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AIDS Testing
Methodology and Management Issues

Foreword by Walter R. Dowdle

With 38 Illustrations

Springer-Verlag
New York Berlin Heidelberg London Paris
Tokyo Hong Kong Barcelona Budapest

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Serologic Tests for the Detection of Human Immunodeficiency Virus Infection

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Persons infected with the human immunodeficiency virus (HIV) have a spectrum of clinical features, ranging from persons who are infected but appear completely healthy to those with rapid disease progression and mortality. All groups, regardless of clinical stage, possess several biologic indicators of viral infection or replication (Figure 5.1). These include viremia, antibodies against viral proteins, circulating viral proteins, and nonspecific markers such as neopterin, beta$_2$-microglobulins, and T_4 cell counts. A variety of immunologic tests currently exists for the detection of viral antibodies and protein antigens. These assays have permitted testing programs to protect the blood supply from infected units and to conduct seroprevalence surveys to define the epidemic. More recently, the presence or absence of viral and nonspecific markers have been used to predict the onset of clinical disease.

Of the tests available for HIV diagnosis (Table 5.1), enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA) for HIV-1 antibody are the most widely used. They provide a low-cost, effective screening test for HIV-1 infection. To date, the U.S. Food and Drug Administration (FDA) has licensed 10 EIA tests for HIV-1 antibody; however, 1 test is no longer in production. When properly used, sera are screened by EIA using a single determination. Specimens found to be nonreactive are reported as negative. Specimens that are reactive by the initial EIA are retested in duplicate. If either of the duplicate tests is positive, the sera are said to be repeatedly reactive and must then be tested by a supplemental assay, such as immunofluorescence (IFA) or western blot.[1–4] Patients are informed that they are positive only when they are repeatedly reactive by EIA and are IFA or western blot positive. Results from the EIA alone should never be reported.

Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the the U.S. Department of Health and Human Services.

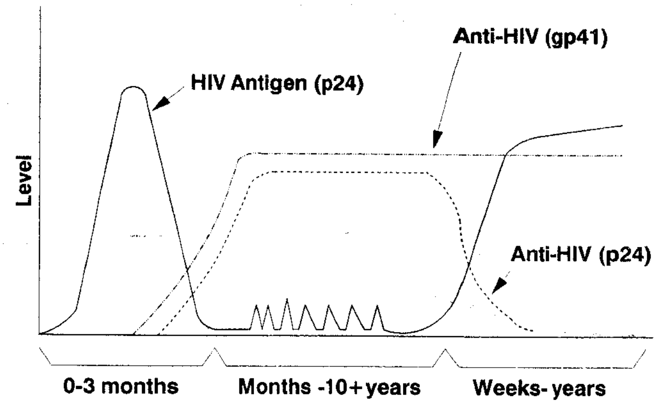

FIGURE 5.1. Natural history of HIV-1 infections.

Enzyme Immunoassay for HIV Antibody Detection

In 1985, the first five HIV-1 antibody kits were licensed by the FDA. Since that time, HIV testing for diagnosis and seroprevalence in the United States has been based on the use of these kits. Then, in 1987, the first confirmed case of HIV-2 was found in the United States.[5] Subsequently, a total of 21 additional cases have been confirmed, all from

TABLE 5.1. Tests for detection of HIV infections.

ANTIBODY TESTS
Enzyme immunoassays (EIA)
Whole virus lysate antigens
Recombinant protein antigens
Chemically synthesized antigens
Other types of screening assays
Particle agglutination assays
Hemaaaglutination assays
Latex agglutination assays
Solid-phase immunoassays
Supplemental tests
Immunoblotting (western blot)
Immunofluorescence assay (IFA)
Radioimmunoprecipitation assay (RIPA)
Virus neutralization
TESTS FOR VIRUS OR VIRAL ANTIGENS
Enzyme immunoassays (antigen capture)
Radioimmunoassay
Antigen capture
Competitive inhibition
Virus culture

individuals who had recently migrated from or visited West Africa.[6] In 1989, the first U.S. native infected with HIV-2 was identified in the northeastern United States.[7] These discoveries led to a reevaluation of the need to screen blood donations for HIV-2 in the United States and to expand HIV-2 serosurveys. Currently, most blood collection centers rely on the cross-reactivity between HIV-1 EIAs for HIV-2 antibodies to detect HIV-2-infected units. HIV-1 EIAs have bveen found to detect between 9% and 91.0% of HIV-2-infected specimens.[8,9] Obviously, there is a chance that infected units could be missed using this approach. Therefore, it became necessary to develop independent tests for HIV-2 antibody. The first of these kits was licensed in April 1990.

Several companies have similar tests under development using either recombinant or chemically synthesized antigens. Most experts believe that future assays will be combination assays that simultaneously detect HIV-1 and HIV-2 antibodies in a single well. These combination tests are proving to be quite popular in Europe where HIV-2 testing is more common.[10,11] Because of the low prevalence of HIV-2 in the United States, the FDA has not yet mandated that the blood supply be screened for this virus. However, if the situation in the United States parallels that of Europe, testing for HIV-2 may eventually be necessary. Several U.S. blood collection centers have begun voluntary screening with HIV-2-specific EIAs.

Commercial enzyme immunoassays for HIV-1 and HIV-2 antibody use several types of antigens, that is, whole virus lysate, recombinant proteins, and chemically synthesized peptides. The first FDA-approved assays using a whole virus lysate antigen[12] remain the most commonly used antigen preparation in the United States. These assays are thought to be more sensitive than EIAs using fewer recombinant or chemically synthesized antigens, because whole virus lysate antigens contain all the viral proteins present in the virus. On the other hand, they also contain proteins from the cells used to grow the virus as well as from the cell culture medium. Therefore, these assays tend to be less specific than assays based on recombinant or synthetic antigens.

Enzyme immunoassays for HIV antibodies can be configured in a variety of ways, the most common of which is the indirect assay (Figure 5.2). The viral, synthetic, or recombinant antigens are bound to a solid support, and the patient's serum or plasma is incubated with the fixed antigens. If any of the antibodies (immunoglobulins) bind to the antigens, this will be detected when an antihuman immunoglobulin G (IgG) antibody is added. The antihuman IgG is labeled by conjugation with an enzyme (e.g., alkaline phosphatase or horseradish peroxidase) so that when a substrate is added an enzymatic reaction will produce a color reaction of an intensity proportional to the amount of human antibody to HIV present.

The competitive assay is a second method of configuring an EIA for the detection of HIV antibody (Figure 5.3). Competitive assays use the same types of antigens as the indirect EIA. These antigens are bound to a solid support, usually a plastic microplate well. The patient's sample (serum or plasma) is added to the well simultaneously with enzyme-labeled HIV antibody. For competitive assays to be accurate and precise, the same amount of antigen and conjugated antibody must be present in every

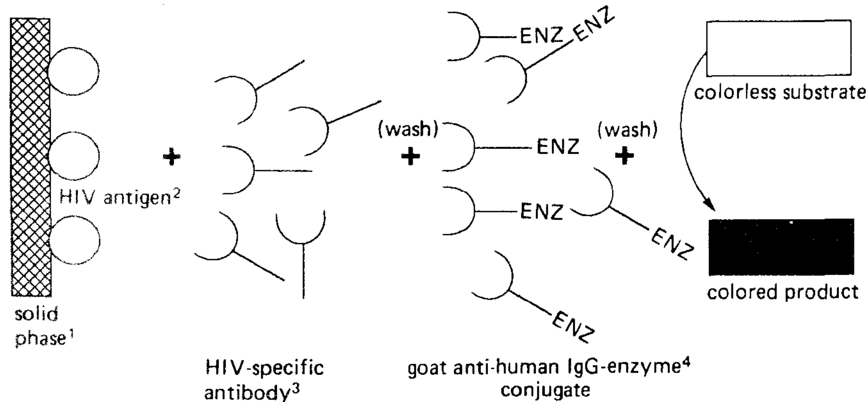

FIGURE 5.2. Configuration of enzyme-linked immunosorbent assay (ELISA), or indirect enzyme immunoassay (EIA), for antibodies against HIV. Key: 1, microplate well or other surface; 2, whole virus lysate, recombinant, or chemically synthesized; 3, patient's serum or plasma; 4, horseradish peroxidase or alkaline phosphatase.

