CHAPTER 9

LABORATORY DIAGNOSIS OF HIV INFECTION

Abstract

Tests for diagnosis of HIV infection include specific tests for HIV infection, detecting immune deficiency, and diagnosing opportunistic infections and malignancies. The *specific* tests for diagnosis of HIV infection are *screening tests*: Enzyme linked immunosorbent assay (ELISA), rapid assays, and simple agglutination assays, collectively known as "E/R/S"; *confirmatory* or *supplemental assays*: immunoblot or Western blot (WB), immuno-fluorescence assay (IFA), radio-immuno precipitation assay (RIPA), and radio-immuno assay (RIA); and *tests for detecting HIV antigen, viral nucleic acids, viral components, or the virus itself*: detection of p24 viral antigen, RT assay, virus culture, and detection of HIV nucleic acids. HIV-infected women tend to have higher CD4 cell counts as compared to their male counterparts, even when the viral loads are similar. HIV-infected women also tend to develop opportunistic infections at higher CD4 counts and this fact should be considered while initiating ARV treatment.

Key Words

ELISA, HIV testing policy, HIV testing protocols, Polymerase chain reaction, Rapid assays, Simple assays, Strategies for HIV testing, Supplemental assays, Western blot

9.1 – PURPOSE OF HIV TESTING

Mandatory Screening: All donated blood, blood products, semen, cells, tissues, cornea, bone marrow, kidney, and other organs are screened for the presence of HIV antibodies, as required by the law of the country. Antenatal screening may also be carried out. An individual found to be positive for HIV antibody should *never* donate blood or any body parts. Though HIV antibody tests may be negative during the early stage of infection, mandatory screening can eliminate large majority of HIV positive persons among potential donors. An individual who tests positive in early stage of infection is infectious to others.

Sero-Epidemiological Studies: Serological tests are useful in epidemiological studies to study incidence, prevalence, geographical, and demographical distribution of HIV infection.

Diagnosis: HIV antibody tests are used to determine when infection has occurred after accidental occupational exposure or in cases of rape. The antibody tests may be negative in acute illness (CDC Group I) and also in late stages when

immune suppression has occurred. The test kits should be able to detect antibodies to both HIV-1 and HIV-2.

Prognosis: Absence of detectable anti-p24 antibodies indicates clinical deterioration. This is associated with increase in HIV antigens and viral load in peripheral blood. The following tests are used for monitoring the course of HIV infection: (a) CD4 cell count: less than 500 cells per mm^3 of peripheral blood indicates progression of disease and the need for ARV treatment; less than 200 cells per mm^3 indicates risk of severe opportunistic infections; (b) viral load: measurement of HIV RNA by reverse transcriptase (RT)-PCR); and (c) measurement of beta-2-microglobin and neopterin in serum or urine: increasing levels indicate progression of HIV disease

9.2 – TYPES OF TESTS

9.2.1 – Laboratory tests for HIV infection and related conditions

1. *Specific tests* for the diagnosis of HIV infection are grouped as follows: (a) screening (E/R/S) tests, (b) confirmatory or supplemental assays, and (c) tests for detecting HIV antigen, viral nucleic acids, viral components, or the virus itself – detection of p24 viral antigen, RT assay, virus culture, and detection of HIV nucleic acids.
2. *Tests for detecting immune deficiency* are not specific for detecting HIV infection alone. Abnormal results may also be obtained in other diseases and/or infections. They provide extra information about the health status of the patient. These comprise: (a) surrogate markers: serum prolactin (increased), dehydro epiandrosterone (decreased), anti-infectivity factor protein to HIV-1 (present), serum IgE (increased); and (b) indirect predictors: total leukocyte count (less than 400/mm^3), CD4: CD8 ratio (normal = 2:1; AIDS = 0.5:1), CD4 cell count (decreased), thrombocyte (platelet) count (decreased), beta-2-microglobulin (increased), serum neopterin (increased), alpha-1-thymosin (increased), IgG and IgA levels (both raised), tuberculin and other skin tests (indicate reduced CMI).
3. *Tests for diagnosing opportunistic infections and malignancies*: most opportunistic infections are diagnosed by microscope and culture. Serology is unreliable due to immune suppression.

