cystein-Cystein linked chemokine receptor</td>
</tr>
<tr>
<td>CD:</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CDC:</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>cDNA:</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS:</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRF:</td>
<td>Circulating recombinant forms</td>
</tr>
<tr>
<td>CXCR4:</td>
<td>Cystein-X-Cystein linked chemokine receptor</td>
</tr>
<tr>
<td>CTL:</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DNA:</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dnTP's:</td>
<td>Nucleotide building blocks</td>
</tr>
<tr>
<td>EIA:</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>EIAV:</td>
<td>Equine infectious anemia virus</td>
</tr>
<tr>
<td>ELISA:</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
</tbody>
</table>
env: HIV-1 envelope gene
Fc: Fragment crystallizable
FITC: Fluorescein isothiocyanate conjugated
FIV: Feline immunodeficiency virus
gag: HIV-1 group antigen gene
gp: glycoprotein
HAART: Highly active antiretroviral therapy
HIV-1/2: Human immunodeficiency virus type 1 or 2
HMBA: Hexamethylene-bisacetamide
HTLV-III: Human T-cell lymphotropic virus type III
ICAM: Intracellular adhesion molecule
IFA: Immunofluorescence assay
IN: Integrase
Ig: Immunoglobulin
JICA: Japan International Co-operation Agency
KB: Kilobases
Kd: Kilodaltons
KEMRI: Kenya Medical Research Institute
LAV: Lymphadenopathy-associated virus
LTNP: Long term non-progressors
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTR:</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MA:</td>
<td>Matrix</td>
</tr>
<tr>
<td>MHC:</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>ml:</td>
<td>millilitre</td>
</tr>
<tr>
<td>µl:</td>
<td>microlitre</td>
</tr>
<tr>
<td>MA:</td>
<td>Matrix</td>
</tr>
<tr>
<td>mg:</td>
<td>milligram</td>
</tr>
<tr>
<td>MOH:</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>MOLT-4/HIV KS158:</td>
<td>MOLT-4 cells infected with HIV-1 subtype D</td>
</tr>
<tr>
<td>MOLT-4/HIV MN:</td>
<td>MOLT-4 cells infected with HIV-1 subtype B</td>
</tr>
<tr>
<td>mRNA:</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTCT:</td>
<td>Mother-to-child-transmission of HIV</td>
</tr>
<tr>
<td>NC:</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>NACC:</td>
<td>National AIDS Control Council</td>
</tr>
<tr>
<td>NASCOP:</td>
<td>National AIDS and STDs Control Programme</td>
</tr>
<tr>
<td>NK:</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NPV:</td>
<td>Negative predictive value</td>
</tr>
<tr>
<td>NSI:</td>
<td>Non-syncytium inducing</td>
</tr>
<tr>
<td>PA:</td>
<td>Particle agglutination</td>
</tr>
<tr>
<td>PBS:</td>
<td>Phosphate buffered saline</td>
</tr>
</tbody>
</table>
PCR: Polymerase chain reaction
PBMCs: Peripheral blood mononuclear cells
pol: HIV-1 polymerase gene
PPV: Positive predictive value
PR: Protease
RIPA: Radioimmunoprecipitation assay
RNA: Ribonucleic acid
RP: Rapid progressors
rpm: Revolution per minute
RT: Reverse transcriptase
SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SI: Syncytium inducing
SIV: Simian immunodeficiency virus
STC: Special treatment clinic
STDs: Sexually transmitted diseases
STI: Sexually transmitted infections
TBE: Tris-boric acid EDTA
T_{H1}: T helper 1
TMB: Tetramethylbenzidine
Tris-sodium EDTA buffer

Joint United Nations programme on HIV/AIDS

Voluntary Counselling and testing

Western blot

World Health Organization
Screening of blood and blood products for human immunodeficiency virus (HIV) is routinely performed using the enzyme-linked immunosorbent assay (ELISA), and the results confirmed by western blot (WB). However, western blot is expensive and mostly performed in developed countries. A technique more superior or comparable to WB and adaptable to developing countries must be sought.

In an effort to identify such a technique, this study determined the efficiency of indirect immunofluorescence assay (IFA) to detect antibodies to HIV-1. Blood obtained from 400 patients seeking treatment for sexually transmitted infections at a special treatment clinic (STC) in Nairobi were tested for anti-HIV-1 antibody by ELISA, particle agglutination (PA) and IFA. Samples that were discordant were further tested using WB and polymerase chain reaction (PCR). The statistical analysis was done using STATA version 6.0 software.

The overall prevalence of HIV-1 in the study group was 38.7%. The overall sensitivity and specificity of IFA was 98.7% and 99.6% respectively, while positive and negative predictive values were 98.7% and 99.6% respectively. The efficiency of the IFA test was 99.3%.

Two hundred and twenty samples (57.5%) were PA and ELISA HIV seronegative. One hundred and fifty samples (37.5%) were PA and ELISA seropositive. The 30 samples (7.5%) that were discordant between PA and ELISA were further tested using WB. A further six samples that were concordant between PA and ELISA but IFA
discordant were tested with WB. Out of 160 samples that were HIV antibody positive by PA, 10 (6.3%) were HIV antibody negative by PA. Out of 240 samples that were HIV antibody negative by PA, 1 (0.4%) was HIV antibody positive by IFA. Out of 170 samples that were HIV antibody positive by ELISA, 22 (5.8%) were HIV antibody negative by IFA. Out of 230 samples that were HIV antibody negative by ELISA, 3 (1.3%) were HIV antibody negative by IFA.

All samples that were HIV seronegative by WB were also HIV seronegative by IFA. However, two (5.6%) samples were HIV seronegative by IFA but seropositive by WB. These samples were further tested using PCR. One sample was PCR positive and the other negative.

The IFA was superior to WB with respect to the ease of use and rapidity. This study demonstrates that IFA can be used as a primary test with western blot as a second confirmatory test to confirm HIV serostatus. The IFA technique can also be used as a serological assay for both screening and epidemiological purposes.
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Acquired Immune Deficiency Syndrome (AIDS) is caused by two viruses namely HIV-1 and HIV-2. These viruses are transmitted from one person to another through blood transfusion and sexual contact. In addition, infected pregnant women can pass HIV to their babies during pregnancy or at delivery, as well as through breast-feeding (Stanley & Madhavan, 1999). Diverse clinical features, including profound immunosuppression with associated opportunistic infections and malignancies, wasting and central nervous system degeneration characterize the disease. Human immunodeficiency virus infects a variety of cells of the immune system including CD4+ T-lymphocytes, macrophages, dendritic cells and monocytes (Stanley & Madhavan, 1999).

Since the early 1980's, the spread of the disease has been dramatic. As the number of reported cases escalated, the acquisition of scientific information exhibited a comparable surge, with reports in the scientific literature increasing logarithmically from 1982 to the present day (Figure 1). In Kenya, HIV was first reported in 1984 (Obel et al., 1984) and it is estimated that 1.5 million Kenyans have died due to AIDS (MOH, 2001a).
The first cases of unusual immune deficiency are identified among gay men in the USA.

In Africa, a heterosexual AIDS epidemic is revealed.

The Human Immuno-deficiency Virus (HIV) is identified as the cause of AIDS.

Acquired deficiency Syndrome (AIDS) is defined for the first time.

At least one case of HIV/AIDS has been reported from each region of the world.

The World Health Organization (WHO) launches the Special Programme on AIDS.

In the USA, the first HIV antibody test is approved by the Food and Drug Administration and HIV screening of blood donations starts.

The first therapy for AIDS-azidothymidine (AZT) is approved in the USA for use.

In 1991-1993, HIV prevalence in young pregnant women in Uganda begins to decrease, the first major downturn in a developing country.

The first efficacy trial of a potential HIV vaccine in a developing country starts in Thailand.

Highly Active Antiretroviral Therapy (HAART) is discussed for the first time.

Scientists develop the first treatment regimen to reduce mother-to-child transmission.

UN AIDS is created.

An HIV outbreak in Eastern Europe is detected (among injecting drug users).

The International Council of AIDS Service Organizations (ICASO) and the Global Network of People Living with HIV/AIDS are founded.

Brazil becomes the first developing country to provide antiretroviral therapy through its public health system.

The first therapy for AIDS-azidothymidine (AZT) is approved in the USA for use.

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Brazil becomes the first developing country to provide antiretroviral therapy through its public health system.

Adapted from UNAIDS, 2001.
The realization that Kenya is losing 700 of its people daily to declare HIV/AIDS a national disaster.

Human immunodeficiency virus antibody testing was implemented in response to the emerging AIDS epidemic and widespread about the integrity of blood supply (Stephen et al., 1989). Screening such as ELISA and particle agglutination (PA) (Yoshida et al., 1987) normally used. The reactive sera/plasma must be confirmed by Western blot (WB), which is expensive and laborious requiring long period. Alternative confirmatory tests for anti-HIV-1 antibody are IFA (Gallo et al., 1986).

1.2 Literature review

1.2.1 Discovery of acquired immune deficiency syndrome

The global epidemic of AIDS is one of the most pressing public emergencies. In 1981 several cases of Pneumocystis carinii pneumonia among young homosexual men and drug users were reported to the CDC in the United States (CDC, 1981). Such diseases normally associated with immune deficiencies (Gottlieb et al., 1981). 
Pneumocystis carinii had been known as a widespread, generally fungi associated with pneumonia. Kaposis sarcoma had been recognized a rare tumor of blood vessel tissues associated with aging. The p
these conditions in young and previously healthy men was alarming. A more complete evaluation of the patients showed that they had a marked deficiency in cellular immune responses and a significant decline in the sub-population of T cells that carry the CD4 marker. More reports describing an identical immunosuppressive syndrome among hemophiliacs (CDC, 1982d), blood transfusion recipients (CDC, 1982a), sexual partners (CDC, 1982c) and infants of mothers with the disease (CDC, 1982b) were reported. This new transmissible disease was named Acquired Immunodeficiency Syndrome, AIDS.