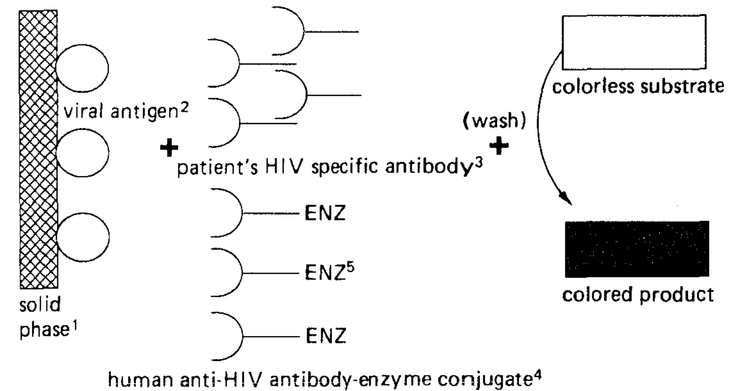

FIGURE 5.3. Configuration of competitive enzyme immunoassay for antibodies against HIV. Key: 1, microplate well or other surface; 2, whole virus lysate, recombinant, or chemically synthesized; 3, patient's serum or plasma; 4, human polyclonol or monoclonal, quantity limited and fixed (added simultaneously with 3); 5, horseradish peroxidase or alkaline phosphatase.

well. Usually the amount of conjugated antibody added is sufficient to saturate available antigen binding sites. During incubation, the patient's antibodies compete with the enzyme-labeled antibody for the limited number of antigen binding sites available on the walls of the microplate. If there are no HIV antibodies present in the sample, all antigen binding sites are occupied by enzyme-labeled antibody and after incubation with substrate give the maximum possible absorbance. As the concentration of antibody increases in the patient's sample, the enzyme-labeled antibody is displaced from the antigen binding site and the color development is less than observed with antibody-negative specimens. In contrast to the indirect assays, the intensity of the color change observed in the competitive EIA is inversely related to the amount of HIV antibody present in the test sample.

The sensitivity and specificity of commercial EIAs were found to be between 98.1% and 100.0% in a study that compared their results to western blot.[13] It has been estimated that when properly performed, the average specificity of EIA tests is 99.8.[3] In routine blood bank practice, currently 0.21–0.66% of samples are initially reactive and 0.03–0.33% are repeatedly reactive.[14] The percentage of repeatedly reactive specimens found to be positive by supplemental tests varies according to the true antibody prevalence of the tested population. As a national average, the prevalence of western blot-positive samples among blood donors has been estimated to be 0.017%.[14]

While commercial EIA tests are very sensitive, it should be noted that false-negative results may occur. Seroconversion usually occurs 2 to 3 months after exposure to the virus[15–20] but may not occur for 5 months[21] or longer if the individual is immunocompromised.[22] FDA-licensed EIAs and western blots primarily detect IgG antibody to HIV and would be negative in early infections before the switch from IgM to IgG antibody.[23,24] This switch has been reported to take as long as 41 weeks.[23,25] Tests to detect these antibodies are discussed later in this chapter.

False-positive EIAs do occur and can cause considerable anxiety if these results are reported before performing a supplemental test. In general, the probability of an EIA-positive result being false positive is inversely related to the intensity of the reaction. In a low-prevalence population (volunteer blood donors), 86.7% of the strongly reactive EIA results were also western blot or culture positive. On the other hand, sera that were EIA weakly or moderately reactive were western blot or culture positive only 1.9% of the time.[26] False-positive EIA results have been attributed to a number of conditions and practices. False-positive results may result from the intrinsic variation of EIA tests, which has been estimated to be as great as 13.0% for antibody levels close to the cutoff.

Heat-treated, lipemic, and hemolyzed sera have also been implicated in false-positivity. HLA antibodies with specificity for HLA DQ3 and HLA DR4 antigens (found in H-9 cell lines used in the production of viral antigen) and probably other contaminating antigens from cell lines[27,28] can also cause the EIA test to be positive. False-positive results have also been found to occur in 19% of hemophilia patients,[29] 13% of alcoholic patients with hepatitis,[30] 4% of hemodialysis patients,[31] and 24% of patients with a positive rapid reagin test.[32] For these reasons, and because of the considerable psychologic and social implications of being HIV antibody positive, it is inappropriate either to counsel people or to make medical decisions about them based on EIA testing alone. Persons who are initially reactive by EIA are retested in duplicate. Repeatedly reactive specimens are then tested with a supplemental tests such as IFA or western blot before test results are revealed to the patient.

Other Screening Assays

Agglutination Assays

Other than EIA, the particle agglutination assay SERODIA-HIV (Fujirebio Inc., Japan) is the most widely used screening test for HIV antibody. Because this test has not been licensed by the FDA, it is more widely used in Asia, Australia, South America, and Africa. The assay is simpler to perform than the standard EIA and requires practically no equipment (Figure 5.4). The assay is, however, well suited for use in large seroprevalence surveys. It is performed by mixing 25 μl of a diluted serum with a suspension of gelatin particles coated with purified HIV antigen. The

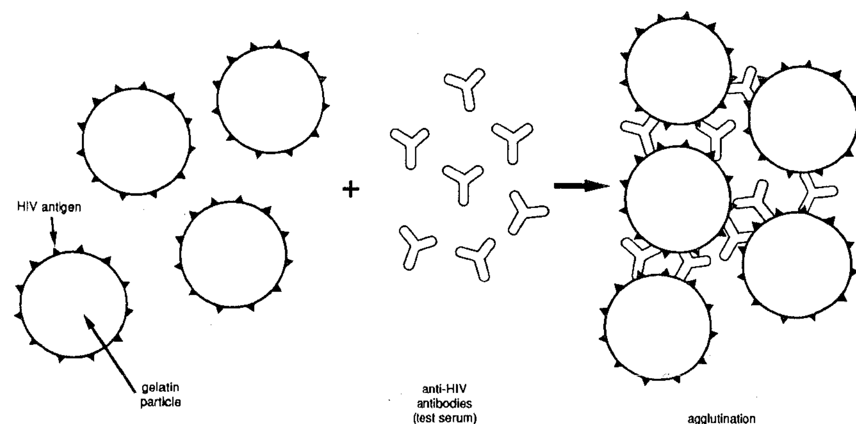

FIGURE 5.4. All agglutination tests have essentially the same mechanism. Particles such as gelatin (shown) or latex are coated with viral antigen. In the presence of HIV antibody, particles are cross-linked and agglutination occurs.

assay is performed in a microplate and can be read after incubating for 2 hours at room temperature. Sera that agglutinate coated particles alone are read as positive; sera that produce no agglutination are negative; sera that agglutinate both coated and uncoated particles are retested after absorption with a suspension of uncoated particles. Positive specimens should be confirmed by the use of a supplemental assay such as western blot.