In most cases, the duration of viraemia is approximately less than 1 week before the appearance of anti-HIV antibodies. Anti-HIV antibodies are detectable by commonly employed tests within 4–6 weeks of infection and in virtually all infected individuals within 6 months and these antibodies persist for life. The level of viraemia is a predictive marker for progression of HIV disease (WHO, 2003).

9.2.2 – CD4 Cell Counts

The normal absolute count of CD4 cells is 950–1,700 per mm^3 of peripheral blood. The percentage of CD4 and CD8 cells is 20–45 per cent and 30–60 per cent, respectively. These normal counts are based on Western literature. Studies

conducted in Lucknow and Vellore show variations in these counts for the Indian population. Hence, a nationwide baseline study is necessary. 3 mL of blood is collected aseptically by venepuncture in a vaccutainer containing EDTA (an anticoagulant). The blood is collected after 10–12 hours of overnight fasting. Ideally, the sample should be processed *immediately* to prevent erroneous results due to disintegration of T-lymphocytes. CD4 and CD8 counts are usually determined by flow cytometry. This method measures the wavelength of light emitted by cells as they flow in a stream, to determine the type of cell. The percentage of cells of each type is multiplied by the total lymphocyte count per micro litre to calculate the absolute number of CD4 or CD8 cells per mL of blood. Manual count kits, enzyme immunoassays, and microsphere assays are also used for CD4 and CD8 cell counts. Though easier to perform and less expensive, these methods are less accurate, compared with flow cytometry. CD4 counts show considerable interassay variation.

As compared with adults, all counts of T-lymphocytes are higher in children. In women, the CD4 counts are influenced by the phase of the menstrual cycle. The CD4 cell counts are higher in the morning and day-to-day variations are also seen. It varies during stress, exercise, acute illness, and drug therapy. Therefore, any large unexpected change in the CD4 cell counts should be confirmed by repeat testing, few days later. The *percentage* of CD4 cells is less variable than the absolute count and may be more reliable in evaluating response to ARV therapy or progression of HIV disease. HIV-infected women tend to have higher CD4 cell counts as compared with their male counterparts, even when the viral loads are similar. HIV-infected women also tend to develop opportunistic infections at higher CD4 counts and this fact should be considered while initiating antiretroviral treatment.

9.2.3 – HIV Diagnosis in the New Born

Conventional antibody tests cannot establish the presence of HIV infection in babies born to HIV-infected mothers because presence of anti-HIV antibodies in newborns may be due to either primary infection or passive transfer of anti-HIV antibody from the mother to the uninfected baby. These anti-HIV antibodies may persist up to 18 months. Appropriate methods for diagnosing HIV infection in babies less than 18 months are by detection of viral DNA or RNA, viral culture, and detection of IgA antibodies to HIV. IgA does not cross the placenta (WHO, 2003).

9.3 – SPECIFIC TESTS

This is the simplest and most frequently used diagnostic technique. Antibodies to both *core* (p24) or *envelope* (gp120, gp41) proteins are detected. There are two types of serological tests: screening (E/R/S) tests and supplemental or confirmatory tests. Among the tests for detecting HIV infection, antibody

detection remains the method of choice (Joshi & Chipkar, 1997). Antibodies can not be detected during the "window period" when initial viral replication occurs (Table 1). IgM antibodies appear in circulation only about 3–4 weeks after infection. Subsequently, IgG antibodies appear. IgM antibodies disappear after 8–10 weeks, but IgG antibodies remain throughout. With the onset of immune suppression, some anti-HIV antibodies such as anti-p24 may disappear.

9.3.1 – Screening (E/R/S) Tests

The following three tests are collectively known as "E/R/S" and includes (a)enzyme linked immunosorbent assay (ELISA); (b) rapid assays – dot blot assay, particle agglutination (latex, gelatine), HIV spot, and comb tests; and (c) simple tests based on ELISA principle.

