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CURRENT TRENDS IN IMMUNODIAGNOSIS OF HIV INFECTION

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Since the recognition of HIV infection and its worldwide spread beginning in the late 1970's through the 1990's, prevention of HIV infections and AIDS has become the major focus of health agencies around the world. World Health Organization (WHO) currently estimates that as many as 20 million people are infected with HIV worldwide with massive new infections occurring in many African and highly populated Asian countries. Recent WHO/GPA surveillance data suggest geographic expansion of the epidemic in south-east Asia, as observed earlier in Thailand, also being repeated in Cambodia and Vietnam. Situation in the Indian subcontinent is already alarming with estimates of 1.5 to 2 million infections and continuing rapid spread of the virus. Worldwide by the year 2000 more than 30 million people could be infected with the virus. Since most afflicted individuals are adults or young adults, the economic and social impact of this epidemic to affected countries could be enormous. Of this approximately half will be women, most of them of child-bearing age, with a potential to transmit virus to as many as 2 to 4 million children via perinatal route. Early diagnosis of HIV infection in these individuals can help to provide proper counseling and treatment, and to prevent the further spread of HIV.

NEED FOR TESTING/DIAGNOSIS

Testing for HIV infection is important for safety of the blood supply, for surveillance purposes and for medical diagnosis of HIV infection. Testing is essential for the development of guidelines for prevention programs and antiviral therapies and research leading to the preventive vaccines. Laboratory diagnosis is also important because it provides uniform criteria for identification of HIV infection for which diagnosis based solely upon clinical criteria is usually too complex and not precise. During the healthy asymptomatic period, which could last for 10 or more years, laboratory diagnosis is essential before any therapy for HIV infection can be initiated.

Ten years ago, in 1985 the first tests became available to detect HIV infection by detection of HIV-specific antibodies present in the blood. Since that time, HIV antibody testing has become an essential tool of public health efforts to stop the epidemic of HIV infection. Importance of testing for the safety of blood supply can be clearly illustrated by the fact that in the United States of America (USA), during the period of 1978 and 1985, before the testing was implemented, approximately 12,000 individuals were infected with HIV due to transfusion of contaminated blood or blood products. After 1985, when the testing of blood supply became mandatory, only rare cases of transfusion related transmissions have occurred in the USA. Currently, the risk of transmission due to transfusion of contaminated blood is approximately 1 in 500,000 donations in the USA. In addition to blood bank screening, testing may be recommended in several other situations such as a) to individuals with high risk behaviors (homosexuals, prostitutes, injecting drug users), b) to those with clinical conditions suggestive of HIV infection, c) to recipients of unscreened blood or blood products, d) to people with sexually transmitted diseases, e) to pregnant women, f) to children born to high risk women or to women known to be infected with HIV, g) to health care workers and h) to those with accidental or occupational exposure to HIV infected body fluids.

Additional benefits of testing include opportunity to provide appropriate counseling to promote behavior change that is necessary to reduce HIV transmission, referral of HIV positive individuals for medical evaluation and proper treatment and advise to HIV positive women to reduce perinatal transmission. Although at present molecular approaches (e.g. polymerase chain reaction [PCR]) are being developed to detect th virus directly, immunologic diagnosis still remain the basis for almost all testing done worldwide. In this review, I will try to bring together incremental developments in immunodiagnosis, including simple and new approaches that are appropriate for developing

countries and address the issues that may be critical in such situations.

BASIS FOR DIAGNOSTIC TESTS

HIV infection in an individual results in early viremia followed by elicitation of HIV-specific antibodies (**Figure 1**). Within weeks after infection, IgM antibody response can be detected which peaks rapidly but is rather short lived. Almost all infected individuals mount a detectable IgG antibody response to the viral proteins within 1-3 months after infection and seronegativity beyond 6 months is not common. Antibodies to viral proteins, specially to envelope glycoproteins, persist for lifetime. Detection of these virus specific antibodies forms the basis of all immunodiagnostic testing methods. Although these methods do not detect the presence of virus or virus components directly, this indirect approach to HIV diagnosis has been found to be quite reliable, sensitive and specific. Extensive research has been carried out to better characterize the humoral immune response, to evaluate the kinetics of early IgM and IgG antibodies and to map the antigenic epitopes that are important for the immunity. The research has resulted in newer tests that are more sensitive and specific than previous versions of tests and capable of early diagnosis (Roziers et al., 1995).

METHODS OF TESTING

Detection of anti-HIV antibodies in serum or plasma of an individual is considered an indirect but definitive test for the diagnosis of HIV infection. The basic goal of detection of HIV antibodies is accomplished by the use of viral antigens that interact with specific antibodies, if present. The resulting antigen-antibody complexes are further detected directly or, as is more common, indirectly by a variety of means such as a change in color (soluble or precipitated), aggregation of particles (agglutination), fluorescence or other properties (detection of endpoint). The assays are optimized to favor the formation of

antigen-antibody complexes and thus achieve high sensitivity. Different tests like enzyme immunoassay, Western blot, dot-blots, various agglutinations tests, or immunofluorescence assays represent similar underlying principle but with different formats and detection endpoints and have been developed as alternatives or for various testing needs.

A. Screening tests for Diagnosis of HIV-1 and HIV-2

Enzyme immunoassay (EIA): The most widely used assay format is the EIA in which HIV antigens are coated on the surface of microtiter wells **(Figure 2)**. Diluted serum or plasma is incubated to allow binding of HIV antibodies to the antigen. The unbound antibodies are then washed off. The bound specific antibodies are then detected by the use of a secondary antibody (for example. goat antibodies to human IgG) conjugated to peroxidase or alkaline phosphatase enzyme followed by addition of appropriate colorless substrate that changes its color when acted upon by the enzyme. The washings that are needed between different additions are usually accomplished by automated washers, while the final change in color is quantitated by spectrophotometer (EIA readers). The available automated or semi-automated instrumentation and application to the mass screening as required by the blood banks has made EIAs the preferred assay format. First generation EIAs used HIV-1 viral lysate as the antigen. Sensitivity and specificity of newer versions are highly improved by the use of purified recombinant viral proteins and synthetic peptides. In addition, there are several variations of basic EIAs which may include competitive EIAs, antigen-sandwich EIAs **(Figure 2B)** and IgG capture EIAs **(Figure 2C)**. In essence, the second and third generation EIAs have evolved to narrow the window period - the time between HIV infection and detection of positivity - while at the same time reducing the false positive results. With the discovery of HIV-2, a closely related but distinct virus, tests were developed to detect HIV-2. Subsequently most HIV-1 EIAs were modified to incorporate HIV-2 antigens to reduce the

cost and labor of two separate assays. Testing by combination HIV-1/2 EIA may require a modified algorithm which should be followed in testing of specimens and reporting of results (Holloman et al. 1993).