The particle agglutination assay has been found to be slightly less sensitive[33,34] and less specific[33–35] than EIA. However, the differences were not large and with some specimens (early seroconverters) the assay was more sensitive. This was thought to be because of the presence of IgM or IgA antibodies being detected by the agglutination assay and missed by the IgG-specific EIA tests. Samples that often gave false-positive results by EIA, including heat-treated specimens, specimens containing HLA antibodies, specimens containing autoantibodies, and specimens from individuals with a history of multiple blood transfusions, were found to be negative by particle agglutination.[35]

A second agglutination test, Recombigen HIV Latex Agglutination assay, has been licensed by the FDA for use as a screening assay. This assay has not gained wide acceptance because of its cost, difficulty in reading, and lack of sensitivity (95.2%) and specificity (96.1%) as compared to EIA.[33] The assay is very easy to perform and produces results in less than 10 minutes. However, it is difficult to read even when the appropriate high-intensity light and magnification are used as recommended by the manufacturer. When performed by well-trained, experienced technologists the results have been found to be reliable.[36]

A passive agglutination test (Quick PHT-HIV, Salck Industries, Sao Paulo, Brazil) has seen limited use in laboratories outside of Brazil. This assay is intended for use in the same situations as a the SERODIA-HIV particle agglutination tests, and the methodology is very similar. However, the Salck test has been found to be very insensitive and less specific than SERODIA-HIV.[37]

Other Rapid Tests

Several solid-phase EIAs in which HIV antigens (whole virus lysate, recombinant, or synthetic) are immobilized on a membrane (HIV-CHEK, Du Pont, Wilmington, Delaware; Abbott Test Pack HIV-1/2, Abbott Park, Illinois; and Genie 1/2, Seattle, Washington) are in use in many laboratories around the world. These assays are often referred to as "rapid tests" because results can be obtained in 10–15 minutes. However, none of the tests have yet been licensed in the United States. The antigens are reacted with the patient's sera, and the resulting immune complexes are trapped onto filters. Subsequent detection of immune complexes is accomplished by the sequential reaction with enzyme–antihuman IgG conjugates and then a precipitating substrate. A color reaction develops at the site where immune complexes are present and stains the membrane. The tests are read as positive or negative on the basis of the presence of the color reaction as compared to positive and negative control spots.

These assays have been gaining in popularity because they are simple and provide rapid results. They are targeted for situations in which

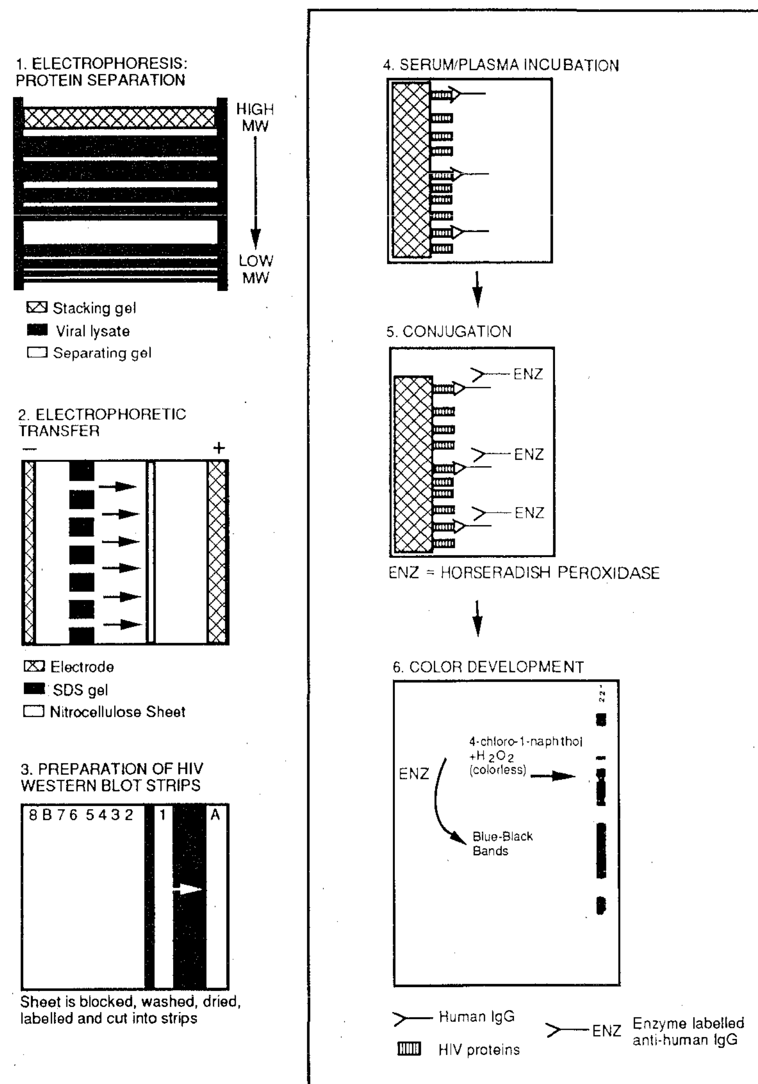

FIGURE 5.5. Step-by-step performance of western blot supplemental test for antibodies against HIV.

standard EIAs cannot be performed or immediate test results are required. Their use in developing nations has been limited because they cost more ($2–$10 per test) than the EIA (<$1). However, as with EIAs, they produce false-positive results and must be confirmed by a supplemental test before notifying the patient of the results. Most of these tests are new, and few data are available on their performance. However, published reports have found HIV-CHEK to be 95% sensitive and 99% specific[37,38]; the GENIE 1/2 is 99.8% sensitive and 95% specific.[39]

Western Blot

The western blot (WB) assay[40] is a more informative followup test for EIA. The WB uses separated HIV proteins immobilized on nitrocellulose paper to determine which antibodies in the sample are responsible for the reactivity observed in the EIA. Partially purified HIV viral proteins are fractionated according to their molecular weight by electrophoresis on a polyacrylamide separating gel (Figure 5.5). The smaller, lower molecular weight proteins are able to move through the gel more readily than the larger, high molecular weight proteins and migrate to the bottom of the gel.

A sheet of nitrocellulose is next applied to the surface of the gel. An electric current is applied, causing the proteins to migrate from the gel to the surface of the nitrocellulose. After blocking and washing, the nitrocellulose page is cut into strips. An appropriate dilution of serum or plasma found to be repeatedly reactive on an HIV EIA is incubated with a strip. Human antibodies to HIV proteins, if present, will bind to the viral antigens bound to the surface of the nitrocellulose. After washing off unbound materials, the strips are incubated with goat antibody against human IgG that has been conjugated with an enzyme, either horseradish peroxidase or alkaline phosphatase. If antibody is bound during the initial incubation, the conjugate will bind to the immune complex. In the final step, an appropriate substrate is added to the strip. Following conversion of the colorless substrate by enzyme, colored bands will appear wherever human IgG was bound to proteins on the strip. The identity and position of these proteins are established by comparison to a standard sera known to react with all the viral bands listed in Table 5.2 and shown in Figure 5.6.

