Laboratory diagnosis of human immunodeficiency virus infection

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S U M M A R Y

Current routine diagnosis of human immunodeficiency virus (HIV) infection is based on the detection of specific antiviral antibodies in body fluids, mainly serum samples. Enzyme immunoassays and agglutination tests are the most widely used basic screening serological methods. Since the specificity of screening tests is limited, all anti-HIV reactive screening test results have to be confirmed, using confirmatory tests such as Western blot or immunoblot tests. Diagnostic problems, which cannot be resolved with serological methods, were cleared using two classical methods for direct detection of viral infection: detection of p24 viral antigen or HIV culture. Polymerase chain reaction (PCR) and other amplification methods are currently the methods of choice for direct detection of HIV infection. The number of copies of the HIV RNA present in blood measured by quantitative amplification methods (viral load) is used as prognostic marker of disease progression, for monitoring antiretroviral treatment and for diagnosis of acute HIV infection before seroconversion.

Introduction

Infection with human immunodeficiency virus (HIV) types 1 and 2 causes a serious disorder of the immune system in which the normal immunity against infection breaks down, leaving it vulnerable to a variety of life-threatening infections/conditions including unusual malignancies. Acquired immunodeficiency syndrome (AIDS), the final stage of HIV infection, was officially recognized for the first time in June 1981 (1).

HIV-1 and HIV-2, the viruses that cause AIDS, are members of the genus *Lentivirinae* of the *Retroviridae* family. They are enveloped viruses containing positive-sense, single stranded RNA that is reverse transcribed to DNA and then integrated into the human genome. HIV persists within different cells for years and cannot be completely eradicated from host with any of the currently available antiretroviral drugs (1).

HIV-1 virions contain two identical copies of RNA and a number of viral proteins. Antibody response to a number of viral proteins can be detected, including products of viral genes *env* (gp160, gp120, and gp41), *gag* (p24,

p17, p7, p9), and *pol* (p32, p66, p51, and p11). HIV -1 can be divided into three groups: M (main), O (outlier) and N (non-M, non-O). HIV-1 group M can be further classified into different HIV subtypes or clades, A to K. Sequences of *the env* gene vary by 20% or more between different HIV-1 subtypes. HIV-1 groups O and N are more distant from all other HIV-1 subtypes but less in comparison to HIV-2 (1).

HIV-2 differs from HIV-1 in its lower pathogenicity and higher level of intrasubtype strain diversity. Although HIV-2 infection is associated with plasma HIV-2 RNA viral loads significantly lower than those found in HIV-1 infection, HIV-2 DNA proviral load is relatively high, with the values similar to the HIV-1 proviral load (1).

Cell free or cell associated HIV enters the human body most frequently via infected blood, semen, and vaginal secretions. HIV immediately targets cells displaying viral receptors: CD4 molecule and chemokine receptors. These receptors are present on CD4-positive T lymphocytes, macrophages, dendritic (Langerhans') and other antigen-presenting cells (1).

Approximately 50-70% of patients with primary HIV infection present flu-like or infectious mononucleosis-like symptoms approximately 2 to 6 weeks after infection (2-4). During the acute phase of infection there is a significant reduction of CD4 cells and virus levels may be as high as 10^6-10^7 viral copies/ml (3,4). During acute infection, p24 antigen is also detectable in plasma. In the following phase virus levels in plasma decrease as a result of the emergence of host antiviral immune response. Both cell-mediated (production of HIV-specific cytotoxic lymphocytes T) and humoral (production of complement fixing and neutralizing HIV-specific antibodies) immune responses are responsible for the significant decrease of plasma viral load. Although the immune response succeeds in downregulating the viremia, HIV is never completely eliminated from the human body and progression to chronic phase of HIV disease occurs in almost all patients (1,4-6).

Laboratory diagnosis of HIV infection

HIV infection/AIDS is a complex infectious disease since it is lifelong, the outcome is invariably fatal and no vaccine is available so far. Since HIV is acquired most frequently through unprotected sexual contact, a number of moral, ethical, legal, and psychological issues are related to HIV testing. It is important to understand that laboratory diagnosis is the only way to establish the HIV infection status of an individual.

The main purposes of HIV testing are:

1. to identify of asymptomatic and symptomatic HIV-positive individuals

2. to assure safety of blood and blood-related products
3. to motivate for behavior changes through counseling among those high-risk behavior individuals who tested anti-HIV negative
4. to induce behavior change and prevent further HIV transmission by counseling in those individuals who tested anti-HIV positive
5. to monitor trends of HIV epidemic.