Particle Agglutination Test: There are other screening assays, such as particle agglutination test, that are used as primary test in some countries. As shown schematically (**Figure 3**), microparticles (e.g. latex beads) coated with viral antigens are mixed with the test serum. If the specific antibodies are present, the particles are cross-linked due to bivalency of antibodies and thus agglutinate. In test like SERODIA-HIV (Fujirebio, Japan), a widely used agglutination test in Japan and some parts of Asia, free particles settle down at the bottom of the well forming a tight button with defined boundary. Antibody cross-linked particles settle much more slowly and cover the whole surface. Other tests using agglutination as the endpoint are RETROCELL HIV-1 (Abbott Laboratories, Illinois, USA), Recombigen (Cambridge Biotech Corp. Massachussetts, USA) and Capillus HIV-1/2 (Cambridge Biotech Corp. Galloway, Ireland). Capillus is a simple and rapid test and is discussed further later. Agglutination tests are fairly easy to perform, however interpretation of results may require some skill. For some tests (e.g. SERODIA-HIV) appropriate instrumentation has been developed to automate the procedure and reading of the results.

Although these assays are quite sensitive as primary screening methods, the positive results have to be confirmed by a more specific test (supplemental test).

B. Supplemental Tests

It was recognized early on that diagnosis of HIV infection should be confirmed by a second test that is more specific and has a different format and test design. Thus, confirmation of a positive EIA by a second supplemental test like Western blot (WB) or immunofluorescence (IFA) became a routine and standard practice for immunodiagnosis of HIV infection.

Western blot (WB) Assay and other Line immunoassay (LIA) :

WB assay is a procedure in which 1) first, viral proteins are separated by their molecular weight on a gel by electrophoresis, 2) proteins are subsequently transferred to the surface of a thin membrane, such as nylon or nitrocellulose, 3) cut strips of this membrane are used for immunoassay using serum or plasma specimen to detect antibodies to virus proteins (**Figure 4**). Assay steps usually involve specimens incubation, conjugate incubation and precipitating substrate, separated by washes, all performed on a rocker for optimal binding and reaction. Antibodies to specific viral proteins are visualized as colored bands. High specificity of the Western blot is attributable to its ability to identify viral protein-specific antibodies present in a given serum or plasma. Due to the specific locations where viral proteins migrate when separated by electrophoresis, specific and non-specific reactions can be recognized. A positive interpretation on WB usually requires presence of antibodies to two or more viral specific proteins (see **Table 1**). There are some differences in interpretation criteria depending on the recommending agency or manufacturers of the WB. A positive WB interpretation is usually regarded as a definitive test for HIV infection. If antibodies to viral proteins are present that does not satisfy positive criteria, the results are termed as indeterminate. Such situation can arise if a) the person is recently infected with HIV and has not yet developed a full complement of antibodies; or b) the person has non-specific cross-reactive antibodies. A follow-up testing at a later date (3-6 months later) can usually clear up this indeterminate test results.

Table 1.
Interpretation criteria for positive Western blot.

Recommending Agency	Presence of Antibodies to at least
CDC/ASTPHLD	any two of p24, gp41 and gp120/160
American Red Cross	at least one band from each of the gene coding for: gag, pol and env.
WHO	Any two env bands ± any other viral proteins

In addition, commercial WB test kits may have their interpretive criteria and in some cases intensity reference band.

As an alternative to WB, which uses purified virus preparation as the antigen source, line immunoassay have been developed using recombinant proteins and/or synthetic peptides representing different gene products. These proteins or peptides are applied as discrete individual lines on the nitrocellulose or nylon membrane. Strips of antigen-coated membrane are used in an assay to detect antibodies to viral proteins. A number of commercially available tests have used this format (for example RIBA from Chiron Corporation, California, USA; INNOLIA from InnoGenetics, Belgium; PeptiLAV from Diagnostic Pasteur, France). Some of these tests also incorporate antigens specific for HIV-2 and thus help differentiate between HIV-1 and HIV-2 infection (see below).

Immunofluorescence Assay (IFA):

IFA involves use of inactivated HIV-1/2 infected cells fixed on a glass slide. Cold acetone or acetone/ether mixture is usually used to remove lipid and fix the cells without destroying cell morphology. HIV-infected cells express viral proteins in the

cytoplasm and on cell membranes and provide antigens for antibody recognition. Adjacent uninfected cells serve as controls. HIV-specific antibodies, if present in a specimen, bind to infected cells and unbound antibodies are removed with repeated washings. Bound antibodies are detected indirectly by the use of fluorescently tagged secondary antibody (e.g. fluorescein isothiocynate-conjugated anti-human IgG) which can be observed under a fluorescence microscope. Most of the indeterminant results obtained by the WB can be resolved by IFA. IFA requires a fluorescence microscope and well trained personnel for interpretation of the fluorescence results. Thus, although it offers an alternative to WB, the test may be appropriate only for laboratories trained in the use of IFA.

With discovery of HIV-2, additional need to confirm and type HIV-2 infection was recognized. Although WB assays made using HIV-2 virus preparations are available, this supplemental test (and HIV-2 IFA as well) can not be used to type infections due to high cross-reactivity between various HIV-1 and HIV-2 proteins. Therefore, typing of virus infections is usually achieved by other methods (see below).

