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MONITORING HIV EPIDEMIOLOGY USING ASSAYS FOR RECENT INFECTION: WHERE ARE WE?

F Barin¹, A Nardone (anthony.nardone@hpa.org.uk)²

1. Université François-Rabelais, Inserm ERI 19, Centre National de Référence du VIH, CHU Bretonneau, Tours, France

2. HIV and Sexually Transmitted Infections Department, Health Protection Agency Centre for Infections, London, United Kingdom

This issue of *Eurosurveillance* is dedicated to the widespread advances being made in Europe in the implementation of Serological Testing Algorithms for Recent HIV Seroconversion (STARHS). With the increasing interest in and the use of STARHS to estimate HIV incidence, we believe that the articles regarding the types of assays available [1], the implications for converting laboratory-based data into a real epidemiological measure of incidence [2] and the experiences from France [3], Germany [4] and Portugal [5] of incorporating STARHS methods into national HIV surveillance systems make this issue of *Eurosurveillance* opportune and of keen interest to a wide readership.

To date, the most important measure to monitor the HIV epidemic has been the reporting of newly diagnosed infections and national surveillance systems are now in place in nearly all European countries [6]. However, the major limitation of this measure is that it does not give an accurate picture of the evolving status of the epidemic as it comprises both people with recent infection and people with infection of several years' duration. In recent years this limitation has been brought into sharp relief in many European countries. Does the increase in many western European countries of diagnosed cases of HIV among men who have sex with men [6] represent a real increase in transmission or a reflection of a greater willingness to test for HIV? In countries such as Portugal, does the shift of newly diagnosed cases of HIV away from injecting drug users and towards those infected by sexual transmission [6] represent the true transmission dynamics of the epidemic? The anticipated benefit of STARHS is to provide answers to these questions by estimating HIV incidence, the number of new infections in a defined time period, and thus enable public health authorities better to target prevention campaigns and resources.

A decade ago, a new strategy based on a testing algorithm that combined two assays, one sensitive and one less sensitive, was proposed to identify a person in the period of early infection, when the antibody titre is increasing but before peak and persistently high antibody response [7]. This strategy requires the use of a commercially available enzyme immunoassay (the sensitive assay) and "detuning" it by increasing dilutions and decreasing incubation times (the less sensitive assay). A blood specimen from a person with early infection is reactive with the commercial assay, but non-reactive with the less sensitive detuned version. The detuned approach has been described using the Abbott HIVAB 3A11 and the BioMérieux Vironostika HIV-1 assay. Unfortunately, both assays

were of the early generation immunoassays for HIV antibody screening and, as neither corresponds to the high sensitivity that is demanded, production of both assays has now ceased.

Another approach to identify recent HIV infections is to quantify the avidity of antibodies by modification of third generation anti-HIV assays that run on random access analysers [8]. A similar methodology has been successfully applied to diagnose primary infection by rubella virus, cytomegalovirus or toxoplasmosis during pregnancy, in order to provide individual counselling [9]. Although it is not common medical practice, improving the detection of recent infection by combining STARHS results with clinical and laboratory data may have benefit for the patient, by providing an opportunity to discuss enrolment in early intervention studies, and reduce the possibility of onward transmission, by enhancing partner notification procedures [10].

There are a number of important obstacles and threats to the widespread use of STARHS in Europe and globally. The first is to assure the long-term supply of assays. The detuned and avidity STARHS assays require modifications of commercially available assays, and their long-term availability cannot be guaranteed. Alternatively, assays can be developed that are designed specifically for the purpose of identifying recent infection. Such assays can be developed commercially, such as BED-CEIA, or by collaboration between national reference laboratories and public health surveillance institutes, as has been done in France [3,11].

A second obstacle is that a window period must be defined for each assay, and then used for either determination of the frequency of recent infection in a given population or for incidence measurement. In a perfect world, one could imagine that every assay should identify a recent infection based on an identical window period. However, the few comparisons of the existing (past or present) assays clearly showed that there are many discrepancies between assays, particularly because the window period is not similar [12]. This is complicated by the fact that, even when using a single assay, the window period frequently differs when applied to a population different from that used initially for the development, especially in areas where non-B subtypes predominate [13,14]. There still remains important work to be done for the validations of the assays and algorithms for estimating incidence from cross-sectional blood specimens.

A third threat is the expertise required to implement the laboratory methods. As outlined in this issue in the article by Murphy and Parry [1], various quality control measures need to be implemented including external quality control procedures. This includes not only assuring and maintaining the operational characteristics of the assay, as outlined in the paragraph above, but also the logistics of rolling out the assay to a wider laboratory network beyond the currently small specialised group of laboratories.

A fourth limitation is the application and integration of STARHS data into routine public health practice. The proportion recently infected is often reported [10,16], but this measure is dependent on HIV testing patterns. The calculation of HIV incidence in the population is much more difficult, as highlighted in this issue by Le Vu et al [2], and will require significant enhancements and changes to current surveillance systems established to monitor the HIV epidemic. Not only will public health authorities need to obtain improved denominator data, but they will also need to enhance their knowledge of HIV testing patterns in different populations and develop current surveillance datasets to include more laboratory and clinical information with which to validate the results of any tests for recent HIV infection [10].

Although all the assays for recent infections have shown limitations, they have been already used in many circumstances to estimate either HIV incidence or, at least, the proportion of recent infection in various populations. Even if they cannot be recommended for routine use worldwide because of insufficient data on their performance to provide precise incidence in different populations, a few studies have already illustrated their usefulness [15,16,17].

The increasing momentum to incorporate STARHS methodologies within HIV national surveillance systems, particularly with the recent release of American estimates of the national HIV incidence [18], highlight the need for a European strategy to be formulated under the auspices and with the financial support of the European Centre for Disease Control and Prevention. Such a strategy should define the additional studies required not only to ascertain the operational characteristics of the assays but also the epidemiological needs for estimating incidence, thus providing best quality data to health policy makers for the implementation and evaluation of prevention campaigns. It is with the development of such a coordinated strategy that a European voice can provide a vital input into global STARHS initiatives.

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Review articles

ASSAYS FOR THE DETECTION OF RECENT INFECTIONS WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

G Murphy (gary.murphy@hpa.org.uk)¹, J. V. Parry^{1,2}

1. Virus Reference Department, Health Protection Agency Centre for Infections, London, United Kingdom

2. Department of Public Health and Policy, London School of Hygiene and Tropical Medicine, London, United Kingdom

The Serological Testing Algorithm for Recent HIV Seroconversion (STARHS) is a generic term for several laboratory techniques that can be used to differentiate recent from long standing infections with human immunodeficiency virus-1 (HIV-1). There are several other approaches that identify acute seroconverters, but STARHS methods are distinguished by their ability to identify infections that occurred during an extended period of 4-6 months prior to sampling. While the STARHS techniques have been employed on an individual basis, their main usefulness lies in the potential of estimating the rate of acquisition of new HIV infection, or incidence, in a population by application to cross-sectional sero-surveys. This is substantially simpler and less expensive than cohort studies. As such, STARHS techniques facilitate the timely monitoring of the impact on HIV incidence of factors such as interventions, demographic factors and behavioural patterns.

The major STARHS techniques currently available are described. Furthermore, the principles behind the methods used are discussed and the limitations of the current assays and the confounding factors that may affect assay specificity are described. A model algorithm for the application of a STARHS assay is shown. Finally, we outline recommendations for laboratory quality systems that will improve the efficiency of STARHS testing, reproducibility of results and reliability of incidence estimates.

Introduction

The ability to segregate recently-acquired human immunodeficiency virus type 1 (HIV-1) infections (RHI), i.e. infections acquired in the previous few months, from long-standing ('prevalent') infections is a valuable tool for real-time measurement of the changing patterns of HIV transmission. Although the HIV infection process and the immune response to HIV afford opportunities to recognise recent HIV infection it is only in the last 10 years that these have been exploited to aid the determination of HIV incidence in populations. Differences between individuals present challenges to the application of serological tests of RHI on an individual patient basis.

Virological and Serological Events following infection

The typical evolution of viral and host markers of HIV infection are illustrated in Figure 1. Investigation of virological and serological events that occur during the very early phase of HIV infection indicate that, following local replication in proximity to the inoculation site a high titre viraemia occurs, generally during the second to third week after exposure [1,2]. This allows generalised seeding of the virus in susceptible tissues throughout the body. HIV genomic RNA is present before the patient has developed

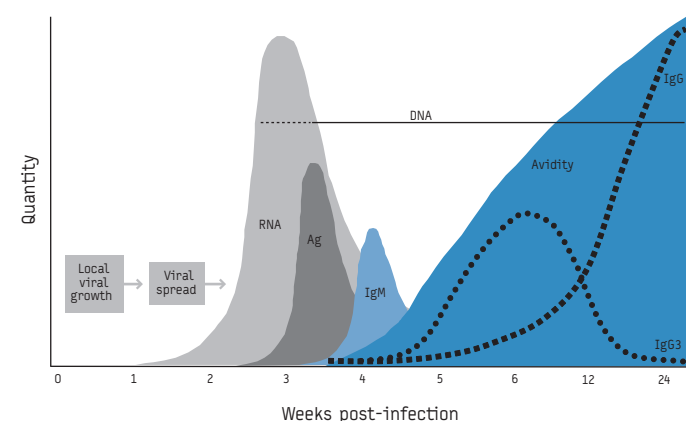
detectable anti-HIV antibodies and is therefore a powerful marker of recent infection. This phenomenon has been used to identify recent HIV-1 infection by some groups [3-6].

A protein component of the virus core, p24 antigen (p24Ag), is usually detectable [1] within a few days of the onset of viraemia [1]. As the host's immune system initiates a response, levels of both the virus and p24Ag fall. The p24Ag usually becomes undetectable until the degradation of the host immune system associated with progressive HIV-related disease, typically around 10 years later. In most cases HIV RNA remains detectable, albeit usually at levels much lower than in the acute phase. Detection of p24Ag in the absence of anti-HIV antibody may also be used as a marker of recent infection but its presence is unreliable and short-lived (1-2 weeks) and therefore has limited utility for measuring incidence.

The short duration of early p24Ag may in part be explained by it being masked due to complexing with the emerging anti-HIV antibodies. Heat or chemical treatment is able to disassociate antibody-antigen complexes, perhaps allowing extended detection of p24Ag further into anti-HIV seroconversion. However, many individuals with established HIV infection also have complexed

FIGURE 1

Typical evolution of key viral and serological markers during the first weeks following infection with HIV-1 (schematic diagram)



Viral markers: RNA, Ribonucleic acid; DNA, Deoxyribonucleic acid; Ag, Antigen. Immunological markers: IgM/IgG, Immunoglobulin M/G antibodies.

Tests that distinguish recently acquired HIV-1 infection from those that are long-standing take advantage of these events.

p24Ag [7]. Moreover, late in the infection, as the immune system fails, p24Ag is often produced in excess and can in many cases be detected even without the dissociation treatment [8,9]. The presence of p24Ag and/or a high level of HIV RNA after the seroconversion period are usually indicative of rapid disease progression and a poor prognosis [10].

The initial immune response is typically heralded by a virus-specific IgM response [11-13]. This IgM response is variable both in intensity and duration, generally peaking within 1-2 weeks, falling to background levels 1-2 weeks later [14]. Contemporaneously, the long-lived high-titre IgG response develops. A gradual increase in anti-HIV titre occurs over several months and this is the basis of both the 'detuned' and 'BED' assays, discussed later in the context of the Serological Testing Algorithm for Recent HIV Seroconversion (STARHS).

Standard HIV screening and diagnostic assays

Standard commercial screening and confirmatory tests are mostly unable to distinguish between long-standing and recently-acquired infections. When specimens are taken during the short period, typically no more than 2-4 weeks, between onset of seroconversion and attainment of the maximum signal in the conventional diagnostic tests, it may be relatively straightforward to diagnose an incident infection on the basis of the rapidly evolving serological pattern. However, to be confident, a combination of supplemental tests needs to be done which may include some or all of the following: Immunoblot (Western blot/line immunoassay); and assays for the detection of: HIV RNA; p24Ag; and IgM anti-HIV. The Western blot assay involves the detection of antibodies against specific HIV-1 proteins separated by molecular weight. The presence and relative reactivity of each specific antibody can be identified, and a pattern typical of recent seroconversion may be recognised. A potential hazard of utilising limited Western blot patterns (i.e. reactivity with few HIV-1 proteins) as evidence of RHI, particularly during the earliest phase of anti-HIV seroconversion, is the significant risk of confusing non-specific reactions with HIV seroconversion. Furthermore, the interval during which this approach may be used, perhaps 3-4 weeks after infection, is too short to permit reliable measurement of HIV incidence on realistic population sizes.

The Serological Testing Algorithm for HIV Seroconversion (STARHS)

The typically rapid immunological response to HIV infection means that within less than a month of anti-HIV seroconversion commencing, standard HIV test kits are unable to distinguish recent from long-standing infections. However, a number of adapted or novel techniques have been developed that are able to identify recent infection over a longer time frame than that achievable with conventional assays. These methods are intended to be applied to individual specimens in which the presence of anti-HIV-1 antibody has already been confirmed, and the approach is known generically as the Serological Testing Algorithm for Recent HIV Seroconversion (STARHS).

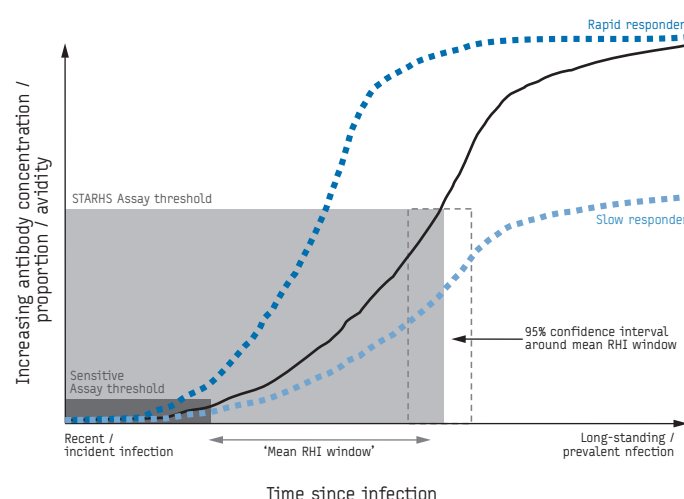
The STARHS approach offers a number of important advantages over other methods for determining HIV-1 incidence. Unlike cohort studies which require repeated testing of individuals, and where results may be biased by people leaving the study, STARHS testing can be carried out retrospectively on stored single specimens from cross-sectional sero-surveys. In comparison with cohort studies, applying the STARHS approach is cheaper, quicker and simpler to perform. Furthermore, STARHS testing can be performed on a

real-time basis thus allowing a measure of recent infection at the time of a study as opposed to incidence derived from a cohort study which cannot be ascertained until after the follow-up sample has been collected and tested.

STARHS/RHI Window Period

The STARHS technique allows HIV-1 incidence to be determined from representative panels of stored anti-HIV-1-positive specimens gathered over a given period from a particular population whose size is known. The duration of the period between seroconversion in the original (sensitive) HIV-1 screening assay and conversion (from recent to long-standing) in the STARHS method must be well-defined and typically in the order of several months, and is critical to a STARHS assay being able to furnish a population incidence rate (Figure 2). The duration of this STARHS window needs to be determined carefully, and this requires panels of specimens from individuals whose date of seroconversion is known or closely approximated. Modelling these data allows the relationship between time since seroconversion and the expected average signal in the STARHS method to be described mathematically. From this, and additional data on known long-standing (>12 months) infections, the chosen cut-point, dividing recent from long-standing, may be set such that it provides an appropriate balance of sensitivity and specificity, and this is typically associated with a mean RHI window in the region of 3-6 months. The duration of the STARHS window is limited by the effects of individual variation on antibody titre and rate of antibody production and maturation. The longer the time after infection, the more pronounced these individual differences become, leading to increasing misclassification [15,16]. Although

FIGURE 2
Principles underpinning the serological testing algorithm for recent HIV infection (STARHS)



The STARHS approaches are applied to confirmed anti-HIV-1-positive specimens and, with the exception of the IgG3 and Inno-LIA approaches, rely on the marker employed, e.g. avidity, increasing over the first several months after seroconversion is detected by a sensitive screening method. If a test specimen gives a result below a pre-determined cut-point, it is deemed to have been a recently acquired HIV-1 infection (RHI). The cut-point is set such that it provides an appropriate balance of sensitivity and specificity, and this is typically associated with a RHI window in the region of 3-6 months. However, the uncertainties around this model should be considered, including the accuracy of the RHI window (95% confidence intervals) and the person-to-person variability shown in the diagram as outliers with either a 'rapid' or a 'slow' response. The former may appear to have a long-standing infection some time sooner than the average RHI window, and the latter may appear to be an RHI some for a considerably longer time.

of lesser magnitude, differences in seroconversion sensitivity between anti-HIV-1 screening tests employed in STARHS should also be taken into account, particularly when moving between generations of screening tests, for which the difference could be more than two weeks [17].

