Current Status of Human Immunodeficiency Virus Testing

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Human Immunodeficiency Virus (HIV, HIV-1) is now known to be the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS). As of March, 1988 there are more than 54,000 cases of AIDS, according to reports of the Centers for Disease Control (CDC). It is estimated that the total number of infected persons at the present time is 1 to 1.5 million in the United States. It is further predicted that there will be approximately 324,000 cases of AIDS in the United States by the end of 1991. Based on advances in immunologic, microbiologic and molecular biologic techniques, there has been remarkable development of several assays for HIV-1 and the current status will no doubt rapidly change in the near future.

Human Immunodeficiency Virus (HIV-1) is a retrovirus, composed of a single strand of RNA admixed with an enzyme, reverse transcriptase, surrounded by a core protein which in turn is covered by a capsule. Of the eight known genes present in HIV-1, the three that are involved in assays for the virus are gag (groups specific antigen, related to core protein), pol (reverse transcriptase activity polymerase regulatory) and env (envelope glycoprotein). These three genes in turn code several antigen products. Those products related to the gag (core) gene are p35, p17, p24 and p17; the env gene (envelope), gp160, gp120, gp41; pol (regulatory) gene, p66, p55 and p31. The numbers correspond to molecular weights in kilodaltons.

Infection is initiated by the virus binding to a specific cellular receptor, related to the CD4 antigen expressed by T4 lymphocytes. Monocytes also appear to have this receptor and they are also susceptible to HIV-1 infection. After entering the cell the virus is uncoated and the single strand of RNA and reverse transcriptase are released. Unique to the retrovirus is the presence of reverse transcriptase which transforms the RNA into double stranded DNA. DNA is then integrated into the host genome becoming an integral part of the host gene. The new virus can be formed from this DNA with transcription to RNA, translation and finally formation of new protein coat with assembly. The host may respond with the production of antibodies to several of the viral products described above.

AIDS may be caused by another virus, human immunodeficiency virus type 2 (HIV-2). One case has been documented in the United States. HIV-2 is considered to be endemic in West Africa, and the U.S. case was a West African who came to the United States in 1987. Several cases of HIV-2 infection have occurred in Europeans and West Africans living in Europe. A collaborative serologic survey of 22,699 persons, including 8,503 asymptomatic blood donors and 14,196 from persons at high risk for HIV-2 infection failed to detect any evidence of HIV-2 infection based on analysis by HIV-2 specific Western Blot. Anti-HIV-1 EIA tests detect 42-92% of HIV-2 infections by cross-reactivity. HIV-2 is transmitted by modes similar to HIV-1.

Natural History of Infection

Most HIV-1 infections start with a mononucleosis-like syndrome which is followed by an asymptomatic stage. This may progress to an AIDS related complex (ARC) or AIDS clinical picture. Initial infection is followed by a transitory presence of viral antigen, which in turn is followed first by antibodies to core antigen p24 and envelope glycoprotein gp120 and gp160. With progression to AIDS there is often decrease to absence of anti-p24. This is associated with a reappearance of p24 core antigen related to increased viral proliferation. The importance of this progression of changes is that tests depending on presence of a single viral protein or antigen may be negative at certain phases of HIV-1 infection. This will be further discussed below.

HIV ASSAYS

HIV Assays fall into two major groups: viral culture and serologic tests. Serologic tests discussed in this article fall into four major categories: (1) measurement of antibodies to whole virus or selected viral antigens; (2) demonstration of viral antigens in serum; (3) demonstration of proviral DNA or viral RNA in peripheral blood monocytes; (4) substitute (surrogate) tests.

HIV CULTURE

Culture of HIV virus is the ultimate gold standard for demonstration of presence of the virus. However it is time-consuming and expensive. The procedure may take weeks to finish. Most important, the test is not as sensitive as serologic tests. Indeed, the virus cannot be grown from 40% or more of patients with HIV infection due to the variability of viremia and problems in sampling. The test may have certain limited research use in determining viral load, monitoring drug therapy, evaluation of CSF fluid in neurologic disorders related to AIDS and possibly evaluation of other tests.
SEROLOGIC TESTS

HIV-1 Antibody Tests

At the present time there are four types of assays available for testing of antibody to HIV-1: (1) enzyme linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA); (2) protein immunoblot (Western blot, WB); (3) radioimmunoprecipitation assay (RIPA); and (4) immunofluorescence assay (IFA).