C. Typing of Viral Infections

In most areas of the world, and even in West Africa where HIV-2 prevalence is relatively high, HIV-1 epidemic has dominated the spread of the virus. If the typing of viral infection is desired following a positive screening test, then appropriate tests should be used. Typing by commercially available WB is not possible due to extensive cross reactivity between HIV-1 and HIV-2 viral proteins, including envelope glycoproteins. It was observed that oligomeric forms of the transmembrane envelope protein cross reacted significantly more than the monomeric glycoprotein, presumably due to common conformational epitopes (Parekh et al., 1991). However, transmembrane glycoproteins of both HIV-1 and HIV-2 were found have immunodominant epitopes which do not cross react (Gnann et al., 1987); synthetic peptides

made from these sequences can be used for the typing purposes (**Table 2**).

Table 2. Immunodominant Peptide Sequences	
Virus	Sequence
HIV-1	LGIWGCSGKLICTT
HIV-2	LNSWGCAFRQVCHT

Therefore a WB assay made with HIV-1 viral proteins and HIV-2 typing antigen (immunodominant transmembrane peptide) applied at the bottom of the strip as a line is able to type and confirm HIV-1 or HIV-2 infections (Pau et al., 1993).

Concerns with high cost of commercial WB assays and their inability to type virus infections have resulted in the development of immunoassay that use immunodominant peptides from HIV-1 and HIV-2 applied as discrete lines or spots. Examples of such tests are PeptiLAV (Diagnostic Pasteur, France), Genie 1/2 (Genetic Systems, Washington, USA), INNOLIA (InnoGenetics, Belgium) and Rapid Testing Device (RTD 1/2) (Cambridge Biotech, USA). Some of these tests have been used for typing of virus infections and examining the extent of dual (HIV-1 and HIV-2) infections in dually seroreactive populations in West Africa (George et al., 1992). Such tests can be used as supplemental assays in a diagnostic algorithm replacing a traditional WB. The assays have good sensitivity, but the specificity to type viral infections vary from about 70% to more than 90% depending on the test.

Although, typing of viral infections (HIV-1 or -2) is useful for epidemiologic purposes to monitor the virus spread in different populations, questions remain about the usefulness of virus typing in developing countries with limited resources.

Infections with either HIV-1 or HIV-2 results in development of AIDS and related opportunistic infections, although low pathogenicity of HIV-2 has been well recognized with longer asymptomatic period. If virus typing is desired, the supplemental test capable of confirming and typing HIV infection, instead of WB, should be used to reduce the cost.

PROBLEMS WITH DIAGNOSIS

Enzyme Immunoassay: Purified virus preparations were the first antigens to be used in EIAs (first generation tests). However, there were limitations of both sensitivity and specificity. Envelope proteins constitute only a minor proportion (less than 10%) of total viral proteins in purified virus, although they elicit an early, persistent and a major humoral response in infected individuals. Thus, enrichment of envelope proteins was important. Moreover, purified virus preparations usually contain significant amounts of cellular proteins which could not be removed easily without additional loss of viral envelope proteins. These impurities contribute to low specificity. These limitations could be overcome by use recombinantly expressed purified envelope proteins. Use of such antigens resulted in the development of second generation tests which are not only more sensitive but also more specific. Thus, tests that use appropriate envelope recombinant proteins are usually more sensitive in detecting early seroconverters than those that use whole viral lysate and false positive rate is often lower due to the absence of contaminating cellular proteins.

Simultaneously EIAs using one or more synthetic peptides, derived from HIV proteins, were also developed as second generation tests. Synthetic peptides have advantages of low cost and high stability. Some of these tests are extremely sensitive in detecting early seroconverters and also exhibit high specificity. Moreover tests based on synthetic peptides can be used for detecting HIV infections or typing HIV infection.

However, concerns remain due to limited epitope sequences presented by peptides and absence of conformational epitopes which could compromise sensitivity.

An example of this limitation became evident when Group "O" viruses were discovered in individuals from Cameroon. Due to significant sequence differences between "O" and "M" (main) group viruses, assays that use recombinant proteins or synthetic peptides occasionally failed to detect antibodies to group "O" viruses but viral lysate based assays were successful in detecting group "O" infections (Schable et al., 1995). This high sensitivity of viral lysate based assays appears to be due to high cross reactivity of Gag antibodies. Since accurate diagnosis and safety of blood supply is of great importance, commercial companies that use recombinant proteins and/or synthetic peptides are modifying their assays to increase their assay sensitivity to detect group "O" viral infections. These modifications, in most instances, involve incorporation of immunodominant synthetic peptide or recombinant protein from group "O" viruses as one of the antigens.

Additional problems with EIAs are need for instrumentation (washers and spectrophotometer) and refrigeration. EIAs are also not suitable as single test units or field testing of specimens. A simple, low technology test would be more appropriate for such applications, specially in developing countries.

Western blot Assays: Although Western blot has been a very useful supplemental test for confirmation of HIV infection, there are several drawbacks. 1) Manufacturing of WB strips has been a very labor intensive process resulting in a high cost, as much as $20 to $50 per test. 2) The interpretation of WB results has always generated considerable debate. Different manufacturers and organizations have relied upon different interpretive criteria which classifies a given specimen as positive based upon the presence of a combination of antibodies to specific HIV proteins (**Table 1**). 3) Moreover, interpretation of FDA approved tests in the United States requires comparison of the test strip results

with intensity of a reference band. This adds to the complexity of interpretation to an already complex procedure. 4) Unlike most other tests, WB testing results in a significant number of individuals being classified as indeterminate and may require additional follow up testing.

Pediatric HIV Infection: HIV infection in infants occurs mainly due to perinatal transmission from infected mothers. Although only about 25% of infants acquire HIV infection, all infants born to seropositive women, irrespective of their HIV infection status, test positive on conventional assays due to transplacentally acquired antibodies. Thus, standard serologic tests are unable to diagnose HIV infection in infants at least for 12 to 18 months after birth till uninfected infants serorevert and loose maternal antibodies.

NEW DEVELOPMENTS IN DIAGNOSIS

Continuously there are new developments in HIV diagnosis. They relate to one or more of the following: improvements in sensitivity and specificity, simplicity and ease of use of the assay, use of alternative specimen other than serum/plasma, less labor/low cost, and rapidity of the assay. In addition, tests are specifically developed to address the issue of early diagnosis of HIV infection in infants.