CLINICAL SPECIMENS: The specimen of choice for HIV testing is serum or plasma. Assays for detecting anti-HIV antibodies in whole blood, saliva, urine, and dried blood spot have also been developed (WHO, 2003).

Saliva: In injecting drug users, blood vessels are collapsed and serum samples can not be easily obtained. Saliva is an acceptable alternative clinical specimen for antibody testing by ELISA. Use of saliva yields comparable results. Collection of saliva is a non-invasive and painless procedure with no risk of needle-stick injuries to health care workers (Frerichs *et al*., 1992). However, tests that use saliva are not very sensitive in relation to recent sero-conversion.

Urine: HIV antibody levels in urine are much lower than that in plasma. This could lead to false negative reaction, especially in early seroconverters. It is also difficult to achieve sterility of urine to prevent microbial contamination and enzymatic activity (Joshi & Chipkar, 1997).

9.3.1.1 – ELISA

ELISA is recommended as a *first-line test* by NACO. ELISA techniques require an ELISA reader and are suitable for use in laboratories where more than 30 samples are tested at a time (WHO, 2003). Direct solid phase ELISA is most

Table 1. Relation of tests to clinical stage

	Antigen detection			Antibody detection	
Stage of infection	p24[a]	RT[a]	Virus isolation	ELISA	Western blot
Window period	+ (< 50%)	+ (< 50%)	++	–	–
Acute HIV infection	+	+	±	+	+ (Partial p24 and/or gp120)
Asymptomatic infection	–[b]	–	–	+	+ (Full pattern)
Symptomatic disease	+	+	+	+	+ (p24 absent)

[a] *p 24 = p24 antigen; RT = Reverse Transcriptase assay*.

[b] *Decline of p24 antigen signals progression of disease and indicates need for ARV therapy*.

commonly used. Most commercially available ELISA kits detect both HIV-1 and HIV-2.

Procedure: The antigen is prepared from viral lysate or recombinant protein and/or synthetic peptides (WHO, 2003). The viral antigen is coated on surface of microtitre wells and the test serum is added. If antibody is present, it binds to the viral antigen; unbound serum is washed away. Anti-human goat Ig linked to a suitable enzyme and a colour-forming substrate are added. Development of colour (detected by photometer and read by ELISA reader) indicates *positive* test.

Advantages: Easy to perform, high sensitivity and specificity, can be automated for testing a large number of samples, reagents have a shelf life of 6–12 months, and economically advantageous over rapid test.

Disadvantages: Takes 3 hours to yield results. In general, higher the prevalence of HIV positive, greater the probability of true positives, greater is the positive predictive value (PPV). Causes of *false positive* ELISA test include blood malignancies, any infection due to DNA-virus, autoimmune disorders, multiple myeloma, primary biliary cirrhosis, alcohol-induced hepatitis, chronic renal failure, positive RPR test for syphilis, vaccination against influenza and hepatitis B, treatment with antisera-containing antibodies, antibodies to class II lymphocytes, and Steven-Johnson syndrome. *False negative* ELISA test results may be obtained when the test is performed during the "window period". Other causes include immunosuppressive therapy, replacement transfusion, malignant disorders, B-cell dysfunction, bone marrow transplantation, use of kits that primarily detect p24 antibodies, and presence of laboratory glove starch powder (NACO Training Manual).

Modifications of ELISA: Various modifications of ELISA are possible, depending on solid phase, source of antigen, enzyme conjugate-substrate detection system, and sequence in which the reagents are used in ELISA (Joshi & Chipkar, 1997). The solid phases used are microtiter plate (common), polystyrene beads (common), nitrocellulose membrane, nylon membrane, red blood cells, gelatine particles, latex particles, and microscope slides.

Sources of Antigen: (a) viral lysate: since this is produced from T-cell culture material, the contaminating cellular antigens can increase the false positive reactions. This is used in the first generation ELISA; (b) recombinant antigen: incorporating the genetic material of HIV into "vehicles" such as *Escherichia coli* produces recombinant antigens. This is used in second generation ELISA; (c) synthetic peptides: these are chemically synthesized amino acid residues corresponding to specific epitopes of viral antigens. They are free from contaminating cellular material and are used in third generation ELISA.

