HIV testing technologies after two decades of evolution

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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 antiretroviral therapy whose viral infection may become resistant to therapy. In addition, modifications to conventional methods have introduced new assays, such as sensitive/less sensitive (detuned) assays that can estimate when someone was infected, thereby providing a useful tool for epidemiologic incidence estimates and enrollment into specific intervention programmes for recently infected persons. Many of the newly evolving technologies are essential for use in resource-limited countries 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 use easily-collected fingerstick blood and oral fluids, and have one-step procedures that are relatively foolproof. Manual CD4 lymphocyte count assays that require only a light microscope and haemacytometer and more simple assays to estimate viral load are appropriate for developing countries where sophisticated instrumentation cannot be supported. Technologic advances with HIV diagnostics continue to address outstanding and new issues associated with diagnosis and the monitoring of infection by providing more simplified, cost-effective, and accurate testing throughout the world.

Key words CD4 levels - HIV screening - HIV testing - molecular methods - monitoring - nucleic acid tests

Laboratory testing to identify infection by the human immunodeficiency virus (HIV) has been used throughout the world for 20 yr, and continues to be a major factor in saving lives. Not only in HIV testing essential for protecting the blood supply and recipients of donor tissues, but it provides valuable information for the ongoing surveillance of infection and for the diagnosis of individuals who can benefit from therapy. More recently, exquisitely sensitive molecular methods have been developed that can identify persons with very early infection and they have been instrumental in giving health care providers information to stage infection, predict outcome, and to successfully manage infected persons who are on antiretroviral therapy. No other methods for detecting an infectious agent have had such an important global impact.

This review is aimed to provide a brief overview of the current technologies available for detecting and monitoring HIV infection. Also presented are newer technologies that can offer important enhancements for diagnosis, surveillance, blood screening, and disease monitoring, particularly for resource-limited countries.
**Evolving technology**

Over the past two decades, a number of important advances have been made in the arena of retroviral testing. In addition to refinement of the classical serologic screening and confirmatory assays, novel serologic methods have been developed to offer advantages in all testing situations. Among the advances, particularly during the last decade, are rapid serologic tests that can be performed on fingerstick blood samples and oral fluids, and require only 1 or 2 procedural steps. These rapid tests have been recognized to have important applications for saving lives through the institution of anti-retroviral therapy in a clinically relevant time during occupational exposure cases and for pregnant women in labour whose HIV status is unknown. They have also now been recognized as important tools for use in public health clinics to identify infected persons immediately, thereby reducing loss to follow up when patients must return at a later date for receiving their results.

ELISA technology has now progressed to fourth generation assays that are now being used in both industrialized and resource-limited countries to increase the sensitivity for detecting early infection in a cost-effective manner. Serologic assays have also been modified to include antigens for the enhanced detection of viral variants, such as HIV-1 Group O virus. Rapid confirmatory serologic tests have been introduced to offer cost-savings, simplicity, and can be applied in public health clinics to confirm infection, thereby allowing for immediate post-test counseling and enrollment into management programmes.

Serologic assays have also been introduced that can provide an estimation of the time that a person was infected. These sensitive/less sensitive (detuned) tests are valuable for incidence estimates for better surveillance and can assist in contact tracing.

Molecular assays for HIV, similar to serologic assays, have evolved over the past two decades, progressing from strictly research tools to routine methods that provide valuable contributions. The implementation of HIV molecular methods to characterize and manage HIV infection has changed the way that HIV medicine is practiced. Polymerase chain reaction (PCR), nucleic acid sequence based amplification (NASBA), and branched DNA (bDNA) methods offer increased sensitivity for early infection to enhance the safety of the blood supply, are used for the monitoring of HIV viraemia to predict disease outcome or death, and can be used to signal viral drug resistance through changes in viral copy number during therapy. Molecular assays also allow for genotyping to determine the mutations associated with drug resistance so that therapies can be changed to more effective ones, and can also be used to determine HIV types, groups, and clades for epidemiologic purposes. Most recently, more simplified methods have been developed and marketed that allow for a close estimation of viral copy number (viral load) in blood, thereby eliminating the need for sophisticated methods such as PCR that cannot be supported in many countries.

Manual methods to determine lymphocyte types (CD4) and numbers are a recent introduction to the HIV testing arena. The determination of CD4 levels and the periodic monitoring of these levels are the most important means to determine when to initiate anti-retroviral therapy and when a person’s immune system is failing. Classical methods to assess CD4 lymphocytes have required sophisticated instrumentation (e.g., flow cytometry); these methods are expensive, and difficult to establish and maintain in countries with limited resources. The availability of simple-to-perform manual methods is now addressing an important void. The world has finally assembled a significant effort to provide treatment for HIV infected persons in developing countries, and more simple and manual methods for monitoring CD4 levels and viral loads will be invaluable.

However, even with the best of methods, none are perfect or free from false positive and false negative results. Therefore, tests must be selected carefully, used appropriately, and their limitations realized.

**Markers during HIV infection**

Host and viral markers that occur during HIV infection are used to identify infection, and monitor viral replication, disease progression, and immune status. These markers appear fairly consistently between different individuals at various times after infection. The kinetics of their appearance (time
intervals) dictate the choice of tests (e.g., antibody, antigen, molecular, or CD4) to use; depending on the purpose of testing; they are based on an understanding of viral dynamics and the kinetics of the host’s immune response.

After HIV infection, the sequence of blood markers to identify infection, in their chronological order of appearance, is as follows: viral RNA, p24 antigen, and antibody to HIV antigens. Within 2 wk after infection (10-14 days), viraemia, as measured by viral RNA, appears to increase exponentially until the humoral and cell-mediated immune responses control HIV replication. Viral RNA levels, which are an indication of viral replication, usually reach close to 1 million copies of RNA/ml within a couple of months before gradually decreasing to a fairly constant level known as the set-point. This set-point is important, and has been used to predict the subsequent course of infection and disease. High set-points signal a faster course until the development of AIDS and death, while lower set-points are associated with a longer (slower) disease course. Subsequently, RNA levels gradually increase over time until a point at which viral replication again increases exponentially at the time of AIDS. In most individuals who are not treated with anti-retroviral therapy, the time to AIDS is about 10 yr.

Viral protein (p24 antigen) can also be measured in blood, but its detection is later than viral RNA. It is certain that p24 antigen increases in parallel to viral RNA as the virus replicates, but is detected later because of the methods used for its detection; that is, p24 antigen methods are less sensitive than the amplification methods used to detect RNA. This is supported by the recent development of highly sensitive p24 antigen methods (e.g., p24 boosted methods and Immuno-PCR).