Antigens: Almost all new tests use recombinant proteins and/or synthetic peptides. Since antibodies to both envelope and Gag proteins are believed to be important for diagnosis, new recombinant antigen have been developed incorporating antigenic portions of envelope and Gag into a single molecule. Such chimeric antigens are available for HIV-1 and HIV-2 and are being explored for diagnosis. Use of a single chimeric antigen, instead of two or more individual antigens, eliminates competition for binding and ensures that important antigenic epitopes are bound to the plastic or solid surface in equimolar amounts. Moreover, this would simplify antigen purification, characterization and handling thus helping to reduce the cost.

Similarly chimeric synthetic peptides, incorporating immunodominant portions of both HIV-1 and HIV-2 into a single peptide, are also being investigated for their ability to recognize HIV-1/2 specific antibodies simultaneously (Shah et al., 1996). We have also used various peptide antigens with V3 loop sequences inserted into a mucin backbone for their diagnostic potential (Fontenot et al., 1996). The results suggested that minimum amino acid sequences of epitopes when presented in a manner to achieve the right conformation can have enhanced antigenicity and can act as better antigens. Short peptide sequences derived from the tip of the V3 loop of gp120 were used in immunoassay to determine the prevalence and distribution of two distinct HIV-1 subtypes (clades B and E) in Thailand (Pau et al., 1993). The study provided very valuable epidemiologic information regarding the introduction and movement of two subtypes in Thai population. These different antigens can be used in EIAs, line-immunoassay or other rapid assays suitable for a variety of testing situations.

Formats: As the focus of epidemic is shifting from the western world to Africa and Asia, testing strategies are also changing towards simple and inexpensive methods. The developments in the testing formats have resulted in assays that are easy to perform and interpret, do not require any instrumentation and costs significantly less than the traditional EIA/WB algorithm. Examples of different formats include self-containing flow through devices with dots or lines as markers of positivity, capillary enhanced agglutination, dip sticks or other variations **(Figure 4)**. It should be stated that although many such tests are being developed, only a few have good sensitivity and specificity. Simplicity and rapid assay format sometimes results in inadequate performance. To ensure that simplicity in format does not compromise performance in detecting low antibody levels or local HIV variants, it is important that the test is adequately evaluated in the area of its intended use in parallel with EIA/WB prior to its use in diagnosis or surveillance.

Tests such as HIVCHECK (Ortho Diagnostic Systems, USA), GENIE 1/2 (Genetic Systems, USA), TESTPACK (Abbott, USA), and AccuSpot (Specialty Biosystems, USA) are examples of flow through devices with one or more antigen spots on the membrane. The devices have absorbent material underneath to retain the liquid. Sequential addition of test serum, conjugate, and substrate (few drops each with washes in between) yields colored spots if the specimens contained virus specific antibodies. In some tests, antibody binding is detected by the use of gold-conjugated Protein-A or secondary antibody for direct visualization of positive reaction and does not involve enzyme conjugated secondary antibody or substrate. In one version (Insti-1/2, IntraCell Corp. USA), the secondary reagent was coupled to a colored dye giving a blue spot. This test was found to be very sensitive, although specificity needed further optimization. Another similar test, SUDS (Murex Corporation, USA) has absorbent material placed radially and is approved by Food and Drug Administration (FDA) in the USA. The flow through single unit test devices are self-contained minimizing risk of exposure to potentially infectious agents, do not require any equipment to run, are easy to perform and give results within few minutes.

Another simple and rapid test using a novel format is Capillus HIV-1/2 (Cambridge Biotech Corp. Ireland). It is based on the principle of agglutination as described before, however agglutination reaction is favored by the flow of the latex beads through a narrow capillary as shown by the direction of arrows (see Figure 4). As the homogeneous latex suspension flows, a positive reaction is accompanied by the appearance of particulate aggregates of latex. This simple test has been found to be quite sensitive and specific and has gained acceptance in some developing countries and surveillance programs in Africa.

A recent test, SeroStrip-1/2 (Saliva Diagnostic Systems, Singapore), has a unique dip stick format. Gold conjugate is applied as a dried chemical between the two membrane. When placed in the diluted specimen, gold conjugate with IgG antibody is

wicked towards the antigen (applied as a line on the middle part of the dip stick). HIV-specific antibody-gold complexes are captured by the antigen, resulting in a red line. In the absence of the specific antibody, antigen line remains colorless. Upper line provides IgG control by capturing IgG in the sample to ensure specimen adequacy. Again, this test is very simple to perform and in preliminary evaluation has been found to have good sensitivity and specificity.

Another example of a simple test is Immunocomb, developed by Program for Appropriate Technology in Health (PATH), Seattle, USA. Antigen is applied as spots on a plastic comb (8 notches) with its positions matched to microtiter plate wells. The comb is dipped into the specimen and then gold conjugate. The test takes 20-25 minutes to run with two incubations of 10 minutes each. With the help from PATH, the test is manufactured in several developing countries. Although the test is simple to perform, it generates potentially infectious liquid waste in open containers (during wash steps between incubations) and was found to be somewhat cumbersome.

Simplicity of test formats often results in rapid test results. This may be quite important in many developing countries for screening of the individuals prior to blood donations. Since many blood donations are by relatives of patients and thus are hot transfusions, tests such as these can serve a very important purpose in screening the potential donors and for prevention of transmission. Testing algorithms that use two different simple tests, instead of conventional EIA and WB algorithm, have been evaluated in various settings and have been found to be very cost-effective with sensitivity and specificity equivalent to EIA/WB. Although such tests are very attractive for developing countries, relatively high pricing do not allow their wide-spread use. A few of the tests being evaluated cost significantly less (less than $1.00 per test) and may find widespread use in developing countries.

Alternative Specimens: There has been considerable interests

in the use of specimens other that serum or plasma for detection of antibodies to HIV. Alternative specimens include dried blood spot (collected on a filter paper), saliva or oral fluid and urine.

