TECHNICAL SERIES

HIV Testing

Evolution & Practical Issues

...Setting trends

OBJECTIVE OF THIS TECHNICAL SERIES

Over the past two decades, HIV diagnostics have been essential in detecting and monitoring infection, and continue to play a major role in saving lives throughout the world. As technology evolved, screening, confirmatory, and HIV monitoring assays have been improved and offer better alternatives to address blood screening, surveillance, diagnosis, and patient management.

Molecular methods are critical in detecting early infection and for managing patients on anti-retroviral therapy whose viral infection may become resistant to therapy. Many of the newly evolving technologies are essential for use in resource-limited settings because they can address cost issues, limited infrastructure, and a lack of formally trained personnel. Newer rapid HIV kits can be stored in a wide range of temperatures (2-30°C) to address cold-chain issues, can be performed using easily collected fingerstick blood and also other body fluids like saliva, and have one-step procedures that are relatively foolproof.

Despite all the advancement, developing countries like India face another daunting task; that of correct scientific information available to laboratory professionals involved in HIV testing. Terminologies like “generations of HIV test”, “window period”, “false positives”, “p24 antigen”, “HIV-1 group-O”, “indeterminate results” etc. have become common laboratory jargon. But, what do they actually mean? This publication is an attempt to answer these questions in the correct scientific perspective. The purpose of this technical series is as follows:

1. ***To break myths and remove confusion in HIV testing***
2. ***To understand problems associated with HIV testing***
3. ***To throw light on how to report a positive & negative test result***

BACKGROUND

Human immunodeficiency virus (HIV) is a lymphotropic retrovirus that primarily infects and destroys $CD4^+$ lymphocytes. These cells are crucial for the induction and regulation of the immune response. Their progressive depletion by the virus causes irreversible disruption of normal immune function, leading to immunosuppression and the subsequent development of AIDS. Early medical intervention reduces the risk of transmission and delays progression to AIDS. This necessitates early detection of the infection, which may be asymptomatic for a prolonged time.

HIV is believed to have originated in West-Central Africa and jumped species (zoonosis) from primates to humans. HIV-1 evolved from a Simian Immunodeficiency Virus (SIV_{cpz}) found in the chimpanzee subspecies *Pan troglodytes*. HIV-2 crossed species from a different strain of SIV, this one found in sooty mangabeys. The earliest documented HIV-1 infection dates from 1959, and was discovered in the preserved blood sample of a man from Kinshasa in the Democratic Republic of the Congo.

CHRONOLOGY

1981 - 5 cases of pneumocystis carinii pneumonia (PCP) in Los Angeles
1983 - Pasteur Institute isolates retrovirus (HIV-1) from lymph node
1984 - Gallo's group confirms and extended findings to AIDS
1986 - HIV-2 was isolated

THE GRIM PICTURE

Infection in humans is now pandemic. As of January 2006, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimate that AIDS has killed more than 25 million people since it was first recognized in 1981, making it one of the most destructive pandemics in recorded history. In 2005 alone, AIDS claimed

an estimated 2.4-3.3 million lives, of which more than 570,000 were children. A third of these deaths are occurring in sub-Saharan Africa, retarding economic growth by destroying human capital. Current estimates state that HIV is set to infect 90 million people in Africa, resulting in a minimum estimate of 18 million orphans. It's a similar situation in India as well. As of April 2006, National AIDS Control Organisation (NACO) reported an estimated 5.21 million HIV-positive adults in India.