TABLE 5.2 Identification of major structural gene products of HIV-1 and HIV-2 by their molecular weight in western blot and their recommended nomenclature.

Gene	Gene product	HIV-1 nomenclature	Gene product	HIV-2 nomenclature
ENV	gp160	Precursor[a]		
	gp120	External envelope	gp125[b]	External envelope
	gp41	Transmembrane	gp36/41[c]	Transmembrane
			gp70[d]	Dimeric form of Transmembrane
POI	p66	Reverse transcriptase	p68	Reverse transcriptase
	p51	Reverse transcriptase	p56	Reverse transcriptase
	p32	Endonuclease	p55	Reverse transcriptase
			p34	Endonuclease
GAG	p55	Precursor		
	p40	Precursor		
	p24	Core	p26	Core
	p17	Matrix	p16	Matrix

[a] Usually not present. Often confused with oligomeric form of gp41.
[b] Identified as gp105 by some manufacturers. Usually present but often obscured by oligomeric forms of transmembrane glycoprotein.
[c] Different virus strains may produce one or both forms of transmembrane glycoprotein.
[d] Sometimes seen on some commercial reagents.

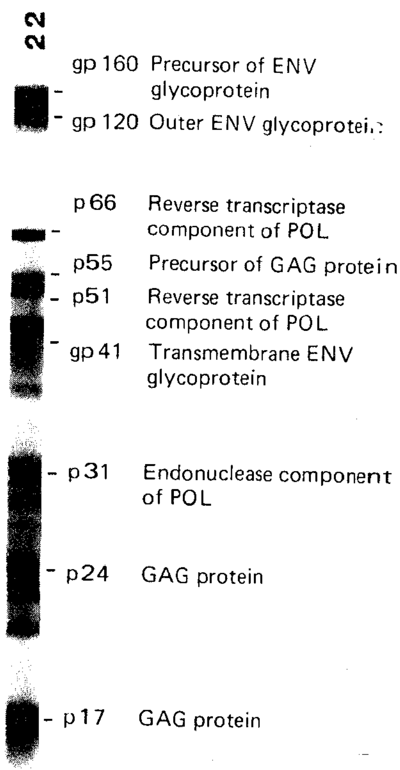

FIGURE 5.6. Major structural proteins seen on HIV-1 western blot.

Interpretion of western blot tests relies on the structural proteins coded for by the *env*, *pol*, and *gag* genes (Table 5.2). Most reports indicate that antibodies to *gag* proteins appear first on the western blot test. For HIV-1 infections, these antibodies appear to decrease or become undetectable with the onset of clinical symptoms.[40–45] In contrast, antibodies to the external glycoprotein and the transmembrane proteins can be detected in nearly all HIV-1-infected patients regardless of clinical stage.[46–48] Antibodies to the *pol* gene product are often detected if the antigens are present on the strips. Their diagnostic value is low compared to the *gag* and *env* proteins. Antibodies against other HIV proteins *tat*, *vif*, *nef*, and other *gag* proteins are detected less often and are not routinely used as markers of infection.

It is interesting to note that considerable confusion remains over the accurate identification of *env* glycoproteins present on commercial HIV-1 and HIV-2 western blots. Recent reports regarding HIV-1 western blots[49,50] have reported that a number of commercial western blots misidentified the 120-kd and 160-kd bands on their strips as representing the external glycoprotein and the envelope precursor glycoprotein, respectively. In fact, these bands represented trimeric and tetrameric forms of the transmembrane glycoprotein (TMP), gp41. These studies showed that the true precursor and external glycoproteins were not present on most commercial western blots. On those strips where the external envelope was present, it banded at the same position as the trimeric form of the TMP and could not be distinquished from this glycoprotein.

Similar observations have been made for the glycoproteins of HIV-2 (Figure 5.7). The TMP of HIV-2 was found to exist as a full-length glycoprotein (gp41) in some isolates and in a truncated form (gp36) in others. Some isolates can produce both forms. Studies have shown that both forms of the TMP often are present on commercial western blots as trimers, again binding at the same position as the external glycoprotein. The envelope precursor has not been found to be present at significant levels in commercial western blots for HIV-1 or HIV-2. This often leads to misinterpretation by existing interpretive criteria and precludes the correct identification of antibodies to the viral glycoproteins.

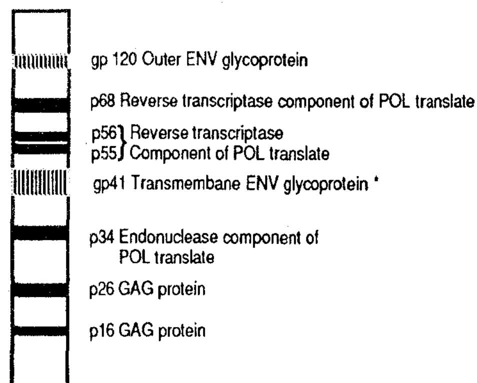

FIGURE 5.7. Major structural proteins seen on HIV-2 western blot.

Despite the overall sensitivity and specificity of western blots there has been extensive debate over the best interpretive criteria. This debate increased when it was found that considerable cross-reactivity exists between HIV-1 and HIV-2 antibodies by western blot.[51] This has been found to be especially true in West African countries centering around Cote d'Ivoire. Cross-reactivity between antibodies against *gag* and *pol* is expected and was predicted based on the sequence homology that exists for HIV-1 and HIV-2. It was, however, somewhat surprising to discover that extensive cross-reactivity occurred between the *env* transmembrane glycoprotein. In a study of 1362 consecutive tuberculosis (TB) patients and 2127 consecutive blood donors in Abidjan, Cote d'Ivoire, it was found that 73% and 83%, respectively, of all EIA positives were dually reactive by western blot.[51] Efforts to classify infections as HIV-1 or HIV-2 has had considerable influence on the selection of interpretive criteria for HIV-1 and especially for HIV-2.

Various interpretive criteria for HIV-1 and HIV-2 are presented in Table 5.3. In the United States, the Centers for Disease Control/Association of State and Territorial Public Health Laboratory Directors (CDC/ASTPHLD) criteria[53] is the most frequently applied standard. A serum is interpreted as positive by this standard when two of the bands p24, gp41, or gp120/gp160 are present. A negative test interpretation requires the absence of all bands. Any other pattern is regarded as indeterminate. As presented in Table 5.3, several other groups have presented interpretive criteria. Some such as the Ortho/Du Pont FDA-approved western blot recommend very conservative criteria. These criteria interpret sera as positive when bands detected include p24, p31, and either a diffuse gp41 or gp120/160 band. This strict interpretation maximizes the specificity of the test and is mainly intended for use with samples from individuals at low risk of HIV infection, such as blood donors, for whom there is no other clinical information. These criteria may not be suited for all situations, especially the testing of persons at increased risk or with symptoms suggestive of HIV infection. Criteria that require the presence of *gag*, *pol*, and *env* gene products for a positive interpretation to be made have a reduced sensitivity for diagnosis in AIDS patients who may have already lost their *gag*/*pol* antibodies.[45,53]

The diagnosis of HIV-2 infection by western blot is based heavily on the specific and sensitive detection of antibodies to the *env* glycoproteins.[54] This definition classifies a patient as HIV-2 infected on the basis of the presence of two glycoprotein bands (gp36/41, gp105, or gp140). Unfortunately, this interpretation is based on the misidentified *env* glycopro-

TABLE 5.3. Criteria for positive interpretation of western blot tests.