Definition of the STARHS window permits measurements of HIV-1 incidence to be made on achievable populations, but their robustness will depend on several factors, not least the accuracy of the mean STARHS window period employed. When applying STARHS on an individual (diagnostic) basis, the duration of the STARHS window period cannot be accurately defined, and arguably need not be. Importantly, it must be borne in mind that the STARHS windows described represent the mean interval between the earliest time at which an HIV-1 diagnosis may be made and conversion to long-standing status in the STARHS assay, and not the upper limit. This is derived by examining specimens from many seroconverting individuals in whom the immune response will mature differently. Accordingly, taking the 155 days' window advised for the BED-CEIA assay (described below), a substantial proportion (roughly one-half) of those infected will already have converted in that assay to a long-standing infection at under 155 days since seroconversion, and the remainder at over 155 days; very few will actually convert on day 155. The consequence of this, when applied to individuals, is that some are likely to be advised inaccurately that, in the former example, their infection is over 155 days-old and therefore long-standing, and in the latter, that it was under 155 days-old and therefore classified as recent.

Furthermore, STARHS results consistent with an RHI are known to arise and be persistent in a small proportion of those infected for years and in those presenting late in the course of infection [18], as discussed below. Because the rate of misclassification as an RHI is a key variable influencing the accuracy of population incidence estimates this is coming under closer scrutiny, and has led to proposed correction factors for incidence estimation [19,20]. However, these do not provide a means to ensure an error-free finding when applying STARHS individually. Nevertheless, an improved understanding of sensitivity, specificity and predictive values associated with STARHS testing should provide an appropriate platform for providing advice, care and public health action on an individual basis. It may be advisable when using STARHS as a diagnostic indicator to communicate the timing of

infection less definitively, e.g. when the result is consistent with a RHI: 'The findings suggest HIV may have been acquired in the last 12 months'.

Assays for recent HIV-1 infection

A number of assays can be used within a STARHS programme (Table).

The 'detuned' assay

The 'detuned' assay was the first assay to be described as being able to identify specimens from individuals recently infected with HIV-1 for the purposes of incidence calculation. Employing the recommended assay cut-off, the technique recognises HIV-1 seroconversions that have occurred on average four to six months prior to collection of the positive specimen [15,16,21]. However, the period during which recent infection can be identified can be altered by changing the cut-off applied to the assay. The method relies on the generalisation that anti-HIV titres in the plasma rise gradually, and at a similar rate in each infected individual, over a period of several months following seroconversion.

The 'detuned' approach takes confirmed anti-HIV-1-positive specimens and re-tests them with an enzyme immunoassay (EIA) that has been made less sensitive ('detuned') by increasing the dilution at which each specimen is tested from 1/76 to 1/20,000 and by reducing the incubation times. Although assay variability is partially accommodated by the inclusion of a calibrator, obtaining accurate results by the detuned approach is technically demanding, requiring precise preparation of high serum dilutions and strict adherence to incubation conditions. Recent seroconversion is inferred if the confirmed anti-HIV-1-positive specimen is negative in the less sensitive EIA.

The 'detuned' approach has been described for two different immunoassays: the Abbott HIVAB 3A11 (Abbott laboratories, United Kingdom (UK)) and the bioMérieux Vironostika HIV-1 microelisa (bioMérieux, UK). Production of both assays has now ceased with the last lot of bioMérieux Vironostika assays expiring in summer 2008. Both these assays use a semi-purified viral lysate antigen adsorbed to the solid phase. In both cases the viral lysate derives from an isolate of the subtype B strain of HIV-1. The Abbott HIVAB 3A11 antigen is also 'spiked' with purified native gp41 antigen. The use of an antigen from a single HIV subtype means that

TABLE

Methods described which may be employed on serum/plasma specimens in serological testing algorithm for recent HIV seroconversion (STARHS) applications to distinguish recent from long-standing HIV-1 infection

STARHS method	Type	Principle	Reference
Abbott HAVAB (3A11)	Modified commercial (withdrawn 2003)	'Detuned' – standard assay, sensitivity reduced to extend seroconversion window	[15]
Abbott AxSYM HIV 1/2 gO	Modified commercial	Avidity of anti-HIV antibodies	[31]
Calypte BED EIA	Commercial	Proportion of total antibodies that are HIV-specific	[23]
bioMérieux Vironostika HIV-1 microELISA	Modified commercial (withdrawn 2008)	'Detuned' – standard assay, sensitivity reduced to extend seroconversion window	[16]
IgG3 anti-HIV	In-house	Transient presence of IgG3 isotype antibodies against HIV p24Ag	[36]
IDE-V3 EIA	In-house	Reactivity with two selected HIV antigens is used to predict likelihood of recent infection	[34]
Inno-LIA HIV	Modified commercial	Relationship of reactivity with various HIV antigens	[37]
Ortho Vitros ECI anti-HIV 1+2	Modified commercial	Avidity of anti-HIV antibodies	[33]
Particle agglutination (SeroDIA-HIV)	Modified commercial	'Detuned' – standard assay, sensitivity reduced to extend seroconversion window	[38]

heterologous antibodies (i.e. those formed against viral subtypes not utilised in the assay) may not bind the antigen as effectively as the homologous antibody. This generally causes an increase in the period during which the assay would determine a specimen to be from an RHI [22]. Reactivity in the 'detuned' assay is standardised against a calibrator specimen to give a standardised optical density (SOD), thus smoothing out run-to-run variability.

BED-CEIA assay

The BED-CEIA (capture enzyme immunoassay) is a commercial product (Calypte Biomedical, United States (US)) designed specifically for the purpose of identifying infections that were acquired recently [23]. Being a class-specific IgG antibody capture EIA, it differs in its mechanism from the 'detuned' assays as its reactivity is dependent, not on the absolute titre of HIV-1-specific antibody, but on what proportion of all the IgG captured from an HIV-infected person's serum is directed against the HIV antigens employed. In early infection, the proportion of HIV-specific antibody is lower than in a long-standing infection. As the BED-CEIA does not directly measure the amount of antibody present it is technically more robust than the 'detuned' techniques, the principle on which it is based being more forgiving about the accuracy of dilution of test specimens, incubation times and temperatures.

The BED-CEIA was designed to overcome some of the subtype differences associated with the 'detuned' assays, utilising a trimeric branched peptide. Each branch comprises a synthetic oligopeptide derived from the immunodominant region of the transmembrane gp41 glycoprotein of HIV-1 subtype B, CRF_01 AE and subtype D, hence the assay name 'BED'. These three peptides were selected to cover much of the breadth of antigenic diversity, in theory allowing a single window period to be used with the BED-CEIA test, whatever the infecting HIV-1 subtype. However, it has been shown that differences in window periods between subtypes do occur in the BED assay, though perhaps less pronounced than in the detuned assays (see below).

Avidity Assays

A further approach to identifying recent infection is to investigate the maturity of the HIV antibody response by investigating its avidity. Antibodies of low avidity are usually indicative of recent infection and this approach has been shown to be valid for many viral infections [24-26]. Although avidity assays have previously been described for use with HIV-1 [27-30], it was not until recently that assays that could be used for HIV-1 incidence determination were described [31-33].

The method described by Suligoi et al. is a modification of the third generation anti-HIV-1/2 assay that runs on the Abbott AxSYM random access analyzer, and is therefore easy to perform. It uses a method whereby the specimen is pre-incubated with the chaotropic agent guanidine (guanidine hydrochloride) [31,32]. Guanidine treatment of the specimen primarily disrupts the hydrogen bonds that help determine the secondary structure of the antibody, although it may also have a residual effect on the subsequent antibody-antigen interaction. The treatment has a greater effect on early antibodies, the active site of which has a less defined structure and can be degraded by mild denaturation so that they are less able to bind their homologous antigen, thus reducing the signal. As the antibody response matures, the active site becomes increasingly resistant to disruption.

When assessing the avidity of an antibody response, the level of signal obtained after chaotropic treatment is compared with the signal produced when pre-incubating the specimen in a neutral diluent such as phosphate buffered saline (PBS). When the antibody is highly avid and therefore largely resistant to the chaotrope, the two signals in the immunoassay will be very similar. The binding of early, less avid, antibodies on the other hand will be much reduced when treated with the chaotrope, and this will produce a reduced signal compared to the untreated aliquot. The RHI window for the AxSYM avidity assay has not yet been determined precisely, but it is thought to be close to six months.

Recently, an alternative antibody avidity assay has been described that also uses guanidine but runs on the Vitros analyzer (Ortho Diagnostics, UK). It has an RHI window of approximately 142 days when employing a threshold avidity index of 80% [33]. Currently no published data exist on the widescale application of these avidity assays, and work is continuing to refine their performance characteristics and the window period, particularly for HIV-1 non-B subtypes.

IDE-V3 assay

The IDE-V3 immunoassay is based on two conserved highly immunogenic epitopes found in the envelope glycoproteins of HIV-1 [34]. One is derived from the immunodominant epitope (hence 'IDE') of the transmembrane glycoprotein gp41; the second derives from the V3 loop of the outer glycoprotein gp120. The IDE antigen comprises two consensus oligopeptides of 30 amino acids, one from HIV-1 group M and one from subtype D. The V3 component comprises a blend of five oligopeptides derived from the HIV-1 subtypes A, B, C, D and CRF_01 AE. The IDE-V3 assay is not available as a commercial kit, but can be assembled by the user from basic ingredients that are available commercially.

Technically the assay is structured as a simple indirect enzyme-immunoassay, employing a 96-well microplate format, with the 8-well columns alternately coated with the IDE and V3 oligopeptides. A dilution of each specimen is tested against both the IDE and V3 antigens. In its current format this assay has to be assembled by the user from individual components and, although its principle is relatively straightforward, its wider availability as a robust STARHS approach awaits further standardisation of the reagents and controls.

To discriminate recent from long-standing infection this assay employs a mathematical formula which draws on reactivity of the specimen with the antigens from each region. The formula was derived from testing panels of specimens known to be from either recent (<6 months) or long-standing infections. Although the authors imply that the assay is able to identify recent infections that date back no more than six months, this appears to have been based on polarised specimen sets: specimens representing RHI, which had mostly been collected soon after seroconversion, and specimens representing long-standing infections, many of which may have been collected considerably later than six months following seroconversion. Consequently, the continuous relationship between the assay output and time since seroconversion has not been mathematically modeled, and the exact duration of the RHI window period has yet to be calibrated.

Sakarovich et al., applying STARHS assays to seroconverting individuals in Cote d'Ivoire found that the IDE-V3 assay, while having good specificity (96.3%), had poor sensitivity (42.3%), and

this suggests strongly that its seroconversion window is likely to be considerably shorter than six months [35]. Currently this assay is being used as part of the French national screening programme to determine the proportion of newly diagnosed HIV infections that were recently acquired. Work is continuing to improve the estimation of the RHI window period for this assay.

Other STARHS approaches

A number of other approaches have been described that distinguish recent from long-standing HIV-1 infection. These include:

IgG3 Anti-HIV: It is known that the IgG isotypes formed in response to an infection may vary during the course of an infection. Research investigating the IgG isotype response to a range of HIV-1 antigens using a Western blot approach identified that isotype IgG3 was usually present transiently during the first few months of HIV-1 infection [36]. The investigators found the antigen against which the IgG3 response was most reliable was p24. These findings were converted into a simple EIA based procedure whereby IgG3 to p24Ag is typically detectable for only the first 1-4 months of infection. Unfortunately, however, this method has not yet been translated into a commercial kit.

Inno-LIA HIV Adaptation: The Inno-LIA™ HIV I/II Score is a line immunoassay, similar to a Western blot but employing only a limited selection of synthetic oligopeptides and recombinant antigens of HIV-1 and HIV-2. Its routine application is as a confirmatory test to investigate whether screen-reactive specimens are true or false. For the STARHS application the intensity of each band in the Inno-LIA test is read using a slightly modified scoring system. An algorithm is applied to the scores which allows the segregation of the results into recent or long-standing HIV infection [37]. The approach is expensive, but may have utility where it is already routinely employed as the confirmatory diagnostic test.

Several other approaches have been described, including one based on a particle agglutination test in a 'detuned' format [38] and an oral fluid assay [39], but neither of these assays has been applied on a large scale and the RHI window periods have not been established.

New STARHS approaches are under development and should be expected to become available over the next few years.

Limitations of STARHS assays

The accuracy of STARHS assays is affected by a number of factors that are likely to be encountered when testing populations of HIV-infected individuals, and these are outlined below:

Infecting HIV subtype

The detuned STARHS methods have been based on the use of HIV-1 clade B antigens. Because the immunodominant epitopes differ between HIV-1 clades it is likely that the heterologous antibody responses may show lower binding affinities and that this, in turn, could alter the RHI window period, in most cases extending it. Should this be the case, HIV incidence would be over-estimated unless the RHI window is adjusted. This presents serious difficulties when dealing with epidemics of mixed clades, such as are now established in at least some European Union countries.

Studies on populations infected with non-B viruses have indeed revealed that the period during which an infection is identified as recent is significantly different to that for clade B infections. For example, employing an SOD threshold of 1.0 in the Vironostika

detuned assay the average RHI window is 170 days, whereas for the CRF_01 AE virus it is 356 days [22] and for clade C it is 360 days [40]. Comprehensive findings are not available on this issue, and few are actually published.

As discussed above, the BED-CEIA method was designed to overcome problems associated with the lower affinity of heterologous antibody responses by employing a multimeric antigen representing much of the antigenic diversity associated with the immunodominant region of gp41. The manufacturer's product insert for the BED-CEIA advises the use of a single mean RHI window period of 155 days [41]. However, studies have demonstrated that the mean RHI window period for clade C is substantially longer, at 181 days, and for CRF_01 AE it is much shorter, at 115 days [42]. The impact on more recently described methods like the avidity, IDE-V3 and IgG3 methods is as yet unknown.

Acquired immunodeficiency syndrome (AIDS)/Low CD4 Count

The failing immune system associated with advanced HIV disease has long been known to be associated with a decline in anti-HIV antibody levels [8], and this would be expected to impact the specificity of those STARHS methods that depend primarily on the quantification of antibody. Indeed, misclassification rates for the detuned methods have been published, and for Vironostika it has been estimated that approximately 5% of AIDS cases will be misclassified as a recent infection [16]. For the BED-CEIA approach, the AIDS misclassification rate has been estimated at 2-3% [41]. Misclassification of AIDS cases by the IDE-V3 assays is approximately 9% [34]. On the other hand, as the avidity of antibody binding is not related to the quantity of antibodies, it would be expected not to be similarly affected, and preliminary evidence suggests this may be so.

Antiretroviral Therapy

It has been observed that combination anti-retroviral therapy (ART) leads to misclassification of long-standing infections as recent. The exact mechanism has not been elucidated, but simplistically, it is likely that the ART suppresses viral replication to such a degree that the chronic stimulus to the humoral immune response is removed, leading to a decline in anti-HIV antibody titre. The effect is most pronounced during the first few months after ART initiation (authors' unpublished findings). However, in comparison to the very high anti-HIV titres typically found in HIV-infected individuals this effect is modest and would not be sufficient to render state-of-the-art HIV screening tests negative.

Other Confounders

In some cases there is no clear common factor associated with a misclassification by STARHS. In an extensive study among HIV-1-infected men who have sex with men in a UK city, several long-term infected individuals with naturally suppressed viraemia (<50 copies/ml) were flagged as a recent infection by the detuned assay [18]. There is some evidence that the BED-CEIA approach misclassifies a substantial minority of long-standing infections as recent and consequently leads to inflated incidence rates [19,20,43].

