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Laboratory diagnostics for HIV infection

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Summary. Laboratory diagnosis of human immunodeficiency virus (HIV) infection is fundamental for detecting and monitoring infection. Many diagnostic tools are available that are based on both detection of HIV-specific antibodies and virus antigen, or nucleic acid. As technology evolves, HIV testing assays are being improved providing better sensitivity and specificity. In this short review, we summarize the common and new methodologies that are being used in laboratories, from the HIV antibody-based assays to the new tests for the detection of HIV nucleic acids.

Key words: HIV, diagnosis, biological assay, laboratory techniques and procedures.

Riassunto *(Test di laboratorio per la diagnosi di infezione da HIV).* La diagnosi di laboratorio è uno strumento fondamentale per identificare e monitorare l'infezione dal virus dell'immunodeficienza umana (HIV). Oggi sono disponibili molti saggi diagnostici, basati sulla determinazione sia degli anticorpi HIV-specifici, sia degli antigeni o degli acidi nucleici virali. Con l'evolversi della tecnologia, la diagnosi di laboratorio di infezione da HIV avviene attraverso saggi sempre più sensibili e specifici. In questo breve articolo vengono riassunte le metodologie utilizzate in laboratorio, dai saggi basati sugli anticorpi, ai nuovi test per la determinazione degli acidi nucleici del virus.

Parole chiave: HIV, diagnosi, saggio biologico, tecniche e procedure di laboratorio.

INTRODUCTION

Blood tests for HIV infection have been introduced in most of Western Countries in the middle of '80s. Since then, the quality of HIV screening tests has been improving. There are many purposes associated with HIV testing. For example, since 1985 screening of the blood supply has resulted in the protection of countless individuals from HIV infection. In addition, HIV testing can be used for the diagnosis of infection to individuals who whish to know if they are infected by HIV. Finally, HIV tests are also utilized for epidemiologic surveillance, providing health officials with information about the extent of the infection among communities, thereby allowing them to target populations for vaccines and treatment, to assess economical concerns, and to provide counselling to prevent the infection of other people.

The continuous improvement of diagnostic tests is a consequence of the great progresses in the knowledge on the immunopathogenetic mechanisms of HIV infection, and the virus/host interaction obtained in almost 30 years of research on HIV/AIDS. The discoveries on mechanisms of HIV replication, as well as of the immune response in the HIV infected individual over the whole course of the disease, have been fundamental to develop tests able to detect either HIV-specific antibodies, or HIV antigens and nucleic acids.

This short review is aimed at providing a brief overview of the technologies available for detecting and monitoring HIV infection and at introducing newer technologies that can offer important improvements on diagnosis, surveillance, blood screening, and disease monitoring.

IMMUNOLOGICAL AND VIROLOGICAL MARKERS DURING INFECTION

During the course of infection there are several markers from both the virus and the host that can be monitored and used to identify HIV infection. An extensive description of these markers has been reported elsewhere in this issue (reviewed in [1]). The kinetics and times of their appearance are fairly consistent among the different individuals and must be taken into consideration when choosing a diagnostic test. The choice of the markers to test depends on the purpose of the diagnostic test.

After HIV infection, early immunological and virological blood markers appear in a chronological order and, in particular: HIV RNA, HIV p24 antigen (a protein coded by the *gag* gene), and antibodies to HIV antigens *(Figure 1)* [2]. Viral RNA (viremia) is measurable in plasma as early as within 2 weeks after infection (in general in 10-12 days). Its titres increase exponentially, up to about to 1 million copies

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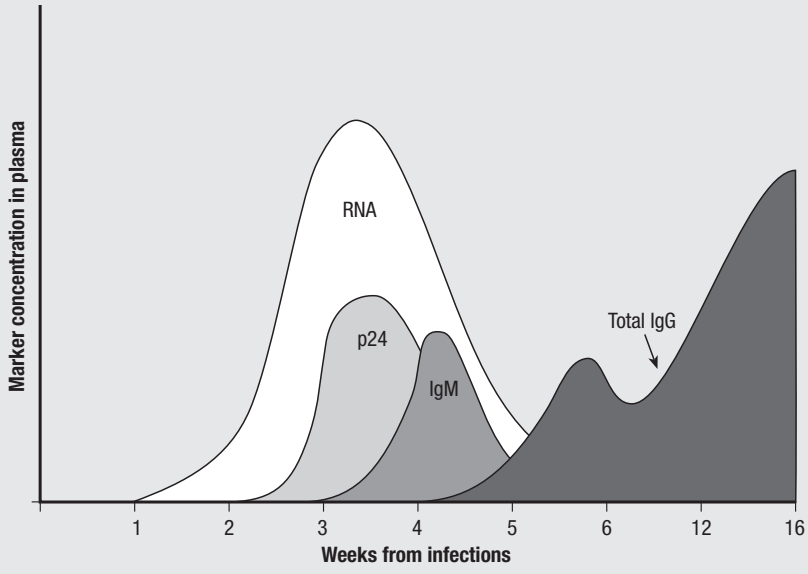