Indirect or serological detection of HIV infection

Current routine laboratory diagnosis of HIV infection is mainly based on the detection of specific anti-HIV antibodies. Blood (whole blood, serum or plasma) is the preferred specimen for the detection of anti-HIV antibodies since it has a higher concentration of antibodies than urine or oral fluids. Antibodies to HIV usually begin to be detectable 3 to 6 weeks (on average 22 days) after infection. The time from infection to first reactivity of screening tests (seroconversion) is called the "window period". During this period the infected individual is highly infectious but anti-HIV seronegative. The window period may be in some individuals somewhat shorter or several weeks longer. Persistently undetectable antibodies more than three months following infection are rare (1,5-8).

All samples are first screened for anti-HIV using basic screening serological methods such as the enzyme immuno assay or agglutination assay. Since the specificity of screening tests is limited, all anti-HIV reactive screening test results have to be confirmed, using confirmatory tests such as Western-blot or immunoblot tests (1,8,9).

Screening tests

Enzyme immuno assay (EIA) is the most commonly performed screening test for detection of anti-HIV antibodies. Briefly, in classical EIA immobilized HIV antigens are used to bind anti-HIV antibodies from the patient's sample. Bound anti-HIV antibodies are then complexed with enzyme-labeled anti-human IgG and are detected in a colorimetric reaction. The resulting colour change is quantified spectrophotometrically and is proportional to the concentration of antibodies in the original sample (1,8,10,11). The improved EIA test formats used for anti-HIV antibody screening are the antibody capture assay and the double antigen sandwich assay (1,8).

Since the introduction of HIV screening assays in 1985, the performance of EIA has continued to improve. First-generation antibody screening assays were very sensitive but not specific since whole viral lysates were used as a source of HIV proteins (antigens). The time interval between infection and antibody detection has been substantially shortened by the introduction of 3rd

generation double antigen sandwich assays, which use recombinant and synthetic HIV peptides as antigens. An even earlier laboratory diagnosis of HIV infection is possible by the detection of p24 antigen or HIV RNA, which are present prior to or during the early stages of seroconversion. Thus, HIV infection can be detected on average 9 days earlier by p24 antigen testing than with 3rd generation assays. 4th generation EIAs or combo-screening assays, which are now available, enable simultaneous screening for anti-HIV antibodies and p24 antigen in a single assay. 4th generation EIAs shorten the window period to an average of 7 days and can detect antibodies against HIV-1 group O (1,8).

Agglutination assays, which may be considered as a variant of the double antigen sandwich assay, incorporate a variety of antigen-coated carriers like red cells, latex or gelatin particles. HIV antigens that are based on viral lysate are attached to the carrier particles by non-specific attachment. Since antibodies are multivalent, in the case of a positive result a sort of lattice network is formed which can be visualized macroscopically or microscopically. Agglutination assays are highly specific and sensitive, simple to perform and do not require sophisticated equipment (1,8).

The reported sensitivity of licensed anti-HIV screening tests under optimal laboratory conditions is greater than 99% (1,8,11). False negative EIA results can occur during primary HIV infection, in immunocompromised patients and due to errors in specimen handling and labeling (11-13). The specificity of repeatedly reactive screening tests is approximately 99%. False reactive (better term than false positive) results can occur due to human error, in individuals with acute Epstein-Barr virus or human cytomegalovirus infection, autoimmune disorders, hipergamaglobulinemias, multiple myeloma, hemophilia, and hemodialysis patients. The percentage of false reactive EIA results decreases as the prevalence of HIV infection in a particular population increases (1,11-14).

The current anti-HIV screening strategy in Slovenia recommends testing of every sample obtained from all individuals except blood donors for whom two different anti-HIV screening tests are used. At the Institute of Microbiology and Immunology, Medical Faculty of Ljubljana which serves as, the Slovenian HIV reference laboratory, every sample is tested using one 3rd generation EIA assay and one 4th generation EIA or combo-screening assay since August, 1998 (15).

Supplemental or confirmatory tests

As mentioned before, anti-HIV antibody screening assays are highly sensitive but prone to false reactivity, while supplemental tests are both sensitive as well as specific and are mainly performed to rule out false reactive results of screening assays. However, supplemental tests do not always give conclusive results. In these cases other tests for direct detection of HIV infection have to be performed or a follow-up strategy is used (1,8).