Quality Control Measures

As with any laboratory diagnostic method STARHS assays must be performed within an appropriate quality system. This includes the documentation of processes, use of standard operating procedures, appropriate training of staff and evidence of competency. In the authors' experience, the type and condition of equipment can significantly impact on the transferability of STARHS methods

between laboratories and lead to inconsistent results. Several of the key elements are discussed below:

Robust and Reliable Methods

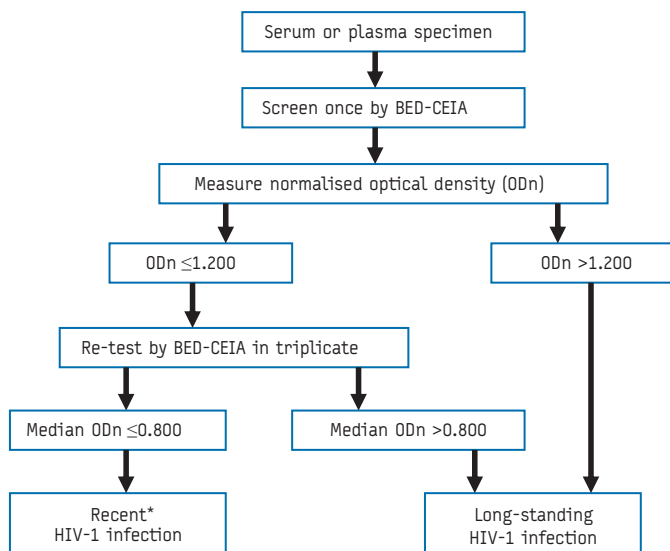
With the exception of the BED-CEIA the methods currently available are either modifications of commercial kits, or 'in-house' assays. Whichever sort of STARHS method is employed, it is important to select an assay that suits the laboratory's resources and skills and the population to which it is to be applied. The method should be capable of providing findings of acceptable accuracy and reproducibility. The use of modified or 'self-assembly' techniques is more vulnerable to inconsistency of performance and in those circumstances validated production and quality control processes must be in place to verify consistency of performance.

Confirmatory Algorithms

In common with other diagnostic methods, the results of a STARHS assay will show some variability. To improve the reliability of the test result, the well-established methods ('detuned'; BED-CEIA) include an algorithm of triplicate retesting of specimens whose reactivity is in the range associated with recent acquisition, and a defined margin above, e.g. for the BED-CEIA a normalised optical density (ODn) of up to 1.200 (Figure 3). However, while this improves the accuracy of the STARHS measurement it does not identify the samples misclassified due to the factors discussed above such as advanced HIV disease. Similar approaches need to be developed for the other STARHS techniques.

FIGURE 3

Example of the STARHS testing process, employing the BED-CEIA procedure



* The duration of the recent HIV infection window that is advised in the BED product insert is 155 days. This is the mean duration, which is an important value when estimating population incidence rates. It is not the upper limit of the STARHS window. Consequently, when interpreting STARHS findings on an individual basis it must be borne in mind that a substantial proportion of those whose ODn is ≤ 0.800 will actually have been infected more than 155 days earlier. Similarly, some whose ODn is > 0.800 will have been infected less than 155 days earlier. As one might expect, findings in close proximity to the cut-point of 0.800 are more likely to be a misclassification.

Assay Calibrators and Assay Controls

A common approach to smoothing out lot-to-lot and run-to-run variation in performance is to employ one or more calibrator specimens which would show reactivity in the mid-range. They are employed to adjust the signal obtained with each test specimen against the reactivity of the calibrator, and thus control variations over time. At present, only the 'detuned' and BED assays incorporate a calibrator, generating respectively a 'standardised optical density' (SOD) and a 'normalised optical density' (ODn). In addition, other controls are normally included (e.g. non-reactive; long-standing). Even when all controls are supplied as part of a commercial STARHS kit it is best practice to include further controls of expected reactivity, either from a third party supplier or produced by the user laboratory, to provide the means to monitor assay performance independent of the kit manufacturer. Such controls provide a tool to ensure the assay is performing within expected parameters, and provide the basis for acceptance or rejection of each set of results.

External Performance/Quality Assessment (EPA/EQA)

An important component of ensuring laboratories' performance is adequate is the blinded examination of small panels (typically 4-8 members) of specimens of unknown status. Such schemes require significant investment to establish and maintain. At present, EPA/EQA schemes exist only for the 'detuned' and BED assays. Furthermore, there is arguably a need for larger panels to qualify laboratories embarking on the application of STARHS methods.

Concluding Remarks

A wide range of STARHS approaches have been described and new methods are under development. They clearly have a potentially important role both in public health monitoring and individual diagnosis. The evidence indicates that the current methods are generally able to distinguish recent from long-standing HIV-1 infections. However, the rigors of assigning an accurate duration to the interval between infection or seroconversion and the time at which the transition to a long-standing infection is assigned by STARHS remain challenging. This is due to the diversity both of the host immune response and of the antigenicity of HIV-1. When applying the method as an epidemiological tool to estimate incidence these variables may be controlled if there is a single prevalent HIV-1 subtype and its associated mean window is accurately known. In many parts of Europe, however, the HIV-1 epidemic is already heterogeneous. The BED-CEIA was designed to accommodate this, but despite this it has emerged that the mean RHI window, even for the small number of clades for which it has been derived, ranges from 115 to 181 days and this alone could lead to over- or under-estimates of incidence of approximately 50% [42].

It remains to be seen whether the assays currently being developed will provide improved accommodation of HIV-1 diversity. The complexities of the multiple variables involved in designing broadly applicable STARHS methods, optimising them, calibrating their performance and recognising their limitations present enormous challenges. A global initiative led by the WHO/UNAIDS has been created which is pooling the experience and resources of laboratory scientists, epidemiologists and statisticians working in the STARHS field. We should therefore expect improved STARHS methods and applications to emerge over the next few years.

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Review articles

PRINCIPLES AND USES OF HIV INCIDENCE ESTIMATION FROM RECENT INFECTION TESTING - A REVIEW

S Le Vu (s.levu@invs.sante.fr)¹, J Pillonel¹, Caroline Semaille¹, P Bernillon¹, Y Le Strat¹, L Meyer², J C Desenclos¹

1. Department of Infectious Diseases, HIV/AIDS-STI-HCV Unit, Institut de veille sanitaire (French Institute for Public Health Surveillance, InVS), Saint-Maurice, France

2. Department of Epidemiology, Institut national de la santé et de la recherche médicale/Institut national d'études démographiques (National Institute of Health and Medical Research/National Institute for Demographic Studies, INSERM/INED/Paris XI U569), Le Kremlin-Bicêtre, France

Since the 1990s, the development of laboratory-based methods has allowed to estimate incidence of human immunodeficiency virus (HIV) infections on single samples. The tests aim to differentiate recent from established HIV infection. Incidence estimates are obtained by using the relationship between prevalence, incidence and duration of recent infection. We describe the principle of the methods and typical uses of these tests to characterise recent infection and derive incidence. We discuss the challenges in interpreting estimates and we consider the implications for surveillance systems.

Overall, these methods can add remarkable value to surveillance systems based on prevalence surveys as well as HIV case reporting. The assumptions that must be fulfilled to correctly interpret the estimates are mostly similar to those required in prevalence measurement. However, further research on the specific aspect of window period estimation is needed in order to generalise these methods in various population settings.

Introduction

Estimating HIV incidence, the number of new infections during a time period, is critically important for assessing the dynamics of human immunodeficiency virus (HIV) transmission and evaluating the impact of prevention policies. A conceptual improvement in surveillance methods has been made in the past ten years to make incidence estimation more feasible. By using a biomarker measurement to identify seropositive individuals who have recently been infected, incidence estimates can be obtained from a single specimen. This laboratory-based method can take advantage of the collection of specimen intended to assess prevalence (the proportion or number of persons cumulatively infected at a given time) and to obtain valid incidence data without the expensive and logistically complex requirement of following a cohort of uninfected individuals over time. However, as for other methods based on repeated prevalence data and mathematical modelling, the use of biomarkers to estimate incidence requires a substantial number of assumptions, some being difficult to assess, and an appropriate definition of the population the incidence is estimated for.

In this article based on the literature, we attempt to give an overview of the methods that allow estimating HIV incidence based on biomarker detection at the early stage of infection. After defining the principles, we review some typical uses of serological incidence assays and the challenges for each type of application.

Principles

Incidence based on detection of virological markers before seroconversion

In 1995, Brookmeyer and Quinn introduced a simple approach for estimating HIV incidence from a cross-sectional survey [1]. They used a two-step algorithm combining diagnostic tests for the p24 antigen and HIV-1 antibodies to determine the prevalence of p24 antigenaemia among antibody-negative individuals (Figure 1). The HIV incidence rate was then calculated by using the classical epidemiologic relation between prevalence, incidence, and duration of the period between the onset of detectability of p24 and the first HIV antibodies.

The disadvantage of this approach was that the time during which p24 antigen is detectable prior to seroconversion is short (the mean duration of this period was 22.5 days in 1995 and has become shorter since then due to the development of new diagnostic assays that allow to detect antibodies earlier [2]). The first consequence of this is that the estimation of this period comes with a considerable uncertainty which can have a large impact on the incidence estimate. The second consequence is that large samples and/or high HIV incidence are required to identify a sufficient number of individuals with detectable p24 antigen who have not seroconverted. Nevertheless, Brookmeyer and Quinn provided the conceptual framework for subsequent laboratory-based methods to estimate incidence from single cross-sectional surveys.

Within the range of methods to identify early infection through virological markers before seroconversion, testing of pooled HIV RNA now seems to be the most appropriate approach because RNA can be detected earlier than p24 antigen, which allows characterisation of a longer time period (Figure 1). Moreover, pooling of specimens improves the predictive value of the amplification assays and substantially lowers the costs. However, in order to obtain accurate incidence estimates, this method requires the inclusion of very large sample populations, such as those provided by blood donations [2] or by the large testing programme in the United States (US) described by Pilcher *et al.* [3].

Serologic incidence assays

Janssen *et al.* were the first to describe in 1998 an approach based on a test specifically developed for the purpose of estimating incidence [4]. This approach named "Serologic testing algorithm

for recent HIV seroconversion (STARHS)” aimed at detecting a transient state reached after the antibody conversion. It thus offered the advantage of testing only positive individuals and defining a period sufficiently short to fulfil the requirements of stationarity of the incidence over the study period, while sufficiently long to minimise the inaccuracy in its estimation. The work of Janssen *et al.* can be considered as a milestone for the concept of serological methods for the estimation of HIV incidence.

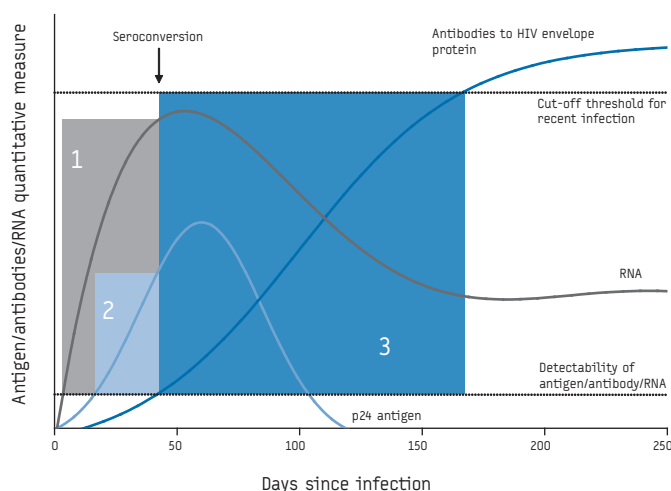
Following the same principle, various applications of laboratory-based incidence estimation from cross-sectional population surveys have been described and a growing number of assays have been developed (see the article of Parry *et al.* in this issue). These assays measure the immunological response against the virus, based on specific HIV antibody concentration [4-6], proportion [7], isotype [8] or avidity [9]. This measure should define a transient state from the onset of detectability by a standard HIV screening test to the cut-off value defining the “established” infection status of the test for recent infection (Figure 1). This period is called the window period. Because of the individual variability in antibody response, window periods may differ widely from person to person. Their mean duration is measured in advance by testing serial specimens from infected individuals with known dates of seroconversion [10]. The STARHS methods have been compared to classical incidence measurements obtained in cohorts to assess their validity [4,11,12]. Provided that the compared estimates are not affected by population sampling bias, the estimates are reported to be similar [10,12].

Incidence estimation

The incidence estimation is calculated as the frequency of the transient state (i.e. the prevalence of recent infection) divided by its duration (the mean window period). As stated above, this calculation is based on the relation “prevalence = incidence * mean duration”. This relation assumes that the condition, in our context “recent HIV infection”, is a rare event so that the prevalence odds

FIGURE 1

Kinetics of virological markers and host immune response used to define transient states in the early phase of HIV infection



1: RNA-to-seroconversion transient state as defined by Busch *et al.*, 2005 [2]
2: p24-to-seroconversion transient state as defined by Brookmeyer *et al.*, 1995 [1]
3: Antibody-based mean window period as defined by Janssen *et al.*, 1998 [4]

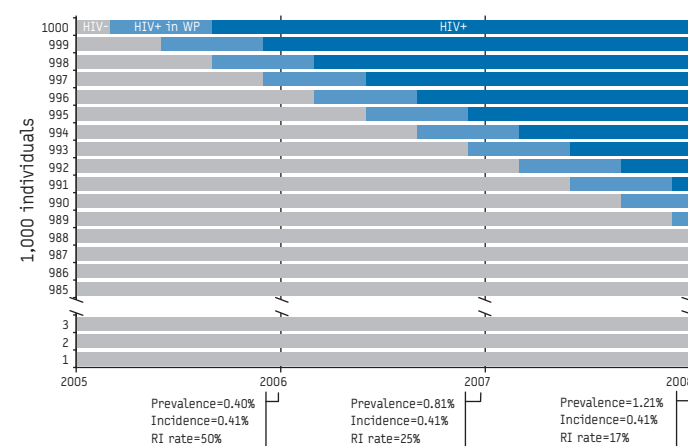
can be approximated by the prevalence [13]. And the relation is valid for a stationary population with a constant level of incidence during the study period [1]. In Figure 2, we present an example of an incidence calculation using the formula developed by Janssen *et al.* with a window period of 180 days [4].

Various adjustments have been made to Janssen’s formula in order to correctly express the number of people at risk and to account for misclassification of long-term infections. The first adjustment consisted in varying the assumed number of people at risk of having had a recent HIV infection during one year. As in the estimation of incidence in a cohort, HIV-negative individuals are considered at risk during the whole period, while infected individuals can be considered at risk during half a year on average [14].

In addition, concerns have been expressed that the mean window period for the BED capture enzyme immunoassay (BED-CEIA) does not properly take into account people who have a very long individual window period and can be falsely classified as

FIGURE 2

Relation between HIV prevalence, recent infection rate, and the incidence estimation in a cross-sectional survey



A constant incidence rate of 0.4% persons/year is observed in a population of 1,000 individuals seronegative from the beginning of year 2005. Prevalence, incidence and rate of recent infection are estimated cross-sectionally at the end of 2005, 2006 and 2007. The number of HIV-positive individuals includes those with recent infection, tested within window period (NR), and those with established infection, tested after the window period (NE), represented respectively in light blue and dark blue in the figure. HIV-negative individuals (Nneg) are represented in grey. While incidence estimates are nearly constant over the years, the recent infection rate, being influenced by the prevalence of established infection, is decreasing.

Estimates are calculated as follows [4]:

$$\text{Prevalence} = \frac{N_R + N_E}{N_{\text{neg}} + N_R + N_E}$$

$$\text{Incidence} = \frac{N_R}{N_{\text{neg}} + N_R} \times \frac{365}{\text{mean WP}}$$

$$\text{Recent infection rate} = \frac{N_R}{N_R + N_E}$$

This illustration was inspired by the presentation of Ruigang Song “Modeling HIV Testing Behavior and Its Impact on Incidence Estimation” at the 15th International AIDS Conference, July 15, 2004, Bangkok, Thailand.

WP = Window period; RI = Recent infection.

recent. This issue is probably a general one, affecting all the tests that have been calibrated using a disproportionate number of short term infections (for less than one year). It should have an impact on incidence estimation since the cross-sectional populations on which the method is to be applied are expected to contain a larger number of long-term infections. Two adjustments have been proposed to correct this issue about the specificity [15]. They share the principle of applying a corrective factor in the incidence formula to compensate for the false recent cases due to very long window period. Other algorithms have been proposed that, rather than correcting the formula, combine two incidence assays in order to avoid misclassification [12,16].

