The HIV pandemic continues to spread with about 38 million people living with HIV worldwide. Globally, approximately 5 million new infections are believed to have occurred in the year 2003, corresponding to about 15,000 new infections per day. About 3 million people have died during the same time period. Through a network of 455 sentinel surveillance sites, National AIDS Control Organization in India estimated in 2003 that 51 lakhs (10 lakh = 1 million) people were infected with HIV. From the early 1990s, there has been a steady increase in prevalence of HIV-1 infection. Prevalence data indicated a decline in new infections from 6.1 lakhs in 2002 to 5.3 lakhs in 2003. Whether the prevention campaigns are having an impact on the spread of HIV-1 cannot be confirmed without the HIV-1 incidence measurements. While HIV-1 prevalence is the accumulated experience of a population with HIV-1
over the years, the HIV-1 incidence is a point estimate of rate of new infections at a given time.

The HIV-1 prevalence can be measured by routine HIV diagnostic assays but direct measurement of HIV-1 incidence has been difficult. The changes in HIV prevalence may not reflect trends in incidence, underscoring importance of HIV-1 incidence\(^3,4\). In a simplified model, HIV-1 incidence can be measured prospectively by following a group of seronegative people and testing them again at the end of one year. The HIV-1 incidence then can be calculated as the number incident cases per 100 persons per year. However, prospective cohort studies are difficult to conduct, may be biased, and are very expensive. Moreover, cohort enrollment, consent process and risk-reduction counselling often results in reduced or lower HIV-1 incidence\(^5\) and may not reflect true population incidence. Cohort studies do not allow sampling of representative populations, which are needed to calculate national incidence. In addition, this exercise has to be repeated every year to monitor the trend and therefore is not practical. Williams et al\(^6\) described a statistical method based on age prevalence data to estimate HIV-1 incidence, with certain assumptions about growth rate of the epidemic and AIDS mortality rate. The method was applied to women attending antenatal clinic (ANC) in KwaZulu-Natal, South Africa to calculate HIV-1 incidence. In the absence of other alternatives, this could be a useful approach. However, with significant prevention interventions including initiatives to expand
antiretroviral (ARV) treatment to people with AIDS in many countries, the assumptions about growth rate of the epidemic and AIDS mortality rate may not be valid. There are several other statistical approaches that have been described but they do not substitute for the reliability of a laboratory test that can be used to detect recent infections.

In the last few years, several different laboratory methods have been identified specifically to detect recent infections either in the pre-seroconversion phase or in the post-seroconversion phase. Cross-sectional specimens, such as those collected for surveillance for assessing HIV prevalence, can be used for this purpose. Laboratory tests during the pre-seroconversion phase include detection of HIV-1 RNA or p24 in the antibody-negative segment of the population, while those in post-seroconversion phase depend on various properties of maturing HIV antibodies to detect recent infection (Fig.; Table).

### Table: Assays for Recent HIV Infection

<table>
<thead>
<tr>
<th>Assay</th>
<th>Test for:</th>
<th>Window from:</th>
<th>Window to:</th>
<th>Window duration:</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV -RNA</td>
<td>Plasma RNA</td>
<td>RNA +</td>
<td>seroconversion</td>
<td>~19 days</td>
</tr>
<tr>
<td>HIV p24</td>
<td>Plasma p24</td>
<td>p24+</td>
<td>seroconversion</td>
<td>~14 days</td>
</tr>
<tr>
<td>LS-EIAs</td>
<td>HIV Ab titer</td>
<td>seroconversion</td>
<td>titer cutoff</td>
<td>130-170 days*</td>
</tr>
<tr>
<td>BED-CEIA</td>
<td>HIV Ab/total IgG</td>
<td>seroconversion</td>
<td>Ab proportion cutoff</td>
<td>153 days**</td>
</tr>
<tr>
<td>Avidity</td>
<td>HIV Ab avidity</td>
<td>seroconversion</td>
<td>avidity cutoff</td>
<td>variable</td>
</tr>
<tr>
<td>Affinity</td>
<td>HIV Ab affinity</td>
<td>seroconversion</td>
<td>affinity cutoff</td>
<td>variable</td>
</tr>
<tr>
<td>IgG3 isotype</td>
<td>IgG3 anti-HIV Ab</td>
<td>seroconversion</td>
<td>undetectable IgG3 Ab</td>
<td>~80 days</td>
</tr>
</tbody>
</table>