The time interval before antibody appears, known as the serological “window period,” is characterized by seronegativity, detectable viraemia (as measured by RNA or p24 antigen), and variable CD4 lymphocyte levels. The detection of specific antibody to HIV signals the end of the window period and labels the individual as seropositive. Antibody to HIV usually appears at about 3-4 wk after infection, but depends on the specific antibody method used and variations in the immune response of different individuals. Nevertheless, antibody is detected in most persons within 1-2 months regardless of the method used, although there are reports indicating that a small percentage of persons may require up to 6 months for antibody to appear. The appearance of antibody is clearly a major mechanism in decreasing viraemia, as noted by the decrease in viral copies and p24 antigenemia as antibody levels rise. This is most likely because of anti-p24 antibody binding viral p24 antigen and limiting viral replication. At some later time, probably because of the slow destruction of the immune system, virus replication increases (RNA and p24 antigen levels increase), anti-p24 levels decrease, and the syndrome of AIDS manifests.

CD4 levels slowly decrease over the course of infection, most likely because of the slow destructive abilities of HIV. When levels reach about 200 CD4 cells/ml of blood, severe immune deficiency occurs, the person is labeled as having AIDS, and the prognosis is poor. The relationship of the viral and host immune markers is depicted in Fig.1.

Purposes of HIV testing

There are many purposes associated with HIV testing. For example, screening the blood supply using HIV antibody tests (e.g., ELISA) has been performed since 1985, and has resulted in the protection of countless individuals from HIV infection. These tests are still useful to protect the blood supply because most infected individuals who have established HIV infection will be detected with antibody tests. HIV antigen and molecular assays can also be applied as screening assays to detect early infection in blood donors and have resulted in the detection of about 5 infected persons per year in the United States (among 13 million donors screened) who were missed by antibody tests. However, in the majority of countries, limited resources restrict the use of antigen and molecular assays because of their cost or requirement for sophisticated instruments. Secondly, testing can be used for the diagnosis of infection, in which case screening test reactive results must be followed by confirmatory tests (e.g., Western blot, indirect fluorescence assay (IFA), Line immunoassay), or...
alternative confirmatory strategies. Confirmation is important so that individuals can be counseled appropriately, and so treatment can be instituted if indicated. HIV antibody tests are also utilized for epidemiologic surveillance, providing health officials with information about extent of the infection among different risk groups, thereby allowing them to target populations for vaccines, for treatment, to assess economical concerns, and to provide counselling to prevent the infection of others. Therefore, diagnostic HIV assays can be divided into two categories: (i) screening assays, which are designed to detect all infected individuals; and (ii) confirmatory (supplemental) assays that are designed to differentiate those persons who test falsely reactive by screening assays from those who are truly infected. Accordingly, screening tests possess a high degree of sensitivity (low false negative rate), whereas confirmatory assays must have a high specificity (low false-positive rate). For most applications, screening and confirmatory tests are performed in tandem to produce results that are highly accurate and reliable.

p24 antigen assays and molecular HIV assays have several important applications. First, they are used as adjuncts to antibody tests to detect early HIV infection; that is, they can shorten the serologic window period\(^a\). In addition, they can be valuable in assisting with the diagnosis of HIV infection in the newborn\(^9\), have proven to be the most important means for monitoring HIV disease progression, and are used to monitor the response to anti retroviral therapy to identify viral resistance for determining when to modify drug therapy\(^{10}\).

The state of the art in HIV testing

Screening assays: Although the goal of screening assays is to detect 100 per cent of all infected individuals, it is impossible because not all infected persons have antibodies or have antibodies at the level
detectable by current tests. Therefore, persons with early HIV infection, before antibody is detectable, will be labeled as negative by antibody tests, when in fact they are positive. However, in most populations, the majority of individuals have been infected for more than 4 wk (no longer in the window period) and will be detected using antibody assays. Therefore, antibody tests are the most appropriate and effective tests for the detection of almost all persons who are HIV infected.

*Enzyme-linked immunosorbent assays (ELISA) for antibody detection:* The initial tests developed for HIV antibody detection were ELISAs. This assay was chosen because these types of tests had been used successfully to detect a variety of other infectious agents, but also because they are generally easy to perform, suitable for large scale screening of blood, do not require the use of radioactive substances, and are sensitive and relatively specific.

ELISAs can be configured to detect antibodies or viral antigens through the use of antigens and antibodies as detection reagents, respectively. Since their introduction, HIV ELISA assays for antibody detection have been modified in different formats and refined and improved, each offering advantages for high-throughput, increased sensitivity and specificity, greater simplicity, and additional cost effectiveness. The two most popular configurations for ELISAs include the indirect ELISAs and the 3rd generation antigen sandwich ELISAs.

Indirect ELISAs are the most commonly used and the most cost-effective configurations to detect HIV antibodies.

Antigen sandwich ELISAs (3rd generation technology) were developed later in an effort to gain sensitivity. They have been shown to meet their claims for having an increase in analytical sensitivity (to detect low levels of antibody such that occur during early infection). In this ELISA configuration, antigen attached to the solid phase binds HIV antibody in the test sample, and is then detected by an enzyme-labeled antigen conjugate. That is, the bivalent antibody will bind to both the capture antigen and the enzyme conjugated antigen. The major advantage of the antigen sandwich technique is that all classes (isotypes) of antibody, including IgM that is sometimes produced early in infection, will be detected, thereby increasing the sensitivity for detecting early infection. Although this antibody test configuration offers sensitivity advantages, it is slightly more expensive.

More recently, 4th generation ELISA tests have been developed that detect both antibody and p24 antigen simultaneously11-12. Although these are more expensive than antibody tests, they offer a cost-savings for those laboratories where separate antibody and antigen assays are performed. That is, the combined 4th generation test is less expensive than performing two individual tests, mostly because of technologist's time-savings. An explanation of how these tests are configured has been published11.

*ELISA to detect p24 antigen:* The p24 antigen assay detects the viral capsid (core) p24 protein in blood which is detected earlier than HIV antibody during acute infection3. The p24 antigen appears at about day 16 after infection as a result of the initial burst of virus replication. Therefore, this marker can be used to shorten the serologic window period by about a week. Although this has been used to screen the blood supply for many years, it will be discontinued because RNA testing has proved to be a more sensitive method8.

Testing for p24 antigen has been of value in the following situations: (i) detecting early HIV infection in persons who have been exposed but are seronegative, (ii) screening blood for the detection of HIV infection before antibody is produced, (iii) diagnosing infection in the newborn, and (iv) monitoring anti-viral therapy. However, in countries that can afford and institute RNA testing, the p24 antigen test offers no advantages; it may be valuable in countries where RNA testing cannot be performed. However, unless an immune complex dissociation method is used, a major limitation is that the test is insensitive when testing blood, both because low levels of antigen are difficult to detect and because antigenemia occurs only transiently during different stages of infection due to the presence of p24/anti-p24 complexes.