Organization	Criteria for HIV-1	Criteria for HIV-2
Organization of State and Territorial Public Health Laboratory Directors/CDC	Any two of: p24 gp41 gp120/160	None
FDA-licensed Biotech/ DuPont Test	p24 and p31 and gp41 or gp120/160	None
American Red Cross	One band from each gene-product group (total of 3): *gag* and *pol* and *env*	None
Consortium for Retrovirus Serology Standardization	Two or more bands: p24 or p31 plus gp41 or gp120/160	None
World Health Organization[a]	Two *env* bands with or without *gag* or *pol*	Two *env* bands with or without *gag* or *pol*

[a] All criteria, except WHO, score blots with no bands as **NEGATIVE**. WHO scores blots as **NEGATIVE** if bands are present that do not correspond to the structural proteins listed in Table 5.2. All other patterns not scored as positive are **INDETERMINATE**.

teins, as previously discussed. It would appear that the best criteria would be based on the presence of *gag* (p26) and/or *pol* (p34) and the transmembrane glycoprotein (gp36/41). The additional presence of the external glycoprotein (gp120) would be supporting evidence of HIV-2 infection, but because of the strain specificity observed this should not be mandatory for a positive interpretation.

In a large clinical study involving screening of a low-risk population, it has been estimated that the false-positive rate of combined EIA and western blot testing is less than 1:100,000.[55]

Indeterminate or "atypical" western blot patterns occur much more frequently and cause greater concern than false-positive results. Indeterminate patterns (e.g., p17 or p24 only) can occur in as many as 15% of samples tested by the western blot, and despite recommendations to the contrary, many uninfected persons may be told that they have an indeterminate result without a clear explanation of its meaning. Cumulative experience from nearly 5 years of testing suggests that indeterminate western blot patterns that do not change for many months or years do not indicate HIV infection.[56–58] Persons with persistently indeterminate western blot patterns usually do not have risk factors for HIV infection, do not manifest laboratory signs of immunodeficiency, and do not develop disease states typical of HIV infection. However, virologic data on long-term followup of persistently indeterminate cohorts were not available until recently.

Two recent studies[59,60] have followed populations of blood donors with persistently indeterminate (>6 months) HIV-1 western blot results up to 2 years and found them to be free of HIV infection. It was shown that none of these uninfected blood donors had detectable HIV proviral DNA when analyzed by polymerase chain reaction (PCR) technique or by culture for HIV-1. On the basis of these results, it is clear that persons having persistent indeterminate western blots should be counseled that they should continue to monitor the band pattern by western blot for at least 6 months; if no additional bands develop, they should then be advised that they are negative for HIV-1 infection.

Blood donors who are repeatedly EIA reactive but are negative by supplemental tests are most likely not infected.[61] However, blood donated by these individuals should not be used for transfusion unless the donors are subsequently found to be negative by both EIA and a licensed western blot on two separate occasions at least 6 months apart. There is an extremely low probability that EIA-positive, western blot-negative individuals from low-risk groups are HIV infected. Therefore, it is not recommended that they be notified of their "false-positive" EIA result. However, this practice is objectionable to some blood banks who think it is less ethical not to inform the donors and to continue drawing units that are discarded.

Immunofluorescence Assay

Many laboratories find the western blot tests to be too expensive for use in high-volume situations. This is especially true in developing nations where the additional problem of availability of western blot must be considered. The immunofluorescence assay (IFA) offers a low-cost alternative as a supplemental assay.[62–67] Many laboratories using this procedure prepare their own reagents. Typically, lymphocytes infected with HIV are inactivated and fixed in acetone on glass slides. Uninfected cells of the same line are either mixed with the infected cells or placed on a separate area of the slide to serve as negative controls. Serum dilutions are incubated with cell preparations, washed, and then incubated with fluorescein isothiocyanate-labeled anti-human IgG. Positive and negative control sera should be included on each slide. Antibodies in the positive sera bind to the viral antigens expressed by the infected cells. A test is interpreted as positive on the basis of the percentage of fluorescent cells and the type of fluorescence patterns observed (peripheral versus cytoplasmic staining). The reports previously cited show good concordance between IFA and western blot results. It should be noted, however, that it is often necessary to absorb sera that give nonspecific staining with uninfected cells[68] before a correct result can be obtained. The nonspecific staining of nearly all sera can be removed by absorption except for a few

that were found to contain antinuclear antibodies. Widespread use of the IFA has been impeded by the lack of FDA-licensed tests and the lack of standardization of in-house and commercial tests.

Radioimmunoprecipitation Assay

The radioimmunoprecipitation assay (RIPA) is another test that is sometimes performed as a supplemental test for EIA. Actively growing cells infected with HIV-1 or HIV-2 are exposed to a growth medium containing radioactive amino acids that become incorporated into the viral proteins.[46,69–72] Cell lysates prepared from these cells are first cleared of proteins that bind nonvirus-specific antibodies by absorbing them with a negative human control serum bound to protein A-Sepharose beads. The lysates are then mixed with the patient's serum and incubated until equilibrium is reached. The immune complexes are then absorbed with Protein A-Sepharose beads. Radioactive antibody–antigen complexes are eluted, and positive eluates are separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to determine specific viral patterns. The binding patterns are similar to those of western blot.

This technique has the disadvantage of being very cumbersome and expensive and is, therefore, primarily a research tool. The advantages of RIPA is that it seems to be more sensitive than Western blot for antibodies to the *env* glycoproteins. Because RIPA uses a cell lysate rather than the lysate of pelleted virus, it contains the true envelope precursor (gp160) and external glycoprotein (gp120). Experience has shown that RIPA is very helpful in confirming HIV infection and in typing the infecting virus as HIV-1 or HIV-2.

Assaying for HIV Antibody in Dried Blood Specimens

For 30 years, state laboratories have screened newborns for metabolic disorders by testing blood specimens obtained by heel-sticks and collected on absorbent paper (Schleicher and Schuell No. 903, Keene, New Hampshire). Recently, it has been shown that antibodies to HIV can be detected in these newborn specimens and, because IgG antibodies are transferred to the fetus, the newborn blood specimens can be used to study the prevalence of HIV in women bearing liveborn infants.[73] More than 2 million infants have been tested by this method (unpublished data) in the CDC HIV Survey of Childbearing Women. A procedural manual describing all the laboratory techniques used in the Survey has been published.[74] Subsequent studies conducted by Genetic Systems (Seattle, Washington) and Organon Teknika (Durham, North Carolina) have shown that testing dried blood samples collected on S&S No. 903 filter paper from finger-sticks gives the same sensitivity and specificity as testing serum and plasma. Subsequent to these findings the FDA has licensed the EIAs produced by these companies for use with dried blood samples.