* refers to mean window period only for subtype B
** refers to mean window for all subtypes A to E
LS-EIAs, less sensitive – enzyme immuno assay; BED-CEIA, BED-capture enzyme immuno assay

**Pre-seroconversion tests**

**HIV-1 RNA or p24 detection:** Detection of HIV-1 RNA or p24 antigen, prior to development of specific antibodies indicates very recent infection (primary HIV infection or PHI). This approach requires that seronegative people are tested by these methods. The duration of this stage is likely to be very short (about 10-20 days), therefore a large sample size is required to detect enough individuals in this short “window period”. Some studies have used these methods to detect recent infections. Brookmeyer et al estimated HIV-1 incidence rates among patients attending sexually transmitted disease (STD) clinics in Pune (India) using prevalence of p24 antigen positivity together with longitudinal follow up data from a subset of patients who returned for testing. The incidence was reported to be very high (18.6% per year) in this population. The consequence of this short “window period” is that any inaccuracy in the window period ...
can lead to a large error when calculations are annualized for incidence estimates. Pilcher et al\textsuperscript{7} used PCR to detect HIV-1 RNA in seronegative individuals in Malawi and demonstrated high rate of acute HIV infection but did not extrapolate their data to calculate incidence, mainly due to uncertainty of "window period". Moreover, these tests are technically more complex, more expensive and require testing of the negative population. Therefore, these are not ideally suited for wide-scale implementation for determination of HIV-1 incidence.

**Post-seroconversion tests**

Several tests have been developed or are being investigated for testing seropositive individuals post-seroconversion to detect recent infections. Because this testing is performed only on seropositive individuals, relatively less testing is required. Subsequently, based on the characteristics (window period) of the test used, annualized incidence can be calculated. This post-seroconversion "window period" refers to mean time period seropositive individuals may stay below a pre-defined threshold on the test following seroconversion.

(i) **Less-sensitive enzyme immunoassay, LS-EIAs (detuned assay):** In 1998, Janssen et al\textsuperscript{10} described a modification of a commercial HIV-1 antibody assay (3A11) from Abbott to detect recent seroconversion. The assay was modified to render it less-sensitive (hence termed 3A11-LS) in detecting HIV-1 antibodies by use of increased serum dilution (1/20,000 instead of 1/400) and shortened incubation times (30 min from 120 or 60 min). When seropositive individuals were tested by this assay, early low-titre HIV-1 antibodies were not detected (remained below a pre-defined threshold cut-off) and therefore, were classified as recently infected. The mean time period that seropositive but recently infected people stay below the threshold of this assay is termed the "window period" (duration from HIV-1 seroconversion). The window is calculated by testing sequential specimens from a number of seroconverters. This duration was found to be 129 days (95% CI 109-149 days). Because this EIA was manufactured as a qualitative test, a calibrator and additional control specimens were needed to determine the threshold cut-off and to monitor the assay performance. Centers for Disease Control and Prevention (CDC) developed these reagents, which were supplied to participating laboratories under "investigational new drug" (IND) agreement. Unfortunately, this first generation EIA was discontinued by Abbott Laboratories. Another commercial assay, Vironostika HIV-1 EIA from Organon Teknika, was similarly modified (Vironotika-LS EIA) and has been used as a replacement to detect recent infections\textsuperscript{11}. The LS-EIAs have been used in several studies to determine HIV-1 incidence\textsuperscript{12-15} and to identify correlates of recent infections.

