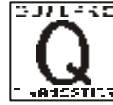


For the use of Registered Medical Practitioners and Laboratories only



TECHNICAL SERIES

Human Immunodeficiency Virus  
Perspectives



**QUALPRO DIAGNOSTICS**

Gitanjali, Tulip Block, Dr.Rego Bagh, Alto Santacruz, Bambolim Complex  
Post Office, Goa - 403 202, India. Tel.:(0832) 2458546-51 Fax:(0832) 2458544  
E- mail: tulip@goatelecom.com, Website:www.tulipgroup.com



...Setting trends



#### Other Technical Series published by TULIP Group

1. Quality Assurance for routine Haemostasis Laboratory
2. Monitoring oral anticoagulant therapy – Concepts & Practice
3. Lupus Anticoagulants – Basic concepts and Laboratory Diagnosis
4. Mycobacterium Tuberculosis – AFB staining, Culture and sensitivity
5. Anti Human Globulin Reagent - Basic Concepts and Practice
6. Syphilis Diagnosis
7. Turbidimetry - An Insight

#### Foreword

**Qualpro Diagnostics** is a part of the innovative **TULIP** Group of companies based at Goa, India.

The group's commitment in building products of international standards, through indigenous R&D has accorded the company virtual leadership in most product segments in the Indian marketplace. Its state-of-art manufacturing facility conforms to the strictest FDA (India) and GMP regulations. In its efforts to build world-class Quality products, the group has recently received the ISO 9001(2000) certification from TUV. It is this commitment to Quality, which has given the group international acclaim.

The products are now exported to over 45 countries globally with an ever-increasing user base. With decades of experience in *in-vitro* diagnostics (IVD), **TULIP** has created a strong knowledge base. **TULIP** believes that in the knowledge-based society of the 21<sup>st</sup> century, regular upgradation of knowledge is essential not only for better diagnosis and patient care, but also to improve the overall quality of life.

Publishing of **Technical Series** is one such initiative to make available to the Laboratory professionals and clinicians updated knowledge that is vital for them to set trends in their day-to-day practice.

## Background

Since its recognition in 1981, the HIV pandemic has spread unabated across continents. The catastrophic potential of the pandemic may still not have been fully realized as it continues to exact enormous toll throughout the world in both human and economic terms.

The demographic characteristics of those affected by the epidemic has changed drastically since the first cases were reported in the year 1981 in the USA comprising overwhelmingly of gay population. Today new cases of HIV infection originate predominantly through heterosexual contacts and injecting drug users. The infection rates amongst women, adolescent girls and children have increased dramatically. It is estimated that in the year 2001 a total of 40 million people worldwide were living with HIV/AIDS and in the year 2001 it is estimated that at least 5 million people were infected with HIV and at least 3 million people perished due to the disease.

The epidemic has negated decades worth of work on controlling diseases, improving nutrition and in many African countries it has become an unprecedented emergency with approximately 25% of the most productive age group between 15 years to 49 years being infected.

It is estimated that in sub-Saharan Africa the life expectancy would reduce from average 64 years to 47 years. If the present warning of epidemiologists go unheeded, South Asia, South East Asia and China may follow the disastrous course of Sub-Saharan Africa. The battle against AIDS has to be fought on multiple fronts such as education, counselling, prevention of infection, voluntary testing, treatment and vaccination programmes.



**TABLE 1:** Summary of the HIV / AIDS epidemic, December 2001.



HTS = Heterosexual, HMS = Homosexual, IDU = Injection Drug Use

**TABLE 2:** Regional HIV / AIDS Statistics, December 2000.

### The HIV Virus

The HIV-1 and HIV-2 belong to the retrovirus family, sub family lentivirinae. Both retroviruses are so named because one step of their replication cycle is the creation of DNA copy of their RNA genome. The genetic information of a retrovirus therefore flows from RNA to DNA at this step, which is in the opposite direction of the flow in most biological systems. HIV is a lipid enveloped positive stranded RNA virus like all Retroviruses.

RNA viruses are distinguished by the possession of an enzyme viral reverse-transcriptase that transcribes the viral RNA into provirus DNA which is integrated into the host cell genome.

### Origin of HIV

Lentiviruses other than HIV have been found in a wide range of non human primates. As a result of molecular analysis and analogy , it is now generally thought that the HIV-2 is similar to SIVsm (Sooty mangabey monkey) and the HIV-1 is similar to the SIVcpz (Pan troglodytes troglodytes chimpanzees), indigenous to the West and West Central Africa.

It is postulated that through Zoonosis these viruses at some point of time crossed over to the humans.

### The HIV Virion

The mature HIV-1 virion measures approximately 100 nm in diameter. The HIV-1 genome consists of two identical, noncomplementary sense (+) strands of RNA, each approximately 9.2 kb long, packaged in a cone-shaped protein core, composed of 24kDa capsid (CA) proteins (p24), surrounded by a unique bilayer envelope derived from the host cell membrane. The viral enzymes, Reverse Transcriptase, integrase, and protease, are packaged with the RNA in the core. The surface (SU) and transmembrane (TM) glycoproteins, gp 120 and gp 41, respectively, form a noncovalent complex. Also incorporated into the envelope are various host proteins including HLA class I and II DR molecules.

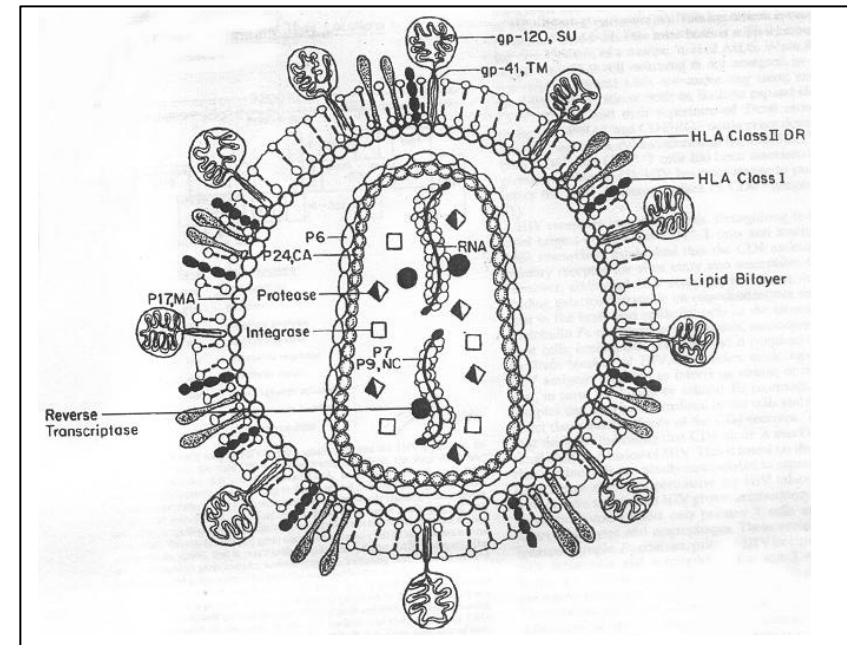


FIGURE 1: The HIV genome.

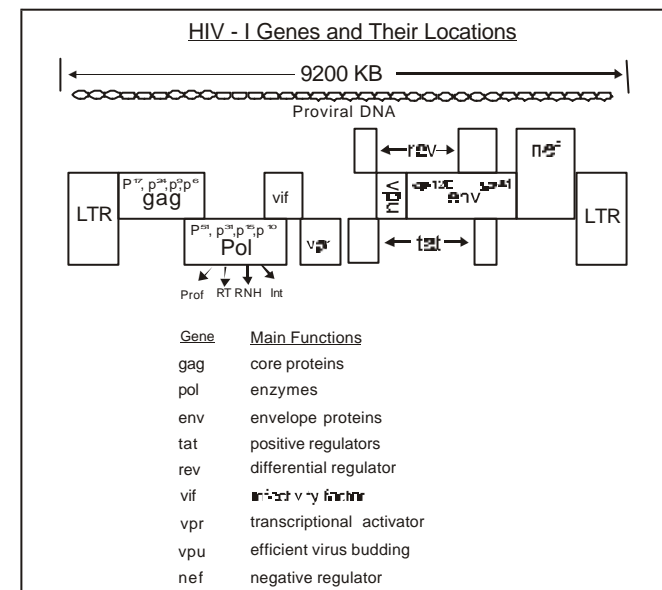


FIGURE 2: Genetic Map of HIV.

Biologically HIV-2 is very similar to the HIV-1, with the genome exhibiting about 60% homology in the gag and pol genes and about 30-40% homology in the other viral genes. It is recognized that HIV-2 is less easily transmitted than HIV-1 and the period between infection and illness is relatively longer in the case of HIV-2. While the HIV-1 is widespread globally, the HIV-2 is primarily found in West Africa.

### HIV Types & variants

The HIV virus is a term used to refer collectively to the HIV-1 and HIV-2 retroviruses which are distinct from one another.

The HIV-1 genome is heterogeneous and the envelope gene with its 5 hyper variable region V3 of the surface glycoprotein gp 120, encoding a central loop structure contains the most important site for antibody binding. The phylogenetic analysis of V3 sequences have lead to the identification of three major groups of HIV-1: M (major), O (outlier) and the most recently described group N (New).