Western blot (WB) and immunoblot (IB) tests are the most widely used supplemental or confirmatory tests for detection of anti-HIV antibodies in Europe, as well as in Slovenia. Both tests, are highly specific, but in comparison with screening tests more laborious and costly. Their high specificity is mainly based on the fact that they allow determination of the reactivity of anti-HIV antibodies with particular HIV proteins. In WB, electrophoretically separated natural HIV proteins derived from whole virus lysates are transferred (blotted) to a solid membrane. HIV viral proteins on WB membrane may contain contaminating human cell proteins. In contrast, recombinant or synthetic HIV proteins mechanically applied onto the solid membrane are used in the IB test. HIV proteins on IB membrane, however, do not contain contaminating human cell proteins and are highly specific.

Briefly, in both WB and IB patient's serum is incubated with a nitrocellulose membrane strip containing separated different HIV proteins. Antibodies directed against particular HIV proteins are identified with enzyme-labeled anti-human IgG, similarly to the EIA method (1,8,16).

In Slovenia WB results are interpreted according to the American Red Cross criteria (1). According to these criteria the presence of anti-HIV antibodies against at least one HIV protein derived from each *env*, *gag* and *pol* regions is required for anti-HIV positivity. The absence of reactivity against any of HIV proteins on membrane is considered as a negative WB result. WB results that do not meet the criteria for a positive result are considered indeterminate (Fig. 1).

In addition to greater specificity in comparison to WB, IB allow also a reliable differentiation between HIV-1 and HIV-2 infection since type specific recombinant proteins and peptides from HIV-1 and HIV-2 are present on the IB membrane. In Slovenia IB results are interpreted according to the manufacturer's criteria of a particular test (Fig. 2).

Patients with repeatedly reactive EIA and indeterminate WB or IB results require serologic and clinical follow-up to determine whether HIV infection is present. There are no clear patterns of antigen reactivity that predict a higher likelihood of seroconversion in patients with indeterminate WB/IB results (17). Additional testing for HIV infection using methods for direct detection of HIV may be indicated, depending on the clinical picture and risk factors for HIV infection (1).

All supplemental or confirmatory anti-HIV tests in Slovenia are performed at the Institute of Microbiology and Immunology, Medical Faculty of Ljubljana which serves as Slovenian HIV reference laboratory since 1991 and had served as Yugoslavian HIV reference laboratory from 1985 to 1991.

Direct detection of HIV infection

Serology represents a highly sensitive, cheap and quick method to screen blood for the presence of HIV infection. However, in certain situations such as in newborns born to HIV-infected mothers, in individuals with indeterminate WB/IB results, in order to determine the HIV status during "window period" and to monitor HIV-infected individuals a direct detection of HIV is required. For these purposes detection of the p24 antigen, virus isolation or detection of viral DNA or RNA by different molecular amplification methods is the most widely used (1,8).

Detection of p24 antigen

The HIV *gag* gene encoded core protein or p24 antigen can be detected in serum or plasma during the acute phase of primary HIV infection ("window period"), during very late symptomatic stages of infection and in the newborns born to HIV-infected mothers (1).

Detection of p24 antigen is mostly performed using EIA. The test principle consists of binding the p24 antigen present in a sample to anti-p24-specific, usually monoclonal, capture antibodies, which coat a solid support. Unbound sample components are washed away, and bound antigen reacts with another p24-specific antibody conjugated with enzyme. For a confirmation of a reactive result, the sample must be subjected to an additional confirmatory neutralization assay (1).

Levels of free p24 antigen decline after the appearance of anti-p24 antibodies due to formation of immune complexes. Only 4% of asymptomatic HIV-infected adults have detectable p24 antigen using standard assays. However, a recently developed assay based on heat denatured p24 antigen tyramide-mediated signal amplification-boosted EIA, coupled with neutralization allowed the detection of p24 antigen in up to 90% asymptomatic HIV-infected adults (18).

The overall sensitivity of standard p24 antigen testing for the detection of HIV infection in infants is 50 to 75% and the specificity is greater than 95% (19,20). The sensitivity of the assay decreases in asymptomatic children and children younger than 6 months of age and ranges 0 to 20% in the first month of life (19-21).

Although p24 antigen assays are very specific, they are relatively insensitive compared with molecular amplification methods, and negative result for p24 antigen does not rule out the HIV infection.