There was significant interest in the implementation of 3A11-LS and Vironostika-LS in other parts of the world. However, two separate studies demonstrated that both the 3A11-LS and Vironostika-LS EIAs had significantly different "window periods" in subtypes B or E infected populations from Thailand\textsuperscript{16,17}. The longer window periods (270 and 350 days, respectively) in E infected persons were attributed to the use of subtype B derived antigens in the assays. This indicated that window periods in other divergent HIV-1 subtypes from Africa and Asia are also likely to be different. Due to presence of two or more HIV-1 subtypes in some parts of the world and their changing epidemiology, it is not practical to use these assays to estimate the incidence. There have been some limited applications of LS-EIAs in Africa\textsuperscript{14} and Asia\textsuperscript{18} but the validity of these estimates are questionable due to poorly defined cut-off and window periods in the circulating HIV-1 subtypes. Therefore, in spite of the importance of estimating HIV-1 incidence, the LS-EIAs have not been implemented widely in other parts of the world due to multiple circulating subtypes and complication of interpreting data.

(ii) **IgG-capture BED-EIA (BED-CEIA):** Limited availability and limitations of the LS-EIAs led many laboratories to investigate alternative approaches to detect recent HIV-1 infections\textsuperscript{19-22}. Based on the development of humoral immune responses, several assays were devised\textsuperscript{20} that examined epitope or antigen-specific responses, antibody avidity/affinity, antibody quantity and conformation dependence of antibodies. From the multitudes of assays examined, we further developed the BED-capture enzyme immunoassay (BED-CEIA) which indirectly measured increasing quantity (proportion) of HIV-
IgG in the serum. Important features of the assay are capture format of the EIA which captured both HIV and non-HIV-IgG in the same proportion present in the serum and inclusion of a multi-subtype derived branched synthetic peptide (termed BED) from the gp41 immunodominant region. Capture EIA allowed serum dilution of 1/100 (instead of 1/20,000 for LS-EIAs) and BED peptide permitted equivalent detection of antibodies to different subtypes. The BED-CEIA detected recent infections in subtype B and E infected individuals from Thailand with similar window periods (mean window of 160 days). Subsequently, the window periods for BED-CEIA have been determined in groups of seroconverters infected with subtypes A/D (Kenya), B (Amsterdam), and C (Ethiopia and Zimbabwe). Data suggest that window periods are somewhat longer in some of the subtypes tested but their 95 per cent confidence intervals overlapped (unpublished data). It is still to be confirmed whether these differences are due to the assay or due to biologic differences between populations and/or HIV-1 subtypes. We have observed that antibody maturation kinetics (as measured by titre, proportion or avidity) are somewhat different between subtype B and E infections in Thailand.

Due to the quantitative nature of the BED-CEIA, the assay is performed under stringent criteria established in our laboratory. We have examined performance characteristics of the assay for multiple variables and have found the assay to be quite consistent. The assay has been transferred to several laboratories in the United States and elsewhere and has been used in multiple cross-sectional specimens to detect recent infections and estimate incidence. The applications include an injecting drug user population in Bangkok, pregnant women in Rwanda and Atlanta, ANC surveillance specimens from Addis Ababa, South Africa and stored surveillance specimens from Cambodia (unpublished data). The results demonstrate strong association between various risk factors and high incidence and provide trends of incidence when specimens from multiple years were available for testing. Incidence data from Cambodia surveillance indicated that incidence dropped significantly in commercial sex workers from 1999 to 2002, consistent with a rapid increase in condom use in this population during that time period. Results also highlighted the problem areas showing that incidence remained high in western part of the country bordering Thailand, possibly due to tourism-related commercial sex. These successful applications have generated widespread interest but availability of reagents remained a limitation. Recently, the assay has been commercialized and is available worldwide for implementation. We are currently developing protocols for the use of dried blood spot (DBS) and serum spot specimens. Our recent data suggest that this application will require controls and calibrator prepared on matching matrix (filter paper). Use with DBS will further simplify surveillance and extend use of the BED-CEIA in populations where specimen collection, processing, storage and transport could be obstacles to testing for recent infections.