1.2.2 Identification of human immunodeficiency virus

There were several hypotheses that tried to explain the cause of AIDS in homosexual men. Some scientists suggested that since sperm was known to be immunosuppressive, then the entry of sperm antigens into blood through rectal tearing might account for the immunosuppression seen in AIDS (Mavligit et al., 1984). The development of AIDS in hemophiliacs who had received injections of factor VIII from pooled and concentrated human plasma and intravenous drug users suggested that AIDS might be caused by a virus. Several viruses were considered but it was not long before a new retrovirus was identified by independent groups as being associated with AIDS (Sonnabend et al., 1983).
Human immunodeficiency virus was first isolated by French researchers from the lymph node of a man with persistent lymphadenopathy syndrome and termed lymphadenopathy-associated virus (LAV) (Barre-Sinoussi et al., 1983). Soon after, the virus was isolated from patients with AIDS and at risk of AIDS and termed human T-cell lymphotrophic virus type III (HTLV-III) (Gallo et al., 1984). Another group of researchers also isolated the virus and termed it AIDS-related virus (ARV) (Levy et al., 1984). The three names were used for the same virus until 1986 when the new virus was renamed human immunodeficiency virus, HIV (Coffin et al., 1986). In 1986, an antigenic variant of the virus was isolated from patients in West Africa and hence the original virus was named HIV-1 and the variant designated as HIV-2 (Clavel et al., 1986).

While both forms of HIV are retroviruses capable of causing fatal AIDS; infection with HIV-2 generally results in a course of disease with a longer incubation period and which is more indolent. Furthermore, maternal-fetal transmission of this variant is limited and seems to provide natural protection, estimated at approximately 70%, against infection with HIV-1 in certain high-risk groups (Shioda et al., 1991). Human immunodeficiency virus type 2 demonstrates a closer genetic relationship and geographic distribution to SIV, long endemic in Central Africa, than to HIV-1 (Markovitz, 1993).
1.2.3 Transmission of human immunodeficiency virus

Transmission of HIV can occur through contact with infected body fluids. It is currently assumed that cellular body fluids such as blood, semen, vaginal secretions and breast milk are effective in transmitting the virus than fluids deficient in cells such as saliva, urine and tears (Stanley & Madhavan, 1999). Transmission may occur across mucosal membranes or broken skin during sexual intercourse; through intravenous exposure such as sharing infected needles, occupational exposure in the health care environment or treatment with infected blood products (Stanley & Madhavan, 1999).

It is estimated that 75% of the cases of HIV transmission are attributed to heterosexual contact (Neal et al., 1997). In populations where prostitution is rampant, some STDs flourish and provide a powerful co-factor for the heterosexual transmission of HIV. The lesions and open sores present in patients suffering from STDs favour the transfer of HIV during intercourse. Vertical transmission from infected mother to child can occur in utero or intrapartum. Postpartum infection can result from the ingestion of breast milk by a nursing infant from an infected mother (Van de Perre et al., 1992).
1.2.4 Characteristics of human immunodeficiency virus type 1

Human immunodeficiency virus type 1 is classified as a retrovirus. Retroviruses are enveloped viruses that possess diploid RNA genomes. These viruses are capable of long-term latent infection of cells and short-term cytopathic effects. Retroviruses produce slowly progressing fatal diseases that include wasting syndrome and CNS degeneration (Levy, 1986).

1.2.4.1 Classification of human immunodeficiency virus

Human immunodeficiency virus is a Lentivirus. Lentiviruses (Latin, lentus means slow) are complex viruses distinguished by the presence of a vase or cone-shaped nucleoid, absence of oncogenicity, and the lengthy and insidious onset of clinical signs. Lentiviruses constitute a genus of the retroviridae family. Retroviruses (retro-from latin turning back) are RNA viruses that replicate via DNA intermediates using the viral RT. Other Lentiviruses include: SIV, equine infectious anemia virus (EIAV), maedi virus (VISNA), caprine arthritis –encephalitis virus (CAEV), bovine immunodeficiency virus (BIV) and feline immunodeficiency virus (FIV). The phylogenetic relationship of HIV-1 with other viruses including HIV-2 is as shown in Figure 2.
1.2.4.2 The structure of human immunodeficiency virion

All the members of Lentivirus family of retroviruses including HIV-1, HIV-2 and SIV share numerous structural and molecular features. These viruses have an RNA genome and two associated molecules of RT that catalyze the reverse transcription of viral RNA into DNA (Wilhelm & Wilhelm, 2001).

Human immunodeficiency virus is a retrovirus of a non-complementary pair of single coding or positive strands of RNA enclosed within an inner, nucleocapsid, and protein core surrounded by a lipid bilayer envelope. An envelope glycoprotein, gp 41 is anchored within the lipid bilayer to which gp120 is noncovalently attached. Inside the lipid bilayer is the internal structural capsid and core proteins, p17, p24 and p7 (Figure 3). The viral
envelope also contains cellular proteins acquired during virus budding, including ICAM (intracellular adhesion molecule), β2-microglobulin and the human major histocompatibility complex (MHC) class I and II molecules (Rizzuto & Sodroski, 1997; Fortin et al., 1997).
1.2.4.3 Genomic organization of human immunodeficiency virus

The genome of HIV-1 is similar to other retroviruses and contains three major genes (flanked by long terminal repeats) that are essential for replication mechanisms (Sodroski et al., 1985). The first gene is HIV-1 group antigen gene \((\text{gag})\), which encodes the precursor protein 55 (p55), which is further cleaved by the viral protease to structural proteins p24, p17, p7 and p6.

The second is the HIV-1 polymerase gene \((\text{pol})\), which encodes for a precursor protein, which is similarly cleaved by protease to three viral enzymes: p11 protease, p66/51 RT, and p32 integrase. The third gene is the HIV-1 envelope gene \((\text{env})\) that codes for the precursor gp160, which is later proteolytically cleaved into the two-envelope proteins gp41 and gp120 (Figure 4).

In addition to these genes, the HIV-1 genome encodes accessory proteins with important functions in viral replication and infection (Table 1) (Emerman & Malim, 1998).
Figure 4. Genomic organization of human immunodeficiency virus type 1

Adapted from http://biology.fullerton.edu
Table 1. Proteins coded for by human immunodeficiency virus genes and their functions

<table>
<thead>
<tr>
<th>Genes</th>
<th>Functions</th>
</tr>
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<tbody>
<tr>
<td><strong>Structural genes</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Gag</strong></td>
<td>Codes for the core and structural proteins p24 (capsid; CA), p7 (nucleocapsid; NC) and p6</td>
</tr>
<tr>
<td><strong>Pol</strong></td>
<td>Codes for p10 (HIV-1 protease; PR), p66/51 (reverse transcriptase; RT) and p32 (integrase; IN)</td>
</tr>
<tr>
<td><strong>Env</strong></td>
<td>Codes for envelope gp 41 and gp120</td>
</tr>
<tr>
<td><strong>Accessory genes</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Tat</strong></td>
<td>Codes for p14</td>
</tr>
<tr>
<td><strong>Rev</strong></td>
<td>Codes for p19</td>
</tr>
<tr>
<td><strong>Nef</strong></td>
<td>Codes for p27</td>
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<td>Codes for p15</td>
</tr>
<tr>
<td><strong>Vpu</strong></td>
<td>Codes for p15</td>
</tr>
<tr>
<td><strong>Vip</strong></td>
<td>Codes for p23</td>
</tr>
</tbody>
</table>

Adapted from Larder *et al.* (1998)
1.2.4.4 Life cycle of human immunodeficiency virus

The stages of the HIV-1 life cycle are summarized in Figure 5. The HIV replication starts with attachment to the target cell. The attachment involves the interaction of the viral envelope protein gp120 with the CD4 molecule (Dalgleish et al., 1984) and chemokine co-receptors (Levy, 1996) on the cell membranes of T-helper lymphocytes. Subsequently, the gp120-CD4 interaction causes conformational changes in the viral protein gp120 that contribute to the exposure of the binding sites of the cellular co-receptors.

After attachment, the viral envelope fuses with the membrane of the target cell and the contents of the virus enter the cytoplasm. Molecules of the virus-derived enzyme, RNA-dependent DNA polymerase or RT, are released into the target cell together with the viral RNA. The RT then directs the synthesis of complementary strand of DNA on the RNA template from the virus in the target cell. Thereafter, the RT directs the synthesis of a second strand of DNA complementary to initial DNA strand. The double-stranded DNA is then randomly integrated into the host's DNA. The proviral form of the HIV integrated into the host chromosome is generally quiescent and replicates co-ordinately with the host cell DNA. When the HIV-1 infected cell undergoes activation or stimulation, the provirus is transactivated, resulting in the production of infectious virions (Stanley & Madhavan 1999).
Figure 5. The lifecycle of human immunodeficiency virus

Adapted from Abbas et al., (2000)
1.2.4.5 Viral phenotypes of human immunodeficiency virus

Primary HIV-1 isolates have been classified into different phenotypic groups according to distinct in vitro properties. Human immunodeficiency virus type 1 isolates have been classified as slow/low nonsynctium-inducing (NSI) macrophage-tropic or rapid/high syncytium-inducing (SI) T-cell-tropic viruses on the basis of virus replication rate, synctium-induction and ability to infect CD4+ cell lines (Schwartz et al., 1989). The finding that chemokine receptors have a critical role in the cellular entry of HIV-1 has led to a new classification of HIV-1 according to co-receptor usage. Isolates with the ability to enter cells using CXCR4 as co-receptor correspond to the previously described rapid /high SI isolates (renamed X4) and isolates which use CCR5 receptor comprise the slow/low NSI isolates (renamed R5) (Berger et al., 1998).

1.2.4.6 Genetic variation of human immunodeficiency virus

The DNA sequence diversity seen in HIV is generated by its RT enzyme which is highly error-prone and gives rise to nucleotide substitutions, insertions, deletions, repetitions and recombinations accounting for the high mutation rates. The turnover of the viral population within an infected individual is remarkably rapid. Early during infection, the patient harbours a relatively homogeneous virus population; over time, the virus population becomes heterogeneous and then, in the late stages of the disease the
virus population becomes more homogeneous again (McNearney et al., 1992).