Applications

While a comprehensive review of applications for serological incidence assays is beyond the scope of this paper, the purpose of this chapter is to point out typical settings in which they may be used.

Typical applications

The most common context in which incidence assays are used are prevalence sero-surveys. Some were dedicated to incidence estimation, but the majority were set up to observe the recent infection status of stored HIV-positive serum specimens.

Numerous serial cross-sectional surveys have been applied in the setting of testing for HIV or other sexually transmitted diseases in countries such as the US [17-19], some European countries [20;21] or Brazil [22]. In these studies, temporal trends in incidence rate could be derived and helped to assess retrospectively epidemic phenomena among high-risk subgroups. But concerns about representativeness and selection bias can be raised about such voluntary testing sites (as reviewed below in the section 'Issues').

Similarly, already existing sentinel surveillance systems have provided insight into underlying trends in transmission in particular risk groups. Specimens gathered at enrolment in syringe exchange programmes or serial street surveys allowed the estimation of trends in HIV incidence among intravenous drug users in New York City, US [23] and San Francisco, US [24] over a long period.

For purposes of precision and as done for prevalence estimation, targeting a more general population than particular high-risk groups requires testing a very large number of people or setting the study in a country with a high incidence level.

At least one of these conditions was met in studies that estimated the HIV incidence by means of recent infection testing in antenatal screening programmes in Cambodia [25], South Africa [26], the US [27] and Brazil [28], in screening programmes for blood donation in the US [2;4], France [29] and the Ivory Coast [30], and a national household survey in South Africa [31].

In all these settings, specimens are collected routinely and can be tested for recent infection retrospectively or prospectively. Some demographic and behavioural data on the targeted population are usually collected along with the specimens, both for positive and negative individuals. Taking advantage of specimens from prevalence serosurveys allows to derive incidence data for these populations with only minor expenses in terms of cost and logistics.

In certain contexts, the most obvious added value of the incidence assays approach is that the incidence could not have been estimated by any other means. This is what happens when no accurate data on prior testing or exposure period can be obtained such as for the population of blood donors screened during their first donation [29].

Identifying recent infection

A particular use of incidence assays is identifying recent infection status per se, for individual patient management such as contact tracing or assessment of primary resistance. It is helpful to bear in mind that characterisation of recent infection was initially a by-product in the method described by Janssen *et al.* which considered incidence derivation as the main outcome. In particular, the use of the mean value of an incidence assay window period assumes that individual window periods are variable and that a certain number of individuals in a given population will have a window period shorter or longer than the mean. Consequently, some misclassifications of established infection (false positives) and of recent infection (false negatives) are to be expected. For the purpose of incidence estimation, the respective misclassifications are supposed to cancel each other out, so that the number of recent infection at a population level is correctly estimated. At the level of individual patients, however, this could lead to serious misinterpretation.

On the other hand, some assays have been developed for the specific purpose of classifying infections in individual patients as recent or established with given predictive criteria. This is the case for the enzyme immunoassay for recent HIV-1 infections (EIA-RI) developed by Barin *et al.* [6]. This assay uses a logistic regression classification algorithm in which the cut-off was chosen to detect individuals infected for less than 180 days with an enhanced focus on the level of specificity of detection. It is to be noted that a lack of specificity, because it affects the population of established infections that is generally larger, should have a wider impact on misclassification than a lack of sensitivity, considering the low prevalence of recent infection status [30]. On-going development of the EIA-RI test aims to re-calibrate it for the purpose of incidence derivation.

Expressing the proportion of recent infection

Some applications define the proportion of recent infection in a population of positive individuals as an outcome. This is the way Puchhammer *et al.* analysed the results of the avidity assay among new diagnoses from case-reporting in Austria [32]. This is also the way that correlates of recent infection among new diagnoses are interpreted in France [33] (see also the article by Semaille *et al.* in this issue). However, this quantity that is somehow related to incidence depends also on the prevalence of non-recent infection and thus can not be considered as a good proxy for incidence. In fact, in the context of diagnostic testing, the proportion of recent infection has a lot to do with the testing framework capacity as well as the incidence rate in the population. Since the prevalence of undiagnosed infection affects the proportion of recent infection independently of any change in incidence (Figure 2), such results are difficult to interpret.

Incidence estimation from HIV case-reporting data

While it seems especially promising to take advantage of recent infection testing among reported HIV diagnoses at province or country level, there are several specific difficulties with regards to deriving a valid incidence measurement. Unlike cross-sectional surveys, a case-reporting system collects information only for individuals with positive test results and generally can not provide information on those who were negative. Therefore, the denominator of the formula, i. e. the number of people at risk, is not available. Another approach is needed to derive an incidence that can be generalised for the population targeted by the surveillance, and to take account of the fact that negative test results are not reported.

Such an approach has been described by Lee *et al.* for the estimation of the national HIV incidence in the US [34]. The statistical framework considers the reported cases identified as recently infected as a sample selected from all annual new cases, with a probability of inclusion related to their testing pattern. According to this probability, each case identified as recently infected is assigned a weight, and the sum of weights provides the incidence count. This approach represents a good opportunity to improve large scale surveillance of HIV dynamics, especially where a framework of HIV case reporting already exists and can provide data on testing patterns.

Finally, another approach has been described to bypass the issue that only positive individuals are reported to the surveillance system. In Ontario, Canada, an enhanced surveillance system has been established that requires diagnostic laboratories to collect information (number and risk factor) on a random subset of individuals with a negative test result in parallel to the information on those that were positive [35]. This system then allows the use of the Janssen's formula to derive the incidence in different risk groups.

Issues

There are issues that pertain to the estimation HIV incidence by characterising recent infections. We can distinguish issues that are related to the determination of recent HIV infection from those that affect the validity of incidence estimation.

Limitations in determining recent infection

The first issues are due to the limitations of the assays in detecting recent HIV infection. As the majority of assays are based on quantitative measurement of the antibody response, factors that affect the patient's immune response lead to some misclassification. Qualitative assays such as the avidity assay may be affected to a lesser extent [36].

Firstly, people with acquired immunodeficiency syndrome (AIDS) may falsely be identified as recently infected due to declining antibody levels. The same appears to be true in some individuals in the late stage of non-AIDS HIV infection. As for the AIDS stage, clinical data or CD4+ T-cell counts would need to be collected in order to exclude these patients from the calculation and avoid overestimation. A correction for misclassification due to late-stage non-responsive patients, has been proposed by Mc Dougal *et al.* and Hargrove *et al.* [15].

Secondly, antiretroviral drugs affect the antibody level by decreasing the viral load [37]. Again, to correctly assess recent infection, patients with ongoing treatment need to be identified and excluded by gathering declarative information (from clinician or patient) or alternatively by detecting drugs in serum specimens by, for example, mass-spectrometry.

Thirdly, test results are affected by the virus subtype and/or the patient's genetic background. It has been shown that all tests that have been developed mainly on specimens from patients infected with subtype B viruses give inconsistent results when used for infections with non-B subtypes. Therefore, an assessment of the test properties (cut-off and window period) in different population settings is needed before applying any method [30].

We have seen how the correct interpretation of test results relies on the availability of clinical data that characterise the population [38]. In order to further interpret incidence estimates, data on

sex, mode of contamination, testing patterns, and possibly virus subtypes must be gathered along with tests results.

Representativeness and selection bias

A general issue of incidence estimation arises from the fact that the populations tested are not randomly selected and may not be representative of the populations at risk of infection. This is particularly the case in the context of HIV testing or sexually transmitted diseases clinics. The bias may go in either direction. People at high risk may seek testing more frequently with the consequence of raising the incidence estimation. On the other hand, people attending HIV testing settings as part of a prevention strategy might be at lower risk than people who do not do a test because they do not recognise the risk or are afraid of a positive result.

Schoenbach *et al.* raised this issue in 2001 and questioned the rationale of inferring HIV incidence in testing settings and in particular, whether it is possible to extrapolate these incidence estimates to a larger population [39]. With regard to generalising incidence, it may be preferable to collect specimens from surveillance settings such as blood donation facilities or antenatal clinics where people are not self-selected but tested in a systematic manner, and where large sample size can be obtained.

Nevertheless, it can be argued that every design of an incidence study suffers from some kind of selection bias, even longitudinal studies [11]. Moreover, studying the level of the infection among the attendees of testing sites can still provide insights over time, especially in conjunction with behavioural data.

Even more problematic seems to be the issue of a selection bias occurring if recently infected people tended to seek testing sooner than expected because of seroconversion illness or identified recent exposure. This leads to an increase in the number of detected recent infections and an overestimation of the incidence. Remis *et al.* refer to this bias as the "seroconversion effect" and proposed a way to measuring it by making different incidence estimates based on varying window periods [40]. Song *et al.* formulated the hypothesis of independence between testing and the occurrence of infection and proposed a procedure to test this hypothesis [41]. All these biases can be found when inferring HIV incidence from case-reporting of new diagnoses which also include individuals seeking testing or health care.

Finally, as it is not always possible to test the whole positive study population for recent infection, the proportion of recent infection obtained among those tested is classically assigned to those for whom a test result is not available. This extrapolation assumes that the availability of specimens for recent infection testing is randomly determined in the population.

Conclusion

Overall, the use of laboratory-based methods to estimate HIV incidence can add remarkable value to surveillance systems based on prevalence surveys or on HIV case reporting. The estimation of HIV incidence provides a clear public health benefit in that it allows better monitoring of HIV transmission and targeting of preventive initiatives. We have seen that the application of those methods in cross-sectional settings have been well described in terms of incidence estimation and limitations, one of the most important limitations being the lack of representativeness. The assumptions that must be fulfilled to correctly interpret the estimates are to a

large extent similar to those required in prevalence measurement. However, further research on the more specific aspect of window period estimation may be needed in order to generalise these methods. In particular, efforts are needed to correctly define the mean window periods for different virus subtypes and stages of infection so that the essential relation between prevalence and incidence holds true in various population settings.

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Surveillance and outbreak reports

FOUR YEARS OF SURVEILLANCE OF RECENT HIV INFECTIONS AT COUNTRY LEVEL, FRANCE, MID 2003 – 2006: EXPERIENCE AND PERSPECTIVES

Caroline Semaille (c.semaille@invs.sante.fr)¹, F Cazein¹, J Pillonel¹, F Lot¹, S Le Vu¹, R Pinget¹, J C Desenclos¹, F Barin²

1. Institut de veille sanitaire (French Institute for Public Health Surveillance), Saint-Maurice, France

2. Université François-Rabelais, Inserm ERI 19, Centre National de Référence du VIH, CHU Bretonneau, Tours, France

New systems of surveillance to better monitor the dynamics of HIV are needed. A national surveillance of new HIV diagnoses which included the collection of dried serum spots (DSS) to identify recent infections (<6 months) using an EIA-RI assay was implemented in 2003 in France. The collection of DSS is based on the voluntary participation by both patients and microbiologists. Multivariate analysis was used to identify factors associated with recent infection (RI). Between July 2003 and December 2006, 14,155 cases newly diagnosed for HIV were reported. A minority of patients refused the collection of DSS (3.3%) and the rate of participation of laboratories was 80%. The test was performed for 10,855 newly diagnosed HIV cases, the overall proportion of RI was 23.1% (95% CI, 22.3%-23.9%). The proportion of RI was higher among men who have sex with men (MSM) (42.8%) than among heterosexuals (16.3%). Among heterosexuals, it varied by current nationality: 27% among French versus 8.4% among Africans. The risk of RI was greater for MSM (aOR=1.8), those of French nationality (aOR=3.9), those with high-economic status (aOR=1.2), those tested after a risk exposure (aOR=1.4), those tested for HIV three or more times during their lifetime (aOR=2.5). The risk of RI decreased with age. A nation-wide implementation of RI monitoring is feasible. The information on RI is very useful for renewing prevention messages, particularly among population in which HIV transmission is on going, such as MSM.

Background

In most industrialized countries, HIV/AIDS routine surveillance is based on case reporting to monitor new diagnoses. Having the characteristics and trends of newly diagnosed HIV or AIDS cases is essential but not sufficient to monitor the dynamic of HIV transmission. Therefore, several countries implemented the surveillance of recent HIV infections at either regional or national level for a given period (e.g. Australia, Austria) or have started this monitoring recently (USA, Germany) [1,2]. To our knowledge, France is the only country where recent infection (i.e. infection acquired in the last six months) has been routinely monitored at the national level since 2003 among patients newly diagnosed with HIV [3].

This monitoring, defined as “virological surveillance” (VS), uses dried serum spots (DSS) taken at the same time as HIV diagnosis and notification. Here, we report the results of the first four years of this virological surveillance from July 2003 to December 2006.

We also discuss the challenges in implementing such surveillance, the possibilities to introduce it in other countries, and the ways to use its results for public health action.

Methods

Case reporting of HIV

Mandatory anonymous HIV case reporting was implemented in France in 2003, and the procedures have been described previously [4]. The following patient characteristics are collected and entered into the national database: sex, age, country of birth, current nationality, region of residency, mode of transmission, socio-professional category, clinical stage at the time of HIV diagnosis (primary infection, asymptomatic stage, symptomatic not AIDS stage, AIDS stage), number of previous HIV tests and reasons for HIV screening. In this article, we analyze new HIV diagnoses dated from 1 July 2003 to 31 December 2006 which were reported to the Institut de Veille Sanitaire (InVS), the French Institute for Public Health Surveillance, up to March 2007.

The estimated proportion of under-reporting of new HIV diagnoses in France varies from 34 to 40%, depending on the year of diagnosis (40%, 37%, 34%, 36% in 2003, 2004, 2005, 2006 respectively). Every year, the estimated proportion of under-reporting and the reporting delay are based on the comparison between the number of HIV notifications and the number of positive serology results reported by all laboratories in France [5]. The case definition of new HIV diagnosis used in both systems is similar.

In this article the proportion of under-reporting and the reporting delay are taken into account when presenting the absolute numbers of recent infections in the results part. These absolute numbers were calculated separately for each year in order to take into account the different proportions of under-reporting which varied each year.

Virological surveillance

DSS was used to determine for each new HIV diagnosis whether or not the HIV infection was recent, i.e. occurred less than six months before diagnosis. For each case, the laboratory that made the original diagnosis was asked to take DSS from the stored serum sample and send it under the patient's anonymous code to the National Reference Center (NRC) by postal mail. Results from the NRC were then sent to InVS and linked to the epidemiological data

in the HIV national database using the patient's anonymous code. Although HIV notification is mandatory, VS is based on the voluntary participation by both microbiologists and patients. The patient's consent for VS is obtained by the reporting clinician through the HIV notification form.

Immunoassay to identify recent infections (EIA-RI)

The characteristics and properties of this assay have been described previously, and are also discussed in the article from J Parry *et al.* in this special issue of Eurosurveillance [6]. Based on early evaluation of EIA-RI we estimated that this assay using DSS would be able to identify recent infections (RI) among all infected patients with HIV-1 (without AIDS) with a sensitivity of 87% and a specificity of 98%. The EIA-RI may misclassify patients at the AIDS stage as recently infected, and therefore patients known to have AIDS (information collected from the HIV reporting form) were classified as established infection whatever the result of the EIA-RI.

Statistical analysis

The chi test for trend was used to analyze the trend overtime of the proportion of recent infections among newly diagnosed HIV cases. The proportions were compared using standard chi-square tests. Variables that were significantly associated with recent infection status in the univariate analysis were entered in a multiple logistic regression model to identify factors independently associated to recent infections (using a global test). The goodness of fit was assessed by the Hosmer-Lemeshow test. All analyses were conducted with SAS® software version 08, and statistical significance was considered for p values < 0.05.