ROUTES OF TRANSMISSION

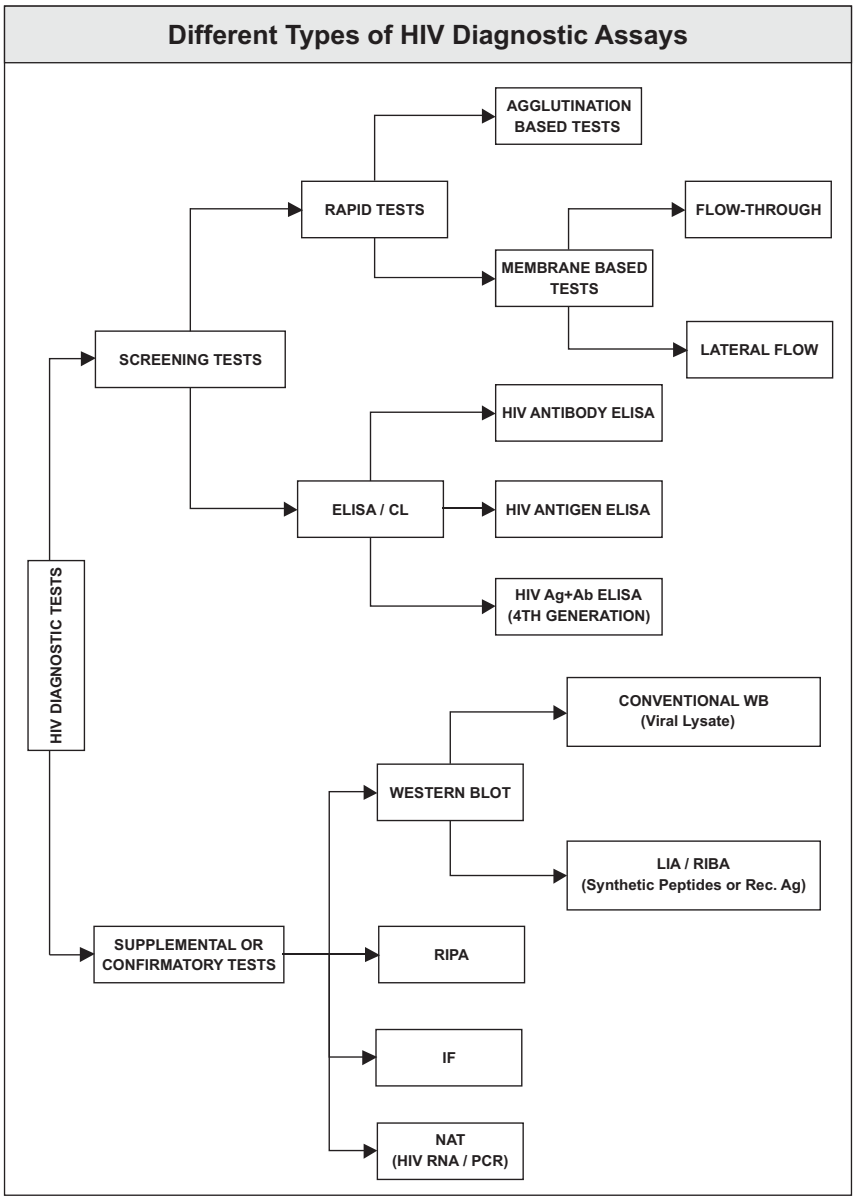
The following chart shows estimated HIV infections through different routes. It is clear that blood transfusions & mother-to-child infections dominate HIV infections. Another interesting point to note is that needle- stick injuries also result in a significant number of HIV infections. This means the healthcare workers; particularly laboratory professionals and surgeons are at increased risk of exposure to HIV.

Exposure Route	Estimated infections per 10,000 exposures to an infected source
Blood Transfusion	9000
Childbirth	2500
Needle-sharing injection drug use	67
Sexual transmission*	73
Percutaneous needle stick	30

* Assuming no condom use

DIAGNOSIS OF HIV

HIV infection can be detected by a variety of tests. The following chart illustrates the different type of HIV tests:



ELISAs or EIA/CL

The most commonly used type of test for screening is the ELISA (enzyme-linked immunosorbent assay) or EIA (Enzyme Immunoassay). When immunoassay is performed using chemiluminescent label instead ofenzyme (as in ELISA), it is known as Chemiluminescent Assay (CL). ELISAs / CL are probably the most efficient tests for testing large numbers of samples per day, as in large blood banks or for surveillance studies.
Since ELISAs require skilled technical staff, equipment maintenance and a steady power supply, they are less suitable for smaller or more isolated hospitals, clinics or laboratories. Regular maintenance of the ELISA equipment is crucial to obtaining reliable results.

The original ELISAs involved the use of viral lysate, and positive specimens were usually confirmed by means of Western Blot technology, which is technically difficult, time consuming and expensive. Second- generation and third-generation ELISAs were developed on the basis of recombinant proteins and synthetic peptides, which increased sensitivity and specificity and considerably shortened the interval between the time of infection and the ability to detect HIV antibodies, i.e. the window period. This period has been further reduced by means of combined antigen- antibody ELISAs, comprising the fourth generation of such tests.

RAPID TESTS

The conventional ELISAs available for the detection of antibodies to HIV require instrumentation (i.e., incubators and mechanical washing and optical reading devices) and generally take 2 to 4 hrs to produce a result. The need for simpler, faster, less expensive, and easier-to-perform tests has become more acute as the HIV pandemic has expanded; thus, a variety of rapid test formats continue to be evaluated worldwide. Rapid tests for the detection of HIV (HIV rapid tests) which provide results concurrent with the patient's visit were preferred and resulted in significant improvement in the delivery of counseling without increasing the cost or decreasing the effectiveness of testing.