The HIV p24 antigen test procedure is a basic antibody-sandwich ELISA where specific monoclonal
antibodies to HIV p24 are attached to the solid phase and “capture” the viral antigen in the sample (which is pre-incubated in a lysis buffer to disrupt the virion). An antibody detector (a biotinylated anti-p24 antibody) is added and incubated, followed by conjugate addition (streptavidin-horseradish peroxidase). If present, the captured antigen is bound by the biotinylated anti-p24, streptavidin HRP binds to the biotin, and the subsequent addition of a substrate results in the production of colour. The resultant optical density (OD) values generated from the colour development are proportional to the amount of HIV-1 p24 antigen in the specimen, and can be quantified by comparing OD values from a standard curve.

The p24 antigen tests are subject to false positive reactions, presumably because of interfering substances (e.g., rheumatoid factor) and other immune complexes. Therefore, specimens that test repeatedly reactive in the antigen test must be confirmed using an antigen neutralization assay to verify specificity. This neutralization assay consists of an anti-p24 antibody that is used to pre-treat the sample before testing in the routine p24 antigen assay. During this pre-treatment, true p24 antigen in the sample will be complexed with the neutralizing antibody, resulting in an antibody-antigen complex. This complex will prevent the p24 antigen from being captured by the solid-phase antibody when tested in the routine p24 test, resulting in a reduction of OD value (usually about 50%) as compared with the OD readings of the non-neutralized sample. If this degree of reduction (inhibition) does not occur, the sample is not confirmed to have p24 antigen, and the reactivity was probably not caused by p24 antigen.

It appears that the main reason for the lack of sensitivity of the antigen test when testing the serum of HIV infected persons is that free p24 antigen in serum is complexed with p24 antibody. To improve the sensitivity of the p24 antigen assay, manufacturers have introduced an immune complex dissociation (ICD) procedure using a low pH buffer or heat (or both) to dissociate p24 antigen/anti-p24 antibody complexes before performing the p24 antigen assay. This ultimately increases the number of p24 antigen molecules available for detection. Using the ICD procedure, an increased sensitivity of the assay can be demonstrated (up to 20% higher detection of positive samples).

**Rapid and simple antibody assays:** Not long after the HIV ELISAs were marketed, rapid and simple assays became available. Rapid HIV tests are defined as tests that can be completed in less than 30 min and do not require instrumentation and can be read visually. These tests gained popularity in the early 1990s, and as technology became refined, proved to be as accurate as the ELISAs.

Rapid tests offer a number of advantages over the more conventional ELISA screening assays. For example, rapid assays are simple to perform (require fewer steps than ELISA), require no instruments, can be performed by persons with limited technical expertise, are often performed at point-of-care facilities such as physician’s offices, and offer results in less than 30 minutes. In addition, a number of rapid HIV tests are considered to be robust (particularly in comparison to ELISAs), as they have a wide range of storage temperatures (4-33°C); many of these can be stored at room temperature. In addition, most rapid assays incorporate an internal procedural control, usually an antibody to human IgG, which detects any immunoglobulin in a sample and is primarily incorporated to eliminate false negative results that could occur because of failure to add a sample (an important attribute that EIAs lack). Some rapid tests are configured to allow the differentiation of viral types (HIV-1 and HIV-2) simultaneously. Even others have separate spots to identify and differentiate HIV-1 group M, HIV-1 group O, and HIV-2.

Rapid assays can be used in a number of essential situations. In United States public health clinics, for example, a large number of clients get tested but do not return for results. In such clinics, where 2.5 million HIV tests are performed each year, over 700,000 (33%) individuals who tested negative and 25 per cent of the nearly 10,000 HIV infected persons did not return to receive their results. Had a rapid HIV test been used that provides results immediately (e.g., rapid oral fluid or fingerstick test), these individuals would have learned their HIV status immediately and been informed and counseled.
appropriately. The use of a rapid test would have also eliminated the need for nearly 2 million negative persons to make arrangements to revisit. It would also allow the 10,000 positive persons to be counseled appropriately on their initial visit about behaviour changes and the potential to receive highly effective anti retroviral drugs that could undoubtedly extend their lives. In occupational exposure cases, rapid testing can occur in minutes, allowing treatment to be started within the two hour recommended time frame to significantly reduce the transmission to the injured person; note that the source patient, not the injured person, is tested to determine if the injured person has been exposed to HIV positive blood from the source. Also of high importance, rapid testing of pregnant women in labour whose HIV status is unknown, can be conducted immediately to make the important decision of the necessity for treatment. Appropriate treatment of both the HIV infected mother and the child in a relevant time frame can decrease transmission by up to 66 per cent. Rapid tests that use oral fluids or fingerstick samples are also valuable in certain populations that are reluctant to give blood (for religious reasons) or for populations where blood is difficult to obtain [obese individuals, intravenous drug addicts (IVDA), etc.] The use of oral fluid or fingerstick samples in combination with the ease of use of rapid assays offers a means to increase compliance for testing, thereby increasing the number of individuals who will learn their HIV status. Rapid HIV tests are becoming widespread in developing countries because they effectively address financial restraints and infrastructure issues (e.g., lack of stable electricity, equipment, and training of personnel) that are omnipresent in developing countries. Rapid tests can also be used in alternative screening and confirmatory algorithms. The use of two screening tests in tandem, if appropriately selected, may offer a predictive value equivalent to that of an ELISA/Western blot algorithm, but with substantial cost and time savings.

The disadvantages of rapid HIV tests are few, but include: (i) errors are common because users become careless with the simple procedures; (ii) interpretation of results are subjective (reader dependent); (iii) they are not effective if the laboratorian is colour-blind; and (iv) the cost is sometimes higher than that of the ELISAs, although cost-savings are realized if a small number of samples are tested per day.

Rapid assays are generally configured as (i) flow-through, (ii) lateral flow, or (iii) agglutination assays. Fig.2 shows a typical dot-blot assay for HIV-1 and Fig.3 depicts an HIV-1/HIV-2 combination assay. Flow-through assays were among the first rapid HIV assays introduced in the late 1980s, and are still popular. Many yield results within 5 or 10 min, some in as little as three minutes. They are easy to perform but do require multiple steps. In typical flow-through assays, the antigens are passively blotted onto the support membranes usually by hydrophobic interactions, and are most often placed as a small circle (dot) or line. These antigens are usually recombinant, synthetic peptides, or a combination of the two. A plastic device holds the solid support and contains absorbent pads under the membrane to collect the serum and reagents after the reaction with the antigens has occurred on the membrane. The captured antibodies are detected by an antibody-labeled enzyme conjugate, or more often using a protein A/colloidal gold conjugate (protein A will bind to human IgG). Most of these dot-blot assays can be stored at room temperature (25ºC) for at least 6 months (controls may need to be refrigerated) and each device is individually packed in the kit. To address interpretation subjectivity, reflectance densitometers have been developed to give an objective readout (Fig.4).