Human immunodeficiency virus type 1 can be divided into three groups, based on the genomic analysis of the viral sequences from both env and gag genes, called major (M), outlier (O) and N (novel or non-M-non-O) (Simon et al., 1998). Ten genetic subtypes (A to J) have been defined within the M group. The prevalence of group O viruses is currently low. Subtype B represents the vast majority of viruses circulating in North America and Europe. In Africa all the HIV subtypes are prevalent (Figure 6). As a consequence of immigration, the original geographical distribution of HIV-1 subtypes is gradually changing. Today, there is no country of the world with a single clade of circulating virus. Human immunodeficiency virus type 1 subtype A is the main circulating strain in Kenya followed by subtypes D, C and G respectively (Neilson et al., 1999).

1.2.5 Pathogenesis of human immunodeficiency virus infection

It is predicted that approximately 95% of HIV-1 infected patients will progressively develop AIDS within 15 years of infection. An acute mononucleosis or flu-like illness characterizes the first few weeks after primary infection. Human immunodeficiency virus/AIDS is characterized by
high levels of viremia and a significant drop in the absolute number of CD4+ cells in the peripheral blood.

Figure 6. Distribution of human immunodeficiency virus subtypes in Africa

Adapted from UNAIDS (1998); www.UNAIDS.org

The acute viremia is followed by activation of CD8+ cells, which may be a response to control virus replication by cytotoxicity against infected cells.
During this stage, seroconversion usually occurs and is associated with rapid increase in circulating viral titers. This is followed by virus dissemination to the lymphoid organs and induction of a rapid turnover of infected lymphocytes (Koito et al., 1995; Schnittman & Fauci, 1994).

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 T<sub>H</sub>1 cells and stimulation of cytotoxic responses including T-cell, NK cell and antibody-dependent cell-mediated activities (Stanley & Madhavan, 1999). While these responses provide temporary control of the infection, they are unable to eliminate the virus.

The HIV patient then enters the stage of clinical latency or the asymptomatic period. A progressive decrease in CD4+ peripheral lymphocytes continues during the latency stage. When the CD4+ counts fall below 200 copies/mm<sup>3</sup>, the disease usually enters a symptomatic phase, characterized by opportunistic infections and other AIDS-defining conditions (Pantaleo et al., 1995).

Several studies have implicated a variety of mechanisms in the progressive depletion of the absolute number of CD4+ cells in peripheral blood. These include: synctium formation between infected and uninfected CD4+ cells;
selective infection or destruction of memory T cells; killing of infected cells by an autoimmune mechanism; Fc receptor-mediated antibody-dependent cellular cytotoxicity; cell-mediated destruction of HIV infected cells by CTL and/or NK cells and programmed cell death or apoptosis (Gouen & Montagnier, 1993). Apoptosis is not restricted to CD4+ cells only in HIV infection: CD8+ T-lymphocytes and B-lymphocytes can also undergo apoptosis (Muro-cacho et al., 1995).

1.2.6 Global epidemiology of HIV/AIDS

It is estimated that 40 million people worldwide were living with HIV infection and/or AIDS at the end of the year 2001. The global estimates can be stratified into 37.2 million adults and 2.7 million children under the age of 15 years. An estimated 5 million new HIV infections occurred worldwide during the year 2001. The total number of AIDS deaths worldwide in the year 2001 was 3 million. Sub-Saharan African countries remain the epicenter of the epidemic with 28.1 million men, women and children infected with HIV (UNAIDS, 2001).

In Kenya it was estimated that 760,000 people had developed AIDS by the end of 1998 (MOH, 1999). The total number of people infected now is estimated at 2.2 million and the cumulative number of AIDS deaths is
estimated to increase from 700,000 currently to 2.7 million by the year 2005 (NACC, 2000).

The HIV epidemic first appeared as a range of opportunistic infections defining AIDS. The counting of new HIV/AIDS cases became a central component of HIV/AIDS surveillance systems. However, there were several important limitations. These counts provided little information about the patterns of HIV transmission because they represented infections acquired over several years. Secondly, AIDS diagnosis required clinical and laboratory expertise that was absent in many countries particularly the developing countries. The advent of the HIV antibody tests in 1985 (Stephen et al., 1989) opened up the possibility of directly measuring the prevalence of HIV infection in populations. Some countries relied on the routine voluntary HIV testing as a means of monitoring prevalence while other countries established comprehensive systems that involved sampling a wide range of population groups at regular intervals. The sentinel surveillance system in Kenya was implemented by NASCOP. It became operational in 1990 and has been conducted annually (MOH, 1999).
1.2.7 Clinical features and treatment of human immunodeficiency virus infection

The signs and symptoms of acute HIV-1 infection appear within days to weeks after initial exposure (Kahn & Walker, 1998). The most common signs and symptoms include fever, fatigue, rash, headache, lymphadenopathy, pharyngitis and myalgia. The clinical manifestations of AIDS include systemic, neurologic, infectious and malignant complications (Table 2).

Some infected individuals progress to AIDS after less than three years of infection (RP), while others can remain asymptomatic for 10 years or more (LTNP) (Pantaleo et al., 1995) (Figure 7). Intermediate progressors, the majority of cases, develop AIDS approximately 10 years after initial infection. Once a patient develops AIDS, survival time is about 2 years.
<table>
<thead>
<tr>
<th>Category A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic: no symptoms at the time of HIV infection</td>
</tr>
<tr>
<td>Persistent generalized lymphadenopathy: lymph node enlargement persisting for 3 or more months with no evidence of infection</td>
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</tbody>
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<tr>
<th>Category B</th>
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<tbody>
<tr>
<td>Bacillary angiomatosis</td>
</tr>
<tr>
<td>Candidiasis, oesophageal (thrush)</td>
</tr>
<tr>
<td>Candidiasis, vulvovaginal; persistent, frequent, or poorly responsive to therapy</td>
</tr>
<tr>
<td>Cervical dysplasia (moderate or severe)/ cervical carcinoma</td>
</tr>
<tr>
<td>Hairy leucoplakia, oral</td>
</tr>
<tr>
<td>Herpes zoster (shingles) involving at least two distinct episodes</td>
</tr>
<tr>
<td>Idiopathic thrombocytopenic purpura</td>
</tr>
<tr>
<td>Listeriosis</td>
</tr>
<tr>
<td>Pelvic inflammatory disease, particularly if complicated by tubo-ovarian abscess</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
</tr>
</tbody>
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<table>
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<tr>
<th>Category C</th>
</tr>
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<tbody>
<tr>
<td>Candidiasis of bronchi, trachea, or lungs</td>
</tr>
<tr>
<td>Candidiasis, esophageal</td>
</tr>
<tr>
<td>Cervical cancer (invasive)</td>
</tr>
<tr>
<td>Coccidioidomycosis, disseminated or extrapulmonary</td>
</tr>
<tr>
<td>Medical Condition</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>Cryptococcosis, extrapulmonary</td>
</tr>
<tr>
<td>Cryptosporidiosis, chronic intestinal (&gt;1 month’s duration)</td>
</tr>
<tr>
<td>Cytomegalovirus disease (other than liver, spleen, or nodes)</td>
</tr>
<tr>
<td>Cytomegalovirus retinitis (with loss of vision)</td>
</tr>
<tr>
<td>Encephalopathy, HIV-related</td>
</tr>
<tr>
<td>Herpes simplex, chronic ulcer(s) (&gt;1 month’s duration), bronchitis, pneumonitis, or esophagitis</td>
</tr>
<tr>
<td>Histoplasmosis, disseminated or extrapulmonary</td>
</tr>
<tr>
<td>Isosporiasis, chronic intestinal (&gt;1 month’s duration)</td>
</tr>
<tr>
<td>Kaposi’s sarcoma</td>
</tr>
<tr>
<td><em>Mycobacterium avium</em> complex or <em>Mycobacterium kansasii</em>, disseminated or extrapulmonary</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em>, any site (pulmonary or extrapulmonary)</td>
</tr>
<tr>
<td><em>Pneumocystis carini</em> pneumonia</td>
</tr>
<tr>
<td>Progressive multifocal leukoencephalopathy</td>
</tr>
<tr>
<td><em>Salmonella</em> septicemia, recurrent</td>
</tr>
<tr>
<td>Toxoplasmosis of brain</td>
</tr>
<tr>
<td>Wasting syndrome due to HIV</td>
</tr>
</tbody>
</table>

Adapted from Bradley & McCluskey (1997)
Figure 7. Viral dynamics over the course of human immunodeficiency virus type 1 infection

Adapted from Larder et al., (1998)

develop AIDS approximately 10 years after initial infection. Once a patient develops AIDS, survival time is about 2 years.
Primary therapy for HIV infection involves a combination of various antiretroviral drugs. The mainstay of HIV treatment has been the use of RT inhibitors. These RT inhibitors prevent the virus derived enzyme, RT, from synthesizing viral cDNA into the host genomic DNA. These drugs include ZDV (Retrovir), didanosine (Videx), lamivudine (Epivir), stavudine (Zerit), zalcitabine (HIVID), efavirinz (sustiva), delaviridine mesylate (Rescriptor) and nevirapine (viramune) (Carpenter et al., 1996; 2000).

Another class of antiretroviral drugs is the protease inhibitors that block critical steps in virus assembly that are dependent on proteolytic cleavage. The protease inhibitors include indinavir (crixivan), ritonavir (Norvir), nelfinavir (Viracept) and saquinabuir (Fortavase invirase) (Carpenter et al., 1996; 2000).

In addition to the primary therapy, other management methods of HIV infection include the use of antibiotics, antivirals and antifungals for secondary or opportunistic infections, vaccination against other etiologic agents, prophylaxis of pneumocystis pneumonitis and psychological support (Stanley & Madhavan, 1999).

1.2.8 Prevention of human immunodeficiency virus infection

A vigorous education program to promote safe sex practices is paramount in reducing transmission of HIV (Beekmann & Henderson, 1995). Barrier
protection with latex condoms is helpful. Screening of blood and blood products from donors for anti-HIV antibodies has improved the safety of blood supply (Burnouf & Radosevich, 2000).