Results

Description of new HIV-1 diagnoses

Between July 2003 and December 2006, 14,155 newly diagnosed HIV cases were reported to the InVS. Males accounted for 61% of cases. More than half (53%) of the newly diagnosed HIV infections were attributed to heterosexual contact, whereas men who have sex with men (MSM) accounted for 25% of the cases (Table 1). Those infected through drug use constituted a low percentage of all cases (2%, n=306). Concerning the nationality of cases, one third (31%, n=4,383) came from sub-Saharan countries, and were mainly infected by heterosexual contact. The reasons for screening and the clinical stage at the time of HIV diagnoses varied by transmission categories. The proportion of cases that have undergone voluntary screening after an exposure is

TABLE 1

Newly diagnosed HIV cases by sex and transmission category, France, July 2003 – December 2006 (n=14,155)

Transmission category	Women N (%)	Men N (%)	Total N (%)
MSM		3,579 (41.6)	3,579 (25.3)
Heterosexuals	4,384 (79.1)	3,168 (36.8)	7,552 (53.4)
Sub-Saharan Africa	- 2,359	- 1,281	- 3,640
France	- 1,150	- 1,234	- 2,384
Other/unknown	- 875	- 653	- 1,528
Drug users	64 (1.2)	242 (2.8)	306 (2.2)
Other *	9 (0.1)	9 (0.1)	18 (0.1)
Unknown	1,084 (19.6)	1,616 (18.7)	2,700 (19.0)

MSM = men who have sex with men

* Hemophilia or transfusion recipient

greater among MSM than among heterosexuals (33% vs 19%), and it is higher among French heterosexuals than African heterosexuals (22% vs 18%). The proportion of cases newly diagnosed at the time of primary infection ('primary infection' as filled in by clinicians whatever the results of the test of recent infection) was greater among MSM than among heterosexuals (19 vs 5%).

Recent infections among new HIV-1 diagnoses

From July 2003 to December 2006, the test for RI was performed for 10,855 new HIV diagnoses. Results were not obtained for 3,300 patients either because the laboratory did not submit DSS to the NRC (2,834 cases representing 20% of all new HIV diagnoses) or the patient did not consent to participation (466 cases, 3.3%). These cases were excluded from further analysis. Among the excluded cases, the proportion of MSM and of French nationals was lower than among the cases included in the investigation (19% vs 27% and 39% vs 46%, respectively), whereas the proportion of cases with unknown mode of transmission and unknown nationality was higher than among the included cases (24% vs 18% and 16% vs 13%, respectively).

The proportions of patients who refused to participate and of laboratories that did not send DSS for analysis were stable over time.

Among the newly diagnosed HIV-1 cases that were included in the analysis, 2,511 were identified as recent with the EIA-RI test (23.1%, 95% CI= 22.3 – 23.9). After adjustment for under-

TABLE 2

Proportion of recent infections among new HIV-1 diagnoses, France, July 2003 - December 2006 (n=10,855 newly diagnosed HIV-1 cases, of whom 2,511 were identified as recent)

	Number of recent infections	Proportion of recent infections	[IC 95 %]	p*
Sex				p<10 ⁻⁶
Male	1863	27.8	[26,8 - 28,9]	
Female	648	15.6	[14,5 - 16,7]	
Age group (years)				p<10 ⁻⁶
15 - 29	756	26.0	[24,4 - 27,6]	
30-39	969	24.3	[22,9 - 25,6]	
40-49	499	21.0	[19,4 - 22,7]	
> = 50	287	18.1	[16,2 - 20,0]	
Transmission category*				p<10 ⁻⁶
Homosexual	1263	42.8	[41,0 - 44,6]	
Heterosexual	939	16.3	[15,4 - 17,3]	
Drug users	33	14.6	[10,0 - 19,2]	
Other/Unknown	276	14.3	[12,8 - 15,9]	
Current Nationality				p<10 ⁻⁶
France	1707	34.4	[33,1 - 35,7]	
Europe (outside France)	59	24.1	[18,7 - 29,4]	
Sub-Saharan Africa	285	8.4	[7,4 - 9,3]	
North Africa	40	18.6	[13,4 - 23,8]	
Other/Unknown	420	20.7	[18,9 - 22,5]	

* chi² test

TABLE 3

Factors independently associated with recent infections among new HIV-1 diagnoses. Results from the multivariate analysis
France, July 2003 - December 2006 (n=10,855 newly diagnosed HIV-1 cases, of whom 2,511 were identified as recent)

		Univariate analysis				Multivariate analysis			
	Number of subjects	OR	95%	CI	p value ^a	aOR	95%	CI	p value ^a
Sex and transmission category									
Male heterosexual	2,414	1			<0.0001	1			<0.001
Male homosexual	2,949	4.07	3.57	4.65		1.85	1.59	2.15	
Other/unknown male	1,332	1.10	0.92	1.32		0.85	0.71	1.04	
Female heterosexual	3,340	1.10	0.96	1.27		1.12	0.96	1.32	
Other/unknown female	820	0.62	0.48	0.80		0.50	0.38	0.66	
Age group (years)									
≥ 50	1,587	1			<0.001	1			<0.0001
15 - 29	2,905	1.59	1.37	1.85		1.92	1.62	2.28	
30 - 39	3,991	1.45	1.25	1.68		1.43	1.22	1.67	
40 - 49	2,372	1.21	1.03	1.42		1.12	0.94	1.33	
Current nationality									
Sub-Saharan Africa	3,405				<0.0001	1			<0.001
France	4,962	5.74	5.02	6.57		3.95	3.36	4.64	
Other/unknown foreign country	2,488	2.89	2.47	3.37		2.59	2.18	3.08	
Reasons for HIV testing									
Pregnancy & systematic screening	1,934	1			<0.001	1			<0.001
Clinical symptoms or biological data	3,677	1.51	1.31	1.74		1.20	1.02	1.40	
Exposure	2,382	2.39	2.06	2.78		1.38	1.17	1.63	
Others	1,768	1.37	1.16	1.62		0.86	0.72	1.04	
Unknown	1,094	1.49	1.24	1.80		1.16	0.93	1.43	
Professional category									
Unknown and non-professional activity	4,816	1			<0.001	1			0.014
Employee	2,079	1.70	1.51	1.92		1.10	0.95	1.26	
Blue collar	1,454	1.03	0.89	1.20		0.91	0.77	1.09	
High level staff	2,506	2.16	1.94	2.42		1.17	1.02	1.35	
Testing frequency (during the whole life)									
One HIV test	3,804	1			<0.001	1			<0.001
Two HIV tests	2,731	1.65	1.45	1.87		1.47	1.28	1.68	
Three or more HIV tests	1,474	4.42	3.85	5.08		2.51	2.16	2.93	
Unknown	2,846	2.15	1.90	2.43		1.91	1.66	2.20	
Year of diagnosis									
Second semester 2003	1,628	1			0.65				
2004	3,160	0.97	0.84	1.11					
2005	3,397	1.04	0.90	1.19					
2006	2,670	0.98	0.85	1.14					
Region of residency									
Outside Paris area	5,661	1			0.0007	1			0.14
Paris area	5,194	1.17	1.07	1.28		0.93	0.84	1.03	

^a global test, CI confidence interval

Note : Hosmer- Lemeshow statistic: $\chi^2 = 10.53$; d.f. = 8; p = 0.23

reporting and reporting delays, the number of recent infections that occurred from mid 2003 to 2006 was estimated at around 4,000. Half of these cases (estimated at 2,010) were among men who have sex with men (MSM): representing 550 to 600 MSM per year. The number of drug users recently infected was very low (52 cases over the whole period). From 2003 to 2006, the adjusted number of cases newly diagnosed and identified as recent was greater among French heterosexually infected persons (805) than among sub-Saharan Africans living in France (454 cases).

The proportion of RI was higher in MSM (42.8%) than in heterosexuals (16.3%) (Table 2). Among heterosexuals, it varied by current nationality: 27.0% among French versus 8.4% among Africans ($p<0.001$). The year of diagnosis was not associated with recent infection in the univariate analysis. In the multivariate analysis the risk of recent infection was greater for MSM (aOR=1.8), those of French nationality (aOR=3.9), those of a high socioeconomic status (aOR=1.2), those tested for HIV after a risk exposure (aOR=1.4) and those who had undergone three or more tests during their lifetime (aOR=2.5) (Table 3). However, the risk of RI decreased with age. Although the region of residency was not independently associated with recent infection ($p=0.14$), this variable was maintained in the model because it improved the goodness of fit ($p=0.23$).

Discussion

We found that a little less than one quarter of the newly diagnosed patients included in the study had been infected with HIV within the last six months. Among the newly diagnosed MSM, half had been infected recently. This is consistent with results reported in several more restricted studies: the proportion of recent infections among new HIV diagnoses was 27% in Austria in 2002-2003, 26% in Switzerland in 2005-2006, 20% in ten cities in the United States in 1997-2001, and 45% among MSM in United Kingdom in 2005, and 36% in a study which mainly involved MSM in the Victoria region of Australia in 1999-2000 [1,2,7-9].

The proportion of recent infections should be interpreted with some caution because it depends on both testing patterns and HIV incidence. This is consistent with our analysis which found that the number of lifetime HIV tests performed is strongly associated with RI, and that the chance of detecting recent infections increased with the number of tests. Similarly, people screened for HIV after a risk-exposure are more likely to be diagnosed as a recent infection (aOR=1.4) than those screened for pregnancy.

Our results indicate that the largest population diagnosed as recently infected in France is the MSM population. This may result from both a relatively high HIV incidence and a more frequent testing among MSM. These findings are supported by other sources of epidemiological data which indicate that MSM have been engaging in high-risk sexual behaviors in recent years in France: (i) increase in the proportion of unprotected anal intercourse from 19% in 1997 to 33% in 2004 (Enquête Presse Gay 1997 and 2004) (ii) outbreak of syphilis ongoing since 2000, and (iii) emergence of rectal lymphogranuloma venereum in 2004 [10-12]. Behavioral surveys have also shown that MSM are more frequently tested for HIV: half of MSM were tested during the last 12 months before the study, whereas in the general population only 11% underwent testing during the last year [10,13,14]. However, the multivariate analysis, taking into account the variable "testing frequency" has identified MSMs as the subgroup with the highest risk of being recently infected.

Current nationality was also found to be strongly associated with RI. Persons of African origin were less likely to be diagnosed as a recent infection than French and other foreign nationalities. This may reflect the fact that HIV-positive Africans living in France are mostly immigrants who could have been infected with HIV many years before in their country of origin where HIV prevalence is high, and diagnosed only recently in France. A survey conducted in 2005 among the African community living in the Paris area showed that the testing frequency in this group was higher than expected: 65% of African respondents had been screened for HIV at least once in their life, compared to 51% in the general population (in 2004) [14,15]. However, the proportion of recent infections among newly diagnosed Africans living in France which we estimated to be 8% indicates that HIV transmission also occurred in this community while living in France.

The proportion of recent infections among drug users was found to be very low, and while surveys have shown that most drug users are aware of their HIV serostatus, these results reflect the positive impact of the harm-reduction strategy implemented in France since the beginning of the 1990s [16].

Socio-professional categories associated with high economic status were also independently related to the fact of being diagnosed as recent. This may reflect both a better access to HIV screening and a better assessment of the risk of HIV infection in this well-educated population with ongoing risk behaviors.

The proportion of cases with recent infection at the time of HIV diagnosis was also found to be higher among younger age groups, which can be explained by the fact that the probability of having a recent infection at the time of diagnosis increases with shorter exposure to the risk. Also, we need to take into account that in France young people are more frequently tested for HIV than older people (17% among 18-24 years old vs 4% among 45-54 years old) [14].

Our results have shown that the proportion of RI was stable between 2003 and 2006, and in the univariate analysis the year of diagnosis was not associated with the recent infection diagnosis. In parallel, HIV screening policies did not change during this period in France, and the rate of HIV screening per 100,000 population did not vary considerably (range from 79 to 81 per 100,000 depending on years) [17].

How feasible is the implementation of monitoring of recent infections among new diagnoses in other developed countries?

Our report summarizes the results of four years of long-term national monitoring of HIV infection by combining the surveillance of recent HIV infection with HIV case reporting. To our knowledge, France is the first country to have implemented such an integrated system at a national level. This was made feasible by using an assay which could be performed on samples collected on filter paper thus making the management and the cost of recent infections monitoring reasonable. The costs were estimated at around three euros per case (including filter paper, a hermetically sealable plastic bag for transportation, reagents, and the time spent by a technician to perform the test). This amount did not include the cost of validation and data entry performed at InVS. Furthermore, a DSS can also be used to determine the group, type and subtype of the virus by a serotyping method, and to genotype the virus in order to monitor the diversity of circulating viruses more closely

[3,18,19]. The EIA-RI assay used in France can also be used in other countries. Although the technique is transferable, other assays could also be chosen (see the article of J Parry *et al.*).

The nation-wide implementation of recent infections monitoring seems feasible in other countries. It has been possible in France with the collaboration of a very high number of laboratories (around 4,300) that perform HIV diagnosis and send the DSS to the NRC. However, the project requires a lot of continuous effort to inform and encourage thousands of laboratories to participate and sustain their collaboration overtime. The participation of laboratories in the voluntary virological surveillance is good (around 80%) and it is also well accepted by the patients (only 3% do not consent to participate). Knowing that other European countries do not have so many laboratories that perform HIV testing (their numbers range from a few dozens to a few hundreds), the implementation of a similar surveillance should, therefore, be more feasible than in France.

When starting the project we also had to resolve the ethical issue of informing or not the patients and their physicians about the patient's RI status. Considering that: a) the test for RI was designed for public health purposes and not for establishing an individual diagnosis as the positive predictive values are not high enough for diagnostic purposes; b) the information flow within the HIV notification channel is anonymous by law whereas giving back the results would mean maintaining correspondence between the anonymous code and the name of the patient; c) it is not clear whether the result (recent infection or not) would have an impact on the individual health since there is non consensus yet on the long term benefit of HAART during the early months of infection and contact tracing is not done in France; it was decided, after a collective discussion with patient associations and clinicians, not to inform either patients or physicians. Although this question was still being discussed at the time of implementation of the system, it ceased to be controversial when the first results of virological surveillance were reviewed with clinicians and patient associations and published in December 2003.

What is the impact of these results in terms of public health?

Nearly half of MSM newly diagnosed with HIV (43%) were shown to have been recently infected. Subsequently, these findings were actively communicated to the gay communities in France and had a major impact. The feedback of this group to associations for the fight against AIDS is critical. Moreover, the findings were used in several prevention campaigns and prompted the Ministry of Health to renew the prevention messages. Nevertheless, the extent of HIV transmission in MSM remains alarming, illustrating the difficulty to target and sustain prevention in this usually well-educated population. However, the high proportion of RI also indicates that screening strategies have been effectively adopted by the gay community: MSM more often than other groups undergo testing for HIV soon after a risk exposure. The data on reasons for screening show that the proportion of those who undergo voluntary testing after an exposure is higher among MSM than among heterosexual individuals (33% vs 19%). Therefore, the proportion of RI among MSM could also be an indicator for screening patterns among this population that would be interesting to monitor.

Although we showed that the proportion of recent infections among the newly diagnosed HIV cases of African origin is much lower than among the general population, our results indicate that

HIV transmission in this group also occurs after arrival in France. In addition, we found out that one out of five newly diagnosed HIV cases of African origin were infected by subtype B, although this subtype is not common in Africa (data not shown in this article) [3,17]. The combination of these two results (proportions of RI and subtype B) has induced the Ministry of Health to adapt its policy regarding the African community living in France and encourage HIV screening and prevention within this population.

While the incidence of HIV infection has been previously estimated in defined risk groups such as prostitutes, IDUs, MSM attending STI clinics, this has not been done at the country level [9,20,21]. We are currently working on assessing the HIV incidence at the country level by combining, through mathematical modeling, the results of the test for recent infection with other factors such as screening patterns (see the article by S Le Vu *et al.* in this special issue of *Eurosurveillance*).

Conclusion

The information on recent infections is very useful for renewing prevention messages, particularly among populations in which HIV transmission is still ongoing, such as the gay community in France, and for promoting HIV testing among populations in which few recent infections have been identified. An overview of new testing strategies is ongoing in France in order to better define the use of the rapid HIV test on whole blood or serum samples, notably in a community context.

