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Current and future assays for identifying recent HIV infections at the population level

Joanna Smoleń-Dzirba, Tomasz J. Wąsik

Department of Virology, School of Pharmacy and Division of Laboratory Medicine, Medical University of Silesia, Katowice, Poland

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Summary

The precise diagnosis of recent human immunodeficiency virus (HIV) infection is crucial for estimating HIV incidence, defined as the number of new infections in a population, per person at risk, during a specified time period. Incidence assessment is considered to be a tool for surveillance, public health and research. Differentiating recent from long-term HIV infections is possible thanks to the evaluation of HIV-specific immune response development or viral markers measurement. Several methods that enable the recognition of recent HIV-1 infection with the use of a single blood specimen have been developed, and their value for use in population level studies has been demonstrated. However, they are still inadequate due to a variable window period and false recent rates among HIV clades and across populations. Application of these assays at an individual level is far more questionable because of person-to-person variability in the antibody response and the course of HIV infection, and because of the prospective regulatory approval requirements.

In this article we review the principles and the limitations of the currently available major laboratory techniques that allow detection of recent HIV infection. The assays based on the alteration of serological parameters, as well as the newest method based on an increase of HIV genetic diversity with the progress of infection, are described.

key words: **recent HIV infection • Serological Testing Algorithm for Recent HIV Seroconversion (STARHS) • Recent Infection Testing Algorithm (RITA) • surveillance • incidence**

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Author's address: Joanna Smoleń-Dzirba, Department of Virology, Medical University of Silesia in Katowice, Narcyzów 1 Str., 41-205 Sosnowiec, Poland, e-mail: jsmolen@sum.edu.pl

Background

In older studies, the number of new HIV diagnoses was used as an indicator of the epidemic's development and the marker of incidence [1,2]. However, available standard HIV diagnostic tests are unable to distinguish between recent and established HIV infections, and for this reason an observed increase or decrease in the number of patients with newly detected HIV infection does not necessarily reflect the real trends in viral transmission, but rather may represent changing patterns of HIV testing in the population. In fact, an unknown proportion of newly diagnosed individuals may be HIV-infected for years, although their infection was not recognized.

In order to obtain consistent incidence estimates, there is a great, global need for reliable assays permitting identification of specimens that have been collected from individuals recently infected with HIV within a defined period after transmission or seroconversion [3]. However, accurate diagnosis of recent HIV infection still poses a challenge for clinicians and laboratory workers, and the difficulties with determination of the infection date impede not only the incidence evaluation and monitoring of HIV transmission patterns, but also hamper proper patient management [4,5].

Clinical manifestation of HIV infection does not allow for the recognition of the exact time of infection, since primary HIV infection may be asymptomatic or the non-specific symptoms may go unrecognized [6,7]. Conventional techniques for the identification of recent seroconverters, such as a prospective follow-up of a cohort of HIV-negative individuals and assessing the seroconversion time as the median time between last negative and first positive result of the HIV diagnostic tests, are expensive, long-term, difficult to conduct, and are not representative for all at-risk populations. Such prospective cohort studies may be unrepresentative because they are frequently conducted in young cohorts. A significant loss to follow-up during the study period (e.g., caused by migration of participants) also may contribute to biased results. Additionally, being in the study may lead to behavior changes called the "Hawthorne effect" that results in a lower rate of HIV incidence in this population than in the general population [3,5,8–10].

Viral markers of HIV infection such as viral RNA and p24 antigen can be detected earlier than HIV-specific antibodies [11]. For this reason, a 2-step laboratory algorithm may be adopted for the detection of early HIV infection. The starting point is standard diagnostic HIV antibody testing. In the next step, samples that tested negative for the HIV-specific antibodies may be subjected to testing for presence of HIV RNA [12] or p24 antigen [13]. The presence of viral RNA or p24 antigen in the absence of HIV-specific antibodies indicates acute or primary HIV infection. However, the disadvantage of this approach is the short time of detectable viremia and antigenemia prior to seroconversion, as well as the requirement for large sample sizes to detect enough individuals during this short period of time [14]. Consequently, the value of these methods in incidence estimations is limited.

As an alternative, another 2-step algorithm has been developed to detect recently HIV-infected patients, based mainly on the evaluation of HIV-specific immune response development or viral markers measurement among individuals who were found to be seropositive by a standard diagnostic assay (Table 1). Thus far, several laboratory techniques that measure HIV-specific antibody titer [15–18], proportion [19], avidity [20], or isotype [21] have been proposed and evaluated. These methods are collectively known as the Serological Testing Algorithm for Recent HIV Seroconversion (STARHS) assays. Currently, the term STARHS is being replaced with a new acronym – RITA – which stands for Recent Infection Testing Algorithm [5]. This new term was introduced to represent more laboratory methods for the recognition of recent HIV infection, not necessarily based on serological markers. In fact, a new technique based on viral markers among HIV-positive individuals has recently been presented [22].