Health care workers and laboratory workers are at increased risk of exposure to HIV. Because of this occupational hazard, public health authorities have implemented the practice of universal body fluid precautions. The use of latex gloves and face shields has been recommended to reduce the risk of occupational exposure. Recapping of hypodermic needles presents a high risk and this habit must cease. All contaminated needles and sharp instruments should be placed directly into secure, closed containers for subsequent sterilization (Beekmann & Henderson, 1995). Several studies have demonstrated benefit from post-exposure chemoprophylaxis of HIV infections (Perlmutter & Harries, 1997). The CDC has developed guidelines for a 4-week course of chemoprophylaxis in the event of an occupational exposure to HIV (CDC, 1998).

Complete avoidance of breast-feeding is the surest way to avoid MTCT of HIV but replacement feeding exposes infants, especially in the developing countries, to the added risk of other infectious diseases with consequent increase in childhood morbidity and mortality (Gaillard et al., 2001). However, the prevention of MTCT is possible and it has been shown that two doses of nevirapine only can significantly reduce MTCT. It has also
been shown that combination therapy for the management of infected children is efficient (Maddocks & Dwyer, 2001).

The control of STIs has been shown to reduce HIV transmission. Most countries have developed strategies to control these infections. The challenge at the moment is to devise ways to overcome barriers that prevent effective implementation of the STI programme. This requires the formation of new partnerships between the private and public sectors and between governments and the communities they represent (Mayaud & McCormick, 2001).

Voluntary counseling and testing (VCT) is an essential component of an effective response to the AIDS epidemic. In view of the ever-increasing HIV/AIDS epidemic in sub-Saharan Africa, the expansion of VCT as an integral part of prevention strategies is a reality. The Ministry of Health in Kenya has developed guidelines for voluntary counseling and testing (MOH, 2001b). Most of the people who are infected are not aware of their HIV status and it is hoped that the service will be able to identify those who are infected early enough for proper care and the majority who are not yet infected.
1.2.8.1 Human immunodeficiency virus vaccines

The guiding principle in control of HIV stems from the natural immunity, which is part of the recovery process. In addition to stimulating protective antibodies against HIV, an ideal vaccine should also induce specific CTL against HIV-infected cells and activate non-specific NK cells. Candidate HIV vaccines are composed of synthetic, recombinant or highly purified subunit epitopes spanning various regions of the HIV proteins gp120, gp 41, Tat, Rev and Nef (Stanley & Madhavan, 1999).

Several types of vaccines that have been designed include: live attenuated, recombinant viruses carrying HIV genes, cloned CD4, cloned envelope glycoproteins and inactivated whole virus. Early vaccine trials focused on a subunit of HIV-1 envelope protein (Stanhope et al., 1993). Subunit antigens are considered much safer than inactivated vaccines because they carry no risk of reversion to virulence but are generally less immunogenic. Various adjuvants such as detoxified lipid A, adjuvant emulsions, liposomes, biodegradable microspheres, muramyl peptides and saponin are being evaluated for their ability to increase immune responses to HIV vaccines (Stanley & Madhavan, 1999).

Live attenuated HIV-1 vaccines have been shown to provide good protection in macaques (Desrosiers, 1998; Johnson & Desrosiers, 1998). However, live attenuated vaccines have a risk of reversion to a pathogenic form. Recombinant viral vectors expressing HIV-1 antigens have been
developed to overcome the safety concerns of live attenuated vaccines. The expression vector results in antigen processing through the MHC class I pathway, which induces CD8+ CTL (Belshe et al., 2001).

The discovery of the X-ray crystallographic structure of gp120, the subsequent revelation of the gp120-CD4 interface, and identification of the conserved site for the chemokine receptor (Kwong et al., 1998) may provide needed information to develop an effective and safe HIV vaccine.

Development of a potent vaccine against HIV has been complicated by a number of factors which include: the genomic variability of the virus, lack of satisfactory surrogate markers of protective immunity, the intracellular mode of HIV transmission and the persistent nature of infection (Stanley & Madhavan, 1999).

While the search for an effective HIV vaccine remains elusive, there are unique clinical observations that suggest there are intrinsic host protective mechanisms against the virus that can be upregulated by vaccination. It has been shown that some African prostitutes who are evidently at high risk have not seroconverted or do not harbour the virus genome (Fowke et al., 1996; Rowland-Jones et al., 1998). These observations are encouraging and support the continuation of efforts to develop a HIV vaccine.
1.2.9 Diagnosis of human immunodeficiency virus infection

Human immunodeficiency virus infection is diagnosed by detecting antibodies specific to the virus or by detecting the virus itself, either by nucleic acid-based tests or if necessary by culture and virus isolation (Lutz, 1996).

Most of the currently used first-line screening systems to diagnose HIV infection employ ELISA techniques. Enzyme-linked immunosorbent assay detects the presence of antibodies to HIV in blood samples. Automated processing of multiple samples using microwell plates enables the processing of many samples, which is essential to the screening of blood donations. Combination assays that detect both HIV-1 and HIV-2 are often used in countries where both strains may be prevalent. If the initial ELISA result is positive the assay is repeated. After two successive positive ELISA results, the serum sample is then confirmed by Western blot technique (Constantine, 1993). Other methods are also available for diagnosing HIV infection. These include rapid tests; p24 antigen capture method which is an immunoassay, *in vitro* culture of HIV infected cells with susceptible cells and PCR (Phillips *et al.*, 2000; Borkowsky *et al.*, 1992).
1.2.9.1 Serological testing of human immunodeficiency virus

The detection of antibodies specifically recognizing HIV is the most common way to diagnose HIV infection in adults and children over 18 months old. These antibodies are usually detectable within 3-6 weeks after infection, and almost all individuals seroconvert by 12 weeks (Constantine et al., 1994). Antibodies to HIV persist for life, with antibodies specific for the envelope proteins and particularly the immunodominant domain of gp 41 (Gnann et al., 1987) persisting and antibodies for gag (Constantine, 1993) frequently declining. Antibodies to the integrase (p32) can be detected after antibodies to the RT (p51 and p66) and like the envelope antibodies; they persist for life (Lutz, 1996).

1.2.9.1.1 Human immunodeficiency virus screening assays

(a) Enzyme-linked immunosorbent assay/Enzyme immunoassay

The ELISA/EIA were introduced in 1985 (Stephen et al., 1989) and the sensitivity and specificity of the techniques has improved over the years. They are the most commonly used tests in HIV screening because they are simple to perform, sensitive and suitable for testing large number of samples particularly in blood testing centers.

All the varieties of ELISA use enzyme conjugates that bind to specific HIV antibody and substrates/chromogens that produce color in a reaction
catalyzed by the bound enzyme conjugate. The antigen is attached to a solid phase in microtiter plate or beads. The antigen may be a viral lysate, recombinant or synthetic peptides (Constantine, 2001).

The viral lysate has the potential advantage of providing a broad spectrum of viral epitopes, which cross-react with divergent virus strains. However, it may also contain antigens from the cells in which the virus was grown, resulting in some false positive results (Burke, 1989). The use of recombinant and synthetic peptides minimizes nonspecific reactions.

(b) Rapid tests used for human immunodeficiency virus screening

Rapid tests are defined as tests that can be performed in less than 30 minutes. The rapid tests detect antibodies to HIV. For example, the Determine HIV-1/2 test kit is an immunochromatographic rapid test that uses a nitrocellulose strip with a conjugate to selenium colloid and a capture site containing HIV-1 and HIV-2 antigens. If a sample contains HIV-1 and HIV-2 antibodies, the antibodies react with the antigen at the site, with the formation of a red line within 10 minutes. Another class of the rapid tests is the 'dot blot' or 'immunoblot'. Most of these tests incorporate a built-in control that indicates that the test was performed correctly. Other rapid tests include dipsticks in which antigen is attached on the 'teeth' of the comb-like devices (Constantine, 2001).
(c) Simple tests used for human immunodeficiency screening

These tests require more than 30 minutes and involve procedures that can be performed easily without instrumentation. These include agglutination assays in which antigen-coated particles (red blood cells, latex particles or gelatin particles) are allowed to react with antibodies to form visible clumping (agglutination) (Constantine, 2001).

1.2.9.1.2 Human immunodeficiency virus type 1/2 supplemental (confirmatory) antibody tests

Most testing algorithms require the use of very specific assays such as WB, IFA or RIPA, to verify reactive screening test results. These tests are specific and sensitive but they are more labor intensive, more prone to subjective interpretation and more expensive than the screening assays (Constantine, 2001).

1.2.9.2 Viral identification assays

(a) p24 antigen

The p24 antigen, one of the core proteins of HIV-1 capsid, can be detected in the serum/plasma of individuals as early as 16 days post-infection and prior to the detection of antibodies (Lutz, 1996). The HIV-1 p24 antigen assay detects the core antigen p24. The antibodies to HIV p24 coated on
the ELISA plate captures the p24 antigen in patient's serum/plasma. An enzyme conjugated second antibody to p24 is then added and a standard enzyme immunoassay method is used for detection. This assay has been used to improve the screening of blood products obtained from donors who may be in the window period following the onset of viremia before the first appearance of antibodies (CDC, 1996).

(b) Viral culture of human immunodeficiency virus

Although isolation of HIV from an individual definitively diagnoses HIV infection, a single positive culture should be confirmed with a second specimen. This assay is very expensive and less sensitive than antibody testing. A negative culture may be due to technical problems, a defective virus, or the inability of the virus to replicate in culture (Lutz, 1996).

(c) Polymerase Chain Reaction (Qualitative)

The PCR is currently the best known assay for the amplification of nucleic acid. The technique detects the HIV proviral DNA. Diagnosis of neonates born to HIV seropositive mothers poses a special problem using serological tests. As maternal IgG is transferred across the placenta during the last trimester of pregnancy, infants born to seropositive mothers will be seropositive irrespective of their state of infection. Maternal antibodies can remain in circulation for up to one year and consequently confound
serodiagnosis of the child. Thus the HIV-1 DNA PCR is preferred for detecting viral genomes in neonates born to mothers infected with HIV-1. The technique has also been shown to resolve repeatedly inconclusive serologic results (Dennis et al., 1999).