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Research articles

SETTINGS FOR IDENTIFYING RECENT HIV INFECTIONS: THE PORTUGUESE EXPERIENCE

H Cortes Martins (helena.cortes-martins@insa.min-saude.pt)¹, M T Paixão²

1. Laboratório de Referência da SIDA, Departamento de Doenças Infecciosas, Instituto Nacional de Saúde (AIDS Reference Laboratory, Infectious Diseases Department, National Institute of Health), Lisbon, Portugal

2. "Centro de Vigilância Epidemiológica das Doenças Transmissíveis", Departamento de Doenças Infecciosas, Instituto Nacional de Saúde (Communicable Diseases Surveillance Centre, Infectious Diseases Department, National Institute of Health), Lisbon, Portugal

Portugal has been the western European country with the highest rate of notified acquired* immunodeficiency syndrome (AIDS) cases since 1999 and human immunodeficiency virus (HIV) infection cases since 2000. Nonetheless, exact information on the magnitude and trends of recently acquired infections is missing. In a cross-sectional study we aimed to determine HIV prevalence, the proportion of recently acquired infections and the incidence among patients attending a Sexually Transmitted Infections (STI) clinic and among HIV positive cases tested at the AIDS Reference Laboratory (ARL), by using the Avidity Index (AI) of antibodies to identify recent HIV-1 seroconversions. Demographic and behavioural data were collected. At the STI clinic 253 patients were enrolled, 16 were found to be HIV infected (14 HIV-1, 2 HIV-2) and a prevalence of 6.3% was obtained. Four recent HIV-1 infections were identified and the HIV-1 incidence was 3.3% per year. At the ARL, 332 newly diagnosed cases of HIV-1 infection were studied, 59 (17.8%) were recent infections and an annual incidence of 4.1% was estimated. These findings support STI clinics as key sentinel sites for recently acquired HIV infections and illustrate the viability of testing for recent HIV infections in these settings and reinforce the value of this method in the surveillance for better monitoring current trends of the HIV/AIDS epidemic in Portugal.

Introduction

Portugal is the western European country with the highest rate of reported acquired immunodeficiency syndrome (AIDS) and newly diagnosed cases of human immunodeficiency virus (HIV) infection according to the EuroHIV end-year report for 2006 [1]. However, the proportion of recently acquired infections is unknown and information on trends is missing. After a decade (1990 to 2003) during which the epidemic was led by cases of HIV infections linked to intravenous drug use (IDU), the proportion of reported cases associated with sexual transmission (homosexual, bisexual and heterosexual) has progressively grown, accounting for over 60% since 2003, with heterosexual transmission currently being the driving force of the epidemic [2].

Similar to other countries, prevalence studies are used in Portugal for the epidemiological assessment of HIV infection as they provide relevant information necessary for the planning of health and social support services. Nevertheless, prevalence does not distinguish between infections acquired recently, i.e. in the

past six months and established infections acquired more than six months ago, and the picture of recent trends of the epidemic is less clear compared to that obtained by incidence studies. The fact that longitudinal studies to calculate incidence are expensive, time-consuming and difficult to perform is well known [3-5]. Alternative approaches to estimate the incidence of HIV infections have been used worldwide [4, 5] and the interest in cross-sectional laboratory based studies has grown in the past decade. Different methods, based on various properties of maturing antibodies for HIV-1, that allow the identification of recent seroconversion have been described and were used for this purpose [5-10]. These laboratory methods, applied to a single serum sample for each HIV-1 infected case, allow the distinction between recently acquired and established HIV-1 infections. The avidity index (AI) of HIV-1 antibodies has been used for this purpose based on the principle that antibodies produced in the early phase of infection show a low avidity for the antigen [7]. Therefore, a low avidity is likely to indicate a recent infection.

In Portugal, the first cross-sectional study in which recent HIV-1 seroconversions were identified was performed in a group of IDUs entering a low threshold methadone programme for the period of one year [11]. Of those 24.5% were found to be HIV-1 infected and among them 18.4% were infected recently. HIV-1 incidence was estimated 7.2 % per year. These results encouraged us to explore other settings where we expected to identify recent HIV-1 infections and to collect useful epidemiological information.

Sexually transmitted infections (STI) are known to be associated with higher transmission or acquisition rates of HIV and patients referred to STI clinics are usually at increased risk for HIV infection [3]. STI clinics were identified as important settings for measuring the prevalence of HIV infection [12] and can also be important sites for estimating incidence. In Portugal STI clinics are rare. However, in Lisbon, a unique drop-in STI clinic exists at a primary healthcare facility, that provides free of charge same day appointments with specialists, laboratory diagnosis of STI (including HIV), treatment for diagnosed STI and counselling for risk reduction. Attending patients are mainly from the Lisbon area, self-presenting or referred by other primary healthcare services.

Laboratory based surveillance data on the number of new HIV diagnoses per year are available in some European countries such as France, allowing for studies to identify recent infections to be conducted [12,13]. However, such a system does not exist in Portugal where notification of HIV/AIDS cases has always been performed by clinicians, with limited laboratory information. In Lisbon, the AIDS Reference Laboratory (ARL) confirms over 300 new cases of HIV infection per year from various population groups, including IDUs, prison inmates and pregnant women.

The aim of our study was to identify population groups from specific settings where recent HIV infections are likely to be detected, to generate data on HIV prevalence and on the proportion of recent HIV-1 infections and to estimate the incidence of HIV-1 infection.

Methods

Study design and population

To achieve the aim of our study two settings, an STI clinic and the ARL, were selected as settings where recent HIV infections are likely to be detected.

STI clinic

A cross-sectional study was performed in a group of attendees at the Centro de Saúde da Lapa STI clinic, in Lisbon. Between February and August 2004, enrolment in the study was proposed by clinicians to all first time attendees and other patients eligible for HIV testing (i.e. patients reporting risk behaviour). Participation was voluntary and anonymous. Written informed consent was obtained from all participants and archived in the patient's clinical file. One blood sample was collected from each participant at the time of enrolment and data on demographics, behaviour and clinical condition were anonymously recorded by clinicians for each case.

AIDS Reference Laboratory

A cross-sectional study was also performed using anonymised serum samples from cases newly diagnosed with HIV-1 infection at the ARL during the year 2005. Demographical and behavioural data were collected of all cases selected for the study. Second time testers were excluded.

Laboratory methods

Prior testing for identification of HIV infection was necessary for samples from the STI clinic. Pools of five sera were prepared with an input of 100 µL for each sample and each pool was screened simultaneously for HIV-1 and HIV-2 antibodies using the third generation enzyme immunoassay AxSYM® HIV 1/2 gO (Abbott Diagnostics Division, Germany). Sera from pools with a positive result were individually tested using the same kit. Samples identified as reactive were further tested for confirmation using the HIV Blot 2.2 (Genelabs Diagnostics, Singapore), a Western blot assay for HIV-1 antibodies also harbouring a peptide for identifying HIV-2 antibodies. Samples reacting with this peptide were additionally tested with New LAV Blot II (Bio-Rad, France), a Western blot assay to confirm the presence of HIV-2 specific antibodies. All tests were performed according to the manufacturer's instructions.

Identification of recently infected HIV-1 cases

In HIV-1 positive sera the AI of the antibodies was determined following the method described by Suligoi et al [7] in order to identify recently acquired HIV-1 infections, i.e. seroconversions occurred six months or less prior to blood collection. For each

sample a pre-test dilution of 1:10 was prepared on two aliquots of 50 µL: one with phosphate-buffer saline (PBS - PBS aliquot) and a second with 1M guanidine solution (G aliquot). After incubation at room temperature, both aliquots were tested with the AxSYM® HIV 1/2 gO (Abbott Diagnostics Division, Germany) assay following the manufacturer's instructions. The sample's AI was determined by the ratio between the results obtained for guanidine aliquot and buffer aliquot using the formula: $AI = (S/CO \text{ of the G aliquot}) / (S/CO \text{ of the PBS aliquot})$. S/CO standing for sample/cut-off value obtained in the test. The 0.8 cut-off for the AI was used to differentiate between recent ($AI < 0.8$) and established ($AI \geq 0.8$) cases of infection [14].

Epidemiological methods and concepts

Prevalence

For both settings, the STI clinic and the ARL, the proportion of HIV infections overall and by type of virus, as well as the proportion of recently acquired HIV-1 infections was determined.

Incidence

A cross-sectional approach was applied and six months was the window period assumed for the AI test used to identify recently acquired HIV-1 infections. The concept for the estimation of incidence [5, 6, 10] using data obtained from cross-sectional study at both sites is based on the following assumptions:

- cases identified as negative were also negative six months before blood sampling;
- cases identified as recently infected were negative six months before blood sampling;
- cases identified as established infection were positive six months before blood sampling;

The rate of seroconversion in the six months before blood sampling is obtained by the ratio between the number of recent infections and the number of susceptibles multiplied by two to obtain the annual incidence.

The following formula was used to estimate incidence in both groups:

$$\text{HIV 1 incidence (\%)} = \frac{N_r}{N_{\text{neg}} + N_r} \times 2 \times 100$$

N_r Number of recently infected HIV cases

N_{neg} Number of cases with a negative HIV test result

Statistical methods and data analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS) program for Windows, version 12.0. Descriptive analysis was performed for each variable, namely frequencies and proportions. Mean age values were compared using the t test for independent samples. Data from different subgroups were compared using two tailed Fisher's exact test or chi-square independence test. Results with a p value < 0.05 were considered as being statistically significant. Odds Ratios (OR) and their 95% confidence interval (CI) were calculated in order to measure the strength of the associations found.

Results

STI clinic group

A total of 253 participants, 143 men (56.5%) and 110 women (43.5%), were enrolled in the study. The age ranged from 16 to

70 years, with a mean of 31.5 years (95% CI: 30.3-32.8) and a median of 28 years.

The majority of participants (90.9%) stated having only heterosexual contacts, 35.5% had more than one sex partner in the previous six months and only 15.9% declared to always use condoms in sexual contacts with unknown partners. Additional risk behaviours for HIV infection such as illicit drug use (not specified) and prostitution were acknowledged by 13 participants. Of all participants 45.1% had never been tested for HIV. Clinical data showed that, at time of enrolment, an STI other than HIV was diagnosed in 87 cases, with a high proportion of viral infections (43.5%). The majority of cases (81.3%) had no previous STI history.

Sixteen cases were found to be HIV infected 14 with HIV-1 and two with HIV-2. An overall HIV prevalence of 6.3% (95%CI: 3.3-9.3) was obtained. Type-specific prevalence was 5.5% for HIV-1 infection and 0.8% for HIV-2 infection. Prevalence by sex and sexual orientation showed a higher value in men than in women, 7.0% and 5.4% respectively and a high prevalence of

18,2% for homo/bisexual men. The comparative analysis between characteristics of HIV-positive and -negative cases is shown in Table 1.

Increased risk for HIV infection was found for homosexual/bisexual clients (OR = 3.33; 95%CI: 1.17-9.49), for those who had five or more sex partners in the previous six months (OR = 4.69; 95%CI: 1.70-12.82) and for those with an STI history (OR=2.91; 95%CI: 1.09-7.77). Although difference was not statistically significant, mean age in the HIV-positive subgroup (34.8 years) was higher than in the HIV-negative subgroup (31.3 years).

The AI of antibodies determined for the 14 HIV-1 cases ranged between 0.33 and 1.06 with a mean value of 0.85. Using 0.80 as cut-off value we were able to identify four recent HIV-1 infections. HIV-1 incidence in this group, as defined for the purpose of the study, was estimated to be 3.3% per year. No independent statistical associations were found between recent infections and the study variables.

TABLE 1

Comparative analysis of characteristics of HIV positive and HIV negative cases and characteristics of recent HIV-1 infection cases studied at the sexually transmitted infection (STI) clinic, Portugal, February to August 2004, (n=253)

Characteristics	HIV Antibodies			P ^b	Odds-Ratio (95%CI)	HIV-1 Recent Infection ^a		P ^c
	Tested*	HIV Positive				n [†]	%	
	N	n	%					
Sex								
Males	143	10	7.0	0.796	1.30 (0.46-3.70)	3	30.0	1.000
Females	110	6	5.4			1	16.7	
Age group								
<30 years old	144	6	4.2	0.122	0.43 (0.15-1.22)	0	--	0.221
>= 30 years old	109	10	9.2			4	40.0	
Sexual orientation								
Homo/bisexual	23	4	17.4	0.045	3.33 (1.17-9.49)	2	50.0	0.520
Heterosexual	230	12	5.2			2	16.7	
Number of sexual partners (prior 6 months) †								
>= 5 partners	16	4	25.0	0.015	4.69 (1.70-12.82)	1	25.0	1.000
< 5 partners	225	12	5.3			3	25.0	
Condom use								
Always	40	5	12.5	0.147	2.61 (0.86-7.97)	2	40.0	0.580
Occasional / Never	212	11	5.2			2	18.2	
Additional risks for HIV infection								
No	239	14	5.9	0.196	0.34 (0.07-1.70)	3	21.4	0.505
Yes	13	2	15.4			1	50.0	
Prior HIV test								
Yes	139	8	5.8	0.797	1.22 (0.47-3.14)	3	37.5	0.559
No	114	8	7.0			1	12.5	
Prior STI history								
Yes	47	6	12.8	0.040	2.91 (1.09-7.77)	1	16.7	1.000
No	205	9	4.4			2	22.2	
STI other than HIV diagnosed at enrolment								
No	166	12	7.2	0.588	1.57 (0.52-4.74)	3	25.0	1.000
Yes	87	4	4.6			1	25.0	

* Information displayed for those where available

Note: CI - confidence interval

a - Among HIV-1 antibody positive cases

b - Fisher's exact test for associations between characteristics and HIV antibodies status

c - Fisher's exact test for associations between characteristics and HIV-1 Recent Infection status

† Only cases with one or more partners

AIDS Reference Laboratory group

During 2005, 372 (11.8%) of the 3,159 individuals tested for HIV at the ARL had a positive test result. Western blot testing of these positive samples revealed 360 HIV-1 (11.4%) and 12 HIV-2 (0.4%) infections. We studied 332 HIV-1 infections from the 336 cases found to be first time diagnoses. In this group, whose main characteristics are summarised in Table 2, females accounted for 83 (25.0%) cases and males for 245 (73.8%) cases, for four cases information on sex was missing. The age ranged from 17 to 85 years, with a mean of 35.5 years (95%CI: 34.2-36.69) and a median of 33 years. The majority (75.6 %) of cases lived in the Lisbon district.

Data on behavioural risk towards HIV infection were missing in 145 (45.7%) cases. Available information showed that, of 187 cases, sexual risk was present for 29.9% (n=56), in 12 cases associated with homo/bisexual contacts and in 44 (78.6%) with heterosexual contact. Drug use was mentioned in 70.1% (n=131).

After testing 332 samples to determine the AI, values obtained ranged from 0.24 to 1.09, with a mean value of 0.89. Cut-off value of 0.80 was applied and 59 (17.8%) cases were identified as recent HIV-1 seroconversions. No statistical association was found between recent HIV-1 infections and study variables. Even though the difference is not statistically significant, the proportion of recent infections was higher in females than in males (24.1% versus 15.5%). Estimated annualised incidence of HIV-1 infection among cases tested at the ARL in 2005 was 4.1%.

Discussion

Our study illustrates the first application of a cross-sectional approach to identify recent HIV-1 infections and estimate HIV-1 seroincidence in a group of attendees at a Portuguese STI clinic and in a group of newly diagnosed HIV infected cases detected at the AIDS Reference Laboratory in 2005. Recent HIV-1 seroconverters (less than six months) were identified based on the AI of antibodies.

The STI clinic group consisted predominantly of young, sexually active, heterosexual individuals. The fact that a very low proportion (15.9%) of participants stated to use condoms consistently in sexual intercourse with unknown partners and 45.1% had never been tested for HIV may derive from a lack of awareness to recognise the risk of contracting an HIV infection through unprotected sex. In the STI group authors did not only identify cases of HIV-1 infections (5.5%) but also HIV-2 (0.8%) cases, which mirrors the pattern of HIV infection in Portugal [2]. The overall prevalence rate of 6.3% and the 18.2% prevalence rate for homo/bisexual men are among the highest figures published [12]. A higher prevalence of HIV infection in homo/bisexual individuals than in heterosexuals without additional risk behaviour has been described in most European countries [12, 15]. The increased risk for HIV infection found in homo/bisexual participants is therefore consistent with the literature. Our data also show that a high number of sex partners increase the risk of HIV infection. The presence of STI indicates a risky sex behaviour that can lead to HIV acquisition or transmission. Accordingly, for the cases included in this study, a history of STI was also found to be an increased risk factor for HIV infection.

The determination of the AI enabled the identification of recent HIV-1 infections among HIV-1 infected participants. The proportion of recent HIV-1 infections (28.6%) identified in this study and the estimated incidence (3.3% per year), are similar to the highest values observed in published studies [3,16,17]. Nevertheless, caution is needed when comparing results obtained with different laboratory methods used to study other population groups as the window period varies from test to test and consequently the proportion of cases classified as recent.

The HIV-1 seropositive group assessed at the ARL consisted mainly of young individuals (median age 33.0 years) who were predominantly male (73.8%) and the majority of those with available information on risks were drug users (70.0%).