Dried blood spots (DBS): DBS can be collected on filter paper with proper training and instructions and requires a needle stick and very small amount of blood. Thus it can be obtained from individuals resistant to venipuncture or from infants. Moreover DBS has the advantage of ease of shipping and storage. Antibody can be eluted with elution buffer containing detergents and detected using modified EIAs and WB. Centers for Disease Control and Prevention (CDC), in collaboration with participating State Health laboratories, have successfully used the DBS for the large scale surveillance of child-bearing women in the United States for the detection of HIV infection in this population for the last 7 years (Gwinn et al., 1991) . The DBS were collected from the newborns as part of their metabolic screening program. One spot was used for anonymous testing of HIV antibodies. Since antibodies in infants represented mothers' antibody profile, the survey provided valuable epidemiologic and geographic information about HIV infection in child bearing women. More than 5 million infants (and thus women) have been tested so far by EIA (modified and optimized for DBS) and positive results confirmed by a modified WB (miniblot). Although DBS represents a useful alternative specimen, its collection requires some training and testing has to be accompanied by stringent quality control and assay modifications.

Oral Fluid or Saliva: In the last few years, oral fluid (OF) has generated a lot of interest for the detection of HIV-specific antibodies. A number of commercial companies have developed OF collection devices to ensure collection of adequate specimen and preservation of antibodies (OraSure by Epitope, Oregon, USA; Omni-Sal by Saliva Diagnostic Systems, Washington, USA; and Salivette by Sarsdedt, UK). Collection devices usually involve an absorbent pad that is allowed to saturate with saliva in mouth.

The saturated pad is then transferred to tube containing buffer with preservatives that include microbial and protease inhibitors. This, coupled with modified and optimized EIA (e.g. GACELISA by Murex Corp., UK) and WB, has permitted detection of HIV antibody with sensitivity (Granade et al., 1995) comparable to that achieved for testing of serum specimens. Miniature WB (miniblot), used earlier for dried blood spot specimens, was adapted for the confirmation of positive EIA. IgG concentration in the OF varied from <1 µg/ml to more than 100 µg/ml. However, the detection of HIV-specific antibody was not correlated to the level of IgG. Implication of OF collection and testing in developing countries are enormous and represent tremendous advantages over invasive blood collection procedures. This include low cost in personnel, training and equipment and ease of acceptance of noninvasive collection procedures. Moreover, the risk of breakage during collection and transportation is minimized.

Urine: Although urine could be an attractive specimen due to ease of collection, there has been concerns about the stability of the antibodies in urine and effects of its low pH. Recently, tests such as Calypte EIA (Calypte Biomedical, Berkeley, CA) has been specifically developed to detect HIV-antibodies in the urine. After the collection, a urine preservative tablet is added to the sample to stabilize antibody. The Calypte EIA is reported to have sensitivity equivalent to plasma or serum. Occasional detection of urine positivity in seronegative individuals have raised important question about validity of this test or origin of the positive results. Thus, although urine can be a very simple and useful specimens for HIV diagnosis, additional parallel work needs to be done on serum and urine to validate the test or tests and confirmatory assays need to be developed to eliminate false positive results.

DIAGNOSIS IN INFANTS

The need to diagnose HIV infection in children, specially in first few months after birth, has resulted in development of many new approaches. This topic is exhaustive and will require a separate review to do the justice. However, here I will briefly summarize the new approaches for pediatric diagnosis. Although molecular or virologic methods, such as polymerase chain reaction (PCR) and virus culture have been used in many studies to diagnose HIV infection and have been found to be quite sensitive and specific in identifying most infected infants within 30-45 days after birth, at least at present these tests are not commonly available for pediatric diagnosis. In addition to the technical difficulties, specialized facilities, instrumentations and expertise needed, prohibitively high cost will limit their use for diagnosis in most developing countries. Therefore alternative immunologic approaches would be more desirable.

Immunologic approaches to diagnose HIV infections in infants include p24 antigen detection (p24 Ag assay), HIV-specific IgA detection (by IgA-Western blot or IgA-EIA), detection of HIV-specific IgG secreting cells or secreted antibodies (enzyme linked immunospot assay [ELISPOT] and In-vitro Antibody Production [IVAP]) or detection of HIV-specific IgG (by IgG-Capture EIA).

p24 Antigen Assay: Since free p24 antigen does not apparently cross the placenta, detection of this viral antigen in infant is considered an indication of HIV infection (Palomba et al., 1992). Therefore, p24 Ag assay (EIA format) has gained significant attention as a test for diagnosis of HIV infection in children. Microwells coated with p24 antibodies are used to capture p24 that may be present in the serum or plasma. Captured p24 is detected by the use of conjugated anti-p24 antibody (antibody sandwich EIA) and appropriate substrate. A modification of this assay involves disruption of p24 antigen-antibody complexes (immune complex dissociation) with acid or base prior to the assay to increase sensitivity of detection. The assay has been

able to detect p24 antigen only in a subset of infected children. In prospective follow up studies, some infants show p24 antigen peak followed by a decline. However, this trend is not universal and detection of p24 antigen is not always consistent. Therefore a negative p24 Ag assay does not rule out infection. Moreover, occasionally uninfected infants have been shown to have detectable p24 antigen, specially during the first few weeks of life, thus lowering specificity of the assay.

HIV-specific IgA: Although the maternal IgG crosses the placenta, the IgA and IgM do not. Attempts to detect early HIV-IgM in infants have not been very satisfactory, although specific IgA has been found to be useful. In general, overwhelming and competing IgG present in the specimens is first removed by Protein-G Sepharose adsorption which permits the detection of the HIV-IgA. Virus-specific IgA is detected by IgA-Western blot as described (Weiblen et al., 1990). The assay is 30-40% sensitive in first two months after birth but the sensitivity increases to 70-80% by 6 months. Although useful, the assay is highly cumbersome and expensive. As a simple alternative, IgA-capture EIA has been evaluated by us (Parekh et al. 1993) and was found to be quite comparable to IgA-WB assay. The assay did not require any prior removal of the competing IgG. The IgA-capture EIA had sensitivity of almost 75%-80% and specificity of 100% at 6 months of age. Moreover, due to its EIA format the assay is less complex than the IgA-WB, costs significantly less and can be performed in a few hours. Thus IgA-capture EIA has potential utility in developing countries for diagnosis in infants.

ELISPOT and IVAP: ELISPOT uses antigen coated membrane wells and infants' lymphocytes. HIV-specific antibody secreting cells adhere to the membrane during incubation. Location of HIV-antibody secreting cells is determined by anti-IgG antibody conjugate and appropriate substrate and can be seen as colored spots (Nesheim et al., 1992). IVAP involves culturing of lymphocytes in vitro to allow secretion of antibody. The specific antibody is detected by the assay of the supernatant fluid

(Amadori et al., 1990). Although both assays have been able to detect HIV infection in infants, there have reports of lack of specificity during the first two months. Moreover, assays are somewhat more technical in nature and requires separation and culturing of viable lymphocytes.