1.2.10 Human immunodeficiency virus testing strategies

The most commonly used confirmatory test was the Western blot. However, its use has proved to be very expensive and can, under some conditions produce indeterminate results. Similar assays, generally called Line immunoassay (LIA) based on recombinant proteins and/or synthetic peptides capable of detecting antibodies to specific HIV-1 and/or HIV-2 proteins have been developed but they are equally expensive (Constantine, 2001). Simple and rapid tests have been developed to meet a market demand or rapid small-scale or point of testing. Studies have shown that their sensitivity and specificity are similar to those of the standard enzyme immunoassays and Western blot algorithm (Behets et al., 1992; Stetler et al., 1997). The WHO and UNAIDS has therefore recommended that countries should consider testing strategies which use ELISAs and simple/rapid assays rather than ELISA/WB for HIV antibody detection to reduce the cost (WHO, 1998).
However, most of these tests employ recombinant proteins or synthetic peptide antigens derived from subtype B viruses. It has been shown that some of the rapid tests fail to detect some specific HIV subtypes (Phillips et al., 2000). False results have also been reported in some of the rapid assays (Giles, et al., 1999; Makuwa et al., 2002). The use of rapid tests in a specific geographic area should be validated to ensure that the test is adequately sensitive and specific to the circulating HIV-1 subtypes. Therefore there is need to explore the use of other confirmatory tests that can be adapted in the developing countries and the source of the antigen should be from the circulating HIV strains.

1.2.11 Hypothesis

There is no difference in terms of sensitivity and specificity between IFA and WB.

1.2.12 Justification of the study

The most common initial laboratory HIV-1 antibody detection assay is the EIA, which despite its high specificity has a low positive predictive value when used to screen large populations with a very low prevalence of infection. The use of rapid diagnostic tests can result in significant shortening of reporting time and is cost-effective in many settings but it gives false-positive results (Phillips et al., 2000). Although the HIV screening assays detect antibodies in patients with AIDS very effectively,
their use for detecting antibodies in non-diseased individuals has presented a different challenge (Doran & Parra, 2000). The occurrence of even a small number of false results by these tests can have profound implications especially when testing a population at low risk of infection. This is true especially when testing blood donors, since false positive results waste resources in discarded blood units. Furthermore, HIV infection generates a degree of unwantedness that creates psychiatric symptoms such as anxiety and depression. A false-negative or positive result can have serious consequences for blood recipients. A false negative result may also lead to wrong decisions for individuals who are intending to get married, students travelling for studies abroad, couples intending to get children and pregnant women.

There is therefore need to explore the use of cost-effective, reliable and accurate tests for HIV testing. In the present study the efficiency and feasibility of using IFA to detect antibodies to HIV was determined.

1.2.13 Objectives of the study

1.2.13.1 General objective

To determine the accuracy and feasibility of using IFA for the confirmation of HIV diagnosis in Kenya.
1.2.13.2 Specific objectives

(a) To determine the HIV prevalence in the study group.

(b) To compare HIV antigen expression and production between two cell lines.

(c) To assess the sensitivity and specificity of IFA as a confirmatory test for HIV diagnosis.

(d) To assess the positive and negative predictive values of IFA using ELISA, PA and WB as the reference tests.

(e) To determine the efficiency of IFA.
CHAPTER 2

MATERIALS AND METHODS

2.1 Study population

Four hundred patients seeking treatment for sexually transmitted infections (STIs) at the special treatment clinic (STC) in Nairobi were enrolled into the study between June and November 2001. Details of clinical notes, sex, age and district of origin were included in the study form (Appendix 1).

2.2 Laboratory materials and reagents

2.2.1 Materials and equipment

The following laboratory materials and equipment were used: Vacutainer tubes with K3 EDTA as anticoagulant (Becton-Dickinson), Vortex Mixer (Thermilyn Maxi-Mix, USA), Tray Mixer (Kayagaki, KR-IN, Japan), Centrifuge tubes, 15ml and 50ml (IWAKI, Japan), 75cm$^2$ culture flask (IWAKI, Japan), 25cm$^2$ culture flask (Corning), Automatic ELISA washer (Labsystems Well Wash Ascent, Finland), ELISA Reader Multiscan (Labsystem, Finland), Olympus BX50 system Microscope (Tokyo, Japan), Tally counter (Tokyo, Japan), Pipetman Gilson P10, P100, P200 and P1000 (France), microscope glass cover slips 22X50mm (England), Teflon coated slides (Cel-line/ERIE Scientific Co.), Safety cabinet class IIB (Hitachi Japan), centrifuge Avanti J-25I (Beckman USA), Ultracentrifuge (Beckman...
USA), Waterbath (Advantec, Japan), Centrifuge Allegra 6KR (Beckman, USA), Serological pipettes (Falcon, Becton Dickinson, USA), Precision tips 10μl (Gilson), 200μl (Greiner), 1000μl (Labsystem), Gloves (Dermagrip, Malaysia), CO₂ incubator (Forma Scientific, Inc. USA), Sanyo freezer -20°C (Japan), Sanyo Ultralow -80°C (Japan), Sanyo Medicool fridge +4°C (Japan), Pipet-aid (Drummond), Serum vials and Cryoboxes (Nalgene/Nunc), U-shaped plates (Fujirebio, Japan), Flat-bottomed culture plates (Nunc), Hotplate/stirrer (England) and Model 7000 8-12 channel digital micro pipette 40-200μl (Japan).

2.2.2 Reagents and kits

The following reagents and kits were used: Inno- lia HIV confirmation (Innogenetics), Lia Tek HIV III (Organon-Teknika), Vironostika HIV Uniform II plus O (Organon-Teknika), KEMRI HIV-1 PA kit (KEMRI), Anti-human IgG FITC (Dako, Denmark), RPMI 1640 Medium (Nissui pharmaceutical Co., Ltd, Japan), Fetal Bovine Serum (Gibco BRL), L-glutamine 200MM, 100X (Gibco BRL), Tris (hydroxymethyl) aminomethane (Wako), Hydrochloric acid (Wako), Sodium chloride (Wako), Ethylenediamine-N,N,N',N'-tetraacetic acid, disodium salt, dihydrate (EDTA.2Na) (Dojindo, Japan), Sucrose (Wako), Sodium bicarbonate (Wako), PBS tablets (Gibco BRL), Trypan blue (George T.Gurr Ltd, England) and Culture freezing Medium (Gibco BRL).
2.3 Blood collection and separation

Peripheral venous blood (4.5ml) was drawn directly into vacutainer tubes containing EDTA as anticoagulant. The samples were then transported at room temperature to KEMRI/HIV laboratory for subsequent procedures. The samples were given laboratory code numbers. Plasma was obtained by centrifuging blood at 1500 rpm for 10 minutes at room temperature. The plasma (300μl) was aliquoted into 5 plasma vials. Three vials were stored at −20°C and the other two at −80°C until the time the next procedures were carried out. The PBMC's were extracted using ammonium-chloride precipitation method and stored at −80°C (Eggleton et al., 1989).

2.4 Cells for establishing human immunodeficiency virus infected cell lines

The MOLT-4 cells (Kikukawa et al., 1986) were used to establish permanently HIV infected cell lines. The cells are lymphocyte-like in morphology. After infections with HIV, the MOLT-4 cells grow permanently producing large amounts of virus continuously (permanent cell lineage can be established by infecting these cells with HIV).

All the cell lines were cultured at 37°C and 5% CO₂, in RPMI medium supplemented with 10% heat-inactivated fetal calf serum and kanamycin (60μg/ml). The cells were seeded at a concentration of 3x10⁵ cells /ml twice weekly in fresh media.
2.5 Determination of the optimum concentration of MOLT-4 cells

The MOLT-4 cells were cultured for 3 days and the cell concentration determined by the trypan blue exclusion principle. The MOLT-4 cells were harvested by centrifuging the culture fluid at 800 rpm for 5 minutes. The supernatant was discarded and the MOLT-4 cells washed twice with PBS by centrifuging at 800 rpm for 5 minutes. The MOLT-4 cell concentration was then adjusted using PBS to $2 \times 10^6$ cells/ml, $1.5 \times 10^6$ cells/ml, $1 \times 10^6$ cells/ml and $7.5 \times 10^5$ cells/ml respectively. Five microliters (5μl) and 3μl of the adjusted MOLT-4 cell suspensions were then applied onto Teflon coated slides. The slides were air-dried for 1 hour and fixed in cold acetone (4°C) for 1 hour. The slides were stained with trypan blue. The excess stain was washed using PBS. The cell distribution was observed under the microscope (Mg x 200).

2.6 Comparison of antigen production by MOLT-4/ HIV MN and MOLT-4/HIV KS158 cell lines

2.6.1 Human immunodeficiency virus antigen preparation for SDS-PAGE

Sodium dodecyl sulfate poly-acrylamide gel electrophoresis and Western blotting were carried out as described by Haper and Coles (1996) to
determine the cell line with a better HIV antigen production to be used as a source of viral antigen for immunofluorescence assay (IFA).

The two cell lines (MOLT-4/HIV KS158 and MOLT-4/HIV MN) were seeded with the same cell densities (3x10^5 cells/ml) into 75cm² culture flasks containing 40ml of growth media and cultivated at 37°C for 4 days. In one of the flasks, 1ml of 0.3mg HMBA was added to determine its effect on the antigen production. The viable cell numbers and the viability of the 4-day cultures were confirmed to be almost the same using the trypan blue exclusion principle. The 4-day cultures were then centrifuged at 3,000 rpm for 5 minutes to remove the cell debris. The supernatants were filtered through 0.4μm-membrane filters to further remove cell debris.

The filtrates (20ml each) were overlaid on 25% sucrose (10ml) and 55% sucrose (3ml) and centrifuged at 25,000 rpm for 3 hours. The virus fractions were collected carefully. The virus fractions were then diluted with TNE buffer and centrifuged for 1 hour at 3000 rpm. The virus pellets were solubilized with 100μl of loading buffer for SDS-PAGE and used as antigens.
2.6.2 Sodium dodecyl sulfate poly-acrylamide gel electrophoresis and Western blotting

Sodium dodecyl sulfate poly-acrylamide gel electrophoresis and Western blotting were carried out as described by Haper and Coles (1996). The antigen samples were run on 12.5% polyacrylamide gel containing SDS for 2 hours at room temperature. One piece of the gel was stained with coomassie brilliant blue and the second with silver stain. The two stains were used due to their differential staining of proteins. The third gel was used for Western blotting. The nitrocellulose membrane and filter paper were cut according to the gel size and soaked in blotting buffer. The gel was sandwiched between the nitrocellulose membrane and filter paper and then transferred into the electrophoretic apparatus. The transferred membrane was incubated in a HIV positive serum at room temperature for 1 hour. After washing, the membrane was incubated with alkaline-phosphatase conjugated anti-human rabbit serum for 1 hour.