The specificity of RITA assay may be defined as the ability to classify a patient with a long-duration HIV infection as having a non-recent infection (being RITA-nonreactive or RITA-negative). It can be expressed as a proportion of non-recent infections detected as non-recent. Low assay specificity results in a high number of false positives, which means that patients with long-standing HIV infection are misclassified as being recently infected. The fraction of non-recent infections falsely classified as recent is known as the false recent rate. In turn, RITA assay sensitivity may be described as the ability to classify a patient with short-duration HIV infection as having a recent infection (being RITA-reactive or RITA-positive). In other words, it is a proportion of recent infections detected as recent. Consequently, low assay sensitivity results in a high number of false negatives, which means that patients with recent HIV infection are misdiagnosed as having long-standing infection [5,17,23].

In the RITA methods it is important to determine the so-called assay recency window period. This window period is defined as the mean interval between the earliest time at which HIV diagnosis can be made by a standard HIV diagnostic test, and conversion from recent to long-standing status in the RITA assay denoted by the RITA assay's cut-off value [14,24]. The recency window period typically spans several months and is established in advance during the RITA assay calibration process. This calibration requires sets of serial specimens gathered from HIV-infected patients with known or closely approximated dates of seroconversion. For each specimen from such panels, the RITA test is performed and the quantitative test result (e.g. optical density) is related to the time since seroconversion. On this basis, an assay's cut-off value (threshold) at which the test should be read as RITA-positive (recent infection) or RITA-negative (non-recent infection) is selected. Concomitantly, the recency window period (denoting the mean time period from seroconversion, during which the assay result will stay positive) is established. Together, the cut-off value and the mean window period of RITA assay should assure the best assay specificity and sensitivity. A well-defined recency window period is critical for RITA assay-based incidence calculations; however, this period may be different among infected subjects because of the individual variability in antibody titer, maturation, and rate of antibody production [5,24]. Since the recency window period is a term that can be confused with the diagnostic pre-seroconversion window period used in the commercially available assays for HIV diagnosis, the term "mean RITA-duration", denoting the mean duration of the "state of recency", has been proposed [5].

Table 1. Methods applied to discriminate between recent and long-standing HIV infections.

Assay	Basis & interpretation & assay type	Technique*	Test	Reference
	Serological assays			
Detuned	Anti-HIV **antibody titer** is rising gradually after seroconversion.	First generation EIA	Abbott HIV-1 3A11, Abbott Laboratories**	[15]
			Vironostika HIV-1 Microelisa, bioMerieux**	[30]
			Avioq HIV-1 Microelisa system, Avioq Inc.	
	Results of a standard HIV diagnostic assay with a **reduced sensitivity** are below the specified cut-off value for recently HIV-infected individuals, and above this level for long-term infected subjects.	Rapid immunoassays	Determine HIV-1/2 assay, Abbott Laboratories	[32,33]
			OraQuick Advance HIV-1/2 assay, OraSure Technologies, Inc.	[33]
	Modified commercial assay		Uni-Gold Recombigen, Trinity Biotech	[34]
		Particle agglutination	HIV-1/2 particle agglutination test, Serodia	[35]
Avidity index	Anti-HIV **antibody avidity** is increasing gradually after seroconversion. Sample diluted **with a dissociative agent** and separately with a neutral substance is subjected to the standard HIV diagnostic test, and the avidity index (AI) is calculated. AI is below the specified cut-off in samples with low-avidity early antibodies from recently HIV-infected individuals, and above this level in samples with high-avidity mature antibodies from long-term infected subjects. Modified commercial assay	Third generation EIA	HIV-1/2 gO EIA for the AxSYM analyzer, Abbott Diagnosics	[20,41]
			Anti-HIV-1/2 Vitros ECi assay, Ortho-Clinical Diagnostics	[43]
			Genetic Systems HIV-1/2 Peptide EIA, BioRad Laboratories	[38]
BED EIA	Proportion of HIV specific IgG to total IgG is rising gradually after seroconversion. Results of a **quantitative IgG-capture enzyme immunoassay** are below the specified cut-off value for recently HIV-infected individuals, and above this level for long-term infected subjects. Commercial assay	Capture EIA	BED EIA HIV-1 Incidence Test, Calypte Biomedical Corporation	[19]
IDE-V3	Antibody response to the HIV-1 gp41 immunodominant epitope (IDE) and gp120-V3 loop is increasing after seroconversion***. Probability of a non-recent HIV infection is calculated from the reactivity of the specimen with the IDE and V3 in the enzyme immunoassay. In-house assay	EIA	IDE-V3 assay	[17]
Anti-p24 IgG3	IgG3 isotype antibodies are present early in the infection and are not detectable after about 4 months post-infection. Results of an **isotype-specific enzyme immunoassay or Luminex-based assay** are above the specified cut-off value for recently HIV-infected individuals, and below this level for long-term infected subjects. In-house assays	EIA	Anti-p24 IgG3 assay	[21]
		Luminex-based assay	Bio-Plex System, BioRad Laboratories	[61]
INNO-LIA	Anti-HIV **antibody titer** is rising gradually after seroconversion, and different anti-HIV antibodies emerge at different time post-seroconversion. HIV recency information is extracted from the Western Blot confirmatory test by applying the modified interpretation algorithms taking into account intensity of the antibody-antigen band and the specific banding pattern. Commercial assay, modified interpretation algorithms	Western Blot	INNO-LIA HIV-1/2 Score, Innogenetics	[18]