Enzyme Conjugate-Substrate Detection System: A substrate is a reagent, which is degraded in the presence of the conjugate due to enzymatic activity. The enzymes used are alkaline phosphatase and horseradish peroxidase. *Horseradish peroxidase* is the preferred enzyme due to its low cost, easy conjugation to protein and the wide variety of substrates that can be used in combination with it.

Based on the sequence in which the reagents are added, ELISA can be classified as follows:

1. Indirect ELISA: This is the most common type of ELISA used for antibody detection. The HIV antigens are coated on to the solid phase such as wells of microtitre plate or polystyrene beads. Antibodies, if present in the sample will bind to the antigen. This antigen-antibody complex can be detected by enzyme-labelled conjugate. A colour reaction is produced on addition of a suitable substrate. This colour reaction is directly proportional to the concentration of antibodies in the given sample.
2. Competitive ELISA: The sample from the patient and the enzyme-labelled antibody (conjugate) are added simultaneously on to the solid phase. HIV antibodies, if present in the sample, compete with the antibodies in the conjugate and reduce the binding capacity of the labelled antibody on the solid phase. If a colour reaction is produced on adding substrate, it indicates the absence of HIV antibodies in the given sample.
3. Antigen Sandwich ELISA: It is a modification of indirect ELISA, to increase the sensitivity and specificity of the test. This assay detects all classes of HIV antibodies. The components of this test are similar to that of indirect ELISA, except that an enzyme-labelled antigen is used instead of enzyme-conjugated anti-human immunoglobulin.
4. Antigen-Antibody Capture Assay: The solid phase is coated with an antibody agent (usually goat or sheep anti-human immunoglobulin), which "captures" the antibody, if it is present in the sample being tested. The antibody is detected by using either an enzyme-labelled antigen, or antigen followed by an enzyme-labelled antibody. If an antibody is present in the given sample, on addition of the substrate, a colour change is obtained, in presence of the conjugate. This colour change is directly proportional to the amount of antibody present in the specimen.
5. Chemiluminescence: This is a modification of ELISA, involving the use of light enhancing substances with the substrate. A special reader is used, which is able to detect the slightest variation in optical density (Joshi & Chipkar, 1997).

9.3.2 – Rapid Tests ("R")

Rapid tests are visual tests that do not require the ELISA reader. These tests are available in smaller test packs and are therefore suitable for a laboratory processing smaller number of samples (WHO, 2003). Rapid tests include dot-blot assays, particle agglutination (gelatine, erythrocyte, latex, and microbeads), HIV-spot and comb test, and fluorometric microparticle technologies. These tests use HIV-1 or HIV-2 recombinant or synthetic peptide antigens separately. This allows differentiation of HIV-1 and HIV-2 infection.

Advantages: Most rapid assays have a built-in immunological control "dot" to confirm that the test has been performed accurately. Though most rapid tests have sensitivity and specificity comparable to that of ELISA, they are technically

simple to perform and results are available in less than 10 minutes. No specific equipment is required for identifying positive reaction.

Disadvantages: These are expensive tests with subjective end-point. Stored, contaminated, or lipaemic samples can give faulty results. Most of the rapid assays use only one synthetic peptide. Hence, early seroconverters or weak positive samples may not be detected. Automation is not possible and thus, only a few tests can be done at a time (Joshi & Chipkar, 1997).

9.3.3 – Simple Tests ("S")

These are based on the ELISA principle. Simple tests take a little longer (more than 30 minutes), as compared with rapid tests which are used to confirm the diagnosis. The WHO recommends that a sample tested positive by one type of ELISA/rapid test should be retested by another ELISA/rapid test, based on a different principle, and using a different antigen preparation.

9.3.4 – Agglutination Assays

These assays have a solid phase (usually gelatin particles, red blood cells, latex particles, or microbeads are used), which acts as the indicator system. The HIV antigens get non-specifically bound to these carrier particles. HIV antibodies, if present in the given sample, form a lattice network with the antigen-coated particles. This lattice network is visualised as "clumping" or "agglutination".