At the present time the most widely used testing procedure is an EIA assay with supplemental confirmation by Western blot or immunofluorescence assay.

EIA

Enzyme immunoassay (EIA) was the first commercially available test and is by far the most widely used screening test for HIV-1 antibody. The test currently used disrupted virus which has been grown in tissue culture. After purification, the viral antigen material is bound to a solid media such as polystyrene beads or the walls of a plastic microtiter well. Serum or plasma is incubated with the fixed antigen and after washing any antibody (immunoglobulin G) attached to the solid phase will be detected with an anti-G antibody labeled with an enzyme such as alkaline phosphatase or horseradish peroxidase. The enzyme in turn can then be reacted with a substrate resulting in a colored product. The intensity of the color measured spectrophotometrically is related semiquantitatively to the amount of antibody. Positive and negative controls are run and an absorbance (cut-off) value is determined above which a specimen is considered reactive and below which a specimen is considered nonreactive. "Reactive" and "non-reactive" are used to describe the actual test reaction. "Positive" and "negative" describe the interpretation of test reactions. "Positive" describes an EIA which is repeatedly reactive and "negative" an EIA which is non-reactive or not repeatedly reactive. At the present time there are eleven licensed EIA kits. All of the kits achieve sensitivity (the probability that a test result will be reactive if the specimen is a true positive) and specificity (probability that a test result will be non-reactive if the specimen is a true negative) of above 95%, some approaching 100%. In the American Red Cross Blood Services Laboratories a specificity of 99.8% was achieved. False positives may be due to (a) intrinsic variability in test performance, (b) heat and activation of serum, (c) HLA antibodies reacting with T cell antigens (HIV-1 antigen is produced by culture in virus in human T cells), (d) biologic false positives have been reported in patients with hypergammaglobulinemia and autoimmune diseases, patients with multiple transfusions, alcoholic patients with hepatitis, hemodialysis patients, and positive rapid plasma reagent tests. Manufacturers have constantly developed modifications of methodologies to avoid most of these false positive reactions and present EIA methods are considerably improved over previous procedures. More recently developed EIA procedures use synthetic or recombinant viral proteins rather than disrupted virus. This eliminates cross-reaction with host cell antigens and theoretically will provide a more standardized result. Tests developed with these agents must have a sufficient number of antigen components to ensure reactivity with a virus known to have variability in its serologic effect. Nevertheless, these tests offer the promise of greater sensitivity and specificity as well as greater ease of use including recent development of a rapid latex agglutination test. These new methods are awaiting Food and Drug Administration (FDA) approval after sufficient trials.

At the present time EIA testing is the basis of most screening programs because it is the least expensive and most rapid approach to testing with excellent sensitivity and specificity. Because manufacturers are constantly modifying and improving licensed kits, it is difficult to make a comparative evaluation of time.

Protein Immunoblot (Western Blot)

The Western Blot test has become the most widely used supplemental test and is considered the "gold standard" for confirmation of a positive EIA result.