Rapid lateral flow assays typically consist of antigens applied to a membrane which is assembled “laterally” with sample pad at one end and wicking pad at the other end, and offer added advantages over flow-through rapid tests. These advantages include more simple procedures, including some having only “one-step” procedures, room temperature storage, some incorporating an antigen sandwich configuration (third generation) to increase sensitivity, and the practical advantage that these assays are not fraught with the problem of poor flow-through that is noted with some flow-through rapid devices because the reagents wick horizontally or vertically by capillary action. Most of these rapid assays, also called immunochromatographic assays, have most, if not all, reagents contained in the device. The reagents are
contained in a flat or cylindrical cartridge device, usually made of plastic or paper, and include a membrane strip that contains the antigens that will capture antibody in the sample. Whole blood, oral fluid, or serum (depending on the test) is applied at one end of the device (or the strip is placed in a tube containing the sample), and the sample is allowed to diffuse along the strip by the process of chromatography (diffusion or wicking). Impregnated reagents in the strip, often a protein A/colloidal gold reagent, allow the antibody/antigen/conjugate reactions to occur without further additions of reagents, whereas other lateral flow assays require just one other addition of buffer after sample addition. The simplified procedure decreases the chances of technical error, making these assays more foolproof. The results are usually a “test line,” which indicates reactivity of HIV antibody with the immobilized HIV antigens, and a “control line,” which acts as a procedural control. Therefore, these types of rapid assays offer attractive features, are accurate, and are gaining in popularity. Figures 5 and 6 depict two rapid lateral flow assays. The OraQuick HIV-1/ HIV-2 is now approved in the United States to detect both HIV-1 and HIV-2 using oral fluids, fingerstick whole blood, venipuncture whole blood, and plasma.25

Limitations of screening assays: Screening tests to detect antibodies to HIV, although highly effective in identifying infected persons, will not always detect all individuals who are infected and do not always correctly classify persons who are not infected. A less-than-perfect ability to identify all infected persons can be the result of a variety of reasons. This may be the result of an imperfect epidemiologic sensitivity, analytical sensitivity, sensitivity to detect HIV-2 or other HIV variants, or the result of technical errors.6 These limitations of the tests must be recognized, and to minimize these, the most appropriate tests for a particular testing situation must be selected. Manufacturers are required to include in their package specifications that usually include its sensitivity, specificity, and predictive values. These claims may be the result of clinical trials required for test approval but may also take into account in-house studies by the manufacturer (non-clinical trials). Although these values may give a relatively good idea of how the tests perform, they must be viewed with caution because the results of all studies may not be included.

Confirmatory assays: Confirmatory 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 confirmatory tests are from a person who is not infected. Confirmatory tests produce false negative results in persons who are in early stages of infection because confirmatory tests are not as sensitive as screening tests, and they also produce false positive results in some cases. The purpose of confirmatory 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 the great majority of persons.

HIV Western blots, Line ImmunoAssays (LIA), and IFA are the most common serologic confirmatory assays, although others, including rapid and simple confirmatory assays have also been developed. Confirmatory assays are more costly than screening tests, generally require expensive and sophisticated instrumentation, and are more cumbersome to perform. In general, the different confirmatory assays have an equivalent cost. The most common confirmatory assays are presented below:

**Western blot**: The Western blot is probably the most widely accepted confirmatory assay for the detection of antibodies to the retroviruses and many consider it as the “gold standard” for validation of HIV results, although it is sometimes replaced by other assays such as the LIA and IFA. Most Western blots that are used for detecting infection by the retroviruses are supplied by commercial companies in kit form with the antigens already being electrophoresed (separated by molecular weight) and blotted onto a membrane which is then cut into strips. The HIV-1 viral antigens are therefore separated as follows: gp160, gp120, p66, p55, p51, gp41, p31, p24, p17, and p15. It is important to remember that non-viral proteins derived from the host cells in which the virus was grown will also be present on the nitrocellulose membrane. If antibodies to these non-viral bands are present in the patient sample, interpretation may be confusing.

Interpretation relies on the presence or absence of reactivity to the specific antigens. Although a number of organizations have different criteria for what constitutes a positive reaction, interpretation criteria always require reactivity to at least 2 of the following antigens: gp160/120, gp41, or p24. Any reactivity that does not meet the requirements for being positive, classifies the sample as indeterminate. The absence of any reactivity is interpreted as negative.

False positive Western blot results have been reported, although this is rare. In most cases, these
samples will show no reactivity to p31. Hence, caution should be made when reporting results from persons whose sera show this profile, and the laboratory should include a statement in the report indicating that follow up testing should be conducted in 1-3 months. Other causes of false positive Western blot results are vaccination with HIV envelope proteins, and in persons who have some autoimmune diseases such as systemic lupus erythematosus (SLE). Details on Western blot testing and interpretations can be found elsewhere.

**Line immuno assay (LIA):** The principle of the LIA is similar to the Western blots in that it incorporates separate HIV antigens on nitrocellulose strips so that each reaction can be visualized separately; the procedure is almost identical. The difference is that in LIAs, artificial HIV antigens are “painted” on the strips rather than being electrophoresed from viral lysates; these antigens are usually recombinant antigens and/or synthetic peptides.

The LIA offers several advantages over the Western blot. Because the antigens are not derived from lymphocyte-cultured viral lysates, they eliminate the background from the non-specific host cell proteins, thereby decreasing the number of indeterminate results in non-infected persons. Also, the synthetic and recombinant antigens can be better standardized, which minimizes kit lot-to-lot variations. The antigens can be applied in optimal concentrations, unlike Western blots, where poor expression of some antigens (e.g., gp41) is always a problem. Differentiation of HIV-1 and HIV-2, or even groups of virus (e.g., HIV-1 group O), is possible in LIA because of the ability to include antigens from each virus. Finally, the LIA offers better quality control because they include controls on the strips to ensure that the procedure was performed correctly and to aid in the grading of reactions.