Advantages: These tests are easy to perform, have good sensitivity, require little or no equipment, and are relatively cheap. Their results are comparable to that of ELISA, and are less time consuming.

Disadvantages: These tests cannot be automated for mass screening of samples; false negative reactions or "prozone phenomenon" can occur, especially when gelatin particles are used as the solid phase (false negative reactions are prevented by diluting the samples); interpretation of reactions is subjective; and species-specific agglutinins are a problem when red blood cells are used as solid phase. Red blood cells (erythrocytes) have a tendency for *rouleaux* formation, which could be misinterpreted as a positive reaction (Joshi & Chipkar, 1997).

9.3.5 – Confirmatory (or Supplementary) Tests

The WHO recommends that a sample tested positive by one type of ELISA/rapid test should be re-tested by another ELISA/rapid test, based on a different principle, and using different antigen preparation. When a tested sample is reactive once, by a system of E/R/S, the test is repeated by a different system to confirm the diagnosis. When a tested sample is reactive a second time, a confirmatory/supplemental test is used with the same sample to confirm the diagnosis (Joshi & Chipkar, 1997; NACO).

Rationale: Screening tests are highly sensitive, but may lack specificity. False positive reactions can be caused by cross-reactivity with contaminating host cellular antigens or due to technical errors. Therefore, more specific confirmatory tests are essential for discriminating between a true positive and false positive reaction.

Limitations: Confirmatory assays do not always produce conclusive results ("positive" or "negative"). Additional tests should be performed in case the results are inconclusive or "indeterminate". Individuals who test indeterminate should be re-tested after several weeks or months (Joshi & Chipkar, 1997).

9.3.5.1 – Western Blot

This is the standard confirmatory assay, which should be used only to resolve indeterminate results or diagnose HIV-2 infection (WHO, 2003). Combination kits for detection of HIV-1 and HIV-2 antibodies are available, where HIV-1 viral lysate antigens and HIV-2 envelope-synthetic peptides are incorporated into nitrocellulose strips. Individual viral antibody detection kits are also available. The western blot (WB) usually detects antibodies to p24 (*gag* gene, core protein) and gp 120, gp 41, gp 160 (*env* gene, envelope protein). False positive and indeterminate results are avoided since these assays do not contain contaminating cellular components (Joshi & Chipkar, 1997).

Technique: The HIV proteins (antigens) are separated according to their molecular weight by polyacrylamide gel electophoresis. These separated proteins (antigens) are transblotted on to a nitrocellulose membrane. This blotted membrane is cut into thin strips and are reacted with test serum. If antibodies to HIV are present in the test serum, they combine with different protein fragments of HIV. The strips are then washed and reacted with enzyme conjugated anti-human globulin. A suitable substrate is then added, which produces colour bands. Position of the colour band on the strip indicates fragment of antigen with which antibodies have reacted

Interpretation: If the test is *positive*, bands will show multiple protein fragments. Bands representing the 3 genes (gag, env, pol) provides conclusive evidence of HIV infection. WB test is *positive* if strips show bands representing at least *two* out of four HIV proteins – p24, gp160, gp120, gp41. If bands representing p24 or gp120 are seen only at one site, interpretation of WB test is difficult. This situation occurs in early HIV infection or may be non-specific. A reference strip and criteria for interpretation have been issued by the WHO, the American Red Cross, and the Consortium for Retrovirus Serology Standardization (CRSS). (Joshi & Chipkar, 1997).

Line Immunoassay (LIA): The single strip line immunoassay is a modification of the WB. This technique uses synthetic peptides and recombinant antigens coated as discrete lines on a nylon strip. Combination kits for line immunoassay are available for detecting HIV-1 and HIV-2 (Joshi & Chipkar, 1997).

9.3.5.2 – Immunofluorescence assay (IFA)

HIV-infected cells are fixed onto a glass slide and reacted with test serum. Fluorescein-conjugated anti-human gamma globulin is then added. If the test is positive, *apple-green* fluorescence appears when examined under fluorescent microscope. Immunofluorescence assay is less time consuming as compared with the WB technique, but the performance is comparable to that of the WB technique. However, it requires an expensive fluorescence microscope and trained personnel to interpret the results (Joshi & Chipkar, 1997).