Table 1 continued. Methods applied to discriminate between recent and long-standing HIV infections.

Assay	Basis & interpretation & assay type	Technique*	Test	Reference
	Viral marker-based assay			
High-resolution melting	HIV genetic diversity is rising with the progress of infection. Viral genes are amplified and subjected to the LightScaner analysis to receive high-resolution melting scores. Low melting scores are associated with low viral genetic diversity and indicate recent HIV infection, whereas high melting scores are associated with high viral genetic diversity related to chronic HIV infection. In-house assay			[22]

* EIA – enzyme immunoassay; ** assays currently not produced or produced under a new name by another manufacturer; *** gp – glycoprotein.

The purpose of our review is to describe currently available assays for diagnosis of recent HIV infection. We also discuss the relevance, limitations and the main factors that are affecting the results of these assays, and provide insight into some new techniques and algorithms which are under development.

Applications of the Assays for Recent HIV Infection Determination

Development of reliable assays that allow us to distinguish persons with recent HIV infections from persons with long-standing infections is important for a number of reasons. Initially, such tests were designed for HIV incidence estimations [15]. Knowing the number of newly infected individuals in a defined time period enables more accurate description of the HIV epidemic, and helps with identifying current trends in HIV transmission. The ability to recognize recent HIV infection may permit more precise identification of populations at greatest risk for HIV infection, allowing intervention in those populations with targeted prevention programs in order to reduce HIV transmission, and enabling public health agencies to more efficiently use their resources. Assays for determination of recent HIV infection should also give the opportunity to monitor the effectiveness of prevention strategies. Additional uses include estimating incidence for planning and powering clinical trials and also serving as an end-point in community-based prevention trials [3,8,10,25,26].

Another advantage of the recent HIV infection detection system is associated with clinical management, and offers the option of considering early antiretroviral treatment, since the early stage of infection may be the most beneficial time to initiate HIV care services [4,27]. Patients diagnosed as recently HIV-infected may also be recruited for research on early intervention drugs or for therapeutic vaccine trials [25].

Moreover, these assays may enable precise characterization of the recently transmitted viruses, for example, for their genotype and drug resistance profiles [3,25,28].

Features of an Ideal Assay for Detection of Recent HIV infection

The most desirable features of an ideal assay that is able to distinguish between recent and long-standing HIV infection have been specified by the Eurosurveillance Editorial team [3,5,29]. The list includes:

- a well-defined, preferably long, window period,
- consistent discrimination between recent and long-term HIV infections,
- accurate results for different assay cut-off values,
- results' independence of factors such as: viral subtype, opportunistic infections, mode of HIV transmission and patient characteristics (sex, age, race/ethnicity, therapy status),
- possibility of standardization and control of the assay,
- convenient usage (low cost and equipment requirements, ease of handling and storage, no need for an assay calibrator, suitability for small volumes and different types of samples, easily interpretable results, long-term availability of the assay).

Unfortunately, at present no single assay fulfills all points of the desired characteristics.

Serological Assays for Detection of Recent HIV Infection

The assays described below detect recent HIV infection by testing a single HIV seropositive specimen, and are based on the quantitative or qualitative differences in anti-HIV antibodies between recent and long-standing HIV infections. They allow for the retrospective testing of stored samples.

Detuned assay

The first method of identifying recent HIV infection was developed and described in 1998 by Janssen et al [15]. This method, the detuned assay, was originally termed "sensitive/less sensitive testing strategy" and it was designed for the purpose of HIV incidence estimation.