Fig. 1 | *Virological and serological markers during the first weeks following infection with HIV-1. Data indicated in this figure was previously reported from Murphy G. and Parry J.V. and published on Eurosurveillance, Vol. 13, Issues 7-9 (Jul-Sep 2008) (Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=18966).*

of RNA/ml within a couple of months. In the meanwhile, humoral and cell-mediated immune responses develop. These immune responses are able to control HIV replication and to drastically reduce RNA titres to a constant level known as the set-point. Depending on the set-point, it is possible to predict the subsequent course of infection and time of appearance of the disease. The highest the set-point, the faster is the course of the disease and the time to the development of AIDS. Conversely, a lower set-point is associated with a longer (slower) disease course. In later stages of the course of infection, RNA levels gradually increase over time reaching again high levels at the time of onset of AIDS-related symptoms.

The viral p24 antigen can be measured in blood a little bit later than viral RNA, as early as 11-13 days from infection *(Figure 1)*, because the methods used for its detection are less sensitive than the amplification methods used to detect RNA [3, 4]. Viral p24 concentration remains high and the marker is detectable in blood for about one month and half after infection [4].

The time interval before HIV-specific antibodies appear is known as the serological "window period". This period is characterized by the absence of HIV-specific antibodies, detectable viraemia (as measured by RNA or p24 antigen), and variable CD4 lymphocyte levels (reviewed in [1]). The detection of specific antibody to HIV signals the end of the window period and labels the individual as "seropositive" [5]. In most cases, first HIV-specific antibodies to appear are IgM, usually within the first three weeks from infection and peak between the 4th and the 5th week. However, response is strongly dependent on each individual and a broad range of antibody responses have been described [6, 7]. In fact, IgM antibodies can be not detectable in all early infected individuals [8]. Finally, detection of IgM antibodies is strongly dependent on the assay used. By using highly sensitive non-commercial assays, IgM antibodies directed against gp41 have been detected as early as 13 days from infection [9].

HIV-specific IgG antibodies usually appear at about 3-4 weeks after infection, depending on the specific antibody assay used and on the individual immune response variability among different individuals [4]. A very early presence of anti-gp41 IgG (13 days after infection in average) has been detected by using very sensitive non commercial assays [9]. Anti-HIV IgG titres increase soon after their appearance and then show a reduction around 10-12 weeks after infection. The titre reduction is due to a drop of the IgG3 isotype titre, the first IgG isotype to appear, mainly directed against the p24 [10, 11]. After this reduction the total HIV-specific IgG titre increases again reaching very high levels. In any case, within 1-2 months all HIV-specific antibodies are detected, regardless the commercial method used, in almost all the infected individuals, although there are reports indicating that a small percentage of persons may require up to 6 months for antibody to appear [12].

As the HIV antibodies appear, viremia decreases, as a consequence of both a reduced production of p24 and the formation of p24/anti-p24 antibody complexes. Finally, at the AIDS stage of the disease, when the immune system is severely compromised, virus replication again increases, reaching high levels of produced virus.

STATE OF THE ART OF HIV TESTING

HIV assays can be divided into two categories: (i) screening assays, designed to detect all infected individuals; and (ii) confirmatory (supplemental) assays designed to differentiate those persons who test

falsely reactive by screening assays from those who are truly infected. As a consequence, screening tests must have a high degree of sensitivity (low false negative rate), whereas confirmatory assays must posses a higher specificity (low false-positive rate). For most applications, screening and confirmatory tests are performed in tandem to produce results that are highly accurate and reliable.

A HIV infection can be detected by testing the presence of HIV-specific antibodies [13]. HIV-specific antibodies are found in virtually 100% of HIV-infected individuals. Their presence equals the diagnosis of a chronic active HIV infection.