2.7 Particle agglutination assay

This assay was carried out as described by Yoshida et al., (1987). Approximately 30 minutes prior to the test, sensitized and unsensitized gelatin particles were reconstituted with 1.5ml and 2.0ml reconstitution
buffers respectively. The other reagents and the samples were brought out of the fridge and the freezer respectively to attain room temperature.

The plates and the worksheets (Appendix 2) were labelled accordingly. The test plasma was serially diluted in volumes of 25μl per well in a U-bottom microtiter plate. Portions (25μl) of antigen-sensitized particles and unsensitized particles were dropped into the wells containing 1:16 (final dilution, 1:32) and 1:8 (final dilution, 1:16) diluted plasma respectively. The contents of the wells were mixed with a tray mixer and then allowed to stand at room temperature for 2 hours. A result was considered positive when unsensitized particles did not agglutinate and sensitized particles gave a definite agglutination pattern.

2.8 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay was carried out as described by McDougal et al., (1985). The test reagents and samples were allowed to remain at room temperature. The worksheet (Appendix 3) was labelled accordingly.

A strip holder was fitted with 8 microelisa strips. One hundred microliters of the specimen diluent was added into all the wells including the control wells. Fifty microliters of the samples and the controls were added to the wells. The strips were then incubated at 37°C for 1 hour. The wells were
washed six times with phosphate buffer using an ELISA washer machine. One hundred microliters of TMB substrate was then added into each well. The strips were incubated at room temperature for 30 minutes. The reaction was stopped by adding 100μl of sulfuric acid to each well. The plates were read using an ELISA reader within 15 minutes. The cut-off value was calculated and the results recorded as either negative or positive.

2.9 Immunofluorescence assay

2.9.1 Preparation of immunofluorescence assay slides

The MOLT-4 cells and MOLT-4/HIV MN cell lines were cultured in 75cm² flask containing 40ml growth medium for 3 days. The viable cell numbers were determined using a haemocytometer. The cells were then collected by centrifuging the culture at 800 rpm for 5 minutes. The cells were then washed twice with PBS. The cell concentration was adjusted to $1.5 \times 10^6$ cells/ml with PBS. Five microliters of the cell suspension were mounted onto the wells of the Teflon coated slides. The slides were then air-dried for 1 hour and fixed using cold acetone for 1 hour. The slides were then air-dried and stored at -80°C.
2.9.2 Detection of antibodies in plasma

The IFA was performed as described by Lennette et al., (1987). The procedure for the IFA technique was as summarized in Figure 8. Plasma obtained from the patients seeking treatment at the special treatment clinic was diluted 20 times with PBS (20μl plasma: 380μl PBS). An aliquot (10 μl) of the plasma was added to the wells of the prepared Teflon coated slides. The slides were incubated at 37°C for 30 minutes in a humidified chamber. The slides were then washed 3 times with PBS. An aliquot (10 μl) of FITC-conjugated anti-human IgG was added to all the wells. The slides were incubated at 37°C for 30 minutes in a humidified chamber. The slides were washed 3 times with PBS. The cover slips were mounted with buffered glycerol. The cells were observed under the fluorescent microscope.

2.10 Growth curves of MOLT-4 cells, MOLT-4/HIV MN, MOLT-4/HIV KS158 cell lines and determination of antigen expression

The number of MOLT-4/HIV MN and MOLT-4/HIV KS158 cells was adjusted to 3x10^5 cells/ml at day 0. Every 24 hours, the number of viable cells were counted and plotted. At the same time, IFA slides were prepared for antigen expression. The growth culture (1ml) was centrifuged at 800 rpm.
Figure 8. Procedure for IFA using MOLT-4 cells

1. Cell smear preparation
2. Fixation of the smear in cold acetone
3. Reaction with diluted plasma (FITC-conjugated anti-human IgG)
4. Incubation in a humidified chamber
5. Washed three times with PBS
6. Mounted in 90% glycerol
7. Observation under fluorescent microscope
for 5 minutes to collect the cells. The IFA slides were then prepared as described in 2.9.1.

In order to determine the antigen expression, the MOLT-4/HIV MN and MOLT-4/HIV KS158 cells prepared on the different days were reacted with the control plasma as described in 2.9.2. The number of cells expressing and not expressing the antigen per microscopic field at a magnification of x200 was counted.

2.11 Western blot assay

The WB assay was carried out as described by O'Gorman et al., (1991). All the test materials were allowed to remain at room temperature. One milliliter of diluent was added into each trough except the control troughs. Ten microliters of the plasma were added to respective troughs. One milliliter of the negative and positive control was then added to the assigned troughs. One strip with the coated membrane side facing up was added into each trough. The troughs were then covered with a plate sealer and incubated on an automatic shaker for 14-18 hours at room temperature.

The strips were then washed 3 times with the wash buffer. One milliliter of conjugate was then added to each trough and incubated on a shaker for 30 minutes at room temperature. The strips were then washed twice with
wash buffer and once with substrate diluent. One milliliter of substrate buffer was then added into the troughs and incubated on a shaker for 30 minutes at room temperature. The substrate buffer was then aspirated from each trough and 1ml of the stopping solution added to each strip. The troughs were incubated on a shaker for 10-30 minutes at room temperature. The strips were then removed and placed on absorbent tissue and allowed to dry completely before interpretation of the results.

2.12 Detection of proviral deoxyribonucleic acid

Detection of the proviral DNA was accomplished by polymerase chain reaction technique (Saiki et al., 1986) and performed as described by Fransen et al., (1994). The samples that were discordant between IFA and WB and those that were ELISA and P.A concordant but IFA discordant were tested by PCR.

2.12.1 Lymphocyte preparation

The peripheral blood mononuclear cells were obtained by using 0.84% ammonium chloride. Five milliliters of blood were mixed with 30ml of 0.84% ammonium chloride and then incubated for 5 minutes at 37°C. The mixture was then centrifuged for 5 minutes at 1500 rpm and the supernatant discarded. The above procedure was repeated twice and the pellet was washed twice with PBS. The pellet was then stored at -80°C.
2.12.2 Deoxyribonucleic acid extraction

The deoxyribonucleic acid from the peripheral blood mononuclear cells was extracted using DNAzol reagent (Genomic DNA Isolation Reagent). Five hundred microlitre of DNAzol was added into the lymphocyte pellet. The cells were lysed by gentle pipetting. Two hundred and fifty microlitre (250µl) of 100% ethanol was added to precipitate the DNA. The DNA precipitate was removed by spooling with a pipette tip and attached to the wall of the tube. The supernatant was then discarded. The DNA precipitate was then washed twice with 700µl of 95% ethanol by centrifuging at 12,000 rpm for 15 minutes and discarding the supernatant. The DNA pellet was air-dried and then dissolved in 100µl of RNAse free water. The DNA samples were used for amplification (Van Laethem et al., 1998).

2.12.3 Polymerase chain reaction

The PCR was carried out in a 50µl reaction tube containing: 2.5µl 10X PCR buffer, 2.5µl Mgcl, 2.5µl dNTPs, 0.5µl of each primer (M5/M10), 13.3µl water and 3µl DNA. Amplification of the env region was performed on a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer) by using the following cycling parameters: 95°C for 10 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and final extension of 72°C for 10 minutes. For the second PCR the volumes were the same except for the water and the DNA (1st product), which were 14.3µl and 2µl respectively. The primers used were M3 and M8.
For the pol region amplification, the same reaction mixtures were used as above but with different primers (Unipol 5/Unipol 6 and Unipol 1/ Unipol 2 for first and second PCR respectively). The reaction conditions were 96°C for 10 minutes, 35 cycles of 96°C for 10 seconds, 45°C for 30 seconds, 72°C for 1 minute and final extension of 72°C for 10 minutes. The reaction was carried out using Perkin Elmer 9600 Thermal Cycler.

The sequences of the primers used were as follows:

*env* primers;

M5 CCAATTCACCACATTATTGTGCCCATGG
M10 CCAATTGTCCCTCATATCTCTCTCTCTCCAGG
M3 GTCAGCACAGTACAATGTACACATGG
M8 TCCTTGGATGGGAGGGGACATCATG

*pol* primers;

Unipol 5 TGGGTACCAGCACACAAAGGAATAGGAGGAAA
Unipol 6 CCACAGCTGATCTCTGCCTTCTCTGTAATAGACC
Unipol 1 AGTGGATTCATAGAAGCAGAAGT
Unipol 2 CCCCTATT CCTCCCTTCTTTTTAAAA
2.12.4 Polymerase chain reaction product detection

Polymerase chain reaction product detection was realized by carrying out electrophoresis on 1.5 % agarose gel and staining with ethidium bromide. Three grams of agarose was mixed with 200ml of 0.5% Tris-Boric Acid-EDTA (TBE) and heated to dissolve. After dissolving, the gel was poured onto gel plate fitted with combs. The gel was left to solidify and then 3µl of the PCR product applied to each well. A 100 base pair ladder was used as a marker. Electrophoresis was carried out at 100 volts for 30 minutes. The gel was then removed and stained with ethidium bromide for 20 minutes in the dark. The gel was then mounted on an ultraviolet transilluminator and photographed (Van Laethem et al., 1998). Samples were considered positive when bands of between 300 and 330 base pairs were observed and negative when no band was detected for pol primers. For the envelope primers, the appearance of bands at 550 base pairs was considered a positive result.

2.13 Data management and statistical procedures

The HIV status was determined following the UNAIDS/WHO recommended testing strategies (WHO, 1998). All the samples were diagnosed using ELISA, PA and IFA. The results that were concordant were considered
positive or negative. Those that were discordant were confirmed using WB and PCR.