The detuned approach is based on the observation that anti-HIV antibody titer in the plasma is different in recent *vs.* non-recent infection, and in each infected individual the titer is generally rising gradually, at a similar rate during a period of several months after seroconversion. The assay procedure involves dual testing of the same sample with the same standard HIV diagnostic enzyme immunoassay. At first, a sensitive test is performed with a standard protocol, as recommended by the manufacturer. Secondly, samples confirmed to be anti-HIV-positive are submitted to the same immunoassay, but with the modified testing procedure, in order to make it less sensitive (detuned). The assay modification consists of increased sample dilution and reduced incubation times. Specimens that are anti-HIV-positive according to a sensitive test, and non-reactive (tested below the

specified cut-off) on a less sensitive assay, are considered to be derived from recently HIV-infected persons. Individuals with long-standing infection and related high titer antibodies will remain reactive in the less sensitive version of the assay [15,16]. It should be remembered that the high sample dilution required in the detuned approach affects the accuracy of the assay results, and the highest precision here is essential. To reduce run-to-run variability of the detuned assay, the standardized optical density (SOD) is calculated for each specimen with the use of a calibrator [30].

Initially, the detuned approach was developed for use with a first generation Abbott HIV-1 enzyme immunoassay (3A11 assay, Abbott Laboratories), which is currently not being produced. Another first generation immunoassay commonly used in a detuned approach was the Vironostika HIV-1 MicroElisa (bioMerieux, France) [30]. Although the production of this assay had been ceased by bioMerieux, it is still available under a new name – the Avioq HIV-1 MicroElisa system (Avioq Inc., USA). The same commercial assay has been adapted for the detuned approach with the use of oral fluid, an alternative to the former serum-based testing strategies. Although it was shown that there is a good concordance between the oral fluid and serum-based sensitive/less sensitive assay, further large-scale studies on the oral fluid test are necessary to develop a highly accurate assay with known mean RITA duration, which would offer significant advantages over serum-based methods [31].

Newer, simpler rapid HIV diagnostic tests, such as the Determine HIV-1/2 assay (Abbott Laboratories, USA), OraQuick Advance HIV-1/2 assay (OraSure Technologies, Inc., USA) and Uni-Gold Recombigen (Trinity Biotech, Ireland), as well as the HIV-1/2 particle agglutination test (Serodia, USA) have also been modified to render them less sensitive, and their ability to distinguish recent from long-term HIV infection has been tested [32–35]. Results were comparable to those obtained with the Vironostika HIV-1 MicroElisa detuned assay; nevertheless, additional investigation is still required to standardize rapid tests for the detection of recent HIV infection. After full calibration, validation and evaluation in cross-sectional settings, these tests could likely facilitate incidence estimations, as they do not require sophisticated laboratory instrumentation and infrastructure, and provide results in minutes.

A common concern of the detuned assays based on modified commercial tests is their subtype B-dependent performance, which can limit their use in parts of the world in which other HIV clades predominate. Since HIV-1 clades differ in terms of immunodominant epitopes, patients infected with non-B viruses may produce antibodies of reduced binding affinities to the subtype B antigens employed in the detuned assays, leading to false diagnosis of recent infection [36].

Depending on a cut-off value applied to the detuned assay, the period during which recent HIV infection can be recognized may change. With the recommended assays' cut-off values, mean time from seroconversion, during which recent infection is identified in the less sensitive approach, was estimated to be 129 days for the Abbott 3A11 assay [15], and 170 days for the former Vironostika HIV-1 MicroElisa [16]. These window periods' durations were determined based on studies with HIV-1 subtype B seroconversion panels. The use of the same cut-off values may result in significantly longer mean window periods for HIV-1 genetic variants other than B (e.g., 356 days for the recombinant form CRF_01AE [36], and 360 days for subtype C [37]), which indicates that appropriate cut-off values should be applied for different HIV-1 subtypes.

Detuned assay performance may lead to misclassification of patients with long-standing asymptomatic HIV infection as being recently infected. This concerns subjects receiving antiretroviral drugs, indicating that therapy-induced suppression of viremia can lead to decreased seroreactivity, and consequently to false-positive results in the sensitive/less sensitive testing strategy. Similarly, the results of the detuned assay can also be affected in a subset of HIV-infected patients, called elite controllers, who are able to maintain the virus undetectable without antiretroviral treatment [38]. Moreover, such misclassifications have been observed for patients with advanced AIDS who have decreased antibody levels because of strong immunosuppression [16].

While the detuned approach is based on modification of the commercial assays, its next limitation is a difficulty with the assurance of long-term availability of the commercial assays, as well as the calibrators and quality control material [39,40].

Avidity index assay

Another approach to identifying recent HIV infections exploits the increasing antibody avidity during maturation of the immune response in the first year of infection [20,41]; therefore, low avidity of HIV-specific antibodies indicates recent infection.