HIV-1 antigen, usually a viral lysate, partially purified, is electrophoretically separated into viral protein components on a polyacrylamide gel. The proteins are separated by molecular weight and transferred on to nitrocellulose which is cut into strips and incubated with patient and control sera as well as molecular weight standards. Any HIV-1 antibody which reacts with the protein components is detected by an antiglobulin reaction usually tagged with an enzyme which produces a colored band after reaction with substrate. The bands are read visually. The distribution of the colored bands is compared to known HIV-1 controls. The principle of the assay is similar to that of EIA except that several HIV-1 viral antigens are separated and identified. The problem with the Western Blot test is that it is technically demanding, labor intensive and costly. For the most part the materials have not been standardized. Recently a kit for Western Blot has been approved by the FDA (DuPont). Many labs, however, continue to use methods developed independently. Furthermore, interpretive criteria for positive interpretations have varied among authorities. A recent review summarized four different "standard" criteria. The FDA licensed Western Blot test (DuPont) requires the presence of antibodies to p24, p31, and gp41 or gp120/gp160 (antibodies to products of all three major genes). The American Red Cross requires the presence of antibodies to at least one gene product from each of env, pol and gag. The Association of State and Territorial Public Health Laboratory Directors criteria requires the presence of antibodies to any two of the following: p24, gp41 and gp120/gp160. The Consortium for Retrovirus Serology Standardization requires the presence of antibodies to at least p24 or p31 and gp41 or gp120/gp160 (two gene products). Any combination of bands other than those indicated as positive will be termed "indeterminate." A negative specimen requires no bands present. Depending on the criteria used, sensitivity as specificity will vary. Using the strict interpretation of the licensed Western Blot kit, sensitivity is approximately 56% in AIDS patients, since 60% of AIDS patients have antibodies to p24 and 83% to p31. The demonstrated variability of interlaboratory performance of Western Blot is demonstrated by the fact that 10 of 19
laboratories competing for Department of Defense testing contracts failed the required proficiency panel on one or more occasions. A specificity of approximately 99.4% can be obtained by the Western Blot. Furthermore, the specificity of sequentially performed EIA and WB tests can be greater than 99.99% (less than 1/100,000 persons tested will have a false positive test). An indeterminate sample may be repeated in duplicate and if still indeterminate, a new sample should be tested in 2–6 months. The second sample will often test positive.

It is probable that recent developments in EIA testing using recombinant antigens or chemically synthesized peptides will allow sensitivity and specificity greater than the Western Blot as well as the ability to determine individual antigens by testing patterns. These tests may eventually replace the Western Blot as the "gold standard."

Immunofluorescence Assay (IFA)

The Immunofluorescence Assay (IFA) for HIV-1 antibody is a fast, reliable and sensitive supplemental test for HIV-1 antibody. The test technically is less demanding and less expensive than the Western Blot procedure. The IFA uses a lymphocyte cell culture infected with HIV which is applied on slides, air-dried and fixed in acetone. Uninfected cells are added to the suspension before drying to provide a detection system for nonspecific reactions. The slides are stored at -20°C or colder. After incubation with test or control serum and washing, the slides are incubated with fluorescein-conjugated anti-human globulin and observed with a UV microscope. This procedure is similar to other indirect IFA procedures performed in the laboratory. Apple-green fluorescence in infected cells identifies antibody to HIV-1. The test is more sensitive than Western Blot and at least equally specific. The IFA can be performed in less than 2 hours and requires less technical expertise. However, the interpretation of the slides is more subjective. Pretreatment of serum with uninfected cells may be required to absorb nonspecific reactions.

Radioimmunoprecipitation (RIP)

This test is a reasearch method which is used for evaluating other HIV-1 tests and is especially sensitive to envelope proteins gp120 and gp160 which are often not present in Western Blot assays. The test requires culturing HIV-1 in radioactivated cells producing a radioactive viral lysate. The lysate forms antigen-antibody complexes after exposure to a positive test specimen; the radioactive complexes are separated by immunoprecipitation. The test is considered probably slightly more sensitive and specific than Western Blot.

HIV Antigen Detection

Tests are now available but not licensed by the FDA for detection of viral antigen in serum, plasma and cerebrospinal fluid. The assay for antigen captures the antigen with solid phase anti-HIV-1 antibody. After washing there is incubation with a rabbit or goat antibody to HIV-1 antigen. After further washing, anti-rabbit or anti-goat antibody conjugated with an enzyme is added and the reaction determined with addition of an appropriate substrate. The amount of HIV antigen can be quantitated from a standard curve of absorbance. A repeatedly reactive specimen should be confirmed with a neutralization test performed by preincubating the specimen with human antibody to HIV-1 before running the HIV-1 antigen test. Thus, although relatively simple to perform since the assay is an EIA, an overnight incubation step and a possible confirmation step takes 3 to 4 days to complete a positive result. Compared to culture the antigen assay is a more simple procedure, but is less sensitive. This is probably because antigen detects active viral proliferation while culture probably detects latent virus. Although the sensitivity of antigen detection is relatively low, the test may have value in (1) determining infection in the acute phase, prior to development of antibody. Since the reappearance of viral antigen later in an antibody-positive patient suggests the onset of clinically significant disease, antigen detection can be used as a prognostic tool. The detection of antigen may indicate progression towards ARC or AIDS. (2) Monitoring of anti-viral drug therapy for diagnosis of HIV-1 infection in infants where antibody tests are negated by passive diffusion of antibody from maternal circulation. (3) Further evaluation of indeterminant Western Blot tests.