The high prevalence of HIV-1 (11.8%) infection in the ARL group may be associated with the fact that reference laboratories are likely to confirm more infected cases than other clinical laboratories. The proportion of drug users in this group and a 12 to 19% prevalence of HIV-1 infection described in Portuguese IDUs [18] may as well influence the result.

The proportion of recent HIV-1 infections (17.8%) found is lower than the one recently described in France [19] for newly diagnosed cases of HIV infection (24.9%). Possible explanations for this are different testing policies or rates, awareness of HIV risk, sample size and the use of different laboratory tests. The assessment of our group focused on cases of HIV-1 infection cases nonetheless incidence could be estimated since denominator was known, the result being a high value (4.1%).

TABLE 2

Comparative analysis between characteristics of recent HIV (n=59) and established HIV-1 infection cases (n=273) identified at the AIDS Reference Laboratory, Portugal, 2005

Characteristics	HIV-1 Infections				p*
	Recent		Established		
	n	%	n	%	
Sex					
Males	38	15.5	207	84.5	0.195
Females	20	24.1	63	75.9	
unknown	1	25.0	3	75.0	
Age group (years old)					
≤25	7	19.4	29	80.6	0.506
26 - 30	17	27.9	44	72.1	
31 - 35	10	15.4	55	84.6	
36 - 40	7	15.2	39	84.8	
41 - 50	8	16.3	41	83.7	
51 - 60	1	7.7	12	92.3	
≥ 61	1	11.1	8	88.9	
unknown	8	15.1	45	84.9	
Origin of HIV test request					
Anonymous free test site	2	8.3	22	91.7	0.317
Prison clinical services	5	12.8	34	87.2	
External laboratories	7	13.2	46	86.8	
General practitioner	21	19.1	89	80.9	
Methadone programme	24	22.6	82	77.4	
Risk behavioural for HIV infection					
Sexual – Homo/bisexual	1	8.3	11	91.7	0.141
Sexual – Heterosexual	9	20.5	35	79.5	
Drug use	30	22.9	101	77.1	
unknown	19	13.1	126	86.9	

* χ^2 test

We used the AI described by Suligoi et al. [7, 14] for identification of recent infections taking into account our previous experience, the availability of reagents and equipment, as well as being aware that the results would not be affected by disease, clinical stage or antiretroviral therapy [7]. Even if not adequate for individual and clinical use, this method has been found suitable for epidemiological studies, based on its sensitivity and specificity when the 0.8 cut-off value is used [14]. Also, performance with non-B subtypes of HIV was recently assessed and similar results have been obtained [20]. This fact is of the utmost importance for using the method in Portugal where a high proportion of newly diagnosed patients carry non-B subtype viruses [21]. Although other methods for testing for recent HIV infections have been described, most of them are not available on the market and further constraints to their application have been clearly identified [10].

Pooling sera for HIV seroepidemiological surveys has been used before [22-24] and, due to economical reasons, this method was applied for the STI group. The amount of sera per pool is critical when looking for recent infections and our choice of using five was based on published data [22] where six samples per pool was the minimum format assessed. Even though the sensitivity of HIV tests has increased since 1993 we decided to pool five samples because STI patients are generally at higher risk towards contracting HIV infections and HIV-2 is also prevalent in our country.

There are several limitations and biases for this study: the voluntary participation in the STI clinic group and the fact that patients attending STI clinics are at high risk for HIV infection; the fact that reference laboratories are more likely to register a higher proportion of positive cases and detailed behavioural data are rarely collected in the laboratory setting and in our case were frequently missing in the ARL group. All these factors are likely to influence our results and need to be considered in the interpretation. However, it is the higher risk of the STI clinic patients that enables this population to serve as a sentinel for the wider community.

Conclusion

We were able to determine the prevalence of HIV infections and the proportion of recent HIV-1 infections and estimate an incidence for both groups. Determining the AI for identification of recent HIV-1 infections is possible and easy using a simple and automated method based on commercially available reagents. A high prevalence for HIV infection was found in both of our study groups at an STI clinic and the ARL. Detection of recent HIV-1 infections provides evidence of current transmission. The estimated incidences should represent a baseline for further assessments to enable temporal trends analyses in those settings. Due to the nature of our study which uses a convenience sample, the results can not be extrapolated to other similar health care settings or the general population in Portugal. Surveillance for recent HIV infections with serological methods is feasible and desirable for better monitoring current local trends of the HIV/AIDS epidemic.

* Erratum: The original phrase "Portugal has been the western European country with the highest rate of notified autoimmune deficiency syndrome (AIDS) cases..." was corrected on 12 December 2008 to read "Portugal has been the western European country with the highest rate of notified acquired immunodeficiency syndrome (AIDS) cases..."

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Perspectives

COUNTRY-WIDE HIV INCIDENCE STUDY COMPLEMENTING HIV SURVEILLANCE IN GERMANY

J Bätzing-Feigenbaum (baetzing-feigenbaumj@rki.de)¹, S Loschen², S Gohlke-Micknis¹, R Zimmermann¹, A Herrmann¹, O Kamga Wambo³, C Kücherer², O Hamouda¹

1. HIV/AIDS and STI Unit, Department for Infectious Disease Epidemiology, Robert Koch-Institute, Berlin, Germany

2. HIV Variability and Molecular Epidemiology, Robert Koch-Institute, Berlin, Germany

3. Postgraduate Training Applied Epidemiology (PAE), Department for Infectious Disease Epidemiology, Robert Koch-Institute, Berlin, Germany

Serological methods exist that allow differentiating between recent and long-standing infections in persons infected with HIV. During a pilot study in Berlin between 2005 and 2007 methodologies have been evaluated. In a cross-sectional study blood samples, demographic, laboratory, clinical and behavioural data based on a KABP survey were collected from patients with newly diagnosed HIV infections. The BED-CEIA was used to determine recency of infection. Recent HIV infections contributed 54% (CI [95%]: 45; 64) in MSM and 16% (CI [95%]: 0; 39) in patients with other transmission risks ($p=0.041$). Proportions of recent infections were significantly higher in MSM ≤ 30 years ($p=0.019$). The mean age was 33.9 (median 34 years) in recent compared with 38.6 years (median: 38 years) in long-standing infections ($p=0.011$). High-risk behaviour indicated through very low condom use in recently HIV infected MSM could be identified. The results of the pilot study support expectations that the modified application of the method may contribute to improving HIV prevention efforts in Germany. On this basis the Robert Koch Institute implemented a countrywide HIV incidence study to complement HIV surveillance in early 2008. The study is funded by the German Ministry of Health. Data on recent HIV infections and current HIV transmission risks are collected. Design, methods and impact are described in detail.

Background

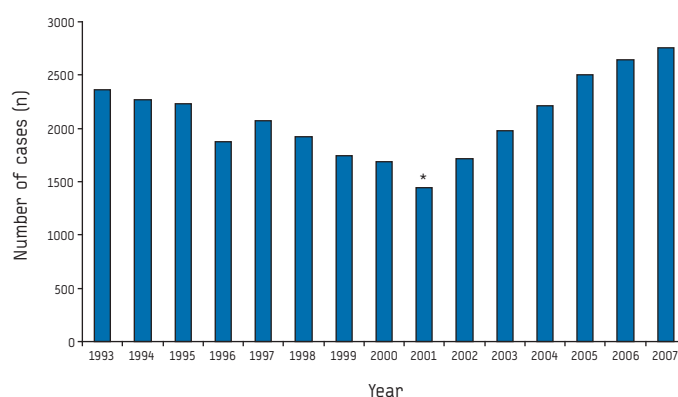
In Germany newly diagnosed human immunodeficiency virus (HIV) infections reached a peak of 2,360 cases in 1993. The number of cases reported to the Robert Koch Institute (RKI), the institution responsible for the national surveillance of infectious diseases in Germany, dropped continuously in the second half of the 1990s, reaching the lowest level so far in 2001 with 1,443 cases. However, since 2001 this trend has been reversed and annual case reports increased to more than 2,750 cases in 2007 [1; Figure 1]. There are several possible explanations for these changes: an increase in HIV transmission ("true" incident infections); improved (earlier) case detection and reporting following the implementation of the "Protection against Infection Act" (Infektionsschutzgesetz - IfSG) in 2001; an increased number of HIV tests performed; changing attitudes towards HIV testing; and more widespread availability of testing facilities and better access to these facilities. The limited data available suggest that the increase in HIV cases is partly due to a rising willingness to test for HIV in groups with a high risk of transmission [2]. The higher number of HIV tests (ELISA and Western blot) performed in German laboratories when comparing the

year 1999 to 2004 and the augmented use of HIV-NAT in primary HIV diagnosis additionally indicate changes regarding HIV testing [3]. The rising number of cases reported between 1996 and 1997 may reflect increased testing for HIV following the implementation of highly active antiretroviral treatment (HAART). Recently the upwards trend in syphilis cases reported in Germany was discussed as a possible cofactor for increased HIV transmission in men having sex with men (MSM) [1]. However, the implications of these trends have not yet been analysed systematically.

The proportion of reported HIV cases without information on the underlying transmission risk decreased from 42% to 13% between 1993 and 2007, primarily reflecting amendments concerning case reporting [1]. In the same period the proportion of cases in MSM increased from 48% to 65%, whilst the proportion of cases with intravenous drug use decreased from 18% to 6%. Heterosexual transmission was constant at around 15-20%; persons originating from high prevalence countries (HPCs) as transmission risk for HIV contributed 11% of the total in 1993 and in 2007, with a peak of 25% in 2002 [1,2].

FIGURE 1

Number of newly diagnosed HIV cases reported in Germany, 1993 – 2007 ($n=31,404$)



* implementation of the "Protection against Infection Act" (Infektionsschutzgesetz - IfSG)

Standard reports of newly diagnosed HIV infections do not permit the differentiation between recently acquired (incident) and long-standing (prevalent) infections, since routinely applied serological HIV tests (screening and confirmatory tests) do not provide such information. The diagnosis of an HIV infection can be delayed by up to several years and the time between infection and diagnosis may be a number of years and vary considerably, thus estimating incidence rates accurately and effectively is difficult. However, incidence estimates are fundamental to understanding the current dynamics of the HIV epidemic.

Several other methods have proved suitable for the identification of recent (incident) HIV infections in patients with newly diagnosed HIV infections. The concept of recent infections in HIV usually covers a period up to six months prior to the diagnosis depending on the diagnostic assay used [4-9]. Testing for recent HIV infections was implemented as an additional component (anonymous and unlinked) of the national HIV surveillance systems in France [10,11], Switzerland [12] and in 22 federal states of the United States of America [13] and was used in selected population groups at risk for HIV infection in the United Kingdom and South Africa [14,15]. Collection of additional data on knowledge, attitudes, behaviour and practices (KABP survey) concerning HIV from patients identified as recently infected with HIV permits analysis of risks and protective factors effective in HIV transmission. Subpopulations at increased risk for acquiring HIV and with limited access to diagnostic services can be identified by comparing KABP data between risk groups.

After encouraging results from a pilot study in Berlin, a nationwide study including, testing for recent HIV infections and a KABP survey was started in Germany in March 2008. The study aims to provide a better picture of the current dynamics and drivers of the HIV epidemic based on incidence estimates. The results are expected to help amend the national prevention strategies.

Pilot Study in Berlin 2005-2007

A pilot study conducted in Berlin from 2005 to 2007 assessed the feasibility of the methodologies described above and the impact of the results for future HIV surveillance in Germany. The design was cross-sectional with voluntary sampling after obtaining patients' written informed consent. Sampling was anonymous and unlinked with no particular risk group being targeted. Exclusion criteria were clinical stage C HIV infection according to the US Centres for Diseases Control and Prevention (CDC) classification [16] and antiretroviral treatment. Clinicians in specialised private practices and clinic outpatient departments (OPD) collected venous blood and clinical data from adults aged 18 years or older with newly diagnosed HIV infections. Twenty of nearly 50 HIV-specialised facilities agreed to participate in the study. To determine a recent HIV infection the blood samples were tested using the BED-CEIA, one of the methods able to detect recent HIV infections serologically in patients with confirmed HIV diagnosis [17]. The BED-CEIA was established using a German HIV seroconverter sample panel with known time of seroconversion. Optimal cut-offs separating recent and long-standing samples in the reference panel were found with an optical density (ODn) of ≤ 0.8 for the BED-CEIA and duration of infection of 20 weeks [18]. KABP data with regards to HIV/AIDS were collected through patients' questionnaires. Test results were not delivered to the patients.

Results

Of 132 cases sampled, 114 were included in the study, 18 did not meet the eligibility criteria.

The 132 cases represent 27% of all newly diagnosed HIV cases reported to the RKI from the Federal State of Berlin during the study period between November 2005 and February 2007 ($n=495$). The total number of cases from Berlin accounted for 15% of all notifications from Germany. As far as data were available, all patients included had HIV-1 subtype B infections. Of the 114 cases meeting the eligibility criteria for the study, 102 were MSM (89%) and 12 had other HIV transmission risks.

Proportions of recent out of newly diagnosed HIV infections were found to be 54% in MSM (95% Confidence Interval (CI): 38-56) and 16% (95% CI: 32-0) in patients stating other risks. Proportions of recent infections were significantly higher in MSM ≤ 30 years ($p=0.019$), mean age was 33.9 (median 34 years) in patients with recent and 38.6 years (median: 38 years) in patients with long-standing infections ($p=0.011$). Symptoms of acute seroconversion correlated significantly with recent HIV infections ($p=0.009$). Mean viral load (VL) was significantly higher in recent HIV infections compared with long-standing infections (1,608,801 copies/ μ l and 141,951 copies/ μ l, respectively, $p=0.009$). A correlation was also found between recency of HIV infection and CD4 cell counts: counts $>500/\mu$ ml were identified in recent HIV infections and counts $\leq 200/\mu$ ml in long-standing infections; however, this correlation was not statistically significant ($p=0.08$).

Patients recruited for the pilot study showed a selection bias with samples from MSM being overrepresented (72% MSM in all cases reported from Berlin compared with 89% in the study sample). However, comparison of basic demographic variables in case reports of MSM from Berlin and MSM in the Berlin pilot study sample did not show statistically significant differences within the study period. High-risk behaviour indicated through very low condom use in recently HIV-infected MSM could be identified: $>90\%$ did not use condoms during sexual intercourse in the six months prior to HIV diagnosis and 19% stated that they did not use condoms despite being aware that their sexual partner had tested positive for HIV [19].

Conclusions

We were not able to produce incidence estimates since essential denominators are currently not available in Germany. Nevertheless, the results of the pilot study support expectations that the modified application of the method will contribute to amending and improving HIV prevention efforts in Germany.

National HIV Incidence Surveillance Programme 2008 - 2010

Since November 2007 the RKI initiated a nationwide study funded by the German Ministry of Health (BMG) to collect data on recent HIV infections and current HIV transmission risks. The results are expected to complement the available data on HIV from the general surveillance by identifying subpopulations presently at increased risk for acquiring HIV infections and the risks most recently having an impact on HIV transmission in Germany.

Design and methods

To obtain the desired information a cross-sectional unlinked anonymous study, with a case control component will be conducted from 1 March 2008 to 28 February 2010. Samples and data are collected over this period through either laboratories or specialised

clinical centres. Information on screening patterns for all cases is gathered in both the laboratory and clinical study arm. As data from the two study arms cannot be linked, overlapping of sampling from patients in both study arms cannot be excluded.

Laboratory study arm Collaborating Institutions

Newly diagnosed HIV cases in Germany are reported to the RKI by more than 200 laboratories. Only 36 labs, however, contribute significant numbers to the reporting of newly diagnosed HIV infections (significant defined as providing each at least 1% of the total number of cases reported nationally). These 36 labs are responsible for almost 70% of all reported newly diagnosed HIV cases in Germany, with the remaining approximately 170 labs reporting another 30%. All 36 laboratories reporting high numbers of HIV infections agreed to participate in the national HIV incidence study (exhaustive sampling). Thirty-five of 51 randomly selected laboratories with HIV case reporting on a smaller scale also agreed to participate (random sample). Thus, a total of countrywide 71 laboratories will constitute the laboratory study arm.

Methods

Participating laboratories will collect plasma or serum samples from all newly diagnosed HIV cases during the study period. Samples are provided as "Dried Plasma Spots" (DPS) or "Dried Serum Spots" [20] and sent every month to the project group HIV Variability and Molecular Epidemiology at the RKI. All samples are tested for recency of HIV infection using the BED-CEIA. Clinical data are limited to information reported according to the national HIV surveillance regulations [21]. Data will allow to estimate recent HIV infections and incidence proportions by using basic demographic data and to analyse the risks to acquire an HIV infection. Data collected in this study arm are expected to be representative for Germany. The sample size is expected to include 1,600 cases annually representing around 60% of all new HIV diagnoses.