A direct diagnosis of HIV infection is also possible through the demonstration of infectious virus, using cell culture, or the identification of viral antigen (p24 antigen) or viral nucleic acid (through NAT, nucleic acid testing). Beside these qualitative tests, assays for the quantitative detection of virus have become very important: the concentration of viral RNA in plasma, the so-called "viral load", has become an indispensable tool for guiding antiretroviral therapy [14].

Depending on the purposes of the test, different algorithms are currently used. For screening of blood donations and for epidemiological studies, a highly sensitive algorithm for detection of anti-HIV antibodies is used. For diagnosis, a positive result in a highly sensitive screening test must be followed by a further investigation using an accessory (confirmatory) test, to confirm the positive results obtained with the previous assay.

Most screening tests are based on the EIA (enzyme immuno assay) principle. Screening tests must be extremely sensitive to minimize the chance of yielding a false negative result. This means that they have to be able to also detect the low titre- and low avidity-antibodies found early in the course of HIV infection. If the result of a screening test is reactive (positive), this has to be confirmed by (at least) one confirmatory assay.

Screening EIA

Today, a variety of manual and automated test methods are available. However, screening assays are often performed on automated systems, so that large numbers of samples can be tested safely and economically.

All antibody assays are based on the principle of a specific antigen-antibody reaction. In 1985, first generation assays appeared. These assays employed "whole virus" antigens, obtained from cell cultures. Detection of antibodies bound to HIV antigens used an "indirect" approach. Briefly, viral lysate is bound to the so-called solid phase, on the bottom of the wells of a microtitre plate. Upon addition of patient serum that contains antibodies directed against HIV antigens, an antigen-antibody binding will occur. A washing step then removes all unbound constituents of the serum, including all antibodies not recognizing the HIV antigens. Remaining bound antibodies are then detected through the addition of a conjugate. This conjugate is a second antibody directed against one or more classes of the human antibodies (generally obtained from goats or rabbits). The conjugate is coupled with an enzyme molecule. A further washing step then removes the unbound conjugate. After washing, a substrate of the coupled enzyme molecule is added. Chemical conversion of the substrate, due to the action of the enzyme, generates the development of a colour that is read at the spectrophotometer. The intensity "optical density" (OD) of this colour reaction is proportional to the antibody activity in the sample. Positive and negative control specimens must be included in each test run and the OD values, obtained with these specimens, are often used to calculate the test's cut-off, to distinguish positive from negative values. The first generation EIA were enough sensitive, but less effective regarding their specificity. Their capacity to detect early HIV antibodies averaged a bit more than 40 days after infection [1] *(Figure 2)*.

In 1987, the second generation EIA appeared. They used the same indirect format as the first generation assays, but the difference was the presence of HIV recombinant antigens and peptides, instead of the full viral lysate, bound in solid phase. The introduction of recombinant antigens increased the specificity of the test and, in the same time, ensured a good sensitivity. These tests reduced the window period [2], being able to detect antibodies as early as 33-35 days after infection *(Figure 2)*.

In the '90s, the problem of the huge variability of HIV became progressively evident. EIA kits started to include also antigens from the HIV-2 virus, in order to ensure recognition of antibodies directed against both HIV-1 and HIV-2. In addition, new antigens from viruses of the HIV-1 groups M, N, and O were included. Determination of antibodies directed against the different HIV-1 subtypes from group M was ensured by the proven cross reactivity of antibodies with all group M subtypes (HIV-1 A to K) [15, 16].

In 1994, third generation EIA were designed on a new format. Recombinant HIV-1 and HIV-2 proteins and/or peptides, bound on the solid phase (either the bottom of a microplate, or a bead), react with the patient serum. Antigen-bound antibody is revealed by the addition of the same viral antigen conjugated with an enzyme molecule. This "sandwich" format ensured higher sensitivity and specificity, since all potential classes of anti-HIV antibodies (IgG, IgM and IgA) could be revealed. This generation of tests drastically reduced the "window period", bringing it to about 22 days after infection *(Figure 2)* [2].