Detection of Pro-viral DNA or Viral RNA

Recent developments in hybridization methods for DNA, utilizing a nucleic acid amplification method known as polymerase chain reaction, automated by instrumentation developed by Cetus Corporation has made analysis of pro-viral DNA a more pragmatic test. Pro-viral DNA is amplified several hundred thousand to a million fold or greater. The procedure is at an early stage of application, and its reliability and significance remain to be determined. It is, however, exciting new avenue in detection of HIV-1.

SURROGATE TESTS FOR HIV-1

The prohibition of use of direct testing for HIV-1 in some states for pre-insurance evaluation requires the use of assays which indirectly detect effects of HIV infection. It is obvious that these tests, being indirect, are not as effective as specific HIV-1 detection procedures. Surrogate tests include: (1) T-lymphocyte subset assay, (2) beta-2 microglobulin assay, and (3) neopterin assay.

T-Lymphocyte Subset Assay

T-lymphocyte subsets are determined by direct immunofluorescence using monoclonal antibody and flow cytometry. The absolute number of total lymphocytes, and T4 (helper) lymphocytes and T8 (suppressor) lymphocytes are determined as well as the T4/T8 ratio. HIV-1 demonstrates an exquisite tropism for T helper cells (T4). HW-1 infects and destroys T4 cells. As infection progresses, the absolute number of T4 cells drops. Also, early in infection the number of suppressor T cells (T8) increases, stimulated by the HIV-1 infectious process. In one study which analyzed differences between sero-positive men and sero-negative homosexual and heterosexual men, the T4 cell count was significantly less in the sero-positive men. Twenty-nine percent of sero-positive men had a T4 cell count less than 400. On the other
hand, there were many sero-positive men with counts in the normal range. The majority of men with HIV-1 sero-positivity had a T4/T8 ratio less than 1. The T4/T8 ratio, similar to the T4 absolute count, progressively decreases from normal control values (1.68 mean) to HIV sero-positive patients (0.6–0.8 mean) and patients with AIDS (0.36–0.46 mean).

**Beta-2 Microglobulin**

Beta-2 microglobulin is a low molecular weight protein that is present on the surface of all nucleated cells and is a subunit of histo-compatibility antigens. As a result of cell turnover, it is normally produced at a steady rate and released into body fluids. Cell beta-2 microglobulin production and turnover rate are increased in both cytomegalovirus infection and HIV infection, resulting in increased serum levels. In studies of men sero-positive for HIV, beta-2 microglobulin was the single most powerful predictor of progression to AIDS. 17

**Neopterin**

Neopterin is a pteridine which is released by activated macrophages which are related to activated lymphocytes. The mediator between these two cells is production of gamma interferon by activated T-lymphocytes. Activated lymphocytes are more prone to cell death by HIV infection with a greater risk of development of AIDS. Neopterin levels demonstrate a significant positive correlation with HIV infection and with increasing immune suppression as measured by the Walter Reed Staging Classification. 21

**Testing for Human Immunodeficiency Virus: General Comments on Evaluation and Application**

No other procedure has stimulated discussion of sensitivity, specificity and predictive value as HIV testing. The immense clinical, social, epidemiological and psychological impact of a diagnosis of HIV infection demands a perfect test which, of course, is not possible. In a recent excellent review, Schwartz, et al. discusses methods of improving evaluation, performance and application of HIV tests. 7

**Evaluation of Tests**

Test evaluation may be inadequate for several reasons which are enumerated by Schwartz, et al. as follows: (1) spectrum bias, (2) use of an inadequate reference standard, (3) failure to use different cutoff points, (4) referral bias, (5) inadequate sample size. 7

**Spectrum Bias**

To eliminate spectrum bias, one must ensure that the HIV test evaluation examines patients with different stages of HIV infection to detect varying levels of antibodies. Also, the HIV-infected as well as non-infected group should have concurrent diseases found in the tested populations, that may possibly affect the HIV test. These conditions may produce false positives, and a lack of inclusion of co-morbid conditions may overstate specificity. Such conditions include sexually transmitted diseases, acute infections, chronic diseases and intravenous drug use.