Advances in technology have led to the development of a wide variety of rapid HIV tests, including agglutination assays, dipstick assays, flow- through membrane assays, and lateral flow membrane assays. Many of these tests are presented as strips or cartridges incorporating the reagents and not requiring additional equipment. They are suitable for the performance of single tests, are easy to use and can be carried out by any health care worker who has received appropriate training. Most rapid HIV test kits can be stored at room temperatures of up to +20 to +30ºC. Furthermore; the diagnostic performance of high-quality rapid tests is comparable to that of traditional ELISAs. WHO has developed testing algorithms showing that sequential combinations of two or three antibody tests (ELISAs and/or rapid tests) can be reliably used to confirm HIV test results.

The lateral-flow tests are currently the most advanced in rapid HIV assays because of its simplicity, convenience, advanced generation, option of whole blood as sample, longer shelf life and storability at room temperature. However, earlier generation flow-through assays that are less sensitive, are still used in some developing countries like India owing to lack of access to advanced technology.

COMMONLY ENCOUNTERED PROBLEMS IN RAPID TESTS

The following section deals with the commonly encountered problems in rapid tests and their solutions.

High Background

Background formation is dependant to a large extent on the quality of sample used. Because of the quality of sample used, there is negligible / no background with some samples whereas some show a background colour. Samples that are:

- turbid,
- lipemic or
- contaminated;

show background colour in membrane based assays. In addition, aged samples i.e., stored samples that have undergone repeated freeze thaw cycles also show background colour. In such cases, samples should be spun hard at 3500 rpm for 15 minutes to pellet the suspended matter. Only clear supernatant should be used for testing. However, as explained in “background due to artifact factor” section, some samples show background despite being centrifuged due to presence of certain artifact.

Background due to the pH factor: Dark background may be due to abnormally high pH of the sample > 7.45. At high pH the colloidal gold conjugate in rapid assays may be precipitated on the membrane and show a strong background colour. The range of blood pH in the first few hours of life is 7.09-7.50 but thereafter is 7.35-7.45. The unusually high pH in some patients may be due to:

- ***Metabolic alkalosis -*** This is a result of increased bicarbonate in blood due to excessive alkali administration or loss of Cl (-) as gastric Hcl due to prolonged vomiting or intracellular K (+) depletion.
- ***Respiratory alkalosis -*** Respiratory alkalosis is one of many acid-base disorders found among critically ill patients. Hyperventilation in hysteria and stimulation of the respiratory center are the usual causes. Patients have fever if respiratory alkalosis is the result of an infectious disorder. Hyperthermia (fever) of any origin may in turn result in respiratory alkalosis.
- ***Pre-analytical error -*** Any pH above 7.45 is considered to be alkalemia. In Metabolic or Respiratory alkalosis, usually the pH goes up maximum to 7.8. Above pH 7.8 indicates a fatal consequence. This error may take place during sample collection (contamination of sample vial by detergents).
- ***Low pH -*** Similarly low pH below 7 renders the sample acidic. This too causes the colloidal gold conjugate in rapid assays to precipitate / aggregate and show a dark background. Therefore, for obtaining proper reactivity and valid results, the ideal pH of the sample should be between 7-8.

Background due to the artifact factor: Background colour may also occur due to the presence of a high molecular weight molecule (or artifact) in sera that blocks the pores of the membrane. As a result the conjugate may not flow adequately leading to its precipitation on the membrane surface. For example, in some cancer patients there may be presence of artifacts (immunoglobulins, high molecular weight proteins like cell debris etc.) in the serum. These artifacts may block the pores of the membrane or interact with gold conjugate to precipitate the same. These samples show background colour even after centrifugation. Therefore, such samples should be tested with other assays like ELISAs and not with membrane based rapid assays.

Improper sample flow in rapid assays

Due to inherent variability of sample, problems related to flow of sample on membrane may not be totally eliminated. That is why all rapid assay manufacturers indicate the possibility of “invalid results” in their pack insert. However, despite all precautions, the following errors may occur with rapid assays:

- ***Dispensing error -*** The sample / buffer / reagents may be added in incorrect quantities. Low volume of reactants may cause inadequate hydration of membrane and lead to improper sample flow. On the other hand, excess liquids may result in overflow and show invalid or variable results.
- ***Reaction time -*** Sometimes reading is taken before reaction time is complete. This error commonly occurs with lateral flow rapid HIV assays. The technologist may not wait till the full reaction time of 15-30 minutes is complete. This may be apparently interpreted as “flow-problem”, because sufficient clearance time has not been allowed.
- ***Damaged foil pouch -*** The flow properties of the nitrocellulose membrane may be affected if the foil pouch containing the device or dipstick is damaged. To avoid this, most manufacturers incorporate a desiccant in the pouch. Therefore, the pouch should be checked for pinholes and the desiccant should be observed for any colour change.

  This is mentioned by the manufacturers in the product pack insert and should be routinely followed as part of GLP (Good Laboratory Practice).