Recently, fourth generation assays have been introduced. These assays are able to reveal the presence of both the antibodies and the p24 major antigen of HIV. This has permitted to further reduce the window period, at almost the levels of the detection of virus RNA *(Figure 2)* [2, 17]. Using fourth generation tests, the definition of "window period" changes a bit, since the reduction of the "window"

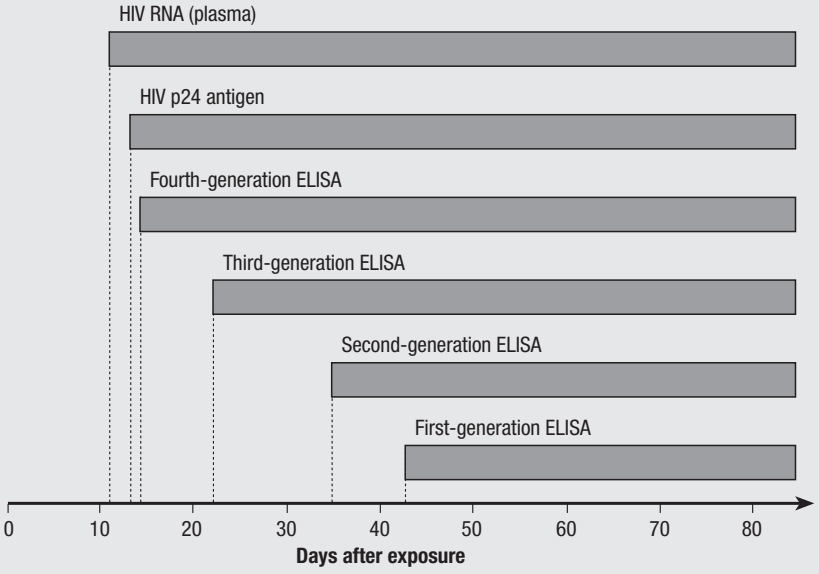

Fig. 2 | *Time of detection of specific markers of HIV infection, according standardised, commercially available kits. Time 0 indicates time of HIV infection.*
Data indicated in this figure was previously reported from Weber B and published on Exp Rev Mol Diagn, Vol. 6, Issue 3, pages 399-411 (2006).

is a consequence of the detection of the presence of HIV antigen, rather than antibodies in the very early phases after infection. Thus, in this case, the term "window period" refers, more properly, to the period occurring from the time of infection to the time of the appearance of a positive result by a fourth generation test. It can be expected that, in the near future, fourth generation EIA will replace the third generation assays due to their importance in the screening of low- and high-risk populations [17, 18]. However, by using fourth generation tests, there is a risk of a "second diagnostic window". This situation is very rare, but it can happen, as consequence of a delayed development of the antibody response. In fact, if p24 antigen levels start reducing before the development of HIV-specific antibodies, it can happen that a sample collected very early after infection can result positive at the fourth generation EIA. This is because the test is recognising the presence of p24 antigen. Nevertheless, a sample from the same subject collected few weeks later can be negative at the same test because the p24 levels are reduced under the detection limit of the kit and a HIV antibody response is not developed, yet [19].

Today's EIA are estimated to be more than 99.9% sensitive. This means that HIV antibodies with even low titres and avidity for the antigen are detected. High sensitivity reduces the chance of a "false negative" test result. These assays are used as first line assays for the detection of HIV-specific antibodies, since their sensitivity ensures that no positive samples are lost. The use of these assays is mandatory when testing blood donations, as any failure to identify a positive sample correctly can have serious consequences for the transfused persons and, more in general, for the entire population.

However, high sensitivity tests can still have a lower specificity. Third and fourth-generation EIA have an average specificity of 99.5% to 99.9%. As a consequence, the assay may occasionally provide a "false-positive" result. In fact, a false positive result can be the consequence of non-specific recognition of HIV antigens. False positive results can happen in patients whose immune system is activated, who can generate factors that can bind to antigen not specifically. False positive results in HIV testing have been described for: subjects with acute viral infections [20, 21], individuals vaccinated for different infectious diseases, including influenza [22, 23], hepatitis B and rabies [24-26] and patients with autoimmune diseases [27-31], in particular individuals with antibodies against class I HLA antigens [32-34]. In addition, also pregnancy, malignant neoplasm, and lymphomas have been described to be responsible to cause non-specific recognition of HIV antigens [20, 21, 34-36].

Due to the possibility of non-specific reactivity inherent in any assay, all reactive screening test results must be confirmed, in order to exclude the risk of reporting non-specific reactivity as "positive" for HIV infection. Only after results of confirmatory testing, made after a defined period (in most cases, about 1 month) from the screening test, are available, should one talk of a positive HIV test.