**Inadequate Reference Standard**

This refers to the lack of a true gold standard in HIV-1 testing. As pointed out above, the cultured virus is evidence of infection, but is a relatively insensitive test, falsely underestimating the prevalence of HIV infection. The Western Blot test, commonly used as a gold standard, has varying methodology and interpretive guidelines which in turn change its sensitivity and specificity. Thus, evaluation of any new test should probably use two or more supplemental tests to give significant support to a HIV positive sample group. Related to this is the need for standard panels, but the relatively small size of these panels of positive samples can lead to false evaluations.

**Failure to Use Different Cut-off Points**

In tests for HIV, where there is a continuous absorbance scale increasing from non-reactive to reactive, a cut-off value can be selected at any number of different points. The cut-off point is selected so that true negatives are separated from true positives with the least number of false positives and false negatives.

Lowering a cut-off point will increase sensitivity but decrease specificity. The selection of a cut-off point must take into account the use of the test and the prevalence of disease in the population tested.

**Referral Bias**

Referral bias refers to sequential analysis such as EIA followed by Western Blot. The sequential testing procedure will tend to underestimate the sensitivity and specificity of the first test relative to the second test.

**Inadequate Sample Size**

For a given degree of sensitivity, a decrease in HIV prevalence in a population markedly increases the number of nonreactive specimens that must be retested by a standard reference test on all samples tested in the evaluation. Use of small samples in populations with low prevalence leads to unacceptably wide confidence intervals (CI); i.e., a test yielding a sensitivity of 0.9 may have a 95% CI of 0.70 to 0.97. 7

**PERFORMANCE OF TESTS**

Test performance is essentially related to the nature of the methodology used and the quality assurance employed by the testing laboratory. Laboratory testing requires that each step be performed properly, including (1) collection, labeling and transport of specimens, (2) laboratory reagents and procedures under constant monitoring, (3) interpretation of analytic results, and (4) communication from the laboratory to the clinician and finally to the person tested. 8 Methodology design which eliminates complicated and technically demanding steps will increase precision and decrease test variability. Monitoring of reagent quality by manufacturers is necessary to ensure good test performance.
Proficiency testing is a useful facet of quality assurance. Unannounced or blinded testing is more effective than non-blinded proficiency testing. Reporting degree of reactivity rather than simply positive or negative can provide information useful in improving interpretation of results. The implications of HIV testing require that a laboratory performing HIV tests be licensed and subjected to thorough proficiency testing.

As discussed above, there is a semi-quantitative relationship of the degree of reactivity of EIA to the amount of antibody present in the sample. In addition, with increasing optical density there is an increasing probability of a result being indicative of HIV infection (true positive). For any given degree of reactivity, the probability that this value represents true infection is dependent also on the prevalence of infection in the population tested. In one example, a degree of EIA reactivity of moderate degree would only have a probability of 1.13% of predicting a true positive (infected individual) in low risk blood donors, while in a high risk population such as Methadone Clinic attendees, the same degree of reactivity would have a 96.9% probability of indicating an infected individual.

TEST USAGE

Mandatory HIV-1 testing of applicants for insurance is a controversial subject. In general, there has been a tendency to permit such testing for applicants to life insurance but not health insurance. Because of the controversy of any type of mandatory testing, the responsibility is on the testor to be sure that the assay and assay sequence chosen gives a maximum degree of specificity with the fewest false positives possible, even at the expense of sensitivity (false negatives). As discussed above, the sequence of EIA and Western Blot testing can achieve a false positive rate of less than 1/100,000 persons tested. With improvement in EIA, Western and other tests, using recombinant antigens and test adjustments, as well as attention to overall improvement of HIV-1 testing as outlined by Schwartz, et al., specificity can be further improved with false positives brought almost to zero. In this way, acceptance of HIV-1 testing in low prevalent populations can be achieved.

References