NOTE: ***Rapid assays need to be discarded after the reaction time is over.*** *A membrane-based rapid assay should not be read after the specified reaction time. This is because some sera contain certain non- specific artifact that do not react at first contact with antigen or antibody in the membrane but may do so after the reaction time is over. This happens due to evaporation (in flow-through) and back-flow (for lateral-flow assays) of the reactants. Again due to evaporation / back-flow, gold conjugate returning from the absorbing pad is very likely to dry out at the capture region because during drying the capture element becomes very hydrophobic. This hydrophobicity causes charged gold particles to bind non-specifically to the Test region and show a false positive result. Therefore, if future documentation of test results is absolutely required, the device / dipstick may be photographed / scanned at the end of reaction timeand the image stored.*

CONFIRMATORY & SUPPLEMENTAL ASSAYS

Due to the inherent variability of HIV and its serological response, it is difficult to consider a particular assay as confirmatory. WESTERN BLOT & HIV-RNA detection by PCR may be at the most termed as supplemental assays; not confirmatory assays. This is because both false positives & negatives have been reported in various studies with these tests. Virus isolation & culture is considered to be the most sensitive & specific test. However, false-negative HIV cultures sometimes are observed, particularly during the newborn period, and either false negative or false positive PCR test results may be noted occasionally. Therefore, for purposes of clinical decision-making, any positive test result should be confirmed with a second HIV culture or PCR test performed on a separate blood specimen.

Therefore, Confirmatory or supplemental assays are designed to offer a greater specificity than screening assays, and therefore are the methods of choice to verify that reactive results from screening assays represent HIV infection. They do not, however, verify that screening test negative results are from non-infected persons, nor do they verify that negative or indeterminate results from these tests are from a person who is not infected. The purpose of supplemental tests is to rule out false positive results by screening tests not to confirm that a person is unequivocally infected with HIV or to confirm that a person is negative for HIV. They are highly effective in great majority of persons.

Western Blot

Western Blot (WB) detects antibodies to HIV qualitatively to viral proteins. Viral antigens are electrophoretically separated and transferred ("blotted") onto a nitrocellulose sheet. Antibodies, which react with antigens on the membrane, are visualized with an enzyme labeled anti-human IgG and an enzyme substrate. WB shows an insoluble coloured product at the site of reaction. Therefore, WB detects only IgG antibodies and is less sensitive than 3rd / 4th Generation assays. However, as mentioned earlier, like any other supplemental test, WB has higher specificity than screening assays. Despite this, as per some reports, WB tests are indeterminate in 20 to 40% of healthy blood donors who are negative on the ELISA test. To improve sensitivity & specificity, there have been some modifications of the conventional WB that uses viral lysate as antigens. Currently, Western Blots using synthetic peptides (Line Immunoassay, LIA) and recombinant antigens (Recombinant Immunoblot Assay, RIBA) have been developed and marketed.

Radioimmunoprecipitation Assay (RIPA)

In this method, HIV Ag is metabolically labeled in HIV culture medium. The labeled cells are then disrupted to release the labeled proteins. The resulting suspension is clarified to obtain the proteins. These labelled proteins are used for testing the sample. The method is not popular because of the use of live HIV particles. Therefore, containment facilities for handling live HIV are critical. Also special facilities for handling radioisotopes and necessary regulatory clearance are essential. Moreover, the need of expensive equipment & skilled personnel makes RIPA one of the least practical assays for diagnosis of HIV infection.

Indirect Immunofluorescence (IF)

Like WB, IF also detects anti-HIV antibodies. Acetone fixed HIV infected lymphocytes are incubated with sample and a fluorescein conjugated anti-human IgG. The results are examined using fluorescent microscopy. If anti-HIV is present a typical pattern of fluorescent staining is seen in the cells. Similar to WB, IF also detects only IgG antibodies. Therefore, as mentioned earlier, its sensitivity is also less than 3rd/4th Generation HIV assays. IF requires expensive fluorescence microscope and highly skilled operator. It is also time-consuming to perform. This is the reason, like RIPA, IF is not a popular TOC (Test of Choice) for HIV diagnosis.

Nucleic Acid Tests (NAT)

In this method viremia is determined by measurement of HIV RNA in sample. The basic idea is that the viral genome copies must stem from virus particles (virions) as free blood nucleic acid is split by nucleases. The number of copies correlates therefore with the amount of the virus, one HIV particle containing two genome copies.

Several NAT technologies are available. All molecular methods have three steps in common, including: (i) a front end step that includes sample preparation and/ or viral nucleic acid extraction; (ii) a middlestep consisting of target nucleic acid sequence amplification or amplification of the signal generated from the detection of target viral RNA; and (iii) a back end step that allows detection and/or quantification of the amplified products. The three major methods are:

(i) the reverse transcription polymerase chain reaction (RT-PCR),
(ii) nucleic acid sequence based amplification (NASBA) / transcription mediated amplification (TMA), and
(iii) branched chain DNA (bDNA).