Within the major group M, there are eight subtypes A through to J. As the virus continues to mutate and the global epidemic continues, new subtypes are expected to develop and be isolated.



The group O, of HIV-1 shares 55% to 70% homology with the major group M of HIV-1.

In Africa, most subtypes are found, although, subtype B is relatively less prevalent.

### Life Cycle of the HIV

Like all lipid enveloped viruses, the HIV must remain moist to be infectious and it is easily inactivated. For this reason, HIV is primarily transmitted sexually, by exchange of blood, or from mother to child perinatally or by breast-feeding or through sharing infected needles during injecting drug use.

After initial contact and attachment to the hosts immune system (e.g. lymphocytes, monocytes) post infection a cascade of intracellular events within the host are unraveled, the end product being the production of new viral particles, death of the infected cells and the ultimate devastation of the hosts immune system. The main steps in the HIV replication cycle are as follows:

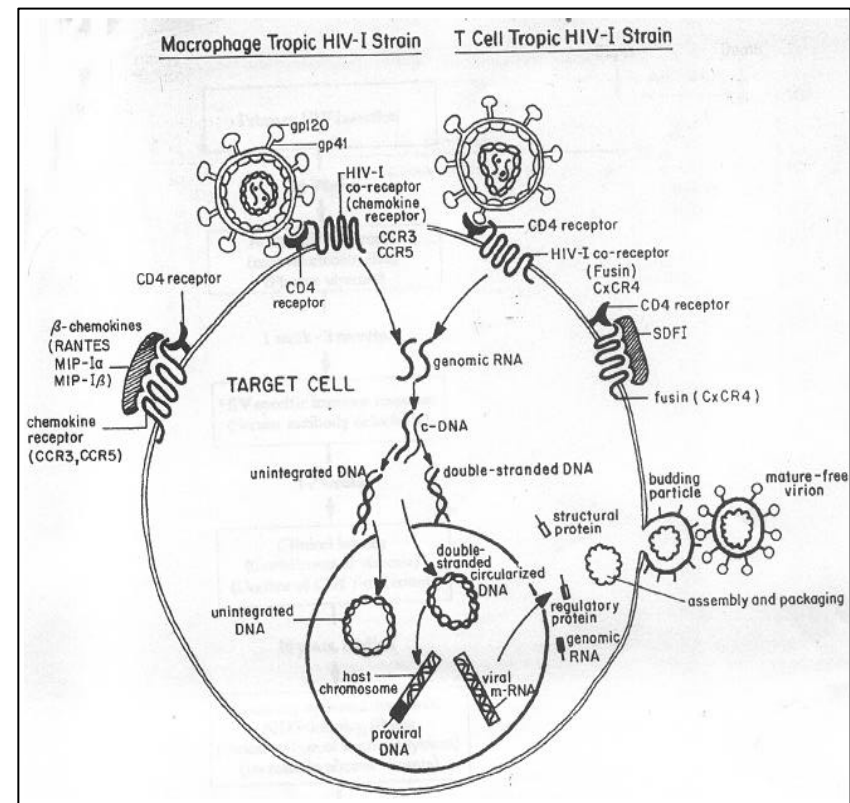


FIGURE 3: The HIV Replication Cycle.

## Functions of the HIV Gene



**TABLE 4:** Functions of the HIV Gene.

**Attachment to the Lymphocyte Membrane:** On the surface membrane of all living cells are complex protein structures called “receptors”. A receptor is often compared to a lock into which a specific key or “ligand” will fit. There are at least two receptors on T-lymphocytes to which the human immunodeficiency virus (HIV) locks on to. The primary receptor is called “CD4”. But a second receptor that loops through the cell membrane seven times is critical for infection to occur.

HIV infection of a lymphocyte requires attachment of the virus to the cell membrane through both of these “ligand receptor” links. In cells whose “7-transmembrane receptor” is different, the HIV “key” no longer matches the lymphocyte “lock” and attachment is incomplete. Such cells may avoid infection with HIV.

**Entry of the Viral RNA:** Tight attachment of the viral particle to receptors on the lymphocyte membrane enables fusion with the cell membrane. The viral contents including viral RNA then empty into the cell’s cytoplasm, like the other viruses.

**Reverse Transcription:** Converting viral RNA into DNA

An enzyme that is part of the human immunodeficiency virus reads the sequence of viral RNA nucleic acids that have entered the host cell and transcribes the sequence into a complementary DNA sequence. This enzyme is called “Reverse transcriptase” . Without reverse transcriptase, the viral genome cannot be incorporated into the host cell, and cannot reproduce.

Reverse transcriptase sometimes makes mistakes reading the RNA sequence. The result is that not all viruses produced in a single infected cell are alike. Instead, they end up with a variety of subtle molecular differences in their surface coat and enzymes. Thus through each replication cycle HIV surface molecules are continually changing.

### **Integration of Viral DNA:**

Once the viral RNA has been reverse-transcribed into a strand of DNA, the DNA can then be integrated (inserted) into the DNA of the lymphocyte. The virus has its own enzyme called “integrase” that facilitates incorporation of the viral DNA into the host cells DNA. The integrated DNA is called a provirus.

### **Transcription: Back to DNA**

As long as the lymphocyte is not activated or “turned-on”, nothing happens to the viral DNA. But if the lymphocyte is activated, transcription of the viral DNA begins, resulting in the production of multiple copies of viral RNA. This RNA codes for the production of the viral proteins and enzymes (translation) and will also be packaged later as new viruses.

### **Translation: RNA -> Proteins**

There are nine genes in the HIV RNA. Those genes have the code necessary to produce structural proteins such as the viral envelope, core and the enzymes like reverse transcriptase, integrase, and another crucial enzyme called protease.

**Viral Protease :** When viral RNA is translated into a polypeptide sequence, that sequence is assembled in a long chain that includes several individual proteins (reverse transcriptase, protease, integrase). Before these

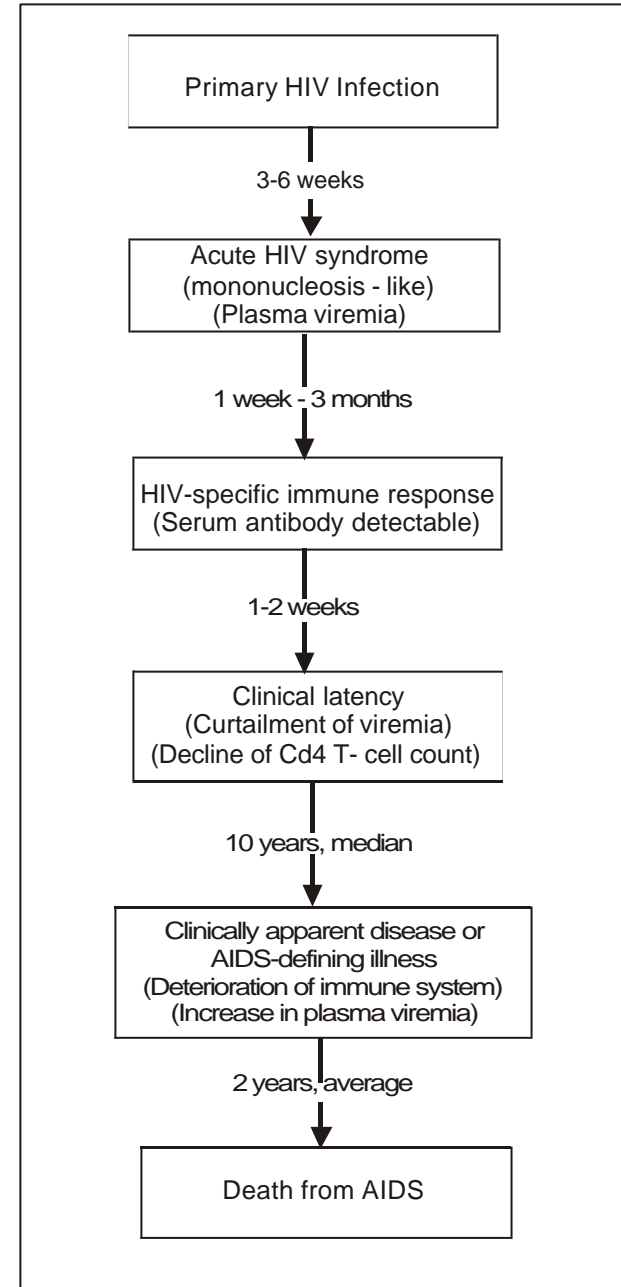
enzymes become functional, they must be cut off from the longer polypeptide chain. Viral protease cuts the long chain into its individual enzyme components, which then facilitates the production of new viruses.

**Assembly and Budding:** Finally, viral RNA and associated proteins are packaged and released from the lymphocyte surface, taking with them a swatch of lymphocyte membrane containing viral surface proteins. These proteins will then bind to the receptors on other immune cells facilitating continued infection and propagation.