IgG-Capture EIA: Although conventional EIAs detect maternal antibody present in the uninfected children upto 18 months of age, IgG-capture EIA was negative in these children by 6 months (Parekh et al., 1993). By this age, almost all immunocompetent infected infants make detectable HIV-IgG as seen by IgG-Capture EIA. Thus, this assay can be used for serologic diagnosis of HIV infection in infants at 6 or after 6 months of age with sensitivity of almost 100% (in immunocompetent infants) and specificity of 100%. Since it takes about 6 months for the loss of most (>99%) of maternal IgG (half life = 23 days), the assay cannot be used prior to 6 months for diagnosis. However, it still represents a great improvement in the capability to diagnose HIV infection by a simple serologic means, since a conventional EIA would be reliable only after 18 months of age. Thus, these assays have increased our understanding of immune response to HIV in infants and have helped to diagnose HIV infection in children.

CONCLUSIONS

There has been continuous developments in the immunodiagnosis of HIV infections. A variety of assays in different formats use natural viral proteins, recombinant antigens and/or synthetic peptides. The goal has been to develop assays that are broadly reactive to identify all different HIV variants, detect antibodies soon after infection and thus are highly sensitive. Improvements in the preparation and design of antigens have also improved the specificity of the assays. In fact, immunoassay for HIV diagnosis have led the revolution in diagnostic industry and have sought to overcome the many challenges that different situations present. Yet there are no simple and inexpensive assays for detection of HIV antibodies in

oral fluid or urine. Ease of specimen collection for OF or urine, coupled with simple, rapid and affordable assays can have a great implications in the diagnosis of HIV infections in developing countries and ultimately in stopping the spread of HIV infection.

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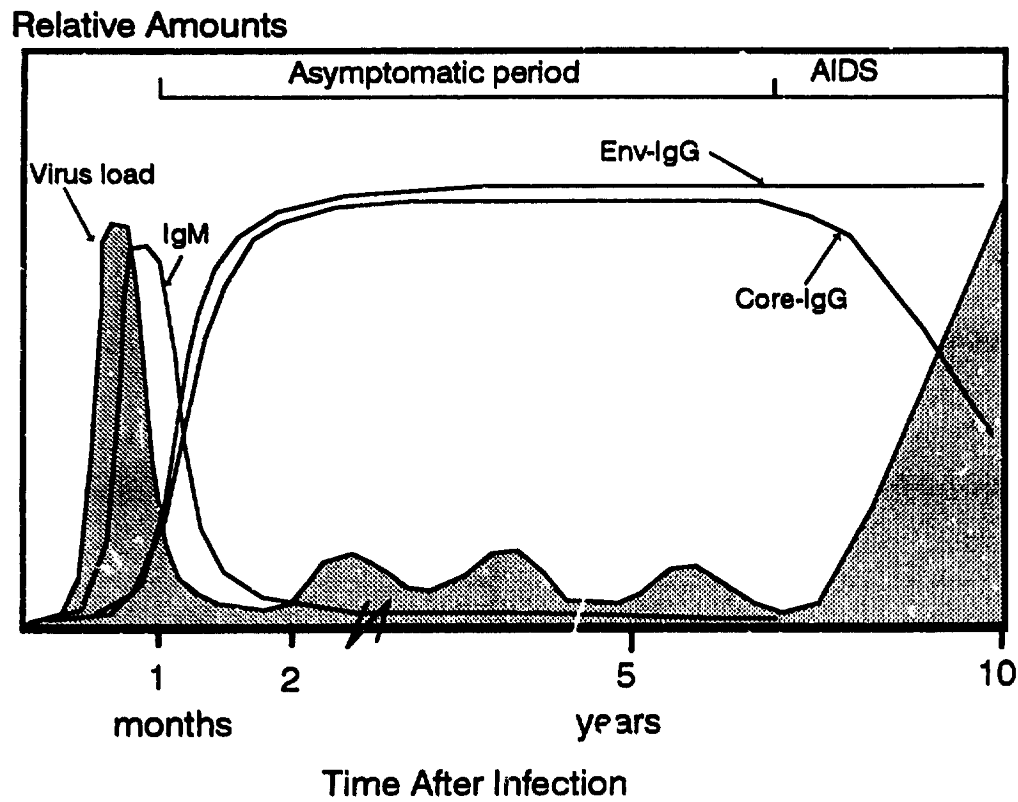

Figure 1. Schematic representation of dynamics of virus load and antibody levels after HIV infection.