Avidity, which refers to the overall strength of antibody-antigen binding, can be investigated by treating the specimen with the dissociative agent and subsequently subjecting it to the commercially available standard HIV diagnostic assay. Dissociative (chaotropic) agents, such as guanidine hydrochloride [20], potassium thiocyanate [42], diethylamine [38], urea [43], or low pH [44], disrupt the hydrogen bonds that maintain the higher levels of the antibody structure, and interfere with antibody-antigen interaction. The effect of the chaotropic treatment is visible for the early antibodies presenting low avidity, whereas mature antibodies of higher avidity are resistant to the mild chaotropic agents and remain bound to the antigen.

In the avidity method, each specimen is divided into 2 aliquots: 1 is diluted with the chaotropic agent, and the other with the neutral substance (e.g., phosphate-buffered saline or wash solution). After performing the standard diagnostic assay, the avidity index (AI) is calculated as a ratio of the signal obtained for the aliquot treated with the dissociative agent to the signal for the aliquot diluted with the neutral substance. Low-avidity early antibodies treated with the chaotropic agent present reduced binding abilities to the antigens, and therefore the signal produced in the diagnostic assay is low when compared to the one obtained with the same sample not treated with the chaotrope. On the other hand, high-avidity mature antibodies become resistant to the treatment with the chaotropic agent, and remain complexed with the antigens, resulting in similar signals in both aliquots, diluted with the dissociative agent, and with the

neutral substance (AI≈1.0). The levels of the specificity and the sensitivity of the avidity assay depend on the selected cut-off value – the higher the cut-off value applied to the assay, the higher the assay sensitivity and the lower the observed specificity [20,41]. Since the avidity assays are not reliant on the antibody titer, they should be less influenced by antiretroviral treatment, unless a treatment is initiated during primary HIV infection, before full-affinity maturation develops [20,40,45]. Effective viral suppression during chronic infection, achieved either through medication or naturally (e.g., in elite controllers) seems not to influence the avidity assays in the way it affects antibody titer-based detuned assays [38]. However, AI values appear to decline in patients with AIDS-defining conditions, leading to misclassification of individuals with advanced disease stage as being recently infected [43]. Among the disadvantages of the AI assay is the fact that an established quality control program is not available.

One of the most popular avidity-based assays used to distinguish recent from long-standing HIV infections is the test described by Suligoi et al. [20], which is a modification of the third-generation commercial HIV-1/2 gO EIA for the Abbott AxSYM analyzer, with guanidine hydrochloride as a chaotropic reagent. With the 0.80 and 0.85 AI cut-off values, the window period of the assay was estimated to be 180 days. Although initially studies with the avidity assay were conducted on subtype B HIV-1-positive samples, it was also shown that this test had been fairly accurate in individuals infected with non-B subtypes [46]. A second guanidine-based assay has been evaluated using the fully automated anti-HIV-1/2 Vitros ECi assay (Ortho-Clinical Diagnostics), in which the AI cut-off values of 0.75 and 0.80 predicted seroconversion within the previous 125 and 142 days, respectively. It was also shown that maturation of the avidity in patients infected with a non-B HIV-1 genetic variants followed similar kinetics to those observed among patients with subtype B infection, thus, the assay performance does not appear to be affected by the infecting subtype [43]. Another avidity index assay uses the Genetic Systems HIV-1/2 Peptide EIA from BioRad Laboratories, modified by the incorporation of a dissociation step with diethylamine [38].

As with the detuned techniques, usage of the avidity index assays depends on the availability of the commercial tests, which may not be accessible in the near future [39].

BED EIA HIV-1 tncidence test

The commercially available BED EIA HIV-1 Incidence Test is a second-generation assay specially designed to discriminate between recent and long-standing HIV-1 infection. It is a quantitative IgG-capture enzyme immunoassay that identifies recent HIV-1 seroconversion by determining the relative ratio of HIV-1 specific IgG to total IgG in the sample. Generally, this ratio is lower in early seroconverters than in individuals with long-term infection, and the proportion of HIV-1 specific IgG to total IgG increases during the first 2 years of infection. In addition, to simplify specimen collection, transport and storage, and to extend the usage of BED assay, a procedure for blood, plasma or serum spots dried on filter paper has been developed [47].

In the assay procedure, all IgG present in the sample (anti-HIV IgG and non-anti-HIV IgG) are captured by goat anti-human IgG antibodies which are covering the wells of a microplate. In the next step, a biotinylated, trimerically branched, synthetic peptide binds to the captured anti-HIV IgG. This peptide is called BED, since its branches include the immunodominant region of the HIV-1 transmembrane glycoprotein gp41 of subtype B, recombinant form CRF_01AE (formerly subtype E), and subtype D, which facilitates detection of HIV-specific antibodies among various HIV genetic subtypes [19,47]. After following 2 incubation steps, with enzyme conjugate and with substrate, the OD values are read and the normalized results are expressed by reference to a calibrator as a ratio specimen OD/calibrator OD, to reduce inter-run variation. Apart from the calibrator, the BED assay is equipped with a quality control program which includes negative control, as well as low and high positive control specimens. Evaluation studies indicate that the cut-off value of 0.8 corresponds to a mean seroconversion duration of 155 days [47].