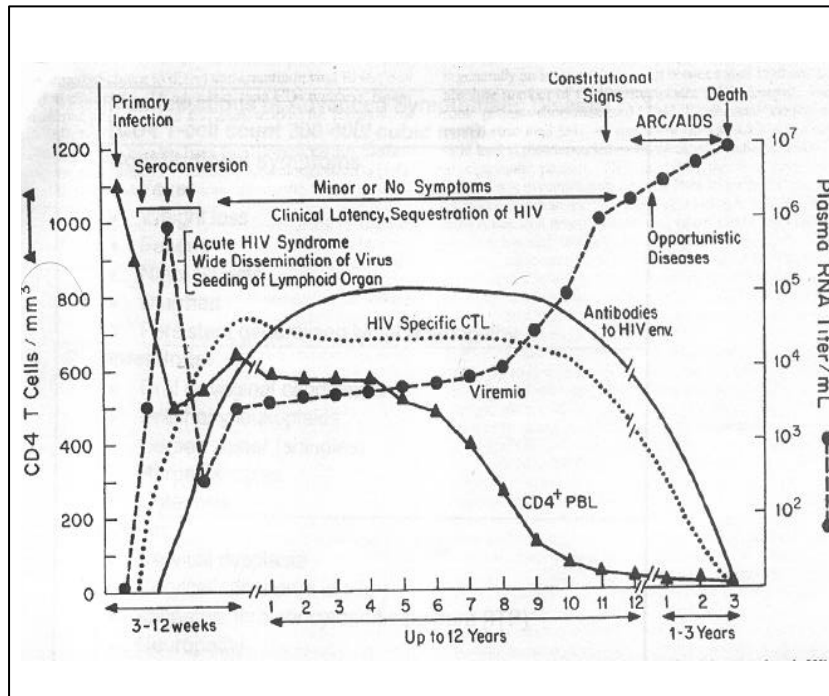
Budding viruses are often exactly like the original particle that initially infected the host, however, the resulting viruses also exhibit a range of variations.

During its replication cycle, the HIV is capable of mutating at a rate of approximately one nucleotide substitution per genome per replication cycle; making the virus capable of wide genetic diversity and flexibility to respond to a wide array of selection pressures.

As a consequence drug resistance and immunologic escape mutants are rapidly generated in infected persons through all stages of infection.



**FIGURE 4:** Natural History of HIV infection



**FIGURE 5:** Immuno Pathogenesis of HIV infection

The duration of clinical latency following primary HIV infection varies widely among individual persons depending upon many factors; such as exposure categories (blood, sexual contact, mother-to-child), portal of entry, viral pathogenicity and mutation, host resistance, intrinsic and specific immune responsiveness, new treatment strategies with combinations of potent antiretroviral drug therapy etc.

There are also some HIV-seropositive, long-term “non-progressors” who have continued to be healthy without evidence of disease or immune defects for ten or more years after primary HIV infection.

Although reliable clinical data are available for only the past 15 years or so, it is predicted that approximately 95% of HIV-1-infected patients will progressively develop AIDS within 15 years of infection. The first few weeks after primary infection are characterized by an acute mononucleosis or flu-like illness. During this stage seroconversion usually occurs and is

associated with a rapid increase in circulating viral titers. This is followed by virus dissemination to lymphoid organs. Adaptive host immunity, including neutralizing antibodies and cytotoxic T lymphocytes (CTL) against virus-infected cells, subsequently develop. While these responses may temporarily control the infection, generally they are unable to eliminate the virus. The patient then enters the stage of clinical latency or the asymptomatic period. A progressive decrease in CD4+ peripheral blood lymphocytes (PBL) continues during the latency stage. When CD4 counts fall below 200/mm<sup>3</sup>, the disease usually enters a symptomatic phase, characterized by opportunistic infections and other AIDS-defining conditions in the host.

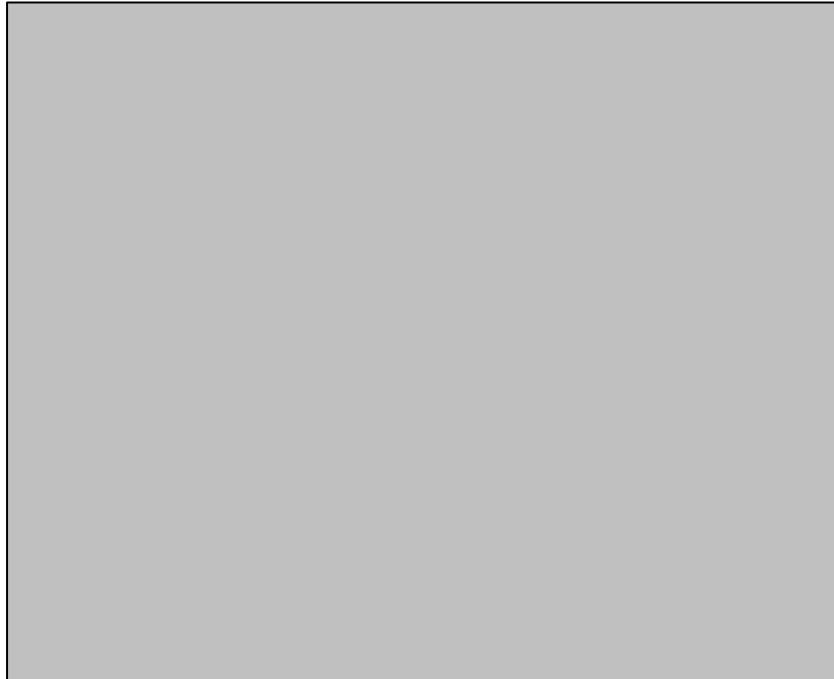
### Clinically apparent disease

This clinically apparent, symptomatic stage is a consequence (secondary manifestation) of the progressive and profound deterioration of the immune system that occurs over time in most patients with HIV infection. The CD4 T-cell count continues downward to the range of 200-400/ cubic mm. Plasma viremia and p24 antigenemia approach high levels such as seen in the primary infection. Some of the constitutional symptoms, opportunistic infections, and other manifestations of advanced symptomatic HIV disease are given in the following table.

TA

--

As the CD4 T-cell count continues to fall, sometimes precipitously, and reaches levels that define AIDS (CD4 T-cell count less than 200/cubic mm.), predisposition to AIDS-defining conditions, such as opportunistic infections caused by viruses, bacteria, fungi and protozoa, neoplastic disease, HIV encephalopathy, wasting syndrome, and progressive multifocal leukoencephalopathy that complicate the clinical course and are often the cause of death.



**TABLE 6:** List of CDC conditions.

### **HIV SPECIFIC PROGNOSTIC MARKERS**

Several HIV specific markers can be used for disease prognosis. Of these declining levels of antibody to p24 (anti-p24) and increasing concentrations of HIV antigen, especially the p24 antigen have been most widely used.

Levels of antibody/antigen however can vary widely between individuals and therefore sequential tests are usually necessary to confirm a trend. Indeed some patients are not measurably antigenaemic until later on in

the disease progression, where as others may be antigenaemic for many years. There is still a debate as to the mechanism of these changes. A decline in anti p24 levels could lead to reduced formation of antigen – antibody complexes and hence allow detection of the p24 antigen. Alternatively, the developing p24 antigenaemia may reflect increased virus replication. As the infection progresses, the amount of viral RNA and infectious virus in plasma increases and the proportion of peripheral blood mononuclear cells carrying the provirus also increases.

The prognostic significance of p24 antigenaemia is indicated by a four-fold increase to the progression to AIDS within 3 years, in those who show circulating p24 antigen. However only about 50 per cent of patients with ARC and approximately two-thirds of patients with AIDS will be antigenaemic.

A decrease in titre of anti p24 antibody is also associated with disease progression and has been shown to occur before the rise in p24 antigen levels. The decline in titre of anti p24 antibody may therefore be an early indicator of disease progression.

### **NON SPECIFIC MARKERS OF HIV PROGRESSION**

Markers other than HIV specific antigens and antibodies can be of use in predicting and assessing progression to AIDS.

At present the most commonly used marker is the CD4 cell count, expressed either as an absolute value (normal adult range 600-1700/mm<sup>3</sup> or as a CD4:CD8 ratio (normal adult ration 1.2 to 3.5).

Similarly, when CD4 cell counts fall to 200/mm<sup>3</sup> or less, prophylaxis against pneumo cystic carinii is generally initiated.

Measurements of cell counts are, however, subject to potentially large biological and laboratory variation and interpretation should be based on more than one result. A recent prospective study has suggested that combining measurements of the levels of either serum  $\beta$ 2-microglobulin or neopterin along with the CD4 cell count improves its prognostic

significance. It is likely that a combination of markers rather than a single marker will prove useful for the monitoring of disease progression. The precise role and practicality of this approach requires further evaluation.