9.3.5.3 – Radioimmuno precipitation assay (RIPA)

This method is a research-type assay, which can be used as an alternative to, or in combination with the WB. Though extremely sensitive, it is expensive and involves radioisotope banding and cultivation of HIV. HIV-infected T-lymphocytes are cultured in presence of radiolabelled amino acids. These T-lymphocytes are then lysed. HIV antibodies, if present, forms antigen-antibody complexes, which are precipitated. This precipitate is subjected to electrophoresis to separate the labelled complexes by their molecular weight. The bands are visualised by autoradiography (Joshi & Chipkar, 1997).

9.3.5.4 – Radioimmunoassay (RIA)

Radioactive iodine^{125} I, which is usually used as a *detector*, is covalently bound to an antigen, antibody, or antibody-binding reagents. A gamma-counter is used to measure the gamma radiation produced by the bound^{125} I. This assay is highly sensitive and specific. However, it is very expensive and measures response to a single viral protein only. It takes a long time to standardise the assay (Joshi & Chipkar, 1997).

9.3.6 – Tests for Detecting Antigen or Virus

9.3.6.1 – Detection of HIV antigen

When a *single* infective dose is high, p24 viral antigen and RT antigen may be detected in the blood after about 2 weeks. The p24 antigen appears early, disappears from the circulation during the prolonged asymptomatic phase and then reappears with the onset of symptomatic disease. Decline of p24 antigen during the asymptomatic phase signals progression of disease and indicates need for ARV therapy. Recurrence of p24 antigenemia corresponds to disappearance of anti-p24 antibody from the circulation. This antigen is detected by p24 antigen ELISA capture assay that uses anti-p24 antibody as the solid phase. Clinical samples for detecting HIV antigen are serum, plasma, CSF, and cell-culture supernatant. This technique is expensive. A "positive" test confirms HIV infection; while a "negative" test does not rule out HIV infection. On incubating the anti-HIV antibody with the patient's serum, free HIV antigen reacts with the anti-HIV antibody. Addition of rabbit anti-HIV p24 antibody and enzyme

conjugate produces a change in colour, which is read at 450 nanometres. The concentration of p24 is determined from a standard curve. Antigen detection tests are used to detect HIV infection in the newborn, when maternal antibodies to HIV-1 confound serological diagnosis and to detect HIV infection during early "window" phase. There is a burst of viral replication, a few weeks after the primary infection. This method is used to resolve equivocal results of WB test. It is also used to: (a) diagnose involvement of the central nervous system or terminal illness. Due to immune collapse during this stage, the HIV antibodies against the viral core antigen may disappear or the HIV antigens may be absent; (b) monitor response to ARV therapy; and (c) monitor progression of HIV disease (Joshi & Chipkar, 1997).

Reverse Transcriptase (RT) Assay: The enzyme RT catalyses the transcription of genetic code of the HIV from its RNA genome to a double stranded DNA, by using radio-labelled thymidine. The activity of RT can be detected as the DNA molecules are synthesised using radio-labelled thymidine, which gets incorporated into them. The amount of radio-labelled thymidine is directly proportional to the concentration of RT, which is directly proportional to the concentration of HIV. RT assay is not used routinely because radio-labelled thymidine, which has short shelf-life, is required to detect RT activity. Handling radioactive material is hazardous. Expensive material and equipment are required, and the assay is not cost-effective.