SOME FAQs IN HIV TESTING

What are "Generations" of HIV Immunoassays?

Since the eighties, HIV immunoassays have constantly evolved. Depending upon the sensitivity and specificity, the assays are grouped into "generations". The following chart shows the different generations of HIV immunoassays.

Nomenclature	Antigen type	What is detected
1st Generation	Uses viral lysate as antigen	Detects only IgG antibody to HIV
2nd Generation	Uses recombinant antigens as antigen	Detects only IgG antibody to HIV
3rd Generation	Uses recombinant antigens and / or synthetic peptidesas antigen	Detects all HIV antibody isoptypes e.g., IgG, IgM, Iga, etc
4th Generation *	Uses recombinant antigens and / or synthetic peptides as antigen and monoclonal and / or polyclonal anti-p24 antibody	Detects all HIV antibody isoptypes and also p24 antigen

**Not available in rapid test format.*

Therefore, from the above it is clear that the most sensitive test is the 4th generation immunoassay. Unfortunately, no 4th generation rapid assay has been developed for testing in resource-poor settings.

What is "Window Period"?

Window period is also known, as SEROCONVERSION PERIOD. Seroconversion refers to the development of detectable antibodies to HIV in the blood as a result of HIV infection. A patient who goes from being HIV negative to HIV positive is said to have seroconverted or is a seroconverter. The time of seroconversion depends upon:

- The host-virus interaction.
- Immune status of the host.
- Route of infection.
- Type / subtype of infecting HIV strain.
- Viral inoculum at the time of exposure.

Therefore, there is no fixed time of the window period. The following chart shows how different bodies have defined window period:

Authorised body	Duration of window period
CDC, USA	2 weeks-1 year; Average: 3-6 months
The Canadian Medical Association	Within 6 months, 95% seroconvert within 3 months
WHO	6 weeks-3 months, unusual cases: 6 months or longer
NACO, India	3 weeks-3 months on an average, can be longer sometimes

The length of the window period varies not only on the factors mentioned above but also on the "generation" of test used. For example, as explained in the subsequent section, the length of the window period, if HIV RNA is detected, is just 1 week. However, the length is increased to 6-12 weeks if the test used is a 1st /2nd generation HIV assay.

What is the detectability of different HIV tests?

As mentioned earlier, several factors influence the time of seroresponse & the titer of antibodies produced. Moreover, the detection of HIV antibodies based on the test sensitivity (Generation) also determines the length / duration of the window period. The following chart illustrate the detectability of different types of HIV assays.

1st & 2nd Generation assays	6-12 weeks after exposure
3rd Generation assays	3-4 weeks after exposure
4th Generation assays	2-3 weeks after exposure
p24 antigen ELISA	2 weeks after exposure
NAT * (HIV RNA detection)	1 week after exposure

* NAT - Nucleic Acid Tests

What are false positive reactions and why do they occur?

False positive reactions are those that show positive in a HIV immunoassay despite the patient being a non-infected subject. This happens with all HIV diagnostic assays including supplemental tests like HIV RNA by PCR. The reasons for such occurrences are debatable and not well understood.

As we are aware, the antigen (lysate or synthetic peptide or recombinant) used in any immunoassay is actually a small portion of the microorganism whether it is a virus or bacteria or protozoa etc. They are proteins molecules with a particular sequence of amino acids the "building blocks" of life on this planet. These amino acids are found in all organisms on earth. That is why cross-reacting epitopes are often found even in apparently unrelated microorganisms.

In case of HIV, the following chart shows how the antigens used in HIV assays worldwide including gp120, gp41, gp160, p18 & p24, shows some degree of cross reactivity with certain sera.

Reference	HIV antigen implicated
Tomiyama T et al. Recognition of human immunodeficiency virus glycoproteins by natural anti carbohydrate antibodies in human serum. Biochem Biophys Res Commun. 1991 May 31; 177(1):279-85.	gp160, gp120 and gp41
Sheikh MJ et al. The gp120 envelope of HIV-1 binds peptides in a similar manner to human leukocyte antigens. AIDS. 1995 Nov; 9(11): 1229-35.	gp120
Gul A et al. Antibodies reactive with HIV-1 antigens in systemic lupus erythematosus. Lupus. 1996 Apr; 5(2): 120-2.	gp18, p24
Esteva MH et al. False positive results for antibody to HIV in two men with systemic lupus erythematosus. Ann Rheum Dis. 1992 Sep;51(9):1071-3.	gp41
ShivRaj L et al. Antibodies to HIV-1 in sera from patients with mycobacterial infections. Int J Lepr Other Mycobact Dis. 1988 Dec; 56(4): 546-51.	p24
Healey DS & Bolton WV. Apparent HIV-1 glycoprotein reactivity on western blot in uninfected blood donors. AIDS. 1993 May; 7(5): 655-8.	gp41
Sayre KR et al. False-positive human immunodeficiency gp41 virus type 1 western blot tests in noninfected blood donors. Transfusion. 1996 Jan; 36(1): 45-52.	gp41

Therefore, all HIV immunoassays are prone to false positivity because all of them use at least one of the "implicated" antigens.