Data processing and statistical analysis was done using STATA Version 6.0 software. The sensitivity, specificity, PPV and NPV were determined using McNemar test as shown below. The NPV measures the probability that a negative result indicates the absence of the disease. The PPV measures the probability that a positive result indicates the presence of the disease. The results of the various assays were correlated by Pearson’s correlation test. The p values < 0.05 were considered statistically significant.

Sensitivity = \( \frac{a}{a+c} \times 100 \)

Specificity = \( \frac{d}{b+d} \times 100 \)

Efficiency = \( \frac{a+d}{a+b+c+d} \times 100 \)

PPV = \( \frac{a}{a+b} \times 100 \)

NPV = \( \frac{d}{c+d} \times 100 \)

Where

- \( a \) = True positives
- \( b \) = False positives
- \( c \) = False negatives
- \( d \) = True negatives
CHAPTER 3

RESULTS

3.1 Study population

Four hundred patients attending the special treatment clinic in Nairobi were enrolled between June and November 2001. All the patients who visited the clinic seeking treatment for various STIs during this period were enrolled. The stratification of study subjects was as shown in Table 2. There was no statistical difference in terms of HIV prevalence within the age groups.

Table 3. Human immunodeficiency virus prevalence within the age groups

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>N</th>
<th>HIV prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 24</td>
<td>82</td>
<td>35.37</td>
</tr>
<tr>
<td>25-29</td>
<td>126</td>
<td>35.71</td>
</tr>
<tr>
<td>30-34</td>
<td>85</td>
<td>42.35</td>
</tr>
<tr>
<td>35-39</td>
<td>52</td>
<td>46.15</td>
</tr>
<tr>
<td>≥ 40</td>
<td>53</td>
<td>33.96</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.68</td>
</tr>
</tbody>
</table>
3.2 Growth curves of MOLT-4/HIV MN, MOLT-4/HIV KS158 and MOLT-4 cells

The growth curves of MOLT-4 cells and HIV infected cell lines are shown in Figure 9. The HIV infected cells had the highest viable concentration on day three while the MOLT-4 clone 8 cells had the highest viable concentration on day 4. The log phases of all the cell lines were maintained for 3-4 days after which the cells lost viability.

3.3 Optimum concentration of MOLT-4 cells

The average number of cells within the photographic field, cell concentration and the amount of cell suspension mounted per well was as shown in Table 4. Five microlitre (5μl) suspension of 1.5 x 10^6 cells/ml and 3μl of 2 x 10^6 cells/ml had a better distribution of cells than 5μl or 3μl of 7.5x10^5 cells/ml and 1x10^6 cells/ml; 3μl of 1.5x10^6 cells/ml and 5μl of 2x10^6 cells/ml (Plate 1).
Figure 9. Growth curves of HIV infected and non-infected MOLT-4 cells

The graph shows the number of viable cells/ml plotted against days post-subculture. Three lines represent different conditions:
- MOLT-4 cells
- MOLT-4/HIV KS158
- MOLT-4/HIV MN

The graph indicates that HIV infection affects the growth pattern of MOLT-4 cells, with a peak at day 4 before a decline.
Table 4. Average number of cells within the photographic field

<table>
<thead>
<tr>
<th>Cell concentration (cells/ml)</th>
<th>Amount per well (μl)</th>
<th>Magnification</th>
<th>Average number of cells within the photographic field</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5x10^5</td>
<td>5</td>
<td>X200</td>
<td>27</td>
</tr>
<tr>
<td>1.0x10^6</td>
<td>5</td>
<td>X200</td>
<td>39</td>
</tr>
<tr>
<td>1.5x10^6</td>
<td>5</td>
<td>X200</td>
<td>58</td>
</tr>
<tr>
<td>2.0x10^6</td>
<td>5</td>
<td>X200</td>
<td>102</td>
</tr>
<tr>
<td>7.5x10^5</td>
<td>3</td>
<td>X200</td>
<td>17</td>
</tr>
<tr>
<td>1.0x10^6</td>
<td>3</td>
<td>X200</td>
<td>23</td>
</tr>
<tr>
<td>1.5x10^6</td>
<td>3</td>
<td>X200</td>
<td>27</td>
</tr>
<tr>
<td>2.0x10^6</td>
<td>3</td>
<td>X200</td>
<td>70</td>
</tr>
</tbody>
</table>
Plate 1. Distribution of MOLT-4 cells

(a) 1.5x10^6 cells/ml (5μl/well)

(b) 2.0x10^6 cells/ml (3μl/well)
3.4 Comparison of HIV antigen expression and production by MOLT-4/HIV MN and MOLT-4/ HIV KS158

The kinetics of HIV antigen expression over one week period of cultivation is shown in Figure 9. The MOLT-4/HIVMN had a higher antigen expression than MOLT-4/KS158. The MOLT-4/HIV MN cell line had higher antigen production than MOLT-4/HIV KS158 cell line (Plate 2). The addition of HMBA into the medium was effective at improving the antigen production of HIV in both strains.

3.5 Antibodies detected by particle agglutination assay

Four hundred plasma samples from patients seeking treatment at the special treatment clinic were tested for anti-HIV-1 antibodies by KEMRI HIV-1 PA kit. The PA results were scored as positive when there was a definite agglutination or negative when no agglutination was observed (Plate 3). Antibodies to HIV-1 were detected from 160 of the 400 patients tested (40%).

3.6 Antibodies detected by enzyme-linked immunosorbent assay

Four hundred plasma samples from patients seeking treatment at the special treatment clinic were tested for anti-HIV-1 antibodies by ELISA.
Figure 10. Kinetics of HIV antigen expression
### Plate 2. SDS-PAGE and Western blotting

<table>
<thead>
<tr>
<th>Marker</th>
<th>MN</th>
<th>MN + HMBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker</td>
<td>MN</td>
<td>MN + HMBA</td>
</tr>
<tr>
<td>Marker</td>
<td>MN</td>
<td>MN + HMBA</td>
</tr>
<tr>
<td>Marker</td>
<td>MN</td>
<td>MN + HMBA</td>
</tr>
</tbody>
</table>

**Protein staining with CBB**

**Silver staining**

**Western blotting with positive serum**

- 204kd
- 120kd
- 80kd
- 50.4kd
- 33.9kd
- 29.2kd
- 21.6kd
- 7.0kd
Plate 3. Particle agglutination results

KEY

A: Human immunodeficiency virus seronegative sample.

B: Human immunodeficiency virus seropositive sample.

The ELISA results were scored as negative or positive after calculating the cutoff value using the negative controls provided in the kit. Plate 4 shows the ELISA results. Antibodies to HIV-1 were detected from 170 of the 400 patients tested (42.5%).
Plate 4. Enzyme-linked immunosorbent assay results

1 2 3 4 5 6 7 8 9 10 11 12

A B C D E F G H

KEY

Wells 1A and 1B are the negative controls.

Wells 1C, 1D and 1E are the positive controls.

The remaining are experimental wells.

3.7 Antibodies detected by immunofluorescence assay

Four hundred plasma samples from patients seeking treatment at the special treatment clinic were tested for anti-HIV-1 antibodies by IFA. The IFA results were scored as positive when there was fluorescence and
negative when no fluorescence was observed (Plate 5). Antibodies to HIV-1 were detected from 151 (37.8%) of 400 patients tested.

3.8 Antibodies detected by western blot

Due to the cost limitations, only 36 samples were tested for anti-HIV-1 antibodies by western blot. The western blot results were scored as positive when there were specific reactions for HIV antigen lines (Plate 6). Antibodies to HIV-1 were detected from 7 (19.4%) of 36 patients tested.

3.9 Polymerase chain reaction testing for human immunodeficiency virus type 1

Human immunodeficiency virus type 1 pol/DNA sequences were detected in peripheral blood mononuclear cells from 5 (5.9%) of 17 patients tested (Plate 7).
Plate 5. Immunofluorescence assay staining of HIV-1 infected MOLT-4 cells

(a) Control spot reacted with a positive plasma (Mg x400)

(b) Control spot reacted with a positive plasma (Mg x1000)

(c) Test spot reacted with a positive plasma (Mg x400)

(d) Test spot reacted with a positive plasma (Mg x1000)
Plate 6. Western blot strips

**KEY**

Strips 1 to 8 were experimental strips.

Strips 9 and 10 were positive and negative controls respectively.
Plate 7. Electrophoretic patterns of PCR products

KEY

Well 1 is 100 base pair DNA ladder.

Wells 2, 3, 5, 10 and 12 are HIV DNA positive samples.
3.10 Determination of sensitivity and specificity of immunofluorescence assay using enzyme-linked immunosorbent assay, particle agglutination and western blot as the reference tests

3.10.1 Comparison of immunofluorescence assay and particle agglutination

Out of 160 samples that were HIV antibody positive by particle agglutination, 10 (6.3%) were HIV antibody negative by IFA. Out of 240 samples that were HIV antibody negative by PA, 1 (0.4%) was HIV antibody positive by IFA (Table 5). The IFA results agreed with those of PA with a consistency of 97.3% (389 out of 400). The sensitivity and specificity of IFA was 93.8% and 99.6% respectively, while positive and negative predictive values were 93.8% and 99.6% using PA as the reference test.

3.10.2 Comparison of immunofluorescence assay and enzyme-linked immunosorbent assay

Out of 170 samples that were HIV antibody positive by ELISA, 22 (5.8%) were HIV antibody negative by IFA (Table 6). The IFA results agreed with those of ELISA with a consistency of 93.8% (375 out of 400). The sensitivity and specificity of IFA was 87.1% and 98.7% respectively, while
Table 5. Sensitivity and specificity of IFA using PA as the reference test

<table>
<thead>
<tr>
<th></th>
<th>IFA</th>
<th>PA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>239</td>
<td>10</td>
<td>249</td>
</tr>
<tr>
<td></td>
<td>99.58</td>
<td>6.25</td>
<td>62.25</td>
</tr>
<tr>
<td>P</td>
<td>1</td>
<td>150</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>0.42</td>
<td>93.75</td>
<td>37.75</td>
</tr>
</tbody>
</table>

Total: 240 160 400

100.00 100.00 100.00

Sensitivity: 93.8%
Specificity: 99.6%
PPV: 93.8%
NPV: 99.6%
Efficiency: 97.5%

Where: N= HIV antibody negative
P= HIV antibody positive
Table 6. Sensitivity and specificity of IFA using ELISA as the reference test

<table>
<thead>
<tr>
<th>ELISA</th>
<th>IFA</th>
<th>N</th>
<th>P</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>227</td>
<td>22</td>
<td>249</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>3</td>
<td>148</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>230</td>
<td>170</td>
<td>400</td>
</tr>
</tbody>
</table>

Sensitivity: 87.1%
Specificity: 98.7%
PPV: 87.1%
NPV: 98.7%
Efficiency: 93.8%

Where: N= HIV antibody negative
P= HIV antibody positive
the positive and negative predictive values were 87.1% and 98.7% respectively using ELISA as the reference test.