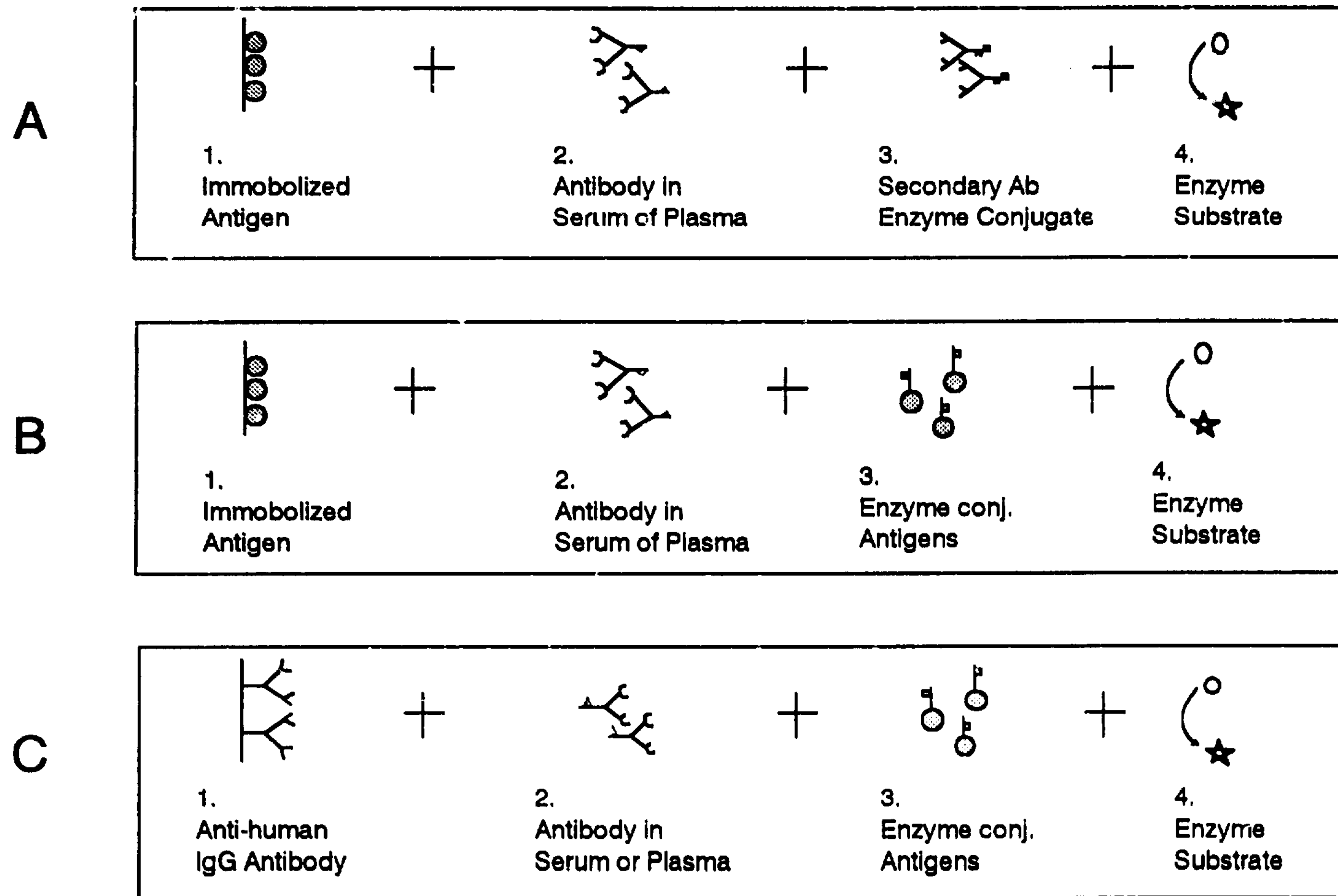

Figure 2.
Schematics of enzyme immunoassays. A: conventional indirect EIA; B: antigen sandwich EIA; C: IgG capture EIA.

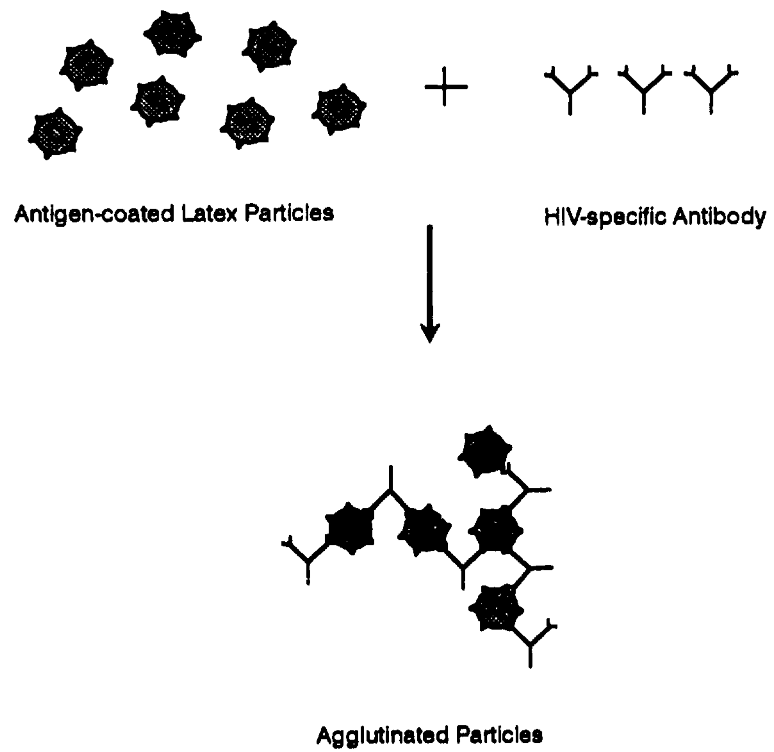

Figure 3. Schematic of agglutination assay.