Isolation of HIV

HIV can be isolated from peripheral blood mononuclear cells (PBMC) or plasma and other body fluids. Briefly, the patient's PBMCs are isolated and incubated with phytohaemagglutinin- and interleukin-2-stimulated PBMCs from healthy blood donors (1). The presence of HIV in the culture supernatant is detected by demon-
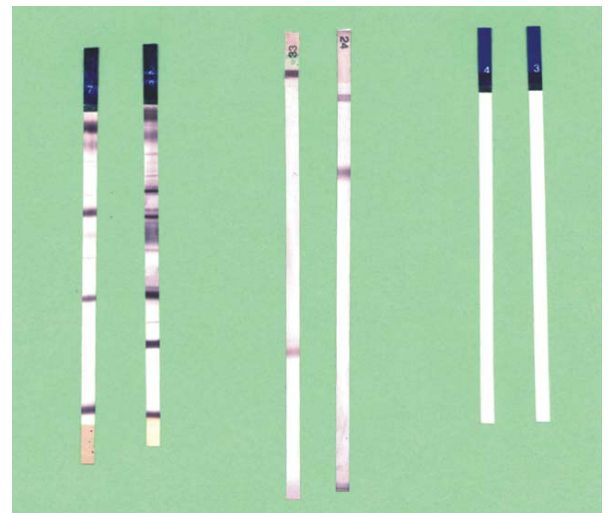

Figure 1. Examples of HIV-1 WB results. Left two membranes: anti HIV-1 positive samples; middle membranes: anti-HIV-1 indeterminate samples; right two membranes: anti-HIV-1 negative samples.

stration of the presence of p24 antigen, reverse transcriptase or HIV RNA (22). The majority of cultures from HIV-positive untreated patients become positive within two weeks (23).

Isolation of HIV is a time-consuming and costly procedure requiring specialized containment facilities and a high degree of expertise. HIV isolation therefore remains mostly an important research and not diagnostic tool.
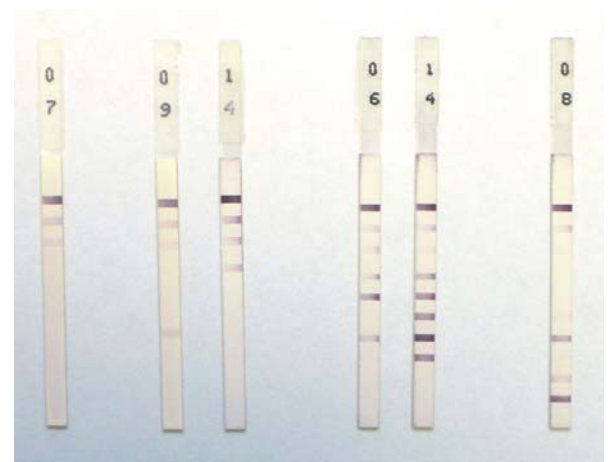

Figure 2. Examples of HIV immunoblot results. Membrane 07: anti-HIV negative sample; membranes 09 and 14: anti-HIV-1 indeterminate samples; membranes 06 and 14: anti HIV-1 positive samples; membrane 08: anti-HIV-2 positive sample.

Molecular amplification methods

Recent studies on the dynamics of HIV-1 replication have enhanced our understanding of HIV/AIDS pathogenesis (24). The discovery of a persistently high level of viral turnover in all stages of disease, even during the period of clinical latency, suggests that sensitive measurement of viral load (reflecting rapid or unchecked viral replication) may be useful in assessing HIV disease stage, disease progression, response to therapy, and risk of transmission (1,8,25-27). HIV RNA quantification or determination of plasma viral load is presently considered the best method for monitoring disease progression and response to antiretroviral therapy (1,8).

Three methods for determination of HIV-1 plasma viral load are widely used at present: two are based on use of enzymatic amplification of target nucleic acids (polymerase chain reaction (PCR) and nucleic acid sequence-based amplification) and the third utilizes branched-chain DNA hybridization signal amplification (bDNA assay). With "ultrasensitive" protocols developed for all three methods the detection limit of 20-50 HIV-1 RNA copies/ml has been already reached (1,8).

The HIV-1 viral load usually ranges between 10^2 and 10^7 HIV RNA copies/ml in untreated individuals. Persistently detectable viremia and high baseline levels are predictors of poor prognosis, while risk of progression of HIV infection to AIDS is relatively low if the viral load is below 10,000 HIV RNA copies/ml. Apart from being an extremely useful predictor of disease progression, viral load measurement is a key laboratory method for monitoring antiretroviral therapy. Determination of HIV RNA has been used by some clinicians also as a diagnostic tool in patients with acute HIV infection, in newborns born to HIV-infected mothers and in individuals with reactive results from screening tests and indeterminate WB/IB results, although this approach is not approved by the Food and Drug Administration (FDA) (1,8).

Conclusion

HIV infection is in almost all instances diagnosed and confirmed by detecting anti-HIV antibodies. Serological assays for HIV infection have been refined over the past years to unprecedented levels of sensitivity and specificity. However, diagnostic problems, which cannot be resolved with serological methods, are usually cleared using methods for direct detection of HIV infection. In addition, molecular amplification methods are currently the methods of choice for prediction of HIV progression and for monitoring antiretroviral treatment.

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