3.10.3 Comparison of immunofluorescence assay and western blot

Out of 7 samples that were HIV antibody positive by WB, 2 (28.57%) were HIV antibodies negative by IFA. All 29 samples that were HIV antibody negative by WB were also HIV antibody negative by IFA (Table 7). The IFA results agreed with those of WB with a consistency of 94.4% (34 out of 36). The sensitivity and specificity of IFA was 71.4% and 100% respectively, while the positive and negative predictive values were 71.4% and 100% respectively.

3.11 Overall sensitivity, specificity, positive and negative predictive values of immunofluorescence assay

The overall sensitivity of IFA for anti HIV-1 antibody detection was 98.7% with a specificity of 99.6%. The positive and negative predictive values were 98.7% and 99.6% respectively (Table 8).
Table 7. Sensitivity and specificity of IFA using WB as the reference test

<table>
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<td>-------</td>
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<td>------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td></td>
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<td>28.57</td>
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</tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>0.00</td>
<td>71.43</td>
<td>13.89</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>29</td>
<td>7</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>100.00</td>
<td>100.00</td>
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</table>

Sensitivity: 71.4%
Specificity: 100%
PPV: 71.4%
NPV: 100%
Efficiency: 94.4%

Where: N= HIV antibody negative
       P= HIV antibody positive
Table 8. Sensitivity and specificity of IFA using the determined HIV status

<table>
<thead>
<tr>
<th>HIV STATUS</th>
<th>IFA</th>
<th>N</th>
<th>P</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>247</td>
<td>2</td>
<td>1.32</td>
<td>62.25</td>
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<td>100.00</td>
</tr>
<tr>
<td>P</td>
<td>1</td>
<td>150</td>
<td></td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>98.68</td>
<td>37.75</td>
<td></td>
</tr>
</tbody>
</table>

Sensitivity: 98.7%
Specificity: 99.6%
PPV: 98.7%
NPV: 99.6%
Efficiency: 99.3%
CHAPTER 4

DISCUSSION

4.1 Characteristics of the study population

The study population comprised patients seeking treatment at the sexually transmitted diseases clinic in Nairobi. These patients formed a high-risk group from which high HIV prevalence rate could easily be recorded. Using a battery of tests, the overall HIV prevalence in this group was found to be 38.7%. This figure contrasts sharply from the overall national prevalence of HIV with the most recent estimates at 13% (MOH, 2001a). This could be due to the fact that this was a high-risk group. The mean age of the patients in the study population was 30.53 years with the lowest being 16 years and the highest 60 years. There was no statistical difference \((p = 0.68)\) in HIV prevalence within the age groups.

4.2 Kinetics of HIV antigen expression and production

Human immunodeficiency virus has been successfully cultured in several cell lines (Farghani et al., 1991; Gallego et al., 1997). In this study, the antigen expression and production between MOLT-4/HIV MN and MOLT-4/HIV KS158 cell lines were compared to establish an ideal cell line to be used as a source of viral antigen for confirmatory IFA in HIV serology. The
data obtained shows that MOLT-4/HIV MN had a higher antigen expression and production than MOLT-4/HIV KS158. This suggests that the MOLT-4/HIV MN cell line can be used as the source of the viral antigen for HIV IFA confirmation. The significance of comparing the antigen expression and production was based on previous reports that have suggested the possibility that one could read a negative pattern of HIV-positive serum samples by IFA because of prozone and limited sensitivity. This effect may be due to limited antigen concentration in the smears (Lennette et al., 1987).

4.3 Comparison of anti-HIV-1 detection by immunofluorescence assay and particle agglutination

The sensitivity and specificity of IFA was 93.8% and 99.6% respectively using PA as the reference test. There was an overall concordance of 97.3% of the results. However, some discrepancies were found between IFA and PA. There were 10 (6.3%) samples that were HIV antibody positive by PA but negative by IFA. These findings are similar to a previous report (Yoshida et al., 1987) where it was found that 5 samples that were positive by PA were negative by IFA. The biological explanation for these discrepancies is not clear but may be due to the different sensitivities of the tests. Whereas the PA test can detect both IgM and IgG, the IFA test detected only IgG (Yoshida et al., 1987). Interestingly, when the 10
discordant samples between IFA and PA were tested by western blot, 9 were negative and one positive. This suggests that results by PA must be confirmed by another test. This positive sample was further tested with PCR and it was found to be negative.

4.4 Comparison of anti-HIV-1 detection by immunofluorescence assay, particle agglutination, enzyme-linked immunosorbent assay, western blot and polymerase chain reaction

There was an overall concordance of 94.4% between IFA and WB. A few discrepant cases were nevertheless noted between ELISA, WB, PCR and IFA. There were 4 samples that were ELISA positive but negative for WB, IFA and PCR. These samples are clearly false positives by ELISA. False positive ELISA results have been reported previously (Auwanit et al., 1991; Doran & Parra, 2000). All samples that were negative by WB were also negative by IFA. However, two samples were IFA negative but WB positive. Further confirmation of these samples by PCR showed that one sample was positive and the other negative. These findings are significant as further evidence of the potential sensitivity and specificity of the IFA technique.

The sensitivity and specificity of IFA was 71.4% and 100% respectively using western blot as the reference test. These findings are similar to
another report (Abraham et al., 1994) where the sensitivity of IFA ranged between 76.7% and 97.6% when the six major western blot interpretive criteria was used (O'Gorman et al., 1991). In this study the overall sensitivity and specificity of IFA was 98.7% and 99.6% respectively. In a previous study (Lennette et al., 1987) a sensitivity and specificity of 100% for IFA as a confirmatory test was reported. Similarly, Abraham et al., (1994) reported a sensitivity and specificity of 97.6% and 97.8% respectively when IFA was compared with western blot.

In this study, IFA had a PPV of 99.6% and 98.7% using PA and ELISA as the reference tests respectively. This is very important for HIV diagnosis since PPV measures the probability that a positive result indicates the presence of the disease. Specificities and sensitivities reported here for IFA show that it could be used for preliminary confirmation.

When compared with WB, IFA has the following advantages. The IFA is superior to WB with respect to the ease of use and rapidity (Asher & Wilber, 1990). The IFA takes less than two hours to perform making it significantly faster than WB test, which is usually an overnight test. The IFA can also be used in quantitative analysis of serum antibody. The in-house IFA reagents are relatively easy to prepare with a shelf life of several months (Lennette et al., 1987).
Furthermore, interpretation of fluorescence patterns in IFA is easy when a control cell is used to assess non-specific fluorescence. The simplicity, quick turnaround time and good reproducibility make the IFA an attractive or supplemental to WB as a confirmatory test for ELISA-positive sera/plasma.

Some limitations of the IFA include the subjective nature of interpretation, the need of a microscope with photographic attachment to document the results and the inability to differentiate the components of the virus to which serological response is targeted.

4.5 Use of polymerase chain reaction as a gold standard test

Out of the 5 HIV positive samples by PCR, 2 (40%) were IFA negative but were both ELISA and PA positive. Out of the 11 PCR negative results, 4 (36.4%) were positive by ELISA but negative by IFA and WB. There was a concordance of 88.2% (15 out of 17) between IFA and PCR. Two samples were IFA negative but were positive by PCR.

The use of PCR method to detect HIV-1 infection prior to seroconversion has been documented for high-risk homosexual men (Wolinsky et al., 1989), intravenous drug users (Farzadegan et al., 1993) and neonates born to HIV-positive mothers (Cattaneo et al., 1999). The PCR technique has been used to confirm HIV-1 infection in individuals with indeterminate
western blot profiles (Sethoe et al., 1995). Polymerase chain reaction can also be used to screen blood donors since it further narrows the window period. This ensures the safety of blood and blood products (Roth et al., 2000; Burnouf & Radojevich, 2000).
CHAPTER 5

CONCLUSIONS

1. The MOLT-4/HIV MN cell line has a better HIV antigen expression and antigen production than MOLT-4/HIV KS158.

2. The overall sensitivity and specificity of IFA were 98.7% and 99.6% respectively. The efficiency of IFA was 99.3%.

3. Immunofluorescence assay can be used as a primary confirmatory test.

4. Immunofluorescence assay is a rapid and reproducible test. It takes less than 2 hours to perform.
CHAPTER 6

RECOMMENDATIONS FOR FUTURE WORK

1. Since the HIV subtype prevalent in Kenya is subtype A, it is recommended that a persistently HIV-infected cell line using this subtype be established to determine its suitability as a source of viral antigen for IFA and other diagnostic tests.

2. It will be important to adapt HIV MN and HIV KS158 on different cell lines such as HeLa and H9 to compare their antigen expression and the suitability as sources of viral antigen for IFA.

3. It will be important to study the apparent intensity of immunofluorescent label and pattern in HIV infected cells using different cell lines depending on the day the smears are made and the serum/plasma antibody titer. This understanding may assist in improving the sensitivity of this test.

4. Since the IFA used in this study detected only IgG, there is need to adapt it to also detect IgM.
REFERENCES


**Centres for Disease Control (1982d).** *Pneumocystis carinii* pneumonia among persons with hemophilia A. *MMWR* **31**: 3667.


APPENDIX 1

STC STUDY FORM

STC No.---------------------

Sex-------------------------

Age-------------------------

District of origin--------------------------

Clinical notes-----------------------------------

101
APPENDIX 2

PA TEST WORKSHEET

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APPENDIX 3

ENZYME-LINKED IMMUNOSORBENT ASSAY WORKSHEET

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