Testing dried blood spot samples requires that the antibodies be eluted overnight in sample diluent provided by the manufacturer of the EIA. A $\frac{1}{4}$-in. spot punched from the filter paper contains approximately 5.0 μl of serum. The dilution of the antibody in the eluate may be more concentrated than that used in the EIA test and will require further dilution (see kit insert) before testing. At this point the assay is performed in the same manner as a test with serum or plasma. As with other specimens, initially reactive specimens are retested in duplicate. If possible, new eluates should be prepared for the retest. However, the blinded nature of the Survey of Childbearing Women means that the repeat testing and the supplemental testing must be performed on the same eluate. Eluates have been found to be stable at 4°C for at least 1 week. Repeatedly reactive specimens are confirmed by testing with western blot. In the Survey of Childbearing Women, because limited quantities of sample were available, a special modification of western blot is used that permits testing of

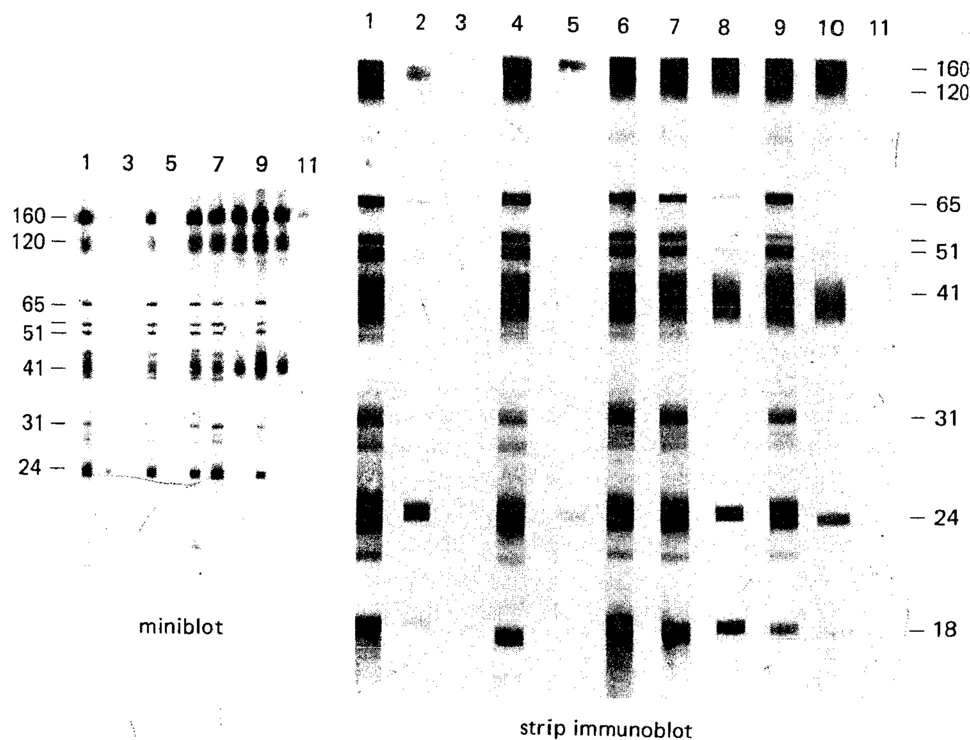

FIGURE 5.8. Comparison of "mini" western blot technique to FDA-licensed Du Pont/Biotech test. Same 11 sera were run in each test.

70 µl of a 1:100 dilution of antibody.[74] An example of this test as compared to the FDA licensed western blot is shown in Figure 5.8.

Collection of blood samples on absorbent paper has unique advantages for large-scale screening programs or for situations in which the collection, processing, and storage of serum or plasma specimens would be difficult or impossible. These samples can be collected by sticking the heel, finger, or ear, eliminating the need for venipuncture. Once collected and completely dried, the dried samples do not require refrigeration, and they can be mailed conveniently and inexpensively to a central laboratory for analysis. When protected from moisture, samples can be stored unrefrigerated for 30 days. For long-term storage, the samples should be placed in gas-impermeable bags with small packets of dessicant and stored at −20°C.

The disadvantages of testing dried blood samples are few but are important to note. Collection of appropriate samples is critical to accurate test results. The circles on the collection samples should be completely filled and saturated. Once collected the specimens must be thoroughly dried before being allowed to contact other surfaces or being placed in plastic bags. Detailed instuctions for collecting specimens have been published[74] and must be closely followed. Testing of samples must include the appropriate filter paper controls,[74] and testing laboratories should participate in a proficiency testing program that distributes specimens dried on filter paper. If all these recommendations are observed, results from testing dried blood spot samples will give the same results as obtained with serum or plasma.

Tests for HIV IgA and IgM Antibodies

Detection of infected infants born to seropositive mothers by serologic assays is difficult because HIV-reactive antibodies produced by the infant are obscured by large quantities of maternal immunoglobulin G (IgG). Passively acquired antibodies may persist for up to 18 months in the infant[75,76] with a mean of about 9 months. Consequently, only tests that can differentiate between antibodies of maternal and fetal origin are of diagnostic value. Assays that are specific for IgM or IgA seem best suited for this purpose because it is known that these antibodies do not cross the placenta.

Implementation of IgM and IgA assays has been hindered by the reported poor specificity of these tests.[77] It is generally agreed that the observed nonspecificity results from the incomplete removal and subsequent detection of maternal IgG. However, it has been shown that this problem can be eliminated by absorbing the antisera with an engineered recombinant form of streptococcal Protein G[78] (GammaBind™, Genex Corp., Gaithersberg, Maryland). Efficiency of removal of IgG was monitored by titration of IgG HIV antibody before and after absorption. In some cases three absorptions were required to completely remove IgG antibody.

Once the IgG antibodies have been removed, the IgA and IgG antibodies produced by the infant can be detected by an immunoblot procedure similar to that described in Figure 5.4. However, the IgA and IgM assays require that a class-specific antibody be used to prepare the conjugate. The assay for IgA HIV antibodies have been promising. Weiblen et al.[78] reported that they detected IgA antibodies in the sera of 66% of infected infants by the age of 6 months. On the other hand, IgM tests for HIV antibodies have been disappointing; only 33% of infected infants were positive for IgM antibodies at the same age. It is not clear whether the observed insensitivity is caused by the lack of early IgM antibody production or the poor sensitivity of the IgM assays. Conjugates for IgM antibodies are clearly inferior in sensitivity and background to IgA conjugates.

Sensitivity, Specificity, and Predictive Value of Serologic Tests

Is the test result positive or negative?—it would seem that this is a simple question with a simple answer. In fact, the answer is complex and requires that we understand several other concepts. First, we must introduce the concept of predictive value. Predictive value is defined by Galen and Gambino[79] as "how accurately a test predicts the presence or absence of disease." The answer to this question is based on the analysis of three variables: (1) *sensitivity*—the incidence of true-positive results in patients with disease; (2) *specificity*—the incidence of true-negative results in patients without disease; and (3) *prevalence*—the incidence (cases per 100,000) of the disease in the population being tested within a given time period.

The predictive value of a positive test (PPV) is defined as the percentage of positive results that are true-positives when the test is applied to a population containing both healthy and diseased subjects. The predictive value of a negative test (NPV) is the percentage of negative results that are true-negatives. As previously suggested this would depend on the number of false-positives and false-negatives produced by the test being used in relation to the number of true-positives and true-negatives and the prevalence of infection in the population. These relationships are illustrated in Table 5.4.

The ideal test would establish the presence or absence of infection in every individual screened. In other words, there would never be any false-positive or false-negative results. Unfortunately, there are no per-

TABLE 5.4. Predictive value of EIA tests for HIV antibody as applied to population containing both infected and uninfected subjects.