Apart from the above, the following is the list of conditions other than HIV that can cause positive results in serological assays as reported in various studies.

Conditions that cause false positives in HIV tests

1. Renal failure
2. Alpha interferon therapy in hemodialysis patients
3. Flu
4. Flu vaccination
5. Herpes simplex I
6. Herpes simplex II
7. Upper respiratory tract infection
8. Recent viral infection or exposure to viral vaccines
9. Pregnancy in multiparous women
10. Malaria
11. Rheumatoid arthritis
12. Hepatitis B vaccination
13. Tetanus vaccination
14. Organ transplantation
15. Renal transplantation
16. Anti-lymphocyte antibodies
17. Anti-collagen antibodies
18. Autoimmune diseases
19. Malignant neoplasms
20. Alcoholic hepatitis/alcoholic liver disease
21. Antibodies with a high affinity for polystyrene
22. Blood transfusions, multiple blood transfusions Ss
23. Multiple myeloma
24. Anti-smooth muscle antibody
25. Anti-parietal cell antibody
26. Anti-hepatitis A IgM
27. Anti-Hbc IgM
28. Haemophilia
29. Stevens-Johnson syndrome
30. Heat-treated specimens
31. Lipemic serum
32. Haemolyzed serum
33. Hyperbilirubinemia
34. Healthy individuals as a result of poorly understood cross-reactions
35. Normal human ribonucleoproteins
36. Anti-mitochondrial antibodies
37. Anti-nuclear antibodies
38. Anti-microsomal antibodies
39. Epstein-Barr virus
40. Visceral leishmaniasis
41. Receptive anal sex
42. Tuberculosis
43. *Mycobacterium avium*

Do more false positives occur in some geographic areas?

Yes, it is reported that more false positive results occur in certain geographic areas. More false positives have been reported in **malaria & dengue endemic areas (like India)**. Normally, diagnostic tests are evaluated in developed countries by using samples locally obtained from well-defined populations in which major parasitic infections are absent. On the other hand, in developing countries, parasitic infections are frequent and lead, in conjunction with poor nutrition to **increased polyclonal antibody stimulation** in the affected individual that can remain throughout life. The increase in nonspecific antibody titers can interfere with the performance of any antibody detection assay like HIV. In an Indian study (a malaria-endemic area) it has been reported that a significant proportion of population shows non-specific false positive serology with various tests like RPR, HIV, WIDAL, RF and DCT in acute other phase of malaria. Studies indicate that false positive HIV serology may occur in low HIV-infection prevalence areas.

What does indeterminate (equivocal) test results mean?

Indeterminate samples are those, which can neither be termed as positive nor as negative. These samples are usually found reactive with initial HIV screening assays (ELISAs or Rapid Tests) and subsequently show indeterminate band patterns in Western Blot. Also, as per WHO/UNAIDS testing strategies, a sample that shows discordant results with the three tests suggested in the algorithm, is also termed as indeterminate or equivocal.

The proportion of repeatedly reactive samples by ELISA but with an indeterminate WB pattern varies 13 to 48% of samples from blood donors in the United States. This proportion seems to be even higher in some regions of Africa. As mentioned in the earlier section, the observation of ELISA-negative samples that render indeterminate WB patterns is surprising, with reports that this result is found for as much as 20% of the ELISA-negative blood donors at the National Institutes of Health (USA).

The long-term outcome and conditions associated with persons identified as indeterminate WB after being repeatedly reactive by screening ELISA are not well characterized and may vary from one geographical region to the other.

A vast number of conditions have been reported by different authors as being associated with indeterminate WB results. They include: systemic lupus erythematosus, presence of rheumatoid factor and polyclonal gamopathy, antibodies to DR-HLA, cross-reactivity to core proteins of other retroviruses like Bovine Immunodeficiency Virus, Mycobacterium leprae infection, heat inactivation of serum samples, in vitro hemolysis, elevated bilirrubin levels and tetanus vaccination.

The significance of the majority of the cases of donors with an indeterminate WB results remains to be determined. Prolonged clinical and laboratorial follow-up of these individuals in reference centers, with addition of molecular tests may be necessary to answer these questions and to improve donor selection and notification strategies.

What are false negative reactions and why do they occur?