9.3.6.2 – Isolation of virus

It is a reference method for identifying HIV infection. Once infected with HIV, an individual remains infected for life. HIV is present in blood and body fluids mostly within CD4 lymphocytes. Clinical samples for isolation of virus are peripheral blood, serum, and bone marrow. HIV titres are *high* in the early phase of infection, before antibodies appear. Antibodies do not neutralise the virus but coexist with HIV. During the asymptomatic phase, HIV titre is *low* and may not be detectable. With the onset of clinical symptoms, HIV titre rises again. A 98 per cent positivity is obtained from confirmed HIV positive individuals. Lymphocytes from patients are co-cultured with an indicator cell-line or peripheral blood mononuclear cells from a healthy, adult, sero-negative donor. Every 3–4 days, the culture has to be supplemented with fresh peripheral blood mononuclear cells, or indicator cells. These cultures are maintained for at least 4 weeks. Assays are done weekly for evidence of viral specific markers like p24 or RT. Viral culture can not be used as a routine assay because the laboratory should maintain strict sterile conditions for the viral culture. Since the virus is directly being cultured, the material is hazardous to maintain. Laboratory staff may be exposed to high concentration of HIV.The culture has to be monitored for 2–7 days and it takes several weeks for the final results. The necessary reagents are very expensive and the assay labour-intensive. The success rate is only about 60–70 per cent. Viral culture is used mainly for characterisation of the virus, determining drug sensitivity, and for vaccine studies (Joshi & Chipkar, 1997).

9.3.6.3 – Detection of viral nucleic acid

Viral nucleic acid can be detected by PCR even in the window period. Being expensive, PCR tests are indicated only when the results of other diagnostic tests are inconclusive. In DNA PCR, peripheral lymphocytes are lysed and proviral DNA is amplified. This test has high sensitivity and specificity. RNA PCR is used both for diagnosing HIV infection and for monitoring level of viremia.

Clinical Samples: peripheral blood, plasma, semen, vaginal or cervical secretions, tissues, CSF, or other specimens such as saliva, urine, breast milk, tears, and amniotic fluid.

It is necessary to amplify target HIV sequences or the molecular probes bound to the target sequences, due to the relatively low viral load in early seroconverters, in individuals in the window period and in infants with transplacental transmission of HIV. PCR, the commonly used amplification technique, is a multistep repetitive process. One PCR cycle takes only a few minutes, and the cycle is repeated about 30–40 times. Theoretically 30 amplification cycles can produce up to a million copies from a single target DNA. Being an extremely sensitive technique, PCR is used to: (a) detect HIV infection in the window period and in neonates born to HIV positive mothers; (b) resolve indeterminate WB test and detect genetic variability among HIV isolates; (c) identify mutations of RT; (d) monitor viral loads in patients on ARV therapy; and (e) differentiate between latent HIV infection from active viral transcription, and HIV-1 and HIV-2 infections. However, PCR can not be routinely used as a screening assay since there may be doubts regarding cross-contamination, and problems of carry-over of amplified products leading to false positive results. It is also an expensive assay, requiring costly equipment and highly trained personnel (Joshi & Chipkar, 1997; NACO).

Viral Load Assay: This involves quantitative estimation of HIV RNA in plasma using PCR. Being highly sensitive, viral load assay is used for determining base-line viral load at start of ARV therapy, for monitoring progress of therapy, and for predicting disease progression and clinical outcome (Joshi & Chipkar, 1997).

9.4 – TESTING PROTOCOLS

9.4.1 – Methods of Testing

1. Unlinked Anonymous Testing: This is an epidemiological method for measuring HIV prevalence which involves the use of already collected blood.
2. Voluntary Confidential Testing: It is mandatory to maintain confidentiality in this diagnostic technique.
3. Mandatory Testing: This is done without consent, as required by law in some countries. In India, mandatory testing is restricted to screening of donors of semen, ova (for in vitro fertilization), blood, tissues, and organs (NACO).

9.4.1.1 – Testing protocols

The procedures should be documented so that the staff members know how to carry out various tests. A sample testing protocol is given below. After pre-test counselling and informed consent, collect the desired specimen from the client and label the tube with a code. Verify the specimen to be collected (whole blood, serum, and plasma), the mode of collection (by venepuncture or finger prick), and procedures for specimen collection. Record a code on the clinic form. The code can be linked to personal identifying information, demographic, and medical history of the client. Perform the HIV test based on the testing strategy (ELISA, or other simple, rapid tests). The exact procedures should be documented. Record the HIV test result on the clinic form. Give the result to the client by name, during post-test counselling (NACO).