Confirmatory assays

Most commonly used confirmatory assays are western blot (WB) and line immune assays (LIA). The WB is a confirmatory assay that is only carried out if the sample is reactive in the screening assay. Many of the commercially available WBs include antigens from both HIV-1 and HIV-2. The WB is a methodology for which HIV denaturated proteins are blotted on strips of a nitrocellulose membrane, which are then incubated with patient serum. If the serum contains antibodies against the various viral proteins, they will bind to the corresponding protein.

This antigen-antibody reaction is revealed using an enzyme-labelled secondary antibody and a matching substrate. A colorimetric reaction will reveal the presence of HIV proteins recognised by antibodies as "bands" on the strip. For HIV-1, proteins detectable by WB can be divided into three groups: the Env (envelope) glycoproteins (gp41, gp120, gp160), the Gag or nuclear proteins (p17, p24/25, p55) and the Pol or endonuclease-polymerase proteins (p34, p40, p52, p68). Most of the commercially available WBs include also a protein from HIV-2 in order to detect both HIV-1 and HIV-2 infections.

The result of a WB may be either positive or negative or (in case of an incomplete pattern of visible bands) "indeterminate", which may reflect borderline or non-specific reactivity. Criteria for the interpretation of HIV Western blot may differ: the Centers for Disease Control consider a positive WB if at least two of p24, gp41, and gp120/160 proteins are present [37, 38], whereas according to WHO recommendations a WB may be judged positive if only two Env bands are found [38]. A more restrictive recommendation is the one from the American Red Cross, which demands at least three bands, one from each group (*i.e.* one protein from Gag, one from Pol and one from Env) [39]. Finally, the Consortium for Retrovirus Serology Standardization recommends the presence of at least one of gp120 or gp160 proteins and one of p24 or p32 proteins [40] for a positive WB.

A great disadvantage of the WB assay is its high price. In addition, the unavoidable subjectivity when reading and interpreting the result and the uncertainty about the criteria of positivity are, often, a great obstacle for a quick and clear result in HIV testing. Finally, the relatively frequent presence of indeterminate results can greatly delay diagnosis and increase costs.

Assays similar to WB, generically called LIA, based on recombinant proteins and/or synthetic peptides capable of detecting antibodies to specific HIV-1 and/or HIV-2 proteins, have been developed. Examples of this technology include the INNOLIA, Pepti-Lav, and RIBA assays. In general, these assays produce fewer indeterminate results as compared to WB, but are equally expensive.

Rapid tests

Today, a number of rapid HIV tests are available, also referred to as rapid/simple (R/S) test devices. These tests are based on one of four immunodiagnostic principles: particle agglutination, immunodot (dipstick), immunofiltration and immune chromatography [41, 42]. In most cases whole blood or capillary blood (obtained from a finger tip) can be used, thus avoiding the centrifugation of a venous blood sample obtained through venipuncture, and test results are normally available within fifteen to thirty minutes. Immunoglobulins may also be eluted from blood spots blotted onto filter paper and dried [43]. Such dried blood spots can be used for the unlinked anonymous screening and in developing countries with insufficient cold storage and transport facilities. Once completely dry, blood from HIV-infected patients does not constitute a relevant infectious risk and the dried blood is stable over long time periods. Urine or oral fluid (*i.e.* "saliva") may also be employed for some assays [44, 45]. Many rapid tests contain a "built-in" internal control, *e.g.* a control band indicating whether the samples and reagents have been added correctly.

At the present, many rapid tests are based on the principles of the second or third generation EIA with antigens from both HIV-1 and HIV-2, and very few are structured as fourth generation tests.

Rapid tests can present some problems of sensitivity. It has been recently reported that, in South Africa, a significant proportion of HIV-infected children have been tested as false-negative using rapid tests [45]. Problems can arise also when these tests are used in a context different from the laboratory. A recent study performed on pregnant women in South Africa has shown that three routinely used rapid HIV tests, which performed well under laboratory conditions, did not show the same performance when used in a clinical setting, giving results of sensitivity as low as 90.2% [47]. Finally, since the negative predictive value of these tests is lower when compared to EIA, special precautions must be taken when using these tests in a population with high HIV prevalence and incidence. In a recent study in Seattle a rapid test used in the population of msm (men who have sex with men) with acute or recent infection, recognised as HIV-positive only 91% of the samples tested as positive in first or second generation EIA, indicating that this test is less sensitive than enzyme immunoassays during early HIV infection [48].