Clinical study arm Collaborating Institutions

Over 80 clinical facilities specialised in HIV diagnosis and care from six regions in Germany will participate in the clinical study arm. The regions selected include those reporting the highest HIV case numbers nationally since 2001 (Figure 2) and they are characterised by a concentration of medical facilities specialised in HIV care compared with other regions. These facilities include private practitioners, clinic OPDs and counselling centres run by local health authorities or non-government organisations (NGO).

Methods

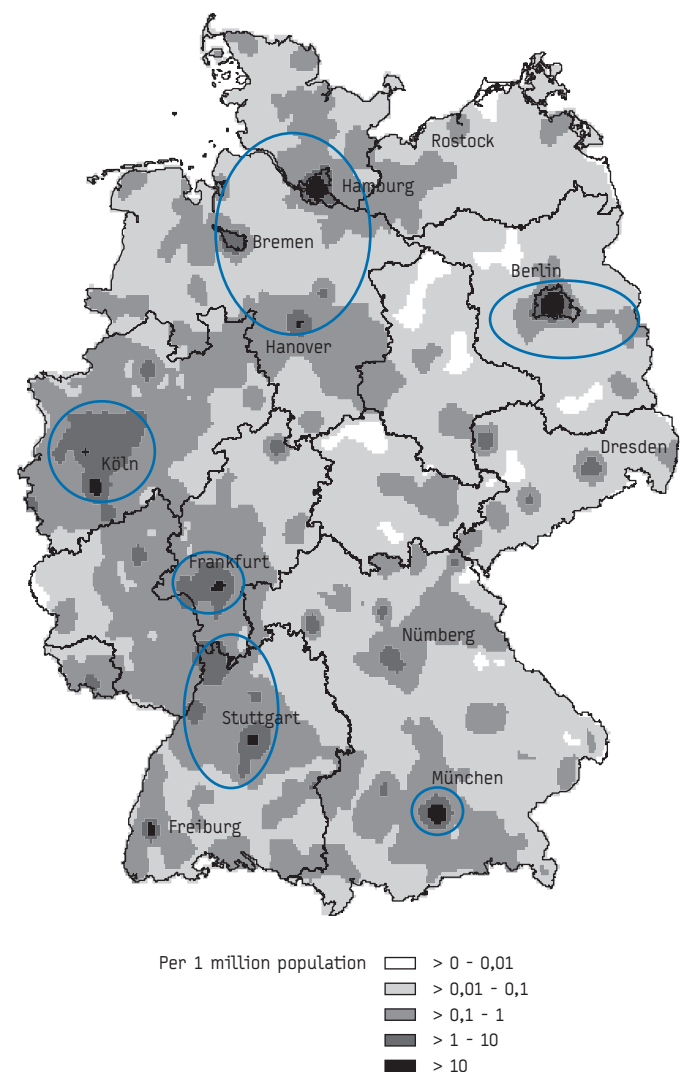
In this study arm clinicians specialised in HIV diagnosis and care will recruit patients with newly diagnosed HIV infections (cases) and patients undergoing an HIV test with negative result (controls). Cases and controls will be matched by basic demographic variables and their risk of HIV transmission. HIV testing for cases and their respective controls has to be performed within a three month period. After obtaining written informed consent, blood samples are collected from case patients as DBS [22]. The samples are analysed for recency of HIV infection by BED-CEIA at the HIV Variability and Molecular Epidemiology project group of the Robert Koch Institute. Clinical and medical history data from case and control patients are collected through a physician's questionnaire. KABP-data are collected from cases and controls by using a self-administered patient's questionnaire. The expected sample size is 600 cases and controls annually. Analyses of the data will allow

comparison between patients with recently acquired HIV infection and persons undergoing HIV tests with a negative test result in the same clinical institutions and in an identical time frame. The analyses aim at obtaining information on the current status of general knowledge about HIV/AIDS, on the behaviour and attitudes towards prevention of HIV transmission, and on the risks taken with regards to HIV transmission.

Impact

The study offers an outstanding opportunity to identify recent HIV infections out of newly diagnosed cases and estimate HIV incidence. As a result of this a deeper insight into the transmission dynamics of the ongoing HIV epidemic in Germany will be available. To prevent further HIV infections, comparative analyses are aimed at identifying the risks for HIV transmission and the relevant behaviour and attitudes. However, the major limitations of our study are insufficient screening patterns that only reflect those patients requesting an HIV test. True incidence estimates will be

FIGURE 2
Cumulative incidence of newly diagnosed cases of HIV in Germany, 2001-2006 and six regions of the clinical study arm, Germany 2008



difficult to obtain as the denominators needed are not available in Germany. Despite these limitations the data are expected to have an impact on amending and improving national prevention efforts and strategies in Germany. Better knowledge of the factors driving the HIV epidemic and of the most recent dynamics of the epidemic revealing subgroups currently at increased risk of acquiring HIV will help to design targeted and prompt interventions.

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Meeting reports

WORKSHOP ON THE SEROLOGICAL TESTING ALGORITHM FOR RECENT HIV SEROCONVERSION (STARHS) AND HIV INCIDENCE ESTIMATES, STOCKHOLM, 11-12 MARCH 2008

Editorial team (eurosurveillance@ecdc.europa.eu)¹

1. Eurosurveillance, European Centre for Disease Prevention and Control, Stockholm, Sweden

The recent development of serological assays for human immunodeficiency virus (HIV) that are able to distinguish recent from long-standing infection has generated an important tool for HIV surveillance. In the European Union (EU), a number of different serological assays are being used, and there is the danger that HIV incidence estimates in different countries, or even within a country, may not be comparable.

The former EU-funded project EURO HIV (http://ec.europa.eu/health/ph_projects/2004/action2/action2_2004_13_en.htm) included a work package on the investigation of several serological assays for recent HIV infection. It investigated the transferability of these tests, their comparative performance and their application in estimating HIV incidence in selected populations. Ten EU Member States contributed to this work.

The HIV experts (both epidemiologists and virologists) came together at a workshop held on 11 and 12 March 2008 at the European Centre for Disease Prevention and Control (ECDC) in Stockholm, Sweden, to discuss different approaches of the Serological Testing Algorithm for Recent HIV Seroconversion (STARHS) and their use for estimating HIV incidences.

EU-wide comparison of HIV serological methods

The EURO HIV 'work package 7' was presented by John Parry (Health Protection Agency (HPA), London, United Kingdom (UK)). It aimed to increase networking and cooperation between reference laboratories with the goal of harmonising surveillance methods in the EU in order to obtain comparable data across countries. It investigated the use of the following serological assays:

- 'Detuned' enzyme immuno-assays (EIA) (modified commercial assays; bioMérieux Vironostika and Abbot HIV AB 3A11)
- BED-CEIA: an antibody capture EIA measuring the IgG proportion (commercial; Calypte Biomedical)
- AxSYM: an antibody avidity assay (modified commercial; Abbot)
- IDE-V3: EIA targeting two antigens at the same time (in-house assay; produced by Francis Barin)

Gary Murphy (HPA, London, UK) gave an overview of the existing serological assays that are able to distinguish between recent and long-standing HIV infection, pointing out the advantages and drawbacks of each method and identifying desirable criteria for an ideal assay. A more detailed description of the individual assays can be found in the article by Murphy and Parry in this issue.

The list of desirable characteristics for a STARHS assay includes a well defined, preferably long, window period, consistent discrimination between recent and long-standing infection, and accurate results for different cut-off values. The result should be independent of factors such as virus subtype, mode of transmission, opportunistic infections, pregnancy, and age, sex, race and therapy status of the patient. On the operational side, cost, availability, equipment requirements, ease of handling and storage, and the suitability for small volumes and different types of samples need to be taken into account, and the assay should ideally not depend on a single company. Moreover, a programme to standardise and control the performance of the assay needs to be in place.

It was concluded that no single assay at present fulfils all the desired characteristics.

All four STARHS methods were compared at the HPA Centre for Infections (CfI), London, though some were in use in other laboratories so that limited further comparisons using the same specimen panels were possible. The panel comprised 374 well characterised samples from England (CfI, London) and France (Université François Rabelais (UFR), Tours), as well as seven panels of around 200 samples from new HIV diagnoses that had been collected in England, Finland, Germany, Italy, the Netherlands, Portugal and Spain, a total of 1,736 eligible specimens. The results of the comparison are available in the final EURO HIV reports (available at: http://www.hpa.org.uk/webw/HPAweb&HPAwebStandard/HPAweb_C/1195733851609?p=1200660013708).

The intra-laboratory reproducibility was found to be satisfactory, with a reasonable correlation between original and repeat test results for the BED, Detuned, Avidity and IDE-V3 assays used at the CfI, and the IDE-V3 assay used at the UFR. However, certain issues were raised such as the need to define a window-period for the AxSYM and IDE-V3 tests, the need to set up a confirmatory test algorithm, particularly for specimens that give results in a critical range around the threshold value, and the evidence that a minority of patients may never develop an immune response sufficient to convert to a long-standing status in some assays. It was also seen as important to take into account the different factors that may bias the results, for instance anti-retroviral treatment, virus subtype, and disease stage.

There was some inter-laboratory variability that the participants thought was due to equipment calibration or maintenance issues and differences in the production lots purchased from the companies. They emphasised the importance of experience and training regarding the equipment and of suitable calibrators and controls, which are still to be developed. The lack of appropriate external assessment programmes to assure the quality of STARHS testing was seen as one of the greatest barriers to the transfer of any given method between different laboratories. It was suggested that it may be necessary to define a reference laboratory responsible for the development and standardised evaluation of new STARHS methods.

Andre Charlett (HPA, London, UK) presented an assessment of whether there was agreement between the four STARHS assays in the identification of recent versus long-standing HIV infection when using different window periods. The classification of the majority of specimens was consistent, but there were also intolerable inconsistencies, and none of the assays was found to be suitable for every specimen.

HIV incidence in the EU

With the laboratory methods still in need of improvement, more uncertainties arise when transferring laboratory data to incidence estimation. Part of 'work package 7' was designed to test the applicability of the STARHS results for HIV incidence estimates in selected subpopulations in different EU Member States. Preliminary results from an HIV incidence estimation in three collaborating countries were presented by Daniela DeAngelis (HPA, London).

The estimates based on data from the four different STARHS assays differed substantially, and it was felt that more discussion will be needed on the interpretation of the results. Three main problems were put forward as possible reasons for the discrepant results: a) the data collection methodology may influence the interpretation of the test results; b) the difficulty of estimating the distribution of the window period, as the estimation procedure involves many assumptions and it might be based on a small panel of seroconverters; and c) misclassification of long-standing infections as recent. Other factors influencing the result include epidemiological data such as the testing pattern, the time since the last negative test and behavioural data.

Ongoing international activities in Europe

The second day of the meeting began with an overview on other ongoing European programmes focused on HIV incidence estimation. After a short presentation outlining the ECDC laboratory strategy, Valerie Delpech (HPA, London, UK) presented the EU-funded project 'Concerted Action on SeroConversion to AIDS and Death in Europe' (CASCADE), a network of epidemiologists, statisticians, virologists and clinicians from leading HIV institutions in 15 European countries, Australia and Canada that collects life-long data from local and national cohorts of seroconverters.

CASCADE's current activities include the ascertainment and follow-up of recently infected people in central and eastern Europe. Since most countries do not have the facilities to start a new large-scale surveillance project, CASCADE plans site visits to laboratories in order to create the infrastructure and train staff in suitable HIV tests. Whether STARHS methods are appropriate in this context, is being discussed.

The session was concluded with feedback from a recent meeting of the WHO working group on HIV incidence assays, a worldwide initiative to establish best practice in the calibration and evaluation of STARHS methodologies, to study the evidence on the use of these assays and to provide guidance on appropriate approaches to measuring HIV incidence. The next steps of the project foresee supporting the establishment of appropriate specimen panels, the calibration of existing and the development of new assays as well as their application, and the determination of a window period. A statistics working group will advise on how to interpret results and determine incidences. The use of incidence assays for purposes other than incidence estimates is being discussed.

Future objectives

In a third session the participants discussed, in two working groups, the laboratory and epidemiological aspects of using various STARHS assays, in order to define the next steps regarding the development and implementation of HIV serological assays and regarding incidence modelling in the EU Member States.

The workshop participants agreed that it is advisable to have at least two satisfactory standard STARHS methods established in all laboratories undertaking STARHS testing, in case one test should be temporarily unavailable. ECDC had hoped to conclude this workshop with a recommendation of one or two of these assays and to discuss the feasibility of their implementation in the EU. However, the experts felt that information for such a decision was lacking, and there was a general agreement that it is at present not possible to make such a recommendation. The laboratory experts were of the opinion that in the medium term it was more likely that five or six different assays would be in use across Europe and stressed that quality assessment programmes would be needed for all of them.

It was agreed that once an agreement has been reached on the test(s) to be used, ECDC should coordinate and fund the development of a framework or guideline for the implementation of STARHS for epidemiological use, detailing what epidemiological data are needed, from which populations, and which sampling strategy should be used. In the meantime, more work needs to be done with regards to the estimation of the window period, and a quality assurance and training programme needs to be developed. Further urgent issues for the near future include the development of an EU-specific panel of seroconverter samples for calibration of the assays, the realistic window period estimates, and a deeper analysis of the epidemiological information including validation of the results in different population groups according to the different factors that may bias the results.

It was decided that the WHO global initiative should be followed closely to avoid duplication of work. The overall conclusion was that, while HIV incidence testing may not become part of routine HIV surveillance in the very near future, all efforts regarding test development and epidemiological sampling frame should be targeted to reach this stage as soon as possible in order to improve the understanding of HIV epidemiology in the EU.

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UNITED STATES CENTERS FOR DISEASE CONTROL AND PREVENTION RELEASE INCIDENCE ESTIMATES FOR HIV

Editorial team¹

1. Eurosurveillance, European Centre for Disease Prevention and Control, Stockholm, Sweden

On 3 August 2008, the United States (US) Centers for Disease Control and Prevention (CDC) in Atlanta released for the first time estimates for HIV incidence based on a STARHS (serological testing algorithm for recent HIV seroconversion) [1]. In their communication, CDC report that the true HIV incidence for 2006 is around 40% higher than the previous estimate of 40,000 HIV infections. They also point out that this new figure of 56,300 does not indicate any increase in the annual number of new HIV infections, which is believed to be relatively stable since the late 1990s. Analysis by transmission category confirms that male-to-male sexual contacts accounted for 53% of the estimated new HIV infections in 2006, high-risk heterosexual contact for 31%, injection drug use (IDU) for 12% and male-to-male sexual contact and IDU for 4%. Further analyses by race/ethnicity revealed an uneven distribution with the highest percentage of new HIV infections occurring in African Americans (45%) followed white Americans (35%) and Hispanics (17%).

The results were obtained after using a STARHS assay, the BED HIV-1 capture enzyme immunoassay (BED-CEIA), to test 6,864 samples from new HIV diagnoses from 22 US federal states in 2006. Whereas standard HIV tests provide no insight into the time when infection was actually contracted, the BED-CEIA is able to identify HIV infections that occurred within around the previous five months. The test thus allows to distinguish between recent and long-standing infections and permits a more precise estimate of the true incidence. A total of 2,133 (31%) tests of the 6,864 were classified as recent infections and the estimated incidence rate for 2006 was 22.8 per 100,000 population. The detailed methods for the calculation of this incidence and an extended back-calculation model to estimate HIV incidence for the period 1977 to 2006 are reported in an article by Irene Hall et al. in JAMA [2].

The CDC state that the implementation of the STARHS-based surveillance system in the US will allow for reliable monitoring of incidence trends in the future, helping to pinpoint the populations at greatest risk and pave the way for more timely interventional measures.

Since it is estimated that one-quarter of HIV-infected individuals are unaware of their infection status and that they account for more than half of all new infections, CDC recommends testing everyone in the US aged 13 to 64 years for HIV. On a more positive note, the stability in the new HIV infections since 2000 is an indicator

that prevention can, and does, work, especially if one takes into consideration that the number of people living with HIV increases over time – due to better survival of infected individuals – and subsequently the overall risk of HIV transmission increases.

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