* CD4 Cell Count
* <b>CD4/CD8 Ratio</b>
CD8 Cell Count
Serum Interferon
Serum IgA
Serum IgE
* Serum $\beta$ 2-microglobulin
Serum/urine neopterin

\* Denotes more commonly used marker

**TABLE 7:** Non-specific markers of HIV Progression

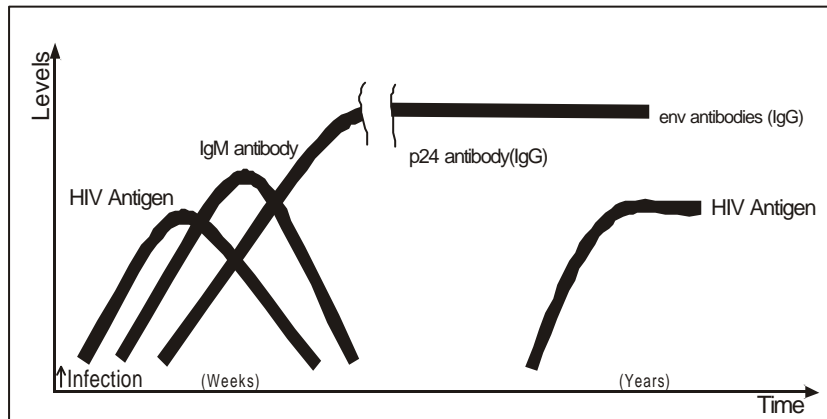


Figure represents the idealized sequence of serological events during HIV infection progressing to AIDS.

**FIGURE 6:** Serological Profile of HIV Infection

Following infection there is evidence of active viral replication as virus can be isolated from cultures of peripheral blood mononuclear cells and p24 antigen can be detected in the serum by immunoassays and viral RNA based assays. This antigenaemia may only last for a week or two and disappears when the antibody response develops following seroconversion.

The timing of collection of any blood samples is crucial as prior to or at seroconversion antigen is detected in less than 50% of samples. Seroconversion occurs in most patients by 4-8 weeks after exposure although in some cases antibody may not appear until 6-9 months later. This delay between infection and appearance of antibodies is termed as the "Window Period". At seroconversion, antibody of the IgM class can be detected. Analysis of the reactivity of the IgG response by immunoblotting or enzyme immunoassay based on recombinant proteins shows that antibodies develop rapidly to the principal structural proteins e.g. the envelope glycoproteins gp 160, gp 120 and gp 41 and the core proteins p18, p24. Antibody to the polygene proteins can also be detected. Antibodies to the envelope glycoproteins gp 160, gp 120 and gp 41 in case of HIV-1 and gp 36 in case of HIV-2 infections may appear before antibodies to the core proteins although the time difference is generally only a matter of days or a few weeks.

After seroconversion there is evidence of continued virus replication as virus can be cultured from the plasma as well as detected by PCR based RNA assays. Indirect evidence is provided by the progressive increase in the level of antibodies to all the major virus components during this time.

In neonates the serological profile is complicated by the presence of the maternal anti-HIV antibody.

For some years after seroconversion the patient may remain well or develop persistent generalized lymphadenopathy. Throughout this period viraemia may increase. A reduction in the level of antibody to the p24 core protein and the appearance of p24 Ag are associated with progression to clinically overt disease. In the later stages of AIDS, as the immune system is destroyed, antibody may not be detectable in some patients.

**HIV Diagnostic Testing techniques**

As has been discussed that virtually all individuals who become infected with HIV mount an IgG antibody response to the virus which current immunoassays will detect by 4 to 8 weeks after primary infection and in

the majority of cases for the remainder of the individuals life. Screening and diagnostic tests thus rely upon the detection of antibodies to HIV. During part of the window period IgM anti-HIV and HIV Ag may be present, but specific screening for them though desirable is not cost effective in low prevalence populations.

During the search for the causative agent of AIDS, tests for anti-HIV detection were developed. Initial testing was by Western Blot analysis. However, the magnitude of the HIV epidemic and the need for extensive screening were not fully understood. By 1983 and 1984, it had become apparent that rapid, simple and inexpensive assays would be required. Research assays were developed and in 1985 several manufacturers introduced enzyme immunoassays for blood donor screening. In the ensuing years these assays have evolved rapidly by utilizing products of modern biotechnology, for screening and confirmation of HIV infection.

**Enzyme Immunoassays (EIA)**

The diversity of EIA formats makes a simple, meaningful definition difficult to formulate, but EIA's can be described as a binding assays that depend upon the antigen-antibody reaction as the basis and the enzyme reaction as a marker for the proof of reaction. Anti-HIV EIA kits in various formats are available. Obtaining high quality results not only depends upon the quality of the kit but also on strict adherence to kit protocols, well-maintained equipments and good performance assurance procedures. Under these conditions many assays currently available will achieve >99% sensitivity and specificity. Typically, a batch of tests takes 1.5-3 hours to complete. Since the EIA's detect Anti-HIV antibodies, the solid phase on the microwell is coated with HIV specific proteins.

**Generation of Assays**

The terms first, second and third generation have been applied to anti-HIV assays according to the source of the HIV Ag used on the solid phase as opposed to sequential improvements.



**TABLE 8:** Generations of Anti HIV Assays

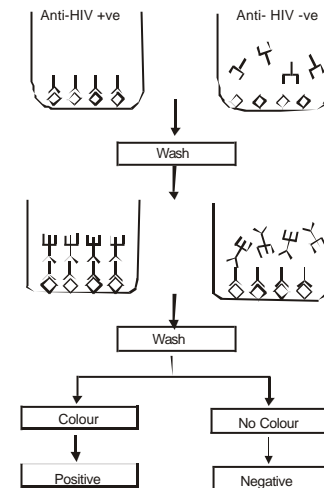
First generation assays may lack specificity because of reactions with contaminating cell proteins and lack sensitivity due to the high sample dilution needed to attain acceptable specificity. For second and third generation assays, the identification and sequencing of important antigenic sites, the expression of proteins of precisely targeted portions of HIV cDNA, and the synthesis of oligopeptides containing immunodominant epitopes has provided new antigens for use in the assay systems. When incorporated into EIAs these recombinant proteins and peptides have led to substantial improvements in sensitivity and specificity and have the added advantage of being safer, easier and more reproducible in performance.

**EIA Assay Formats**

There are four basic assay formats for the detection of antibodies to HIV

**Type 1: Indirect or Antiglobulin**

A relatively crude native HIV antigen preparation is immunoabsorbed to the solid phase by pre-coating with HIV antibody. Anti human globulin conjugated to enzymes is used as a tracer, with a compatible substrate as a chromogenic marker.



**FIGURE 7:** Indirect or Antiglobulin assays

### Type 2: Competitive Assay

In Competitive assays the sample need not be diluted. These assays exhibit high sensitivity. To ensure good specificity and sensitivity the spectrum of anti-HIV detected is predominantly against env. determinants, but because antibodies to env antigens of HIV-1 and HIV-2 show only limited cross reaction the competitive format is unsuitable for combined anti-HIV-1/ HIV-2 screening.

### Type 3: Class Specific Antibody Capture Assay

Class Specific Antibody Capture assays are only available as a research tool. They have proved to be highly accurate for detecting anti-HIV in specimen such as saliva and urine and find important applications in epidemiological research and other situations where venipuncture is impractical.

### Type 4: Double Antigen Sandwich or Immunometric Assay

A commercially available example of this assay detects anti-HIV-1 & anti-HIV-2 by using recombinant HIV-1 antigens and synthetic oligopeptide antigens from the HIV-2.

These assays are sensitive and specific, an added advantage being that they do react with IgM anti HIV antibodies and they are reported to detect seroconversion earlier.

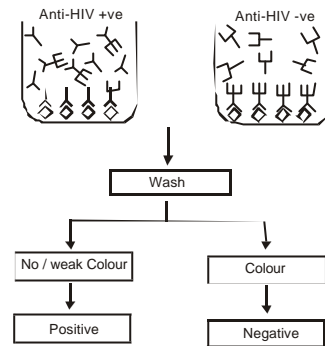


FIGURE 8: Competitive assays

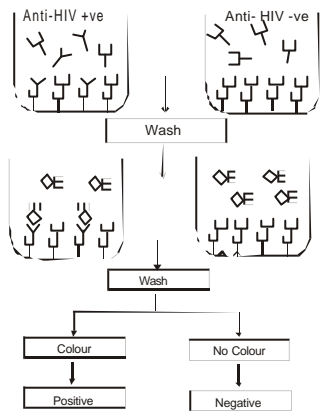


FIGURE 9: Class Specific Antibody Capture Assays

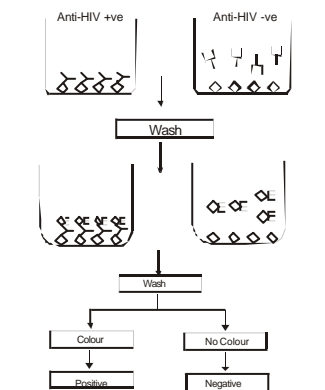


FIGURE 10: Immunometric Assays

### HIV Antigen EIAs

HIV Antigen EIAs have been developed primarily for the purpose of monitoring HIV-infected individuals for antigenaemia, but are also used to monitor growth of HIV in lymphocyte cultures. The methods used are in general modifications of antibody sandwich assays described earlier.