The interpretation of LIA results is simpler than for Western blots because of the absence of so many bands and contaminating host cell proteins. For the majority of LIAs, a positive result for HIV-1 is classified when there is reactivity to p24 and gp41. For HIV-2, if a combination LIA is used, there must be reactivity to the HIV-2 specific peptide and to at least one of the HIV-1 proteins. A negative result is indicated when there is no reactivity to any of the HIV-1 bands or the HIV-2-specific peptide. Indeterminate reactions occur when there are reactions to some bands without meeting the criteria for positive or negative. Fig.7 shows the antigens and controls included on a typical LIA and on a combination LIA.

**Indirect immunofluorescence assay (IFA):** The IFA for detecting antibodies to HIV is used in many laboratories, particularly in Europe, as a confirmatory
test for HIV infection. The IFA detects antibodies to many HIV antigens and is useful as both a screening assay and a confirmatory test. In the IFA technique, cells (usually lymphocytes) are infected with HIV and are fixed to a microscope slide. Serum is added and, if present, the anti-HIV antibodies react with the intracellular HIV before being detected with a fluorescent conjugate (anti-human immunoglobulin coupled with a fluorescent tag). This technique has the advantage of sometimes providing definitive results of samples that have yielded indeterminate results by Western blot analysis, but has the disadvantages of requiring an expensive and well-maintained fluorescence microscope and a subjective interpretation. Therefore, the method requires individuals to be well-trained and to have expertise for interpretation of results. Also, if a patient has received a therapy such as fluorescence angiograms, false positive results may occur. The sensitivity and specificity of the IFA are equivalent to the Western blot.

**Rapid and simple confirmatory tests:** Clearly, there is a major void in the rapid diagnosis of HIV in public health clinics and point-of-care testing venues in persons who have been labeled as positive by screening tests. Currently, rapid screening tests that use fingerstick and oral fluid specimens provide results quickly, but a person found to be reactive must still be recruited back to receive their confirmatory results. In these cases the patient is counseled during the initial visit; however, they must be informed that their result may not be accurate and that they must have further testing before a conclusion is made. This uncertainty of HIV status can be devastating, and it may require weeks before a definitive result can be transmitted to the patient. If a confirmatory result could be obtained at the same time, this uncertainty and the loss to follow up could be eliminated. Also, confirmatory tests require stable electricity and instrumentation, necessities that are not available in most laboratories in resource-limited countries.
Advances in the performance characteristics of rapid tests, improvement in their accuracy, and the availability of multiple recombinant and synthetic peptide antigens, impart rapid assays with a potential to simulate Western blot assays or LIAs, which incorporate separated antigens or distinct artificially-produced antigens to identify specific antibodies to HIV. Accordingly, multiple specific antigens of diagnostic importance (e.g., p24, gp41, and gp120) can be applied to membranes in a rapid test format allowing for the differentiation of antibody reactivity.

We are aware of three rapid or simple HIV confirmatory tests. The Quix HIV-1 Rapid Antibody Identification Test (Universal Health Watch, USA) is a flow-through rapid test that incorporates the diagnostically important antigens, and provides results in 10 min. The Bionor HIV-1 & 2 Confirmatory Rapid EIA (Bionor, Norway) is a simple magnetic bead ELISA that can be performed on a portable magnetic rocker platform and follows a typical ELISA procedure. The HIV 1 & 2 CombFirm (Orgenics, Israel) is a dip-stick method that is simple to perform without the need for instrumentation and results are complete in about 1 h. Rapid and simple confirmatory assays have produced encouraging results and it is probable that they will gain acceptance and wide use in the near future.

Alternative confirmatory strategies: In addition to the traditional screening test/confirmatory test algorithm (e.g., ELISA/Western blot), a number of alternative algorithms have been developed to address cost and infrastructure issues (e.g., laboratories without the capability to use tests that require instrumentation and stable electricity). The strategy behind alternative algorithms relies on the fact that each screening test produces some false positive results, but that not all screening tests produce a false positive result on the same sample. Therefore, the use of two, carefully selected, screening tests used in tandem (one after the other) can increase the positive predictive value of a positive result. Many of these alternative confirmatory algorithms are already in place, and a number of expert organizations recognize their value and are considering their use. These algorithms usually include (i) an ELISA followed by a rapid test, (ii) two ELISAs, (iii) two rapid assays, or (iv) the inclusion of a third test as a tie-breaker if the first two tests produce discordant results.

The use of these alternative algorithms have been shown to provide predictive values equivalent to that of an ELISA/Western blot algorithm, and with substantial (75%) cost and time savings. In fact, the use of a double rapid test strategy has been shown to decrease the cost per patient counseled by almost one half as compared with more classic strategies, and such a strategy can increase the proportion of patients counseled after testing.

There are some basic rules to follow when considering the use of an alternative testing strategy based on two screening tests, and laboratories must evaluate these in their specific testing situations. These rules include, but are not limited to: (i) the different tests must incorporate antigens from different sources (e.g., the first test may use recombinant antigens, while the second test uses synthetic peptide antigens; differences in the type of antigens used in the two tests will help to ensure that an antigen that is responsible for producing a false positive result will not cause the same false positive result in both assays); (ii) if the tests are not based on different types of antigens, the assay configurations must be different (e.g., if two ELISAs are used, they cannot both be based on an indirect ELISA method; instead, if the first is an indirect ELISA, the second must be a competitive or antigen sandwich method); and (iii) the second (and/or third) tests used in an algorithm should possess a higher specificity than the first screening test, while at the same time having an equal sensitivity (such tests are available but may be more expensive). These algorithms should be used with caution, and with a full understanding of sensitivity, specificity, and predictive values of the tests. In addition, it is critical that each laboratory or national programme carefully evaluates a testing algorithm in their settings before adopting it for routine use. This is particularly important if the tests are to be used in areas where HIV variants occur, as tests must be selected that have the ability to detect these variants.

One of the most well known alternative algorithms for cost savings and for addressing limitations in testing facilities is based on performing two screening tests in tandem (as a screening mode), with a confirmatory test performed only if there is a negative result by a second test when testing samples that were
positive by the first test. It was shown that this algorithm provided up to 80 per cent cost savings, with minimum loss of sensitivity and specificity. In these algorithms, a negative result using a highly sensitive rapid screening test is considered as negative (much like all screening test results). A positive result is tested by a second rapid screening test, and if both are positive, the result is considered to be confirmed positive. If a sample that is positive by the first rapid test is negative by the second rapid test, the sample is classified as indeterminate (inconclusive), or is tested by a third screening test or a confirmatory test such as the Western blot; the result of this third test is considered definitive.