Since the BED assay does not directly measure the antibody titer, but rather the proportion of HIV-1 specific IgG to total IgG, its accuracy is less affected by the sample dilution in comparison with the detuned method, and a high level of inter- and intra-user reproducibility has been demonstrated for this assay [47,48]; however, the BED assay misclassifies a significant proportion of individuals as being recently infected [49]. People with AIDS, subjects with late-stage HIV infection and low T $CD4^+$ cell count, as well as people with low viral load, elite controllers, and antiretroviral therapy users are especially problematic [50–53]. Additionally, chronic co-infections with hypergammaglobulinemia and inflammation may lead to false-recent BED assay results [3,5,54].

The use of the multi-subtype BED peptide partially overcomes the problem of HIV-1 variability, and in theory it should allow for the application of the same window period irrespective of the infecting HIV-1 subtype. However, it has been shown that, in the BED assay, differences in window periods between subtypes exist, with 187 days for clade C [55] and 115 days for CRF_01AE [56].

Because BED-derived incidence estimations were shown to be overestimated due to the high false recent rate observed in the BED assay, adjustment factors have been developed, and are recommended to compensate for the misclassification of long-term infections, and to enhance the accuracy of the assay-based incidence estimations [55]. It appears that more accurate measures of incidence may be obtained if adjustment factors are derived and validated locally for each population of interest [57].

IDE-V3 assay

The gradual increase in the titer of HIV-1-specific antibodies during the course of infection was further exploited in the IDE-V3 assay to distinguish between recent and long-term HIV-1 infection [17]. In this indirect EIA format assay, a dilution of each specimen is tested twice on the same 96-well microplate. Antibodies against the HIV-1 immunodominant epitope (IDE) of transmembrane glycoprotein gp41 and the V3 domain of the glycoprotein gp120 are detected separately. Thus, some wells in the microplate are coated with the IDE antigen, which is an equimolar mixture of 2 synthetic oligopeptides representing the consensus of

all HIV-1 group M clades, and the consensus of subtype D only, since it is the most antigenically divergent. Other wells are coated with the V3 antigen comprising an equimolar mixture of 5 oligopeptides representing the consensus sequences of HIV-1 subtypes A, B, C, D, and a recombinant form CRF_01 AE. Since the antibodies against the IDE region of gp41 develop more rapidly than antibodies against V3, the concentration of each IDE peptide is 4 times lower than the concentration of each of the V3 components [17].

The reactivity of the specimens with the IDE and V3 oligopeptides is higher for sera from chronically infected persons (>180 days post-infection) than for sera from recently infected persons (infected within the last 180 days). After performing the IDE-V3 assay, reactivity data for each specimen are subjected to the mathematical (logistic regression) formula which gives a result as a probability (*P*) of being classified as a long-term infected individual. When *P* is >0.5 the sample is classified as being obtained from a patient with a long-standing infection, while when P is ⩽0.5 the sample is classified as being derived from a recent seroconverter [17].

The quantitative detection of antibodies directed against both the IDE and the V3 antigens was introduced to enhance the discriminatory power of the assay. Nevertheless, it was demonstrated that the IDE-V3 assay is not applicable to sera from individuals with AIDS or from patients receiving antiretroviral treatment soon after primary infection, because of frequent misdiagnoses among them [17].

Recently, the assay has been calibrated for the purpose of incidence estimation, and the mean window period was determined to be 180 (167–193) days; however, the duration of the window period may differ between populations infected with various HIV genetic variants [58]. Although the test was designed to overcome the difficulties related to HIV diversity by inclusion of the IDE and V3 oligopeptides of various clades, validation studies with specimens from subjects infected with non-B subtypes are still needed [17,59].

The IDE-V3 assay is not a commercial product, and it can be self-made from commercially available basic components. It was specifically developed to assure the continuous availability of the test for detecting recent HIV infection, independent of any commercial source. The IDE-V3 has been used for surveillance of recent HIV infections and incidence estimations in France since 2003 [58,60].

Anti-p24 IgG3 assay

Another in-house EIA-format assay allowing for the recognition of recent HIV infection is based on the presence of specific IgG antibody isotypes. The assay detects specific anti-p24 antibodies of the IgG3 isotype. It was shown that these antibodies emerge early in the HIV-1 infection, and are not detectable after about 4 months post-infection, which makes them a potential marker of recent HIV infection [21]. An arbitrary cut-off value of 0.5 absorbance units in the anti-p24 IgG3 assay corresponds to the estimated window of 34–120 days following infection [21].