9.4.1.2 – National HIV testing policy

Every HIV testing should be done after the patient's explicit consent and accompanied by pre-test and post-test counselling. Consent is however, *not* required for mandatory screening of donors of semen, ova, blood, organs, and tissues. No individual should be made to undergo a mandatory HIV test. HIV testing should not be imposed as pre-condition for employment, or for provision of health care. India's armed forces are exempted from this provision (NACO).

9.5 – STRATEGIES FOR HIV TESTING

Positive result obtained in any one screening test (E/R/S) has to be confirmed by a supplemental test. The WB test is expensive and time consuming and therefore, different strategies are followed for confirmation. Two different types of ELISA, or one ELISA test along with any of the rapid tests is performed. The clinical sample is considered "positive" if it is positive in both sets of tests. If inconclusive, the test sample is re-tested after 1–2 weeks. The *first* E/R/S test selected for any of the following three strategies should have high *sensitivity*. The *second* and *third* E/R/S tests should have high *specificity* to eliminate likelihood of false positive results.

1. Strategy-I: The clinical sample is subjected once to E/R/S using a highly sensitive and reliable test kit. If *positive*, the clinical sample is considered HIV infected and if negative, it is taken as HIV free.
2. Strategy-II: The clinical sample is subjected to a *first* E/R/S test. If "negative", the sample is taken as HIV free; if "positive", the sample is re-tested with a *second* E/R/S test based on a different antigen preparation and/or a different test principle. If the *second* E/R/S test is also *positive*, it is reported as "positive". Otherwise it is "negative".
3. Strategy-III: This is similar to Strategy-II, with an additional confirmation by a *third* E/R/S test that is based on a different antigen preparation and/or a different test principle. If the clinical sample tests *positive* on all 3 E/R/S, it is reported "positive". A clinical sample that is negative in the *third* E/R/S test is considered "equivocal" and re-testing advised after 2 weeks. If the

second sample collected after 2 weeks also shows "indeterminate" results, it should be tested by a confirmatory assay such as WB. If the confirmatory assay fails to resolve the serodiagnosis, follow-up testing is repeated at 4 weeks, 3 months, 6 months, and 12 months. After 12 months, such indeterminate results should be considered "negative" (WHO, 2003).

The WHO recommends that the strategy for HIV testing should be based on the objective of testing and the prevalence of HIV infection in the population. For ensuring *transfusion or transplant safety*, for all levels of prevalence of HIV infection in the population, Strategy-I is to be adopted. For *HIV surveillance*, Strategy-I is recommended when prevalence of HIV infection exceeds 10 per cent and Strategy-II when it is less than 10 per cent. For *diagnosing* HIV infection in the *presence of clinical manifestations*, Strategy-I is advocated when the HIV prevalence in the population exceeds 30 per cent and Strategy-II when it is less than 30 per cent. For *diagnosing* HIV infection in *asymptomatic* individuals, Strategy-II is to be used when the HIV prevalence in the population exceeds 10 per cent and Strategy-III when it is less than 10 per cent (WHO, 2003).

9.6 – QUALITY ASSURANCE AND SAFETY

The following checklist would be helpful in ensuring quality and reliability of HIV testing.

Pre-Analysis Phase:
1. Trained staff are available and are capable of performing HIV tests.
2. Laboratory set-up is safe and safety issues have been covered during the training.
3. Procedures for specimen collection and labelling are clear.
4. Conditions for transport of specimens are adequate.
5. Specimens are processed correctly before testing.
6. Expiry dates of test kits have been checked.
7. There is a system of rotation of stock of test kits.
8. Test kit reagents are stored at appropriate temperatures, as specified by the manufacturer.

Analysis Phase:
1. A written SOP manual exists.
2. Specimens are processed and stored as specified in the procedure manual.
3. Equipment is maintained and their performance is checked periodically.
4. Reagents are prepared and used correctly.
5. Internal quality controls are included in the HIV test kits.
6. There is a procedure for monitoring quality of tests.

Post-Analysis Phase:
1. HIV test results are correctly interpreted and recorded and records are properly maintained.
2. Data are entered in appropriate records.
3. Quality control is regularly reviewed.

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