Anti-HIV prepared from the serum of HIV infected individuals, animal antisera raised against purified viral or recombinant antigens and monoclonal antibodies have all been used in various combinations as capture and detector antibody as well as a variety of enzymes and substrates.

The sensitivity of HIV Ag assays can be below 50 pg/ml. It should be noted, however, that standards do vary from one commercial kit to another reflecting the unsuitability of standardizing by weight of a complexed antigen, which will be, measured immunometrically.

Estimations of HIV antigen level can, therefore, be regarded as 'semi quantitative'. Most manufacturers do provide HIV antigen standards, which can be included in assay runs in order to produce a standard curve from which specimen HIV antigen levels can be extrapolated. Despite the 'semi quantitative' nature of the estimation, sequential monitoring of titres may be informative regarding disease progression or efficacy of antiviral therapy.

Confirmation of positive findings is recommended and some manufacturers have reagents available for this purpose, usually a blocking antibody.

### Confirmatory / Supplementary Assays

Confirmatory tests are used to confirm whether specimens found reactive with a particular screening test contain antibodies specific to HIV. The most commonly used confirmatory test until recently, was the Western Blot (WB). However, its use has proven to be very expensive and prone to producing large numbers of indeterminate results. The differences in criteria for the interpretation of Western Blot also has lead to classification difficulties of indeterminate samples. Now, studies have shown that combinations of ELISAs or Simple/Rapid assays can provide results as reliable as the WB at a much lower cost.

### 1. Western Blot

The Western Blot was developed to detect antibodies to HIV and to qualitatively determine to which viral proteins the antibody response had been raised. The assay is based on the detection of the reaction of specific antibodies in the sample with viral antigens that have been electrophoretically separated and transferred ("blotted") onto a nitrocellulose sheet on a backing. Antibodies, which react with antigens on the membrane, are visualized with an enzyme labelled anti-human immunoglobulin and an enzyme substrate, which gives an insoluble coloured product at the site of reaction.

Although WB allows the humoral immune response to HIV to be mapped, there are several problems: Not all sera from infected individuals react with all HIV proteins. Sera from some uninfected individuals may react with one or more HIV proteins or with contaminating residual lymphocyte cell proteins. Large differences between batches and particularly between manufacturers occur in the number of proteins and their relative concentrations and positions on the strips. The assay is read visually. To counter these difficulties, some standardization has been attempted, particularly in terms of interpretation of the reactivity observed. Several sets of criteria have been recommended which differ in details, but commonly require an antibody to at least one env. gene product, and at least one band from gag and /or pol for classifying a positive test result.

#### Criteria for Interpretation of Western Blot



TABLE 9. Criteria for Interpretation of Western Blot Assay

Results which neither satisfy 'Positive' nor 'Negative' criteria are termed as 'Indeterminates'.

Recently, immunoblots, which resemble WB but utilize a selection of synthetic HIV antigens applied to membrane strips as discrete bands have been developed (LIA / RIBA). These are reportedly comparable to the electrophoretically manufactured traditional blots, with reduction in the proportion of indeterminate results. However these are equally expensive.

**2. Indirect immunofluorescence: IF** has been commonly employed to detect anti-HIV antibodies. Acetone fixed HIV infected lymphocytes are incubated with sample, followed by a fluorescein conjugated anti-human IgG. The cells are then examined using fluorescent microscopy. If anti-HIV is present a typical pattern of fluorescent staining is seen in the cells. **IF** is usually reserved for confirmation as it is time-consuming and requires highly-skilled operators and an expensive, well-maintained fluorescence microscope.

**3. Radioimmunoassay: RIPA** is used by very few laboratories. Initially, HIV Ag is metabolically labelled by substituting one or more S tagged amino acids in HIV culture medium. The labelled cells are then disrupted to release the labelled proteins and the resulting suspension is clarified. The labelled proteins are used for testing the sample. Containment facilities for handling live HIV and radioisotopes are required along with expensive equipment and consumables for immunochemistry, limiting its usage to few reference laboratories.

### Virus Detection Methods

Virus Isolation has traditionally been achieved by co-cultivation of gradient purified patient's peripheral blood lymphocytes with stimulated lymphocytes from an uninfected individual. To seek evidence of viral replication the cultured cells may be examined by microscopy for cytopathic changes and by HIV immunofluorescence. The culture medium may also be assayed for HIV p24 Ag or reverse transcriptase activity. Isolation of HIV indicates beyond any doubt that the subject is infected with HIV. However, it has a very restricted role to play in confirmatory testing because of its relatively low sensitivity and the cost and complexity of the technique. HIV can sometimes be isolated from fresh or properly stored plasma, but usually a fresh blood sample will be required to provide peripheral blood mononuclear

cells for co-cultivation. Very few laboratories claim nearly 100% HIV isolation from all anti-HIV positive subjects, but most achieve 60-80% success. Virus isolation is generally reserved for research purposes due to its expense, low sensitivity, long incubation time and need for high containment facilities.

#### p24 Antigen and Nucleic Acid Technologies

A number of other assays have been introduced in recent years which assist in the establishment of the diagnosis of HIV infection and may also be used to monitor the progress of the infection and the response to therapy. These include assays that detect virus particles e.g. the HIV p24 antigen ELISA, or the presence of HIV viral nucleic acid sequences (RNA or DNA) by means of nucleic acid amplification techniques. NAT assays have made it possible to detect minute amounts of viral material - as little as a single viral genome (the detection limit for most assays is around 300 copies/ml. These sensitive procedures are well suited for early diagnosis of mother-to-child transmission and for monitoring the viral load of patients who are on antiretroviral therapy. However, in routine practice this technique can have limited specificity, needs sophisticated equipment, rigorous laboratory conditions and highly trained staff, and is still very expensive.

#### Rapid tests for the detection of anti-HIV antibodies

Advances in technology have now made it possible to design rapid HIV antibody assays based on agglutination, immunodot, immunochromatographic and immunoconcentration / filtration techniques, the assays have comparable sensitivity to equivalent generation EIAs.

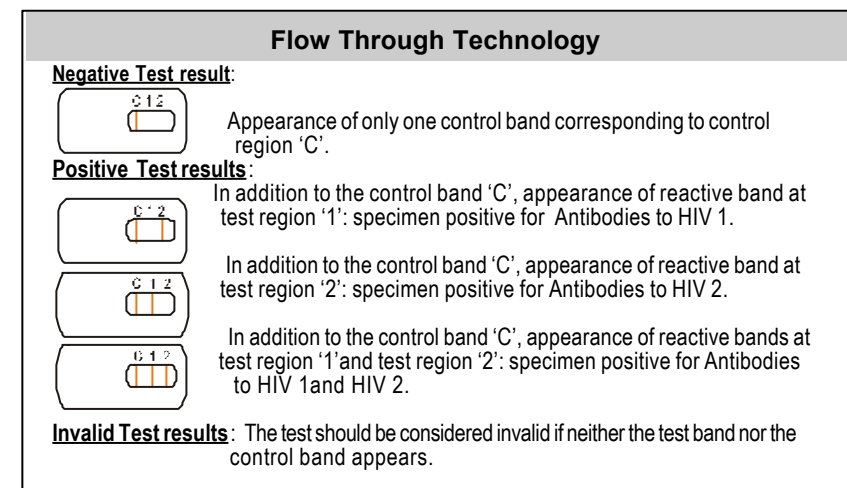
The assays are easy to perform and ideal for low through put laboratories. Since same day results are offered through the use of these tests, they help in timely detection and treatment intervention of HIV infection.

The overall considerations in rapid test assay development are as follows:

1. The tests should pick up antibody response to HIV-1 & HIV-2.
2. The test when used in areas endemic with variants of HIV 1 such as O & M (Cameroon, Gabon) should be reactive with the antibodies to the variants.

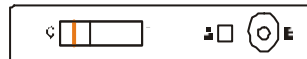
3. The test should increase the sensitivity of detection during the “window period” shortening it as much as possible.
4. The antigenic configuration of the test should preferably use recombinant antigens or peptides from the conserved immunodominant regions of the HIV-1 and HIV-2 genome.
5. The screening test should have sensitivity and specificity in excess of 99%.

Well designed rapid tests based on protein A based tracers that pick up IgG class anti-HIV antibodies bound to the solid phase, in general achieve such specifications. However since protein A based assays necessarily map only IgG class anti-HIV antibodies, they tend to be less sensitive than systems having the ability to pick up IgG and IgM class of Anti-HIV antibodies. Third generation rapid tests now utilize the principle of **double antigen sandwich immuno assays**. In the double antigen sandwich system recombinant proteins from the immunodominant regions of the HIV genome are used on the capture as well as on the tracer reagent. Antibodies to HIV are thus specifically captured and sandwiched during the test specifically between the solid phase and tracer antigens. This third generation system not only improves specificity but also improves sensitivity of the assay since not only IgG but also IgM antibodies react in the system, improving the test sensitivity in the early seroconversion phase of the HIV infection. Such improved third generation rapid tests are being made available now.



### Lateral Flow Technology

#### **Negative test result:**



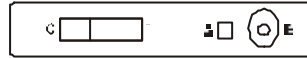
Appearance of only one coloured band at the control region 'C'.