Alternative testing media: Saliva and urine: The use of body fluids other than serum or plasma (alternative fluids) for identifying HIV infection was verified to be accurate in the mid 1990s. For oral fluids (saliva), a better understanding of physiology in the oral cavity led to information that allowed tests to be tailored for accurate detection. Antibodies in the oral fluid are derived from capillaries from the tooth-gum margin; they are IgG antibodies from the blood, not from the salivary glands that contain IgA antibodies. This plasma derived fluid is called a transudate or cervical fluid contains the same antibodies that are detected when testing blood. This transudate from blood constantly leaks into the oral cavity and it is the medium that is tested for HIV antibodies. Specifically designed oral fluid collection devices preferentially pull this fluid from the capillaries. Currently, there are oral fluid ELISA, Western blot, and a rapid test licensed by the FDA for use in the United States.

Intact IgG antibodies are also found in urine, but their exact origin is unknown. The collection of urine is simple, non-invasive, inexpensive, and the sample can be stored at room temperature for one month or refrigerated for extended periods of time. There is an FDA licensed ELISA and a Western blot that use urine.

The testing of alternative fluids to identify HIV infected persons has a number of uses and offers advantages in a variety of testing situations. Oral fluid samples can be collected easily and safely, can be collected in groups, are safer to collect than blood (less infectious), and tend to increase compliance for testing because they can be collected from persons who are resistant to giving blood. In addition, the more recent introduction of rapid assays for testing oral fluid samples and potentially urine combines ease of collection with rapid test results, thereby providing significant capability and flexibility over methods that require venipuncture samples. Finally, the use of alternative fluids allows collection at sites where phlebotomy cannot be performed, such as remote clinics or point-of-care facilities; this can be especially important for areas where individuals must walk or travel long distances for health care. Such collections can even be brought to the individual at their residence.

Molecular assays for monitoring HIV infection: The documented association between the number of HIV virions in the blood (viral load), as measured by viral nucleic acid levels such as RNA, and the clinical course of HIV infection has led to the use of molecular tests as the standard of care for treating patients throughout the world. Molecular tests to assess viral load have also allowed for elicitation of the kinetics of viral replication, an important variable that is used to better understand the natural history of HIV infection. In addition, viral load testing has provided valuable information about the risk of disease transmission after exposure to HIV, and it can be used to diagnose HIV infection prior to seroconversion. In fact, the transmission rate of HIV was 0 per cent among women with plasma HIV RNA levels <1,000 copies/ml, as compared with 41 per cent among women with viral loads greater than 100,000 copies/ml. Most importantly, the viral load in the blood is used in conjunction with CD4 T-lymphocyte counts as the means to stage HIV infection, monitor disease progression, and predict clinical outcome for prognostic purposes. Of paramount importance, viral levels in the blood provide information that will dictate when to initiate and modify antiretroviral therapy. If the HIV viral load increases significantly it is certain that the therapy is failing, and it is likely that viral resistance to the drugs is evolving.

During the 1990s, molecular techniques for the identification and quantification of viral nucleic acid in plasma were implemented and subsequently became known as nucleic acid tests (NAT). These methods were originally developed as more
analytically sensitive methods to detect early HIV infection in infected persons who were falsely negative by serologic methods (during the window period). As quantitative NAT methods became more refined and commercially available, their potential for monitoring viral replication was realized, and the ramifications of HIV replication became apparent.

Although HIV RNA testing is sometimes used for diagnostic purposes, it is not approved for this purpose. There are several reasons for this. For example, there is an absence of available and internationally recognized HIV quantification standards to verify that all types of molecular assays are performing equivalently. Results from assorted sera may vary when analyzed by the different amplification assays; and thus, preclude the validity of results from a particular assay, as well as preventing a comparison of results or use of results interchangeably between different assays. In addition, the performances of these assays sometimes differ when testing different HIV genotypes or clades, thereby raising uncertainty about a correct diagnosis. Also, each assay has inherent limitations (e.g., sensitivity to inhibitory substances that may be present in some specimens) that may cause false negative reactions; and some tests have a higher propensity for false positive results. Finally, the reproducibility of viral load assays (95% confidence limits) is 0.3 log₁₀/ml, indicating that 10,000 copies/ml has a two standard deviations range of 3,100-32,000 copies/ml; this shows that variability in viral load measurements occurs, even in sequential testing, and results should be interpreted cautiously.

Other molecular methods are used to provide additional information for HIV infected individuals. These include in situ hybridization for identifying nucleic acids in tissues, genotyping to determine the type, group, or clade of HIV, and nucleic acid sequencing to assess mutations of the virus that are associated with antiretroviral drug resistance.

Several NAT technologies are available; the most common will be briefly presented. 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 middle step 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).

Polymerase chain reaction (PCR): The PCR technique gains its exquisite sensitivity by artificially replicating nucleic acid sequences of the target (e.g., viral genome) so that millions or billions of the target sequences are made available for detection. Subsequent detection is usually through capturing of the amplified target sequences (amplicons) and visualization through an enzyme/substrate reaction (i.e., colorimetric reaction). HIV RNA detection by RT-PCR requires the use of the reverse transcriptase enzyme that converts the viral RNA to DNA so that the DNA can be replicated for detection. HIV RNA quantification (viral load PCR) uses the same procedure as RT-PCR, but with the inclusion of a standard that allows for RNA copy number to be determined. Different versions of the original RT-PCR method have evolved over the past few years, and the newer versions have a lower detection limit of 50 copies/ml, and a better performance in detecting all HIV clades. To accomplish this increased sensitivity, this ultrasensitive method uses a larger sample volume, and an ultracentrifugation step to concentrate virions before the actual amplification strategy is initiated. Some assays detect amplicons at the same time they are produced (Roche Molecular Systems) and are referred to as “TaqMan” or “Real-time” assays where the amplicons are detected at each cycle of amplification when specific probes that carry a fluorescence marker bind to the amplicons.

NASBA and TMA: TMA and NASBA are isothermal amplification methods that eliminate the need for heat-stable enzymes and the thermocycler instruments that are required for RT-PCR. They involve a one-step,
isothermal amplification reaction that utilizes a continuous cycle of alternating stages of reverse transcription for DNA synthesis using an RNA template, and RNA synthesis from a DNA template adapted for HIV-1 RNA quantification to replicate the RNA target. BioMerieux markets the NucliSens HIV-1 RNA QT assay (http://www.biomerieux.com) based on NASBA technology for use in prognosis and patient management. The assay selectively and directly amplifies HIV-1 RNA without PCR in a one-step sandwich hybridization procedure. A primer is used to synthesize cDNA from the specimen RNA. RNA is then amplified by repeated cycles of synthesis and transcription from this double-stranded DNA intermediate under isothermal conditions. The amount of nucleic acid is determined directly by chemiluminescence. The assay is an automated sample processing, amplification, and detection system that is highly efficient for the amplification of RNA. Three internal calibrators (low, medium, and high RNA concentrations) and total plasma nucleic acids are extracted simultaneously with test samples in each set of testing. The nucleic acid extraction can be performed with a wide variety of sample types other than blood.