Initially, the assay was developed using 17 seroconversion panels obtained from therapy-naive subtype B infected individuals, therefore the impact of the antiretroviral treatment and subtype variation on the assay results is unknown. There was also no data on the assay's performance in patients with late HIV infection and AIDS [21]. Recently, the Luminex technology (Bio-Plex System) was applied to test the IgG3 reactivity for the purpose of recent HIV infection identification [61]. In this technique, the microbeads coated with the HIV-1 antigens (p24 and p66) were incubated with patients' plasma. Specific IgG3 from plasma were detected with the use of phycoerythrin-labeled anti-human IgG3 antibodies. In the evaluation studies, anti-p24 IgG3 reactivity decreased dramatically after 50 days post-seroconversion, while anti-p66 IgG3 reactivity began to decline after 100 days. Nevertheless, the optimal cut-off value for the assay still must be selected, and an estimation of the window period is needed. Since this study included an adequate number of patients receiving antiretroviral therapy, the lower risk of false recent results caused by treatment has been demonstrated. However, misclassifications of long-term non-progressors and individuals with AIDS were observed. Although the assay seemed to perform similarly in subjects infected with subtype B and A/G, further evaluation with multiple HIV subtypes is required.

INNO-LIA HIV-1/2 Score assay

Knowledge that antibody titer rises after seroconversion, and antibodies directed against various viral proteins emerge at different times post-seroconversion, has been applied to detect recent HIV-1 infection using the INNO-LIA HIV-1/2 Score line immunoassay (Innogenetics). This test is the second-generation Western blot assay that measures the antibody reaction against recombinant proteins or synthetic peptides of HIV-1 (p17, p24, p31, gp41, and sgp120) and HIV-2 (gp36 and sgp105). It is originally used as a test for HIV diagnosis confirmation. The antibody reactivity in the INNO-LIA HIV-1/2 Score assay is classified into 1 of 6 possible scores (0, 0.5, 1, 2, 3, or 4) based on the intensity of each antibody-antigen band. Given that the antibody reactivity with the antigens measured by the assay evolves over time after infection, the assay results could provide information which allows discrimination between recent and long-term HIV infection. This information may be attained by applying the proper algorithms to the band intensity scores, which take into account the intensity of each antibody-antigen band and the emergence of specific banding patterns. Since there is no need to modify the assay performance procedure, it may simultaneously serve as both the confirmatory diagnostic test and the assay for recent HIV infection, thereby enabling detection of recent infections with no additional costs in countries that routinely use the INNO-LIA HIV-1/2 Score for confirmation of HIV diagnosis [18].

Estimated window periods for the INNO-LIA HIV-1/2 Score assay employed as a test for recent HIV infection ranged from 36 to 67 days, depending on the algorithm applied to the interpretation of the result. However, authors have stressed that this information was not based on a sufficient number of measurements and is thus unreliable. Such a short recency window period would be associated with the need to test large numbers of individuals in order to attain reliable HIV incidence estimation, since the assay would identify only very recent infections. The specificity of INNO-LIA-based algorithms in people with AIDS was shown to be

high, but the specificity of the method for individuals with advanced disease and those receiving antiretroviral therapy should be assessed. In addition, the influence of HIV subtypes on assay performance needs to be determined [18].

Methods Based on the Viral Markers

High-resolution melting assay for HIV diversity level recognition

A new approach to distinguish recent from established HIV infections has recently been proposed [22]. The basis for this new technique is the observed increase in HIV genetic diversity as the infection progresses from the acute to the chronic phase. Towler et al have developed a high-resolution melting assay for measuring HIV diversity without sequencing. They have amplified the HIV *gag* region and analyzed the amplicons with a LightScaner instrument to produce a melting curve (-d[fluorescence]/d[temperature]) and extracting a high-resolution melting score. The high-resolution melting score was defined as the temperature above which melting occurred, and it was shown that the melting score values differed between viruses derived from individuals with different stages of HIV infection. More precisely, low high-resolution melting scores were associated with the low HIV genetic diversity usually observed in subjects with acute or recent infection, while high melting scores were associated with high HIV genetic diversity related to chronic infection. The median high-resolution melting score for adults with recent HIV infection (4.2) was significantly lower than in subjects with chronic HIV infection (5.1). High-resolution melting scores of >6.3 were highly correlated with chronic HIV infection or AIDS. The main drawback of this promising method of detecting recent HIV infection is the high proportion of chronic infections and AIDS being misclassified as recent. However, authors suspect that the possible factor affecting the assay results was the usage of antiretroviral therapy by patients with chronic HIV infection and AIDS enrolled in the study. Thus far, there is no data on the influence of different HIV subtypes on the performance of the high-resolution melting assay [22].