#### **Positive test result:**



Appearance of a coloured band at the test region 'T' in addition to the band at control region 'C'.

#### **Invalid Test results:**



The test should be considered invalid if neither the test band nor the control band appears.

### **Flow Through v/s Lateral Flow Technology**

Rapid test currently available are based either on the second generation protein A based flow through systems or the third generation lateral flow based double antigen system. The flow through technology based tests are multicomponent, multistep based systems that require cold storage. These tests also require higher degree of laboratorial involvement and can only use serum or plasma as a sample. Since the membrane needs to be rehydrated before use there is a limit on the number of tests that can be run at a time simultaneously.

On the other hand lateral flow technology based double antigen sandwich assays are single step / two step and easier to use. Suitability of whole blood as a sample and room temperature storage in addition to higher clinical sensitivity due to anti-HIV IgM detection capability make these tests a method of choice for most settings. These assays have an overall advantage over flow through technology based tests.

### **Application of HIV Testing**

The donor screening tests for anti HIV have been also adopted for clinical diagnosis and for epidemiological prevalence and monitoring studies. Because the consequences of a positive diagnosis of HIV infection are so serious it is generally considered that people who are tested should be asked to give consent to testing only after counselling, which in simple and sensitive terms explains the background to the screening test, the

implications of a positive finding and advice on risk avoidance. Follow-up clinical and psychological support for those found to be infected is also essential. Of equal importance is a mechanism for distinguishing which of those samples that react in screening assays are genuine, or true positives.

Screening tests for anti-HIV-1 will not detect all HIV-2 infections and the discovery of HIV-2 infections outside of its recognized endemic regions in West Africa has influenced the decision by many countries to introduce tests which can detect antibody to HIV-2 reliably. There are now many 'combined' HIV-1/HIV-2 EIAs and rapid tests that have become the screening method of choice. Simultaneously test that can in addition detect the antibody to subgroup 'O' are considered "complete" as a screening test.

### **Screening**

Anti-HIV screening assays were primarily developed for blood bank screening with the objective of protecting the recipients of blood or its products from infection with HIV. In addition to screening, vetting of donors and phasing out of inducements to donate are now strongly recommended by W.H.O. However, in regions where HIV has spread primarily by heterosexual contacts, vetting of donors is difficult as 'high-risk' groups are not easily recognized and more reliance is therefore placed on anti-HIV screening. Nevertheless, anti-HIV screening alone is insufficient to ensure that the blood supply is free from HIV contamination. Even if the assays were 100% sensitive, technical and clerical errors can lead to reporting errors.

Many considerations affect the choice of screening assay including sensitivity, specificity, ease of performance and cost. Poor sensitivity may jeopardize the safety of recipients of blood or other tissues while, on the other hand, poor specificity is costly in terms of unnecessarily wasted donations, the cost of extra confirmatory testing and the problems of management of donors whose samples are reactive. Limited resources have led some transfusion services to consider the screening test simply as a means to protect recipients. Many however, are also able to assume responsibility for their donors, ensuring that screen-reactive samples are

confirmed before counselling the donor and offering referral to an expert HIV clinic.

Worldwide application of anti-HIV screening assays outside the strictly clinical arena are increasing: many life insurance companies require anti-HIV screening before offering cover to applicants, and some authorities require HIV testing before issuing marriage licences, immigration documents or admitting recruits to armed services. Some controversy surrounds these applications of HIV screening with respect to their moral, ethical and legal justification. If carried out, confirmation of a screen reactive finding is an absolute requirement, especially as these tests are applied to populations in which HIV prevalence is generally low and therefore false positive reactions are common relative to genuine positive reactions.

### **Diagnosis**

When screening tests for anti-HIV first made it possible to identify HIV-infected individuals who did not manifest the classical signs and symptoms of AIDS, there was some debate whether testing was worthwhile for them. However, improvements in predicting the progression of disease by use of prognostic markers and continuous improvements in treatment, both with Antiretroviral and prophylactic treatment of opportunistic infections, now favour the policy of early identification of all HIV-infected individuals.

There are three main diagnostic applications of anti HIV testing:

- (a) To confirm that a patient presenting with symptoms associated with HIV infection is infected with HIV
- (b) To investigate a person who has been exposed to infection because of exposure to body fluids from a known HIV-infected individual, perhaps by sexual intercourse or by inoculation
- (c) And to test those who belong to one of the recognized risk groups, are healthy and have no definite history of exposure, but are anxious and present themselves for testing in the hope of gaining the reassurance of a negative result.

Many diagnostic laboratories now take the precaution of running two screening tests (usually different manufacturers kits) on each sample to

decrease the possibility of false negative findings, whether due to a technical or clerical error or some inherent fault in an assay.

### **Approach to Confirmatory Supplementary Tests**

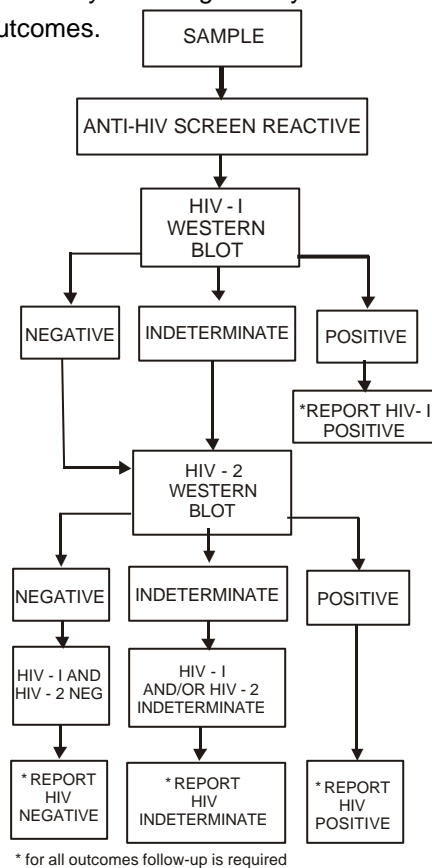
The best of the currently available anti-HIV screening assays will give rise to less than 1 in 1,000 repeatable false positive reactions. Although this represents excellent specificity, when such a test is applied to a low prevalence population only a small proportion of reactive samples will be genuine. To avoid the potentially serious consequences of a false diagnosis, several strategies have been devised for further testing to determine the authenticity of a positive screening reaction. This is referred to as confirmatory or supplementary testing. It is generally accepted that only positive and unexpected negative results in screening assays need to be confirmed. So that unnecessary confirmatory testing is not undertaken, most laboratories first retest reactive samples twice or more in the screening assay. Unrepeatable reactions are reported as negative. Whatever confirmatory strategy is employed, it is good practice to seek a follow-up sample from any individuals found to be reactive. This is to primarily safeguard against a false diagnosis due to technical or clerical errors, but also to check that an unconfirmable positive reaction on a screening assay is not a result of sampling too early during seroconversion. Cases of dual infectivity with HIV-1 and HIV-2 have been reported and thus in some instances this may require confirmation.

### **Western Blot (WB)**

The most widely accepted 'confirmatory' test is Western Blot. A reaction in a screening assay simply indicates that antibody from the specimen is bound to the solid phase: this can be an HIV-specific or non-specific reaction. WB provides additional information about specificity, in terms of reactivity with particular HIV proteins. The reaction pattern obtained may be interpreted as positive, negative or indeterminate according to the application of one of several sets of accepted criteria. The disadvantages of WB are its cost, subjectivity and high rate of indeterminate reactions. Stringent criteria, while achieving high specificity, lead to low sensitivity. Thus, some sera from infected subjects only give indeterminate results. Conversely, low stringency criteria lead to some false positive diagnosis by WB. A further disadvantage is that criteria have usually been determined

using a particular manufacturer's product. When these are applied to a different WB they may not be valid.

Line immunoassays resembling WB and 'dot-immunoblots', each using several synthetic antigens derived from different HIV genes are now being widely used. They are usually more rapid and at least as sensitive as WB. Interpretative criteria vary but are generally similar to WB, with the same three possible outcomes.



**FIGURE 11:** Approach to Confirmatory tests.

### NAT-PCR based Methods

The NAT-PCR based assays detect the nucleic acid of HIV directly in an infected patient's sample and is employed as a confirmatory method. The

expense and technical demands of PCR will, for the foreseeable future restrict its use to carefully selected cases and to providing support to investigations of simpler, less expensive confirmatory strategies.

### Alternative Approach to Confirmatory Testing

This approach seeks to determine if a sample reaction in a screening test will also give a positive signal in at least two further 'independent' screening assays. Assays were originally defined as 'independent' if they were each of a different type. But this concept has recently been broadened to incorporate assays using antigens from different sources, eg. viral, recombinant DNA expression products and synthetic peptides. Just as there have to be trials of WB to develop accurate interpretive criteria, particular combinations of assays must be examined to demonstrate that they are generally independent of each other, i.e. they are not susceptible to the same non-specific effects.

It has recently been shown that a combination of rapid screening assays can also give diagnosis as accurate as WB, and more cheaply. This option should be particularly useful for anti-HIV confirmation especially in the developing world, as a cost effective solution.