Occurrence of false positive tests by using rapid tests has also been reported, in particular in countries with high HIV prevalence of HIV infection. During 2003-2004, rapid tests were included as part of an algorithm to initially screen seronegative male adult volunteers in two randomised trials of circumcision for the prevention of HIV in a rural population in Rakai district of south western Uganda. The 94.1% of samples with weak positive results were negative or indeterminate on confirmation with enzyme immunoassay or western blotting [49]. In a recent study on more than 6000 volunteers during voluntary counselling and testing in Eastern Africa, 24 results were recorded as "weak positive" in rapid tests. However, only 2 of these results were confirmed by conventional EIA [50].

Therefore, given the problems of both sensitivity and specificity that can arise using HIV rapid testing, experts urge that all results with rapid tests have still to be confirmed with a conventional EIA and WB.

The use of rapid tests has been authorised in some countries, such as USA. They may be useful if the result is needed quickly, for instance in emergency

rooms, before emergency operations, after needlestick injuries and to minimize the rate of "unclaimed" test results (if the result is only available after a few days, some of those tested will not return to receive it). Rapid tests, which are easy to perform and require little in terms of equipment, can be useful in developing countries [51], although their poor negative predictive value can create problems when used in populations with high HIV incidence and prevalence. In developed countries, a rapid test should only be used as first guidance, and the patient retested as soon as possible in a regular routine laboratory.

Finally, it must be stressed out that the potentiality of a misuse of HIV rapid tests is a serious problem to date. In some contexts, these tests can be made by inexperienced personnel that can also provide a wrong diagnosis (for both positive and negative results). In addition, since some rapid tests are sold through the internet circuit, a home- and self-made testing can result in little or no assistance when reading the results, thus often misunderstanding the significance of a positive or a negative reaction.

In conclusion, rapid tests can be used in the future for the diagnosis of HIV infection, in both developing and developed countries, but still have to improve in sensitivity and specificity. New formulations are going to this direction and it can be expected that improved rapid tests be part of new diagnostic algorithms, in the future.

HIV antigen diagnosis

An HIV infection may also be diagnosed through the detection of the virus components, rather than virus-specific antibodies. However, HIV antigen is detectable in plasma only intermittently during the asymptomatic period. The detection of viral nucleic acid may be achieved by different laboratory techniques that can determine either proviral cDNA in leukocytes, or viral RNA in the cell-free compartment. The nucleic acid tests (NAT) are commercial tests that can identify HIV nucleic acid (either RNA or proviral DNA). These tests are based on polymerase chain reaction (PCR), branched DNA (b-DNA), nucleic acid sequence-based amplification (NASBA), ligase chain reaction (LCR), or real-time PCR. Most of these assays are generally demanding in terms of costs, laboratory equipment, and staff skills and require stringent quality control. As a consequence, these tests still represent a technical challenge for many laboratories.

NAT can supplement antibody testing for the diagnosis of HIV infection in special situations, such as in suspected acute infection, when antibodies are still undetectable (*Figure 1*), and in newborns of HIV-infected mother, in whom maternal antibodies are still present. The quantitative detection of HIV RNA in plasma ("viral load" testing) is used as a prognostic marker, to monitor antiretroviral therapy and to estimate infectiousness [52]. Today, "ultra"-sensitive tests that detect as few as 50 RNA copies per cubic millimetre of plasma are commercially available. Viral load testing is, therefore, an indispensable clinical tool. Unfortunately, due to the need of skilled personnel and of expensive dedicated instrumentation, as well as the lack of the necessary organisation to monitor HIV-positive ART-treated individuals, many developing countries cannot routinely use the currently available NAT assays [53, 54].

The risk of HIV transmission through blood transfusion has decreased enormously after highly sensitive fourth generation screening tests have been introduced. However, to further reduce the window period, several countries now stipulate HIV NAT in addition to using antibody testing, in screening of seronegative patients in high-risk groups and in certain situations, such as suspected primary infection and in testing babies born to HIV-infected mothers. In fact, it has been seen that the risk of acquiring HIV through transfusion is reduced by approximately 50% using NAT [55].