Multi assay algorithms

The accuracy of RITA tests is affected by a number of factors. For instance, the sensitivity and specificity of the assays identifying recent HIV infections are, to differing extents, influenced by the individual variability of the immune response among HIV-infected subjects, immunosuppression in late-stage AIDS, and antiretroviral therapy usage [16,40,43,45,50,53]. It is also widely accepted that variation exists in the duration of the window period for different HIV-1 subtypes or populations [55,56,59]. The other obstacles that appear to complicate recent HIV infection testing include difficulties with the standardization of window period duration, assays calibration and use of external/internal quality control measures. A problem may also arise with assays that require modification of commercial products, since their availability is unpredictable and cannot be assured. High cost and elaborate performance procedures of some assays may further impede the use of RITA techniques [39].

Since currently there is no agreement which RITA assay is accurate and robust enough for the intended use, and no single test fulfills the required characteristics, the use of a combination of 2 or more assays has been suggested [34,51]. It was also proposed to apply algorithms including clinical status and information such as T CD4$^+$ cell count, and/or HIV RNA testing, and/or determination of antiretroviral use for the purpose of achieving the most accurate detection of recent HIV infection [3,5,38].

Consequently, it was proposed to combine the results of 2 RITA assays based on different biological principles (e.g., BED EIA and avidity index assay, or detuned and avidity index assay) to improve specificity and the overall positive predictive value of recent HIV infection testing [51,62].

The potential improvement of HIV incidence estimation could be also achieved by inclusion of the viral load level measurement at the time of recent infection assay performance, to eliminate persons with well-controlled viremia indicating long-term infection [63].

A more complex algorithm incorporating 2 different RITA assays together with viral load testing and determination of antiretroviral drug use was proposed by Laeyendecker et al. [38]. Here, the HIV-positive samples initially identified by the detuned immunoassay as being from recently infected individuals would be subjected to viral load testing. Next, samples with detectable viral load would undergo an avidity assay, which eventually determines if the sample is from a recently or chronically infected, but immune-compromised, subject. Samples from individuals tested as recently infected by the detuned assay, and with undetectable viral load, would be further tested for the presence of antiretroviral drugs. If the presence of antiretrovirals is confirmed, the specimen is considered to be from a person with viral suppression induced by medication; if antiretrovirals are not found, the sample may be from an elite suppressor [38].

Another suggested testing algorithm for detecting recent HIV infection is based on the assumption that preliminary T CD4$^+$ cell count in HIV-positive specimens should enable the initial recognition and exclusion of samples from immune-compromised people with AIDS [5]. The remaining samples would be tested with the BED EIA, and the ones that appear to be from recently infected individuals would be further submitted to the avidity index assay testing. Samples identified as positive in the avidity assay should then have viral load measured. Only the specimens with a detectable viral load could be considered as being derived from recently infected patients, while those with undetectable viremia should be further tested for the presence of antiretroviral drugs to identify samples from patients receiving antiretroviral treatment (antiretroviral drugs positive), or samples from potential elite suppressors or subjects with well-controlled viremia (antiretroviral drugs negative) [5].

The approach incorporating clinical data might be of special value in populations with a mature epidemic and those with wide access to antiretroviral therapy, allowing for improved accuracy of recent infection testing over a single method alone. Although algorithms that combine the results of more than 1 RITA assay and/or include clinical data seem to reduce the misclassification rate and improve overall predictive values of recent HIV infection testing, there is a lack of significant experience with these algorithms,

and thus far they have not been validated. Moreover, the impact of single components on the effectiveness of the algorithmic approach should be also evaluated [3,5,51,53]. It has been stressed that a standardized approach to development and validation of both new RITA assays and multi assay algorithms is essential to improve accuracy of recent HIV infection testing [23].

CONCLUSIONS

Assessment of HIV incidence in populations is a valuable surveillance tool that helps public health professionals target prevention campaigns directly to the populations at greatest risk of HIV transmission. In order to attain meaningful assay-based HIV incidence estimates in populations, more accurate assays for determining recent HIV infections are needed. The accuracy of existing RITA assays may be influenced by individual variability of the immune response, immunosuppression in individuals with AIDS, co-infections affecting serological profile, viral suppression maintained naturally or by antiretroviral treatment, or infecting virus subtype. These factors should be taken into consideration when using current assays or in developing novel assays for detection of recent HIV infection. Since currently there is no single RITA assay that meets the criteria of an ideal assay for identifying recent HIV infections, the most viable option for detecting recent infections may be the use of multi-assay algorithms instead of a single assay alone. Application of a standardized approach to development and validation of such multi-assay algorithms will increase the validity of subsequent HIV incidence estimates.

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