Disease state	Test results: Number of subjects with positive test results	Test results: Number of subjects with negative test results	Total
Number subjects with disease	TP[a]	FN	TP + FN
Number subjects without disease	FP	TN	FP + TN
Totals	TP + FP	FN + TN	TP + FP + FN + TN

[a] TP = true-positives, number of sick subjects correctly classified by test.
FP = false-positives, number of healthy subjects misclassified by test.
TN = true-negatives, number of healthy subjects correctly classified by test.
FN = false-negatives, number of sick subjects misclassified by test.

$$\text{Sensitivity (positivity in disease)} = \frac{TP}{TP + FN} \times 100$$

$$\text{Specificity (negativity in health)} = \frac{TN}{TN + FP} \times 100$$

$$\text{Predictive value of positive result} = \frac{TP}{TP + FP} \times 100$$

$$\text{Predictive value of negative result} = \frac{TN}{TN + FN} \times 100$$

fect tests. As large numbers of blood donors were screened, it became clear that the distribution of EIA test absorbance values did not assume a normal distribution but was positively skewed.[80] The cutoffs for each licensed test were set within the tail of this distribution such that 0.2–1.0% of random donors were initially reactive. Following FDA recommendations, these initially reactive samples were tested in duplicate. Units that were repeatedly reactive were discarded.

Licensed tests for HIV antibody are extremely sensitive (>99.8%) and specific (>99.8%). However, the prevalence of HIV infection in the populations being screened is low, usually less than 0.1%. The PPV of the EIA tests for screening blood donations (prevalence = 0.02%) using tests with sensitivity and specificity of 99.8% (Table 5.5) is low, 9.0%. This means that for every true-positive, nine other sera will be incorrectly identified as positive in the initial screening. As the prevalence of infection in the population increases, the incidence of false-positive results decreases (Figure 5.9). At a prevalence of 1.0%, the same EIA test will have a PPV of 83.4% (Table 5.6). If only the disposition of the blood was involved, EIA results might be sufficient. However, notification of donors testing positive was predicated on the ability to confirm the EIA reactivity.

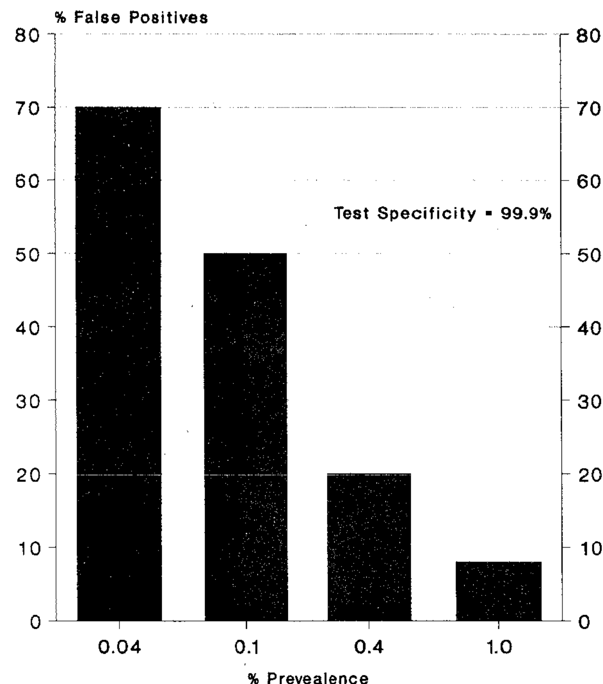

FIGURE 5.9. Percentage of false-positives decreases as prevalence of infection in population increases.

TABLE 5.5. Predictive value of EIA tests for HIV antibody as applied to population with prevalence of 0.02% infection.[a]

Disease state	Test results: Number of subjects with positive test results	Test results: Number of subjects with negative test results	Total
Subjects with disease	199	1	200
Subjects without disease	2000	997,800	999,800
Totals	2199	997,801	1,000,000

$$\text{Predictive value of a positive result} = \frac{199}{199 + 2000} \times 100 = 9.0\%$$

$$\text{Predictive value of a negative result} = \frac{997{,}800}{1 + 997{,}801} \times 100 = 100\%$$

[a] Assay sensitivity, 99.8%; assay specificity, 99.8%.

Table 5.6. Predictive value of EIA tests for HIV antibody as applied to population with prevalence of 1.0% infection.[a]

Disease state	Test results: Number of subjects with positive test results	Number of subjects with negative test results	Total
Subjects with disease	9980	20	10,000
Subjects without disease	1980	998,020	990,000
Totals	11,960	998,040	1,000,000

$$\text{Predictive value of a positive result} = \frac{9980}{9980 + 1980} \times 100 = 83.4\%$$

$$\text{Predictive value of a negative result} = \frac{998{,}020}{998{,}020 + 20} \times 100 = 100\%$$

[a] Assay sensitivity, 99.8%; assay specificity, 99.8%.

The problem of low PPV is addressed by the serial testing of the initially reactive subjects by additional EIAs and supplemental tests such as western blot. Therefore, the PPV of the individual tests become less important in relationship to the probability of a false-positive test (P_{FP}), the probability of a false-negative test (P_{FN}) and the PPV of the recommended HIV testing algorithm. In our example, let S^+ stand for a positive subject and S^- stand for a negative; let E^+ be a positive EIA test result and E^- be a negative EIA test result; W^+ and W^- are the same for western blot results. Then the prevalence is the probability that the subject is positive: $P(S^+) = 0.001$ (0.1%). The sensitivity of the EIA is $P(E^+/S^+) = 0.998$, and the specificity is $P(E^-/S^-) = 0.998$. For the western blot $P(W^+/S^+) = 0.996$ and $P(W^-/S^-) = 0.996$. The formula for the P_{FP} and P_{FN} for one test is as follows:

$$P_{FP} = \frac{P(T/S^-)[1 - P(S^+)]}{P(T/S^-) + P(S^+)[P(T/S^+) - P(T/S^-)]}$$

We need the $P(T/S^+)$ and $P(T/S^-)$ for our entire four-test algorithm to substitute in the formula.

The $P(T/S^+)$ is the probability that the algorithm gives a positive result given that the subject is a true-positive. The possible outcomes of the tests that will lead to a positive conclusion are ++++ OR +−++ OR ++−+. Assuming that the tests are independent, given that the subject is positive, we have:

$$\begin{aligned} P(T/S^+) &= P_{T+} \\ &= P(++++ \text{ OR } +-++ \text{ OR } ++-+) \\ &= 0.998 \times 0.998 \times 0.998 \times 0.996 + 2 \times 0.998 \times 0.998 \\ &\quad \times (1 - 0.998) \times 0.996 \\ &= 0.9940040239, \end{aligned}$$

and if the subject is negative:

$$\begin{aligned} P(T/S^-) &= (1 - 0.998)^3 \times (1 - 0.996) + 2 \times (1 - 0.998)^2 \\ &\quad \times 0.998 \times (1 - 0.996) \\ &= 0.000000031968 \end{aligned}$$

Substituting in the above expressions, we get:

$$P_{FP} = 0.00003212761425452, \text{ or } 32 \text{ per 1 million tests.}$$

The PPV = 0.999967679 or essentially 100%.

The probability of a false-negative tests is $P_{FN} = 0.000007987$ or 8 per million tests. The NPV is essentially 100%.

HIV Antigen Detection

Assay Methods

The first use of HIV antigen detection was for minitoring viral antigen in cell culture supernatants, thereby providing direct confirmation of reverse transcriptase assay results.[81] In 1986, commercial EIA tests for HIV core antigen (p24) became available (Abbott Laboratories, E.I. du Pont de Nemours) for detecting viral antigen in serum, plasma, and cerebrospinal fluid. In 1988, the Abbott Laboratories HIV antigen EIA was licensed by the FDA for diagnostic testing. Several other companies offer tests for investigational use. The principle of detection for HIV antigen tests involves incubating the patient's specimen with anti-HIV-1 capture antibody bound to a solid support, such as polystyrene beads of microplate wells. After washing, the immune complex bound to the solid support is incubated with a second anti-HIV-1 antibody to form a sandwich. This second antibody, in some assays, is conjugated to an enzyme that subsequently is reacted with substrate. The resulting color change can be detected by the increase in absorbance at a specific wavelength, which provides a quantitative measurement of the amount of HIV antigen present in the test sample.