Recombinant and synthetic antigen based immunoassays are very sensitive and specific for the HIV subtypes found in the United States and Europe (mostly HIV-1 subtype B). However, some react poorly with sera from other geographical regions where non-B subtypes are prevalent. (e.g., in India subtype-C is more prevalent) . Also it was reported that some diagnostic kits could not detect the highly divergent HIV-1 subtype O all the time in African sera. This may be due to an inadequate amount or inappropriate sequence of the antigen used, low-affinity antibody in the sera tested, low antibody titer, or inherent feature of the assay system. Therefore, in order to reduce the number of false-negative results it is recommended that manufacturers include in the assay additional peptides from the gag (e.g., p24 antigen) apart from the env region that are also immunodominant and highly conserved. In addition, manufacturers should test sera derived from different geographical locations.

The different causes of false negatives are described in the following chart:

Cause of false-negative results

1. Performance and technical errors
   - Pipetting error
   - Mislabeling of samples or wells
   - Variability in test kits
   - Decreased intensity of EIA reaction by
     - Powder from powdered gloves
     - Storing of samples in serum separator tubes
2. Biologic, pathologic, and pharmacologic determinants
   - Window (preseroconversion) period
   - Delayed antibody synthesis in infants
   - Diminished immune response because of
     - HIV-1-related immune dysfunction
     - Immunosuppressive therapy
     - Concurrent infection with Epstein-Barr virus or cytomegalovirus
3. Congenital or drug-induced hypogammaglobulinemia
   - Formation of antigen-antibody complexes
4. Sensitivity and specificity of the assay
   - Infection by HIV-1 subtype O (if test kit do not use subtype O-specific antigen)
   - Sampling prior to immunoglobulin M to immunoglobuli G class switching (only if 2nd generation assay is used for testing)
   - Hemodilution
   - Nondenatured antigenic epitopes
   - Limited antigenic determinants

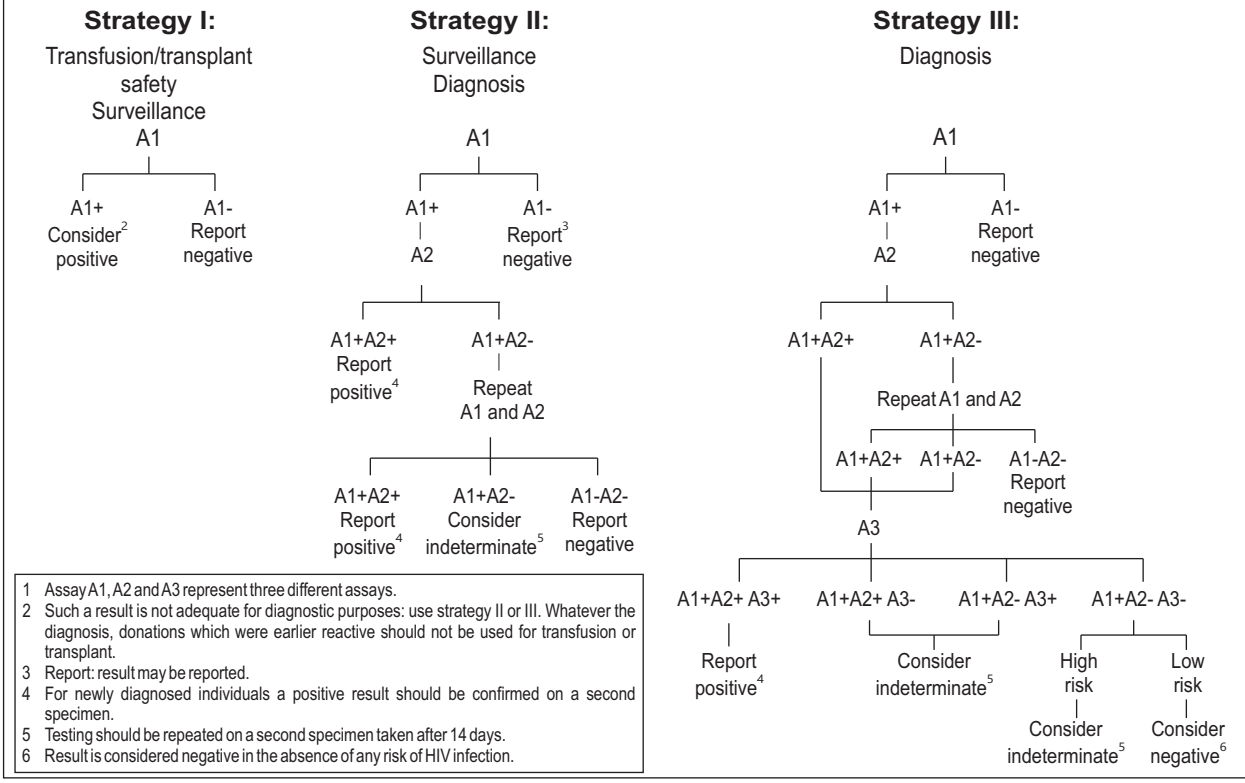
What are the different strategies for confirmation of HIV?