(iii) Antigen-specific responses: The LS-EIAs include viral lysates as the antigens and, therefore, detect antibodies to all viral proteins. Development of humoral immunity to HIV-1 indicate that antibodies to various proteins are elicited at different rates. Western blot profiles suggest that antibody titres to gp120/160, gp41 and p31 are quite different in early versus long-term infections. This is corroborated from the study of seroconversion panels and can form the basis of an assay to detect recent infection. Thus, a combination of assays can be used to detect antibodies to two or more antigens or epitopes to identify recent infections. Antibodies to gag (p24 and p17) and env (gp120, gp41) proteins are usually detected early and get stronger over time after infection. Antibodies to pol gene products (p31/p51/p66) are elicited later in the infection, compared to antibodies to gag/env gene products. Within the envelope proteins, antibodies to gp41 immunodominant region (IDR) are elicited early while antibodies to gp120-V3 loop develop later. Barin and his group from France used a 3-well EIA to detect antibodies to gp41-IDR, V3-loop and p31 (integrase) and showed a good separation of people with early infection from those with long-term infection. This requires that each specimen is tested in 3 separate wells and therefore will increase the testing, compared to LS-EIA or BED-CEIA. This work has not yet been published or applied to cross-sectional specimens to validate this approach.
(iv) **Antibody affinity/avidity:** Antibody affinity is measured by surface plasmon resonance and is defined by an equilibrium constant, $K_A$, which is a ratio of association and dissociation rates of antigen-antibody complex. Antibody affinity increases following seroconversion and usually relates to specific antibody population directed to one antigen or epitope. Kinetics of affinity development are distinct for different antibody populations and can be measured using a single peptide or protein. Using a well-defined immunodominant peptide, such as gp41-IDR, affinity measurement can distinguish early from long-term infections. Since antibody affinity is expected to stay the same even in AIDS, unlike antibody quantity which decays in advanced disease, this approach is less likely to give false recent classification. However, the measurement of antibody affinity is quite complex and not amenable to large throughput. In contrast, overall binding strength of polyclonal antibodies or antibody avidity can be measured by simple modification of an EIA. This is usually done in a two-well EIA; one well is used to perform the regular EIA, while the second well is treated with chaotropic agents such as urea, potassium isothiocyanate (KSCN), diethylamine or simple low pH buffer following serum incubation to dissociate low avidity antibodies. Avidity index is then calculated as a ratio of OD from two wells. Several investigators have explored this approach to examine the avidity maturation following seroconversion and to detect recent seroconversion. The avidity parameter is likely to be more robust in classifying recent infection but requires assay standardization, definition of threshold cut-off and corresponding “window period”. Such an approach can be used as a secondary assay to LS-EIA or BED-CEIA to increase predictive value of recent infection classification. Using microwell plates coated with BED branched peptide, we have developed a BED-avidity EIA using 8 M urea as a dissociating agent (unpublished data). This assay was used in an algorithm to distinguish recent from long-term infection following BED-CEIA as the first test. When the results of the two assays are combined, it improved positive predictive value of classifying recent infection. As present, this assay remains an in-house tool for further investigation in our laboratory.

(v) **Anti-p24 IgG3 antibodies:** Recently, Wilson et al. from Australia have reported that p24-specific IgG3 antibodies are elicited only early in HIV-1 infection but were absent about 4 months post-infection. A simple EIA has been developed to detect p24-specific IgG3. Examination of 17 seroconversion panels indicated that p24-IgG3 was detectable between 34 to 120 days (total duration 86 days). If these findings can be generalized to different populations infected with divergent subtypes, this assay has great potential to be used for HIV-1 incidence estimates.

(vi) **Conformation-dependent antibodies:** One of the features of evolving antibodies is its conformation dependence. Early antibodies in HIV infection preferentially recognize conformational epitopes on proteins such as external glycoprotein, gp120. Later in infection, antibodies are elicited to linear antigenic sites. Antibodies to native protein can be measured using microwells coated with a native protein and compared to microwells that are coated with a denatured protein. Proteins are denatured simply by heating at 100°C or by reduction/alkylation and then using them to coat microwell plates. Differences in conformation dependence of antibodies may provide information to distinguish early from long-term infection. This property of antibodies has been examined in a small study but has not been pursued further. As with the avidity assays, this approach requires at least two-well assay.