TMA is a similar technique where viral RNA is captured on magnetic particles, amplified, and the product added to probes. Gen-Probe (http://www.gen-probe.com) has developed a homogenous, one-tube amplification and detection TMA system that allows a greater than one billion-fold amplification under isothermal conditions using the TMA system which combines the three enzymes reverse transcriptase (RT), RNase H, and RNA polymerase (T7) in a reaction mixture. The viral target sequences are copied by reverse transcriptase using RT to produce a transcription complex. The transcription complex is used to make numerous RNA transcripts (amplicons) of the sequence in 15-30 min, and the process repeats autocatalytically. The detection of amplified product is achieved by a hybridization protection assay technology, whereby probes labeled with acridinium ester (AE) are added to the amplification reaction, a selection reagent is added to differentially hydrolyze the label associated with the unhybridized (unbound) probe, and chemiluminescence (flashes of light) of the hybridized probe is detected by a luminometer following the addition of peroxide and alkali; no light is emitted in the luminometer from the unhybridized probes. There are no wash steps or transfer steps of amplicon to separate reaction vessels, thereby minimizing carry-over contamination.

**Branched DNA (bDNA):** Another popular viral load assay is based on the branched chain DNA signal amplification technique, which is patented by Bayer Diagnostics (http://bayerdiag.com). The bDNA assay is based on the principle of a signal amplification from annealed DNA probes through the formation of controlled networks of synthetic oligonucleotide probes, rather than amplification of the target nucleic acid (signal amplification); that is, the signal, not the target is amplified. Its sensitivity is slightly less than other NAT methods, although the assay has a potential advantage over other methods because of its ability to use a wide range of DNA probes that recognize a broad spectrum of HIV sequences, including HIV viral variants. The HIV bDNA assays can be used for the detection and quantification of HIV virions in plasma, serum, blood cells, or tissue. This method does not require viral RNA purification. Instead, virions are concentrated by centrifugation and disrupted by detergent and proteinase K, releasing viral RNA. A total of 81 target probes are used to bind to different sequences within the pol gene, and a mixture of capture probes hybridizes the HIV RNA to the surface of individual wells in a 96 well plate. An amplifier probe hybridizes to a preamplifier forming a branched DNA complex and generating enough signal sites to be detected directly after labeling. Alkaline phosphatase-labeled probes hybridize to the resultant branched chain DNA and allow for detection of the signal using chemiluminescence technology. Quantitation is achieved by comparing the results of the patient specimen with a standard curve determined by relative light units generated by the standards of known viral concentration.

Molecular methods are limited by both cost (about $80 per test) and their substantial inherent variation in intra-assay, inter-assay, inter-laboratory, and inter-method testing. Therefore, it is recommended that laboratories performing viral load testing consistently use the same type of assay and that physicians make
sure that sequential results on their patients be obtained using the same method performed in the same laboratory.

**New and novel HIV assays**

*Sensitive/less sensitive or “detuning” tests:* During HIV infection, once antibody begins to appear, titres progressively increase until about 4-5 months when levels finally peak and subsequently remain at fairly constant levels throughout the remainder of infection. Also, antibodies usually begin with low avidity (binding strength), which then increases as infection progresses. These parameters have been exploited in the “detuned” or S/LS ELISA methods [also known as Serologic Testing Algorithm for Recent Seroconversion (STARHS)] to estimate the relative time since a person was infected with HIV.

In the classical S/LS ELISA tests, the routine (sensitive part) is performed to verify that the patient is positive for HIV antibodies. By incorporating an extra sample dilution and decreased incubation times into this normal procedure, the sensitivity of the assay is purposely decreased (less sensitive part), allowing for detection of only those individuals who have high antibody titres or avidity. Those detected as positive by the less sensitive part are considered to have an established infection (greater than 4 months) and those not detected in the less sensitive part are considered to have a recent infection (less than 4 months) since their antibody titres are relatively low. This information is important for HIV incidence estimates (which can target high-risk groups who can benefit from the institution of intervention programmes), for studies of HIV pathogenesis, and for enrollment of individuals into intervention programmes that may influence disease progression and outcome.

The avidity assays incorporate a chaotropic agent to disrupt antibody/antigen complexes. If infection is established (infection greater than about 4 months), this agent will have a lesser ability to disrupt the complexes because of high antibody avidity (binding strength). Thus, this pretreatment will not affect the result as much if a person has established infection.

Recently, an ELISA/rapid test S/LS algorithm has been devised to increase the predictive value of results. Also, by determining titres using these assays, it appears that the time of infection can be better defined (unpublished observation).

**Simpler assays to monitor HIV viral load:** Recently, assays have become available that can replace the expensive and rigorous molecular methods for determining HIV viral load. One such assay is aimed to detect and quantify the reverse transcriptase enzyme (RT) activity and can be correlated to the amount of HIV in blood. The ExaVir Load Kit (Cavidi Tech AB http://www.cavidi.com) is a quantitative HIV-RT test for research use only. It is designed to measure HIV RT activity in plasma for the estimation of HIV viral load, and is much simpler than viral load molecular methods. The principle is based on synthesis of a product by the RT enzyme that can be detected by an alkaline phosphatase conjugated antibody. There are two portions to the test, a separation portion and the RT assay portion. The separation portion of the assay inactivates cellular enzymes and then separates the virus particles from the plasma using a gel that binds the virion lipid membrane. The RT portion of the assay is an ELISA with wash steps and incubations between each addition of reagents. A lysate is mixed with RT standards that have been serially diluted to have known concentrations of recombinant HIV-1 RT and will act to provide the means for quantification. The viral lysate and standards are added to a microtiter plate that has been coated with a Poly A reagent that binds an RNA template reagent. A reaction mixture containing a primer and an RT substrate (BrdUTP) is also added. If the sample lysate contains any RT activity, the RT enzyme will synthesize a BrdU-containing DNA strand overnight (DNA-RNA hybrid incorporating BrdU). This hybrid product is detected with an alkaline phosphatase conjugated anti-BrdU antibody and a detection substrate. The procedure is somewhat more cumbersome than a typical ELISA, but simpler and less expensive than molecular methods. The assay requires a vacuum pump, an ELISA plate reader, a rocker, and a 33°C incubator. The assay is a typical ELISA, but requires an overnight incubation plus several hours of incubation during the separation and RT assay steps; the necessity for further incubations before calculating results causes the assay to have a total time of 2-3 days. The RT assay can only be systematically quantified in...
samples with RNA loads of ~300 copies/ml or higher. Although there are no published reports using this assay, there is much excitement in its potential for use in developing countries. Its cost is much lower than that of molecular assays.