### WHO / UNAIDS strategies for HIV antibody screening

These three strategies take into account the objective of testing (i.e. whether for transfusion safety, surveillance or diagnosis) and the local seroprevalence rate. Depending on these factors, three different algorithms for repeat and confirmatory testing have been proposed to confirm initial screening results.

They avoid the use of the Western Blot for confirmation of positive screening test result; although Western Blot confirmation is still the rule in the USA, Germany and other countries, it can be replaced by more economical testing algorithms using ELISA-type assays and / or rapid / simple test devices.

**Strategy 1 requires only one test. It is to be used for:**

- **Transfusion screening** (if no results are to be provided)

- **Surveillance** (if prevalence > 10% and no results are to be provided)
- **Diagnosis** (only if patient is symptomatic and population HIV prevalence > 30%)

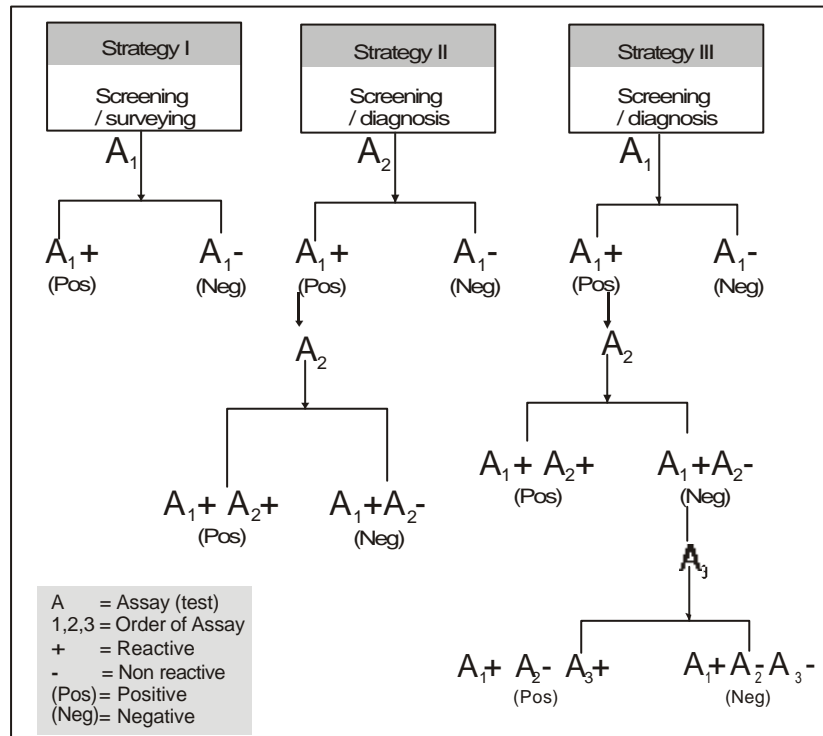
NB: This is not normally adequate for diagnosis!

**Strategy 2 requires up to two tests and is to be used for:**

- **Surveillance** (if prevalence ≤ 10% and no results are to be provided)
- **Diagnosis** if patient symptomatic and population prevalence ≤ 30%, or if patient asymptomatic and population prevalence > 10%.

**Strategy 3 requires up to three tests for:**

- Diagnosis if patient asymptomatic and population HIV prevalence ≤ 10%.



**FIGURE 12: WHO/ UNAIDS Strategies for HIV Antibodies Screening**  
The successful confirmation using these strategies requires the user to distinguish strictly between assay results (non-reactive or reactive) and patient diagnosis (“Positive” or “ Negative”)!

For all strategies, the first (screening) test (Test 1) should be chosen for its very high sensitivity (>99%) (to avoid false-negative results), while the confirmatory tests (Tests 2 and 3) must have a high specificity (>99%). No test performance characteristic must be <95%. Furthermore, the different tests used for each strategy must employ different formats.

**Discrimination Between HIV-1 and HIV-2 Infection**

Tests that claim to distinguish HIV-1 from HIV-2 infection cannot necessarily be regarded as confirmatory tests. Some of them rely on a reaction with a single short length oligopeptide and may lack sensitivity for antibody from all individuals infected by the homologous HIV type. However, most of the confirmatory methods already mentioned will discriminate to some degree between HIV-1 and HIV-2. Cross reactions, between env and gag antigens in WB make it less efficient than other approaches such as utilizing a pair of type-specific competitive EIAs or a line immunoassay employing HIV-1 and HIV-2 specific synthetic antigens. Also, the cost of two WB strips or PCR for HIV-1 and HIV-2 is high and it is doubtful whether such expenditure should be committed to making a differential diagnosis for the small proportion of cases in which more simple and inexpensive approaches fail.

**Paediatric HIV Infection**

Since the first report of an infant with AIDS in November 1982, the incidence of HIV infection in the paediatric population has increased exponentially. By the year 2000, it is estimated that HIV pandemic was responsible for the deaths of 3 million women and more than 2.5 million children worldwide. Additionally about 5.5 million children might have been orphaned because of premature death of their parents.

It has been proposed that when maternal infection rates reach 2-3% globally, HIV infection acquired by the perinatal route could become the most common cause of death in children. Of the reported children, younger than 13 years old in the USA, 90% were born to mothers with HIV or HIV risk factors, 5% received blood transfusions or tissue transplants, 3% were hemophiliacs and 1% had no identified risk factor.

### **Transmission of HIV from mother to infants**

Transmission of HIV from mother to infants is not absolute but relatively efficient. Prospective studies have reported mother to infant HIV transmission in 13% to 39% of infants born to HIV infected mothers. Perinatal transmission may occur before, during or after birth. Many characteristics of the HIV infected mothers may be associated with increased risk of perinatal HIV transmission; women with symptomatic HIV infections or AIDS may have an increased risk of transmitting infection to their infants as compared to asymptomatic pregnant mothers.

Higher transmission rates have been reported for infants delivered vaginally than those by cesarean section. In some studies presence of other STD's during pregnancy, such as syphilis, appear to increase the likelihood of HIV transmission possibly due to placentitis associated with *Treponema pallidum* coinfection. Other ulcerative genital infections may also increase the transmission rates.

Perinatal feeding practices can also affect vertical HIV transmission as HIV infection does occur via breastfeeding. Type of milk ingested (colostrum versus later milk) duration of breastfeeding, duration of mother's HIV infection, her viral load and the antibody content in her milk influences mother to infant transmission rates.

The working definition of intra uterine infection as suggested by experts is a positive PCR or HIV culture or a p24 antigen test on the infants blood within 48 hours of life. Perinatal infection is defined as negative assays shortly after birth and positive results occurring later. Voluntary testing for HIV to recognize HIV infected mothers can benefit through antiretroviral therapies, early prophylaxis of genital infections, providing passive HIV antibody to pregnant women, fetus and the newborn and providing active or passive enhancement of fetal and neonatal anti HIV specific immunity.

### **Diagnostic Challenges: Child born to a HIV infected mother**

Diagnosis of HIV infection in children born to HIV infected mothers is complicated by the presence, in almost all cases, of the maternal anti HIV antibody which crosses the placenta to the fetus. Over all less than

one third of the children are actually infected. Since HIV antibody assays such as EIA, immunoblots primarily detect IgG class of antibodies and in infants, this class of antibody may persist from upto 9-18 months, conventional antibody tests cannot be used to reliably predict the HIV infection in children under 18 months of age. In the first year of life, HIV infection can be reliably diagnosed by PCR and viral culture, since they can correctly identify upto 50% of the infected infants at birth and nearly 100% of the infected infants by 6 months of age. It must be however recognized that false positive PCR results occur in the first week of life and the standard p24 antigen assay is less sensitive than culture or PCR as it fails to detect immuno complexed p24 antigen.

The additional potentially useful methods currently under development and evaluation are:

- a) Test based on in vitro production of virus specific antibodies by patients lymphocytes.
- b) Detection of patient specific IgA antibodies to HIV, since the IgA does not cross the placenta.

### **Anti Retroviral therapy**

The treatment of HIV infection requires an understanding of viral replication, dynamics, antiviral potency, pharmacokinetics and toxicities of individual drugs, interactions between drugs when used in combination and the limitations imposed by failure of one drug on the future use of same and related drugs.

Most of the anti retroviral drugs available today exert their anti retroviral action by disrupting the viral replication cycle of the HIV within the infected host. Broadly these drugs can be classified into:

- Inhibitors of viral reverse transcriptase that block the conversion of the viral RT dependent genomic RNA into double stranded DNA notable amongst those being:
  - Non nucleoside RT inhibitors
  - Nucleoside analogue RT inhibitors, and
  - Nucleotide analogue RT inhibitors.
- Inhibitors of the viral protease enzyme to prevent the cleavage of poly proteins & conversion of viral particle into an mature infectious virion (protease inhibitors).

The inhibitors of viral transcriptase compete with natural enzyme substrates in vivo, leading to perinatal DNA chain termination of the HIV life cycle. On the other hand, action of the protease inhibitors does not let the viral particles mature and remain replication defective.