Other assays (Figure 5.10) use an unlabeled second antibody (rabbit anti-HIV-1) followed by enzyme-conjugated goat anti-rabbit IgG. After the final wash the amount of HIV antigen present in the immune complex is detected as before by the color change resulting from the enzyme conversion of substrate. The second assay described is said to be more sensitive than the first because of the amplification provided by the addition of the second antibody. Both types of assays quantitate the amount of unbound HIV antigen present in the patient's sample by comparison to a standard curve prepared from dilutions of known amounts of HIV antigen. The detectable limit of these assays has been reported to be between 10 and 30 pg/ml.[82–85]

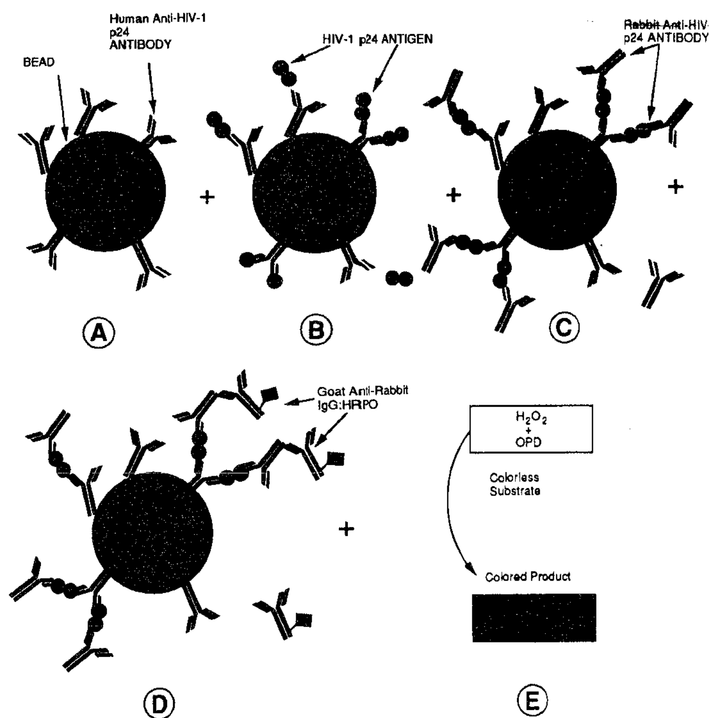

FIGURE 5.10. Configuration for p24 antigen test.

All reactive specimens should be confirmed by a specific neutralization assay to verify the presence of HIV-1 antigen. Neutralization involves preincubating the specimen with human anti-HIV antibody before performing the HIV-1 antigen EIA. If antigen is present, the neutralizing antibody will form an immune complex that prevents binding of the HIV-1 antigen to the HIV-1 antibody bound to the solid support. Reactive specimens are considered confirmed when neutralization results in a reduction of absorbance by at least 50% when compared with the specimen in which human serum nonreactive with HIV-1 is used in place of neutralizing antibody.

Applications of Antigen Testing

Considerable effort has been expended to demonstrate that antigen testing should be added to the battery of tests used to screen blood used for transfusion. Insertion of the HIV antigen test was proposed to close or shorten the window period (see Figure 5.1) between infection and seroconversion. The usefulness of the HIV antigen test in this application will depend on the prevalence of the infection in the population being tested and the length of the window period. In low-prevalence populations, such as blood donors, the routine use of antigen screening does not appear to be justified. Results from three separate studies testing several hundred thousand blood donors showed that antigen detection was no more sensitive than routine antibody screening for detecting HIV-infected donors.[86,87] For high-risk asymptomatic populations, the HIV antigen EIA may prove increasingly useful in diagnostic and prognostic applications. HIV antigen has been detected in early infection of seronegative individuals who are members of a high-risk group or who present with the acute HIV infection syndrome before seroconversion.[88–91] However, most HIV-infected individuals become HIV antigen negative shortly after seroconversion.[91]

Antigen testing has also been shown to be a useful tool in several clinical situations. There is a correlation between the onset of antigenemia and the progression to disease. Antigen testing is also useful in determining HIV infection in neonates born to HIV-infected mothers. And, finally HIV antigen testing can be an effective tool for assessing the therapeutic efficacy of different antiviral drugs. Unfortunately, the low prevalence of antigenemia observed in asymptomatic, HIV-infected populations may limit its usefulness to drug studies involving symptomatic patients in whom the prevalence of antigenemia is much higher. It has been shown that the sensitivity of the test increases with clinical progression of disease, showing 4% positivity in asymptomatic patients, 56% with patients having AIDS-related diseases, and 70% in AIDS patients.[92]

Antigen testing has several recognized limitations. Testing of hemolyzed or lipemic specimens should be avoided. If specimens must be stored, they may be refrigerated at 4°C for 1 week or frozen. Multiple freeze-thaw cycles of frozen samples should be avoided. Furthermore, it is important to emphasize that these tests appear to detect only free p24 antigen. In patients who have a high titer of p24 antibody, p24 antigen appears to be complexed with antibody and is difficult to detect.[92] This is further supported by the observation that persons who are p24 antigen positive after exposure become negative when they seroconvert.[93] Patients again become antigen positive as their p24 antibody falls during progression to the symptomatic state.[91,94]

At the present time, HIV antigen EIA provides a valuable additional tool to antibody testing for the detection of HIV infection. Positive results on the antigen EIA, confirmed by the neutralization test, provide direct evidence of the presence of the virus. However recently developed tests (e.g., PCR) that detect HIV RNA or DNA have surpassed the antigen EIA in sensitivity and specificity. However, antigen testing still has value in situations in which molecular techniques such as PCR are not available.

Conclusions

Sensitive, specific, and inexpensive tests for HIV antibodies have permitted the implementation of effective screening programs to protect the blood supply and to conduct seroprevalence studies to measure the spread of the disease. It has also permitted accurate diagnosis of HIV infection in asymptomatic carriers. These programs have dramatically reduced the rate of spread of the disease in the United States and many other parts of the world. As the AIDS epidemic continues to spread, there will be sustained need for more sensitive and quantitative assays. Tests of higher sensitivity will be needed to permit earlier diagnosis of infection, which will become increasingly important as effective therapeutic interventions are developed to prevent the disease. Tests having greater sensitivity will be needed to reduce the number of false-positives that result from mass screening of low-prevalence populations. Tests should be developed that will detect infection in neonates immediately after birth to allow intervention therapies.

Direct tests that measure viral antigens or the viral genome have progressed rapidly. These tests have progressed from experimental procedures that required several days to perform and cost several hundred dollars to methods that can be completed in a single day. These tests will continue to improve and will assume an important role in HIV testing. However, in the immediate future antibody testing will retain the dominant position in disease control. Considerable progress can be expected in the development of more rapid, sensitive, and specific serologic tests.

Acknowledgment. Statistical calculations performed by Robert H. Byers, Jr., Ph.D., Chief, Statistical Section DHA, CDC.

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