Theoretically, HIV RNA is present in the blood throughout the disease, from seroconversion to AIDS. However, its level is often not as high, lowering to under 400 copies/ml, in particular during the asymptomatic period. A study has demonstrated that in a panel of 35 samples from HIV-positive individuals with low viral load (< 400 RNA copies/ml) NAT failed to detect 4 of these samples [56]. However, today more sensitive tests that are able to detect HIV RNA at almost all times during the disease course, are being introduced.

Finally, the problem of the increasing HIV variability can have a serious impact on NAT sensitivity. NAT screening in geographic regions where multiple subtypes and CRFs (circulating recombinant forms) are present, can result in a failure to detect infection, since primers and probes used can be not the right ones to amplify nucleic acid of some HIV variants. Similar problems with the use of NAT can arise in countries where HIV-2 infection is endemic. All these reasons, together with the still complicated procedure for testing, drastically limit the use of HIV RNA testing in the diagnosis of HIV infection.

Amplification of proviral DNA allows detection of cells that harbour quiescent provirus as well as cells with actively replicating virus. PCR-DNA is particularly useful for the diagnosis of HIV infection in infants and young children (up to 18 months of age). In fact, routine serologic testing of these infants and young children is generally only informative before 18 months due to the presence of HIV-antibodies transmitted by the HIV-positive mother. Virological assays, especially HIV-1 NATs, such as HIV-1 DNA PCR assays, represent the gold standard for diagnostic testing of infants and children younger than 18 months. With such testing, the diagnosis of HIV-1 infection (as well as the presumptive exclusion of HIV-1 infection) can be established within the first

several weeks of life among nonbreastfed infants. The sensitivity and specificity of HIV-1 DNA assays for the diagnosis of HIV-1 infection in infants and young children have been evaluated in several studies with estimates as high as 90% to 100% by 1 month of age [57, 58].

Assays for the detection of recent HIV infections

Identification of recent HIV infections is a formidable tool for evaluating the current pattern of HIV transmission in a community or population. To this aim there are a number of assays that have been recently developed. These assays take in consideration the typically rapid immunological events that take place soon after HIV infection. In fact, the conventional assays for detection of antibodies and/or antigen/nucleic acid, above described, are not able to distinguish a recent from a chronic infection, once production of HIV antibodies has started. New methods have therefore been developed, which are able to discriminate recent and chronic infection once the presence of HIV antibodies has been confirmed. These methods are grouped together under the term of STARHS (serological testing algorithm for recent HIV seroconversion). Each STARHS approach is applied on already confirmed HIV-positive specimens.

A number of antibody-based assays have been developed, which permit to discriminate recent from chronic infections. The "detuned" assay was the first one to be described. It is based on both the low avidity and low titres of HIV antibodies present at the early stages of the disease. As the disease proceeds, both the avidity and the titre of HIV antibodies increase. Therefore, by deliberately combining highly sensitive with less sensitive antibody tests, in particular, a sensitive commercial assay (Abbott HIV 3A11) and a customized, less-sensitive (LS or "detuned") version of this assay, it is possible to identify the specimen from a recently infected individual. In fact, the specimen should be reactive on the sensitive assay, but non-reactive on the LS assay [59, 60]. A similar approach has been performed using also the BioMerieux Vironostika HIV-1 assay (at present no more commercially available) [61].

The BED-capture enzyme immuno assay (CEIA) is a commercial product whose principle is based on the relative proportion of anti-HIV IgG antibodies on the total IgG antibodies present in a specific volume of serum. In the early infection, the proportion of HIV-specific antibodies on the total IgG is lower than in chronic infection, whereas the proportion of HIV-specific IgG antibodies increases during the first two years from infection. BED-CEIA assay utilises a synthetic oligopeptide derived from the immunodominant region of the transmembrane gp41 protein of HIV-1 subtypes B, CRF_01AE and D. The use of this multi-subtype peptide permits to partially overcome the problem of HIV variability encountered with the detuned assay [62].