Since HIV infection is a dynamic process with high rates of replication and great potential for genetic mutation, a major determinant of anti retroviral drug failure is viral genotypic variation conferring drug resistance.

When a patient starts a drug regime, the regime selects for pre existing mutation as well as additional mutants that may appear in different genetic backgrounds. Mutations continue to appear unless rate of replication is curtailed enough to retard genetic variation.

### **Use of highly active retroviral therapy (HAART)**

Combination regimes that produce more potent inhibition of viral replication may delay or prevent resistance from arising by limiting the new mutations from occurring or accumulating. Because of high cost and limited availability HAART is beyond reach of most infected individuals or individuals at risk for HIV infection.

The recent widespread use of plasma HIV-RNA levels to monitor infection and therapy has transformed clinical management of infected individuals. Therapy for persons with HIV infection is based upon CD4 cell counts and HIV-RNA plasma levels.



TABLE 10. Plasma HIV RNA levels.

The level of HIV-RNA reflects the magnitude of viral replication. It correlates with the rate of CD4 T cell decline, the relative risk of clinical disease progression and the time to death.

Baseline RNA levels are measured before the anti retroviral therapy is started and during drug therapy to guide subsequent steps.

A consistent increase in viral load of more than 0.5 log<sub>10</sub> usually suggests a failing anti retroviral regimen. The RNA viral load test is currently available for HIV 1 infection only.

The CD4 T cell count on the other hand reflects the extent of immune damage that has already occurred because of HIV infection. CD4 cell counts correlate with the risk of developing opportunistic infections, neoplasms and with time to death. Base line CD4 T cell counts are measured before anti retroviral therapy is initiated to assess prognosis and guide prophylaxis of opportunistic infections.

### **Vaccines for HIV infection**

It is clear, that attempts to modify high risk behaviour amongst population has met with variable success. Because of the high cost and limited availability, HAART is beyond the reach of most individuals at risk for HIV infection. Effective vaccination probably offers the best hope for containing the HIV epidemic.

The development of vaccines against HIV poses a formidable, scientific, logistic and social challenge and the most important problems are:

- Lack of understanding of the critical parameters of immunity that would protect against the infection / disease or both.
- Substantial genotypic variations occur among the infective HIV strain along with high level of mutation and recombination in the replication of individual isolates.
- The relationship of these genotypic variations to the expression of antigens that might induce protective immunity remains poorly defined.
- An effective vaccine would have to stimulate broadly directed immune response so as to be able to inhibit diverse strains of HIV

A large body of information continues to be generated concerning the immune response to HIV infection as various phase I, II, and III efficacy trials are under way in addition to the development of vaccines based on diverse approaches.

It is hoped that an effective vaccine is closer than never before, to contain the pandemic of HIV and AIDS.

## References and Suggested Reading

1. Detection, Isolation and Continuous Production of Cytopathic Retroviruses (HTLV-III) from patients with AIDS and Pre-AIDS, Mikulas Popovic, M.G. Sarangadharan, Elizabeth Read, Robert C. Gallo, Science, Vol.224, 497- 500, 4 May 1984.
2. Frequent Detection and Isolation of Cytopathic Retroviruses (HTLV-III) from patients with AIDS and at risk of AIDS Robert C.Gallo, Syed Z.Salahuddin, Mikulas Popovic, Gene M.Shearer, Mark Kaplan, Barton F.Haynes, Thomas J. Palker, Robert Redfield, James Oleske, Bijan Safai, Gilbert White, Paul Foster, Phillip D.Markham, Science, Vol. 224, 500-502, 4 May1984.
3. Serological Analysis of a Subgroup of Human T-Lymphotropic Reteroviruses (HTLV-III) Associated with AIDS, Jorg Schupbach, Mikulas Popovic, Raymond V.Gilden, Mathew A.Gonda, M.G.Sarangadharan, Robert C.Gallo,Science, Vol.224, 503-505, 4 May1984.
4. Reteroviruses (HTLV-III) in the serum of Patients with AIDS, M.G.Sarangadharan, Mikulas Popovic, Lillan Bruch, Jorg Schupbach, Robert C.Gallo, Science, Vol.224, 506-508, 4 May 1984.
5. Identification of HTLV-III/LAV *src* Gene Product and Detection of Antibodies in Human Sera, Nancy C.Kan, Genoveffa Franchini, Flossie Wong-Staal, Garrett C.DuBois, W.Gerard Robey, James A.Lautenberger, Takis S.Papas, Science, Vol.231, Reports: 1553-1555, 28 March 1986.
6. Antiserum to a Synthetic Peptide Recognizes the HTLV-III Envelope Glycoprotein, R.C.Kennedy, R.D.Henkel, D.Pauletti, J.S.Allan, T.H.Lee, M.Essex, G.R.Dreesman, Science, Vol.231, Reports 1556-1557, 28 March 1986.
7. Isolation of a New Human Reterovirus from West African Patients

with AIDS, Francois Clavel, Denise Guetard, Francoise Brun-Vezinet, Sophie Chamaret, Marie-Anne Rey, M.O.Santos-Ferriera, Anne G.Laurent, Charles Dauget, Christine Katlama, Christine Rouzioux, David Klatzmann, J.L.Champalimaud, Luc Montagnier, Science,Vol.233, Reports: 343-346, 18 July 1986.

8. Synthetic Peptide Immunoassay Distinguishes HIV Type1 and HIV Type 2 Infections, John W.Gnann, Joseph B. McCormick, Sheila Mitchell, Jay A.Nelson, Michael B.A.Oldstone, Science, Vol.237, Reports:1346-1349, 11Sept.,1987.
9. A Field Test for the Detection of Antibodies to Human Immunodeficiency Virus Types 1 and 2 in Serum or Plasma, Anthony Burgess-Cassler, Gustavo Barriga Angulo, Stephen E.Wade, Patricia Castillo Torres, Willfried Schramm, Clinical and Diagnostic Laboratory Immunology, Vol.3 No.4, 480-482, 1996.
10. Current Concepts in Human Immunodeficiency Virus Infection and AIDS, Stanley A.Schwartz, Madhavan P.N.Nair, Clinical and Diagnostic Laboratory Immunology, Vol.6 No.3, 295-305, May 1999.
11. Diagnosis of Human Immunodeficiency Virus Type 1 Infection with Different Subtypes Using Rapid Tests, Susan Phillips, Timothy C.Granade, Chou-Pong Pau, Debra Candal, Dale J.Hu, Bharat S.Parekh, Clinical and Diagnostic Laboratory Immunology, Vol.7 No.4, 698-699, July 2000.
12. Principle and Practice of Infectious Diseases, Mandell, Bennett and Dolin, 5th Edn., Vol 1-Part II, 1332-1528, 2000,Churchill Livingstone Publications.
13. Genetic Subtypes, Humoral Immunity, and Human Immunodeficiency Virus Type 1 Vaccine Development, John P. Moore, Paul W. H. I. Parren and Dennis R. Burton, Journal of Virology, Vol 75, No. 13, July 2001, p 5721-5729.

14. Pediatric Human Immunodeficiency Virus Infection, Joseph B. Domachowske, *Clinical Microbiology Reviews*, Vol 9, No. 4 , Oct 1996, p 448-468.
15. Global Impact of Human Immunodeficiency Virus and AIDS, Helene D. Gayle and Gena L. Hill, *Clinical Microbiology Reviews*, Vol 14, No. 2 , April 2001, p 327-335.
16. Convergent Evolution within the V3 Loop Domain of Human Immunodeficiency Virus Type 1 in Association with Disease Progression, Nataly Strunnikoba, Stuart C. Ray, Robert A. Livingston, Elizabeth Rubalcaba and Raphael P. Viscidi, *Journal of Virology*, Vol 69, No. 12, Dec 1995, p 7548-7558.
17. Human Immunodeficiency Virus and AIDS: Insights from Animal Lentiviruses, Roger J. Miller, J. Scott Cairns, Sandra Bridges and Nava Sarver, *Journal of Virology*, Vol. 74, No. 16, August 2000, p 7187-7195.
18. Relationship between the V3 Loop and the Phenotypes of Human Immunodeficiency Virus Type 1 (HIV-1) Isolates from Children Perinatally Infected with HIV-1, Fabrizio Mammano, Francesca Salvatori, Lucia Ometto, Marina Panozzo, Luigi Chieco-Bianchi and Anita De Rossi, *Journal of Virology*, Vol. 69, No. 1, Jan 1995, p 82-92.
19. Phenotypic and Genotypic Characteristics of Human Immunodeficiency Virus Type 1 from Patients with AIDS in Northern Thailand, Xiao-Fang Yu, Zhe Wang, Chris Beyrer, David D. Celentano, Chirasak Khamboonruang, Ethan Allen and Kenrad Nelson, *Journal of Virology*, Vol 69, No 8, Aug 1995, p 4649-4655.
20. A Field Test for the Detection of Antibodies to Human Immunodeficiency Virus Types 1 and 2 in Serum and Plasma, *Clinical and Diagnostic Laboratory Immunology*, Vol 3, No. 4, July 1996 , p 480-482.
21. Data on file:Qualpro Diagnostics