A second method for estimating viral load is a p24 antigen ELISA that incorporates a signal-boosting step. It is likely that p24 antigen is present in serum at an earlier time than 14 days post-infection but that the sensitivity of the routine p24 antigen test is just not high enough for detection. This likelihood of detecting lower levels than is currently possible is based on the concept that there are about 3,000 p24 antigen molecules per virion. In the boosted ELISA method, the potential for an enormous increase in signal has been shown. This method uses an ELISA method coupled with a tyramide signal amplification (TSA) system after heat-mediated immune complex dissociation; the heat dissociation step produces more free p24 antigen for detection. The TSA method uses a compound (tyramide) that has the capacity to generate tyramide intermediates upon reaction with a horseradish peroxidase (HRP) enzyme. Upon addition of more peroxidase molecules, these many intermediates in turn, incorporate more HRP molecules with a cyclic generation of more tyramide intermediates and the incorporation of more HRP molecules. The increase in the amount of HRP molecules in this system produces excessive amounts of colour upon addition of substrate, thereby increasing the ability to detect smaller amounts of the antigen. This is a signal amplification method, not an amplification of the antigen. It has been reported that this method has approached the sensitivity of RNA testing (sensitivity of 200-400 copies/ml) for monitoring viral levels in blood after antiretroviral treatment. This could be accomplished at 20 per cent of the costs of RNA tests, thereby being a cost-effective means to monitor HIV progression. The TSA method of p24 measurements was also shown to be predictive of CD4 cell decline, progression to AIDS, and survival. It has also been reported that this method is equivalent in performance to RNA testing for the diagnosis of infection in the newborn, and one study reported that there were no newborn samples that were RNA positive while being p24 antigen negative when using the TSA method, i.e., there was a 100 per cent correlation. This could be a great benefit for monitoring infection in places where RNA testing is limited and cost-effective monitoring is needed, such as in the developing world. The TSA method incorporates an ELISA method known as “ELAST” from NEN Life Science Products, or the HiSens HIV, from PerkinElmer. The TSA p24 antigen test is relatively simple to perform (similar to routine ELISA methods) and can be fully automated. The ELAST amplification system costs about $1 per test for the amplification reagents (biotinyl tyramide solution, diluent, and streptavidin-HRP), but must be purchased in conjunction with the p24 antigen test, which costs about $7 per test.

**Simple assays to quantify CD4 levels:** Quantification of CD4 lymphocyte levels by flow cytometry has been instrumental in providing important information about the immune status of HIV infected persons. It stages infected persons and is a primary tool for determining when to institute and change therapy. However, flow cytometry is a sophisticated method, requiring technical expertise and expensive instrumentation that cannot easily be adapted in resource-limited countries. Recently, alternatives that are much simpler to perform and implement have been refined. A number of methods are briefly introduced below, some that use a more simplified means of flow cytometry, and others that use microscopic analysis or ELISA. The performances of some of these methods have been reported.

First, there are simpler methods that use the principle of flow cytometry and instruments that are easier to operate and at reduced cost. These include the FACSCount (Becton Dickenson, USA), the Cyflow (Partec GmbH, Germany), and Microcapillary Cytometry (Guava Technologies, USA). The FACSCount is a small compact apparatus that quantifies CD4 and CD8 cells without the need for hematology results to obtain absolute WBC counts. It gives absolute CD4 counts and a CD4/CD8 ratio without the need for an external computer. Minimal expertise is required, error conditions are detected and flagged, and all instrument checks are automatic. The instrument operates from a standard electrical outlet and requires no calibration. Reports indicate an excellent correlation with other methods.
The Cyflow instrument performs CD4, CD8, and CD3 counts in an easy to learn and easy to operate instrument system. It runs on 100-240 V and on 12 V DC power (car battery). It analyzes forward and side scatter signals in combination with up to 3 fluorescence channels and data analysis is performed with a PC or laptop computer. The Microcapillary Cytometry system is an affordable, easy to perform assay that can test 100-150 specimens per day by a single operator. It measures CD4 and CD3 cells using a system that is less expensive, more compact, and easy to maintain without the need for sheath fluid. The sample preparation procedure is simple to perform and the instrument automatically calculates the results without further user intervention (www.guavatechnologies.com).

Manual methods for CD4 determination include Cytospheres (Beckman Coulter, USA), Dynabeads (Dynal Biotech, USA), CD4 Count Chip (SemiBio, USA), and the Capcellia (Sanofi Diagnostics Pasteur, France). Most methods are inexpensive and cost between $3-10 per test, as compared with $30-40 per test for flow cytometry. The cytospheres method requires only a light microscope and a haemacytometer. The spheres are inert latex spheres coated with a monoclonal antibody specific for the CD4 cell surface antigen. This binds CD4 cells resulting in a rosette of CD4 cells surrounded by latex beads that is readily recognized by light microscopy. A monocyte blocking reagent (a monoclonal antibody attached to a different color sphere) minimizes the interference from monocytes that contain CD4 antigens because they can be recognized during CD4 cell counting. The procedure is simple and only requires about 15 minutes. The Dynabeads method is another microscopic method, but recommends an epifluorescent microscope (although there is a light microscope version) and a magnet apparatus (costs about $200). Magnetic beads are coated with monoclonal antibodies as a solid phase to isolate CD4 and CD8 cells from whole blood, while CD4 positive monocytes are pre-depleted using CD14 magnetic beads. The assay takes about 30 min and is performed at room temperature. The CD4 Count Chip quantifies CD4 cells in whole blood in a light microscopic method. The technique binds CD4 cells to an antibody-coated solid substrate chip, and after wash

steps, stains CD4 cells for manual counting. The method allows target cells to be fixed on a glass slide as a physical record for future counting and reference. Non-CD4 cells are differentiated by staining with a different colour. The Capcellia is a 2-step ELISA immunoassay that uses paramagnetic particles for CD4 and CD8 and requires a spectrophotometer; the assay can be performed in one hour. It requires a well trained technician.

Conclusion

HIV testing has saved countless numbers of lives during the past 20 yr, and will continue to be instrumental in slowing the pandemic for the years to come. During the past two decades, technology has evolved to produce tests that supplement the classical techniques and have increased the efficiency for detection of infection globally, and for monitoring infection in individuals. With the implementation of important antiretroviral therapy, not only in industrialized countries but also now in resource-limited countries, it is all that much more important to have alternatives to the classical methods and new methods that offer attractive and important features. Technology has and will continues to address many of the outstanding issues.

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