A further approach to identifying recent infections is to investigate the HIV-specific antibody avidity [63]. It is known that antibody avidity to HIV antigens increases over-time during the first year of infection. Thus, low avidity HIV antibodies indicate a recent infection. The avidity index (AI) assay takes advantage of this principle. It is based on the Abbott AxSYM commercial EIA, modified to avoid that low avidity antibodies bind to HIV antigens. For this reason the patient serum is pre-treated with a chaotropic agent, such as guanidine hydrochloride, that both disrupts the hydrogen bonds that maintain the secondary structure of the antibody and interferes with antigen-antibody interaction. Due to low molarity used for the chaotropic agent, the treatment of the serum with this agent, although mild, is very effective for low avidity early antibodies, determining the lack of binding of the antibody to the antigen. On the contrary, in later stages of infection, the increased HIV antibody avidity to antigen determines a higher resistance to the mild treatment with the chaotropic agent, resulting in the binding of the antibody to the antigen. In the test, the level of signal obtained after preincubation of the serum with guanidine is compared with the signal produced when the serum is pre-incubated with a neutral diluent (*i.e.* PBS). If the antibody has high avidity for the antigen, and therefore is resistant to the treatment with the chaotropic agent, the signals with the treated and the PBS-treated serum should be similar. On the other hand, the treatment with guanidine of a serum with a low avidity antibody for HIV antigens (*i.e.* early infection) determines a reduced antibody-antigen binding and, therefore, produces a lower signal when compared to the one obtained with the same serum not treated with guanidine [64, 65]. Recently, the assay has been validated on clade B HIV-1 virus, as well as viruses from non-B subtypes [66].

The IDE-V3 immunoassay is based on two conserved highly immunogenic epitopes of the Env glycoprotein; one is an immunodominant epitope in gp41 and the other one is a sequence in the V3 loop of gp120. IDE-V3 antigens are two gp41 epitopes, representing the consensus sequence of group M viruses and the consensus sequence of subtype D isolates, respectively, and a blend of five V3 oligopeptides from the HIV-1 subtypes A, B, C, D and CRF_01AE. Each specimen is tested against both the gp41 and the V3 antigens. The IDE-V3 assay is an indirect EIA in which each antigen is coated on the solid phase. The assay was developed and validated on panels of informative sera from seroconverters, patients at chronic stage without AIDS, and AIDS patients. Sera were divided into two groups, from individuals with infection ≤ 180 days old and with infection > 180 days old, respectively. Then, based on data of reactivity to these sera, a logistic regression model was used to determine the biomarkers threshold to best detect recent status (the best sensitivity with the best specificity).

Recent infections can be also identified on the basis of the presence of antibody isotypes. As previously described, HIV p24-specific IgG3 isotype is usually present transiently during the first few months of HIV infection [10, 11]. Therefore, an IgG3 anti-HIV EIA based on determination of IgG3 antibodies against HIV p24 has been established, although, at the present, the method has not been translated into a commercial kit.

Several other approaches aimed at identifying recent HIV infections have been developed, which include different principles, from particle agglutination to an oral fluid assay, but these procedures have not been applied in large scale and the recent HIV infection window periods have not been determined.

The accuracy of STARSH assays is affected by a number of factors. In particular the HIV variability is still a major obstacle for many of these assays, since immunodominant epitopes differ between the HIV-1 clades. Another limitation is encountered when sera from individuals with AIDS or very low CD4 counts are tested, since these patients have, usually, low antibody titres. Also the antiretroviral therapy has been associated to a misclassification of long standing infections as recent infections. It was suggested that ART can suppress HIV replication to a degree that stimulus to humoral immune response is strongly reduced, leading to a decline of antibody titres. These factors can influence assays at different degrees, since every assay is based on different principles. A detailed description of the limits and of the applicability fields of these assays goes beyond the purposes of this review.

CONCLUSIONS

During the past 25 years, HIV diagnostics have been essential in detecting and monitoring infection, and their use contributed to save countless numbers of lives. HIV testing technology has been continuously evolving in parallel with new discoveries on HIV/AIDS pathogenesis, and has been offering alternatives to address diagnosis of HIV infection and patient management. Conventional methods for HIV testing, based on detection of HIV-specific antibodies, have been improved over time, gaining in sensitivity and specificity. Kits that allow simultaneous determination of both HIV-specific antibodies and the p24 antigen have been recently introduced, thus reducing the diagnostic window. New rapid kits, which require very few amount of different body fluids and that can determine the presence of HIV antibodies in few minutes are being developed.

In parallel, continuous improvement of technology for determination of HIV nucleic acids has allowed the detection of very low copies of HIV nucleic acid in both plasma and cells, thus providing a formidable tool for diagnosis of HIV infection in very early stages of the disease and for the monitoring of HIV infected patients.

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