As described earlier, because of endemicity of different pathogenic infections in most developing countries, the probability of false positive reactions are high. In fact, the following quote from WHO/UNAIDS document is pertinent in this context - *"When a single screening assay is used for testing in a population with a very low prevalence of HIV infection, the probability that a person is infected when a positive test result is obtained (i.e., the positive predictive value) is very low, since the majority of people with positive results are not infected. This problem occurs even when a test with high specificity is used."*

Therefore, because of the natural cross-reactivity that exists between HIV antigens and other proteins present in the human serum, all immunoassys are considered to be screening tests. This is where the role of supplemental or confirmatory tests becomes important. Results of screening tests in HIV that arouse suspicion, based on clinical symptoms, should be investigated by other appropriate supplemental or confirmatory tests.

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. WHO / UNAIDS has recommended three 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.

Schematic representation of the UNAIDS and WHO HIV testing strategies



1 Assay A1, A2 and A3 represent three different assays.
2 Such a result is not adequate for diagnostic purposes: use strategy II or III. Whatever the diagnosis, donations which were earlier reactive should not be used for transfusion or transplant.
3 Report: result may be reported.
4 For newly diagnosed individuals a positive result should be confirmed on a second specimen.
5 Testing should be repeated on a second specimen taken after 14 days.
6 Result is considered negative in the absence of any risk of HIV infection.

Which strategy to follow?

UNAIDS / WHO also recommends for HIV testing strategies according to test objective and prevalence of infection in the sample population. The following chart explains that:

Objective of testing	Prevalence of infection in the category to which the patient belongs (percentage)	Testing strategy applicable
Screening of blood and blood products	-	I
Surveillance	> 10 < 10	I II
Diagnosis: With clinical signs/ symptoms	> 30 < 30	I II
Asymptomatic	> 10 < 10	II III

For example, in India, HIV prevalence is < 10%. Therefore, as per the above guideline, strategy to be followed in India for "Diagnosis of Asymptomatic Individual" is Strategy-III. On the other hand, for "surveillance" purpose it should be Strategy-II.

How should HIV test results be reported?

As per WHO/UNAIDS recommendations, the following chart elaborates how to report HIV samples that may be of three categories viz., positive, negative and indeterminate or equivocal.

Report	**Negative -** if initial/screening test shows non-reactive result. **Positive -** if the sample shows reactive results concordantly by the three screening tests. **Indeterminate (equivocal) -** if the sample shows discordant results by the three screening tests, the follow up samples are required to retest at two weeks and at three, six and 12 months before the final status of the test results should be conveyed. If the status remains indeterminate after one year, the person is considered to be HIV antibody negative.
Special considerations	Pre-test, post-test counselling service should be provided and confidentiality should be maintained.

From the above chart it is clear that indeterminate results are to be followed up upto at least 1 year after. If still indeterminate then the person should be considered as HIV antibody negative.

HIV TESTING & COUNSELLING

HIV testing and counseling have been recognized as necessarily linked since the first HIV enzyme-linked immunosorbent assay (ELISA) tests became available for the identification of HIV infection in the mid-1980s. Pre-test and post-test counseling were seen as crucial for the testing process because of the seriousness of the news of HIV infection for people receiving a positive result. Additionally, the process of pre-test counseling was designed to ensure that those tested were sufficiently informed about the testing process and the potential consequences: counseling made informed consent possible and ensured that people were not tested in a coercive manner. People with HIV needed the support of post-test counseling in order to manage disclosure and cope with living with HIV. This counseling included the provision of information on preventing the infection of partners and families in the future and on decision-making about pregnancy.

UNDERSTANDING HIV TESTING OBJECTIVES

The main objectives for HIV tests are:

1. Screening of donated blood to reduce transmission of the virus through transfusions
2. To prevent mother to child transmission
3. To detect and prevent sexual transmission
4. Surveillance of HIV prevalence or trends over time in a given population, through unlinked testing of serum (anonymous testing for statistical purposes only)
5. Diagnosis of infection in individuals

Tests with high sensitivity should be used when the objective is to minimize the number of false negative results, such as in screening of donated blood. When the objective is to minimize false-positives, such as in confirming whether an individual is HIV-infected, tests with high specificity should be used. In areas where HIV prevalence is low (like in India), most positive results in initial screening tests are in fact false- positives, so supplemental tests should always be performed if the objective is diagnosis. Today's standards require HIV tests to have a sensitivity of >99% and a specificity of >98%.(Note that the specificity of test kits may vary according to the geographical origin of the serum samples.)

There is no single test suitable for all objectives in all settings. For this reason, different types of tests based on different technologies are often used in combination, depending on the testing objective and the assays cost-effectiveness under local conditions.

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