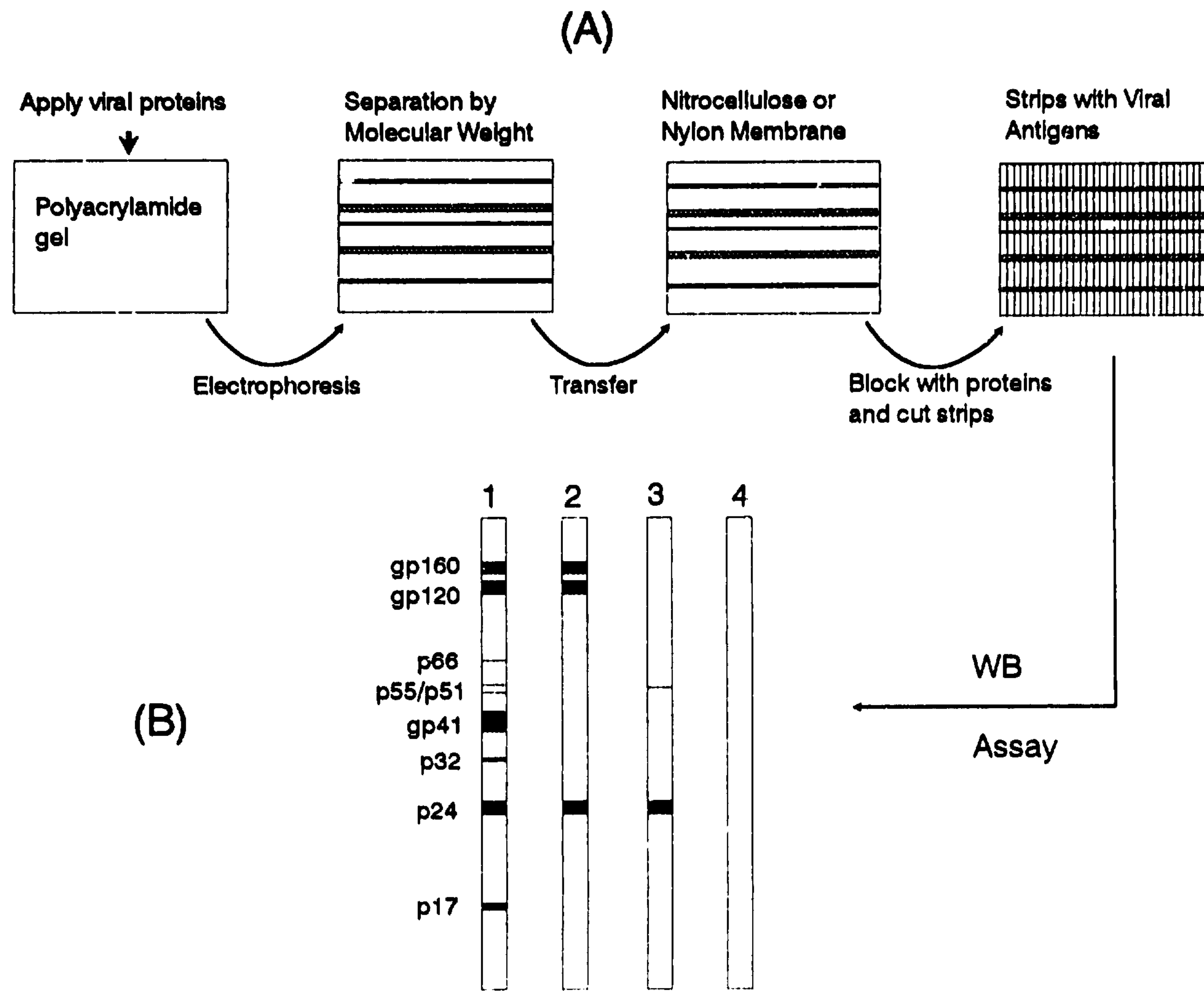

Figure 4.
Preparation of Western blot strips (A) and examples of developed strips (B). Strips 1 and 2 represent results from infected individuals, while strip 3 is an indeterminate banding pattern. Strip 4 shows no bands (uninfected individual).

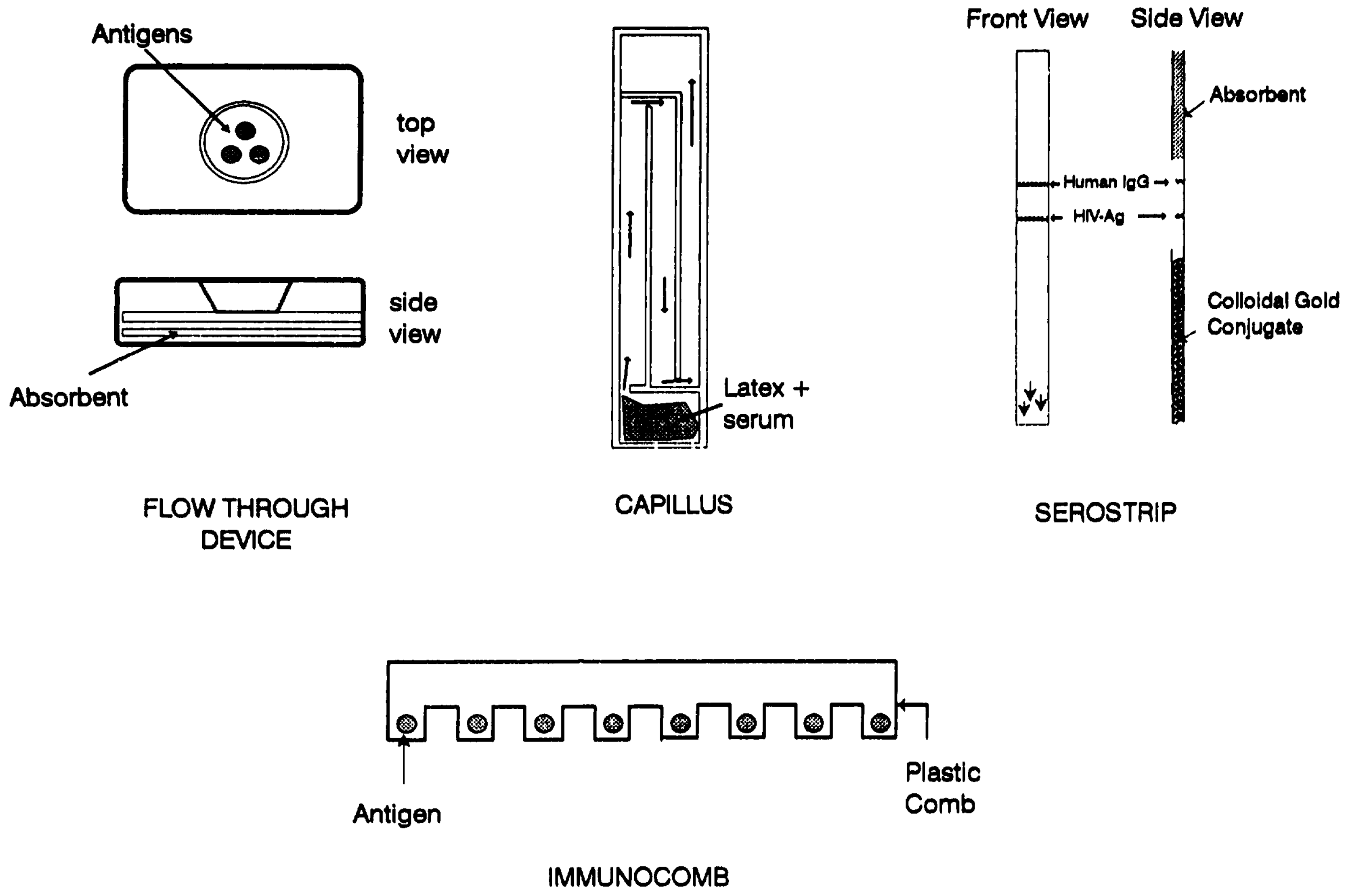

Figure 5. Various simple testing formats.