(vii) **Rapid tests:** Like EIAs, rapid test protocols can be modified such that low titre, early HIV antibodies are not detected by the assays. Constantine et al. modified the Uni-Gold Recombigen HIV rapid assay (Trinity Biotech, Dublin, Ireland) by using 1/115 pre-diluted specimens. The procedure was applied to specimens from known recent or long-term infections. The modified assay correctly classified 83 per cent of recently infected individuals but had high rate of misclassifications among people with long-term infections. It was suggested that it may have an application in an algorithm where those classified as recent infections are further tested by an LS-EIA to increase predictive value. The results indicated that the procedural modification was not optimal for the purpose. Since the RT assays are visual, it requires careful calibration.
We have carefully modified assay protocols of three rapid tests, Determine, OraQuick and SeroStrip ½, by including a pre-dilution step and matching the results to available Abbott 3A11-LS results for a known panel of specimens (unpublished observations). Less-sensitive rapid tests (RT-LS) were then used in several seroconversion panels and other specimens with known 3A11-LS data. There was a high concordance between RT-LS and 3A11-LS in classifying recent and long-term infections with overall agreement reaching >90 per cent. Although this approach is quite attractive, further studies are required to ensure validity of this methodology and consistency of results. Moreover, the impact of subtype variation on RT-LS is unknown but it is possible that they will have different performance as in the case of LS-EIAs. Therefore, application of this approach is not yet recommended.

**Appropriate applications and limitations of the assays**

It is to be noted that these assays, especially those to be used in post-seroconversion phase, are best suited for population incidence estimates and are not meant for individual diagnosis of recent or long-term infection. The threshold cut-offs and mean “window periods” are derived from population data and represent quantitative features of antibody development that are variable in different individuals and possibly in different subtype infections. Predictive values of these assays for individual diagnosis are not well known. Therefore, ideally these methods should be used to compare incidence among different population groups, examine trends of HIV-1 incidence over time in the same population, or assess the effectiveness of interventions, pre- and post intervention. Use of these methods to monitor the trends over time (or pre- and post-intervention) also minimizes the impact of potential biases because these biases will have a systematic effect on the assays.

Some of the antibody-based methods described above may falsely identify about 2-10 per cent of people with AIDS as recently infected due to their declining antibody levels. This points to the caution of using the assays for diagnosing recent HIV infection. Taking clinical status of the individuals or their CD4 levels into account may further help in reducing this misclassification.

Since LS-EIA and BED-CEIA are quantitative assays for measuring HIV-1 specific antibodies, the integrity of serum or plasma specimens is of importance. The blood should be collected and processed optimally, standard serology should be performed within 2 wk while maintaining the sera at 4°C and then frozen at -20° C or below for longer term storage. If specimens are to be transported to another laboratory for prevalence and/or incidence testing, the cold chain should be maintained to ensure specimen integrity. Compromised specimens, such as improperly stored sera, those showing bacterial or fungal growth or highly haemolyzed sera should not be tested for detecting recent infections because of degradation of antibodies. Development of protocols for using DBS or serum spot should simplify specimen processing, storage and shipping to a great extent while reducing the cost.

**Calculation of HIV-1 incidence**

HIV-1 incidence is calculated by using appropriate formula. HIV-1 incidence describes “the rate of new HIV-1 infections per 100 at risk persons per year” and is influenced by the number of people at risk (number negative + number recently infected), and the “window period” of the test used to detect recent infection. Therefore, it is important to know the total number of people in the cross-sectional population. If only a proportion of sera from seropositive individuals are available for incidence testing, the number of seronegatives should be reduced by the same proportion to calculate incidence. Per cent of positives that are recently infected may be useful for demographic correlation but does not define a constant parameter such as HIV-1 incidence that can be compared between populations or over time. If the HIV-1 incidence is to be extrapolated to calculate national or regional incidence, appropriate adjustments should be made for population biases that may exist.

It is important that only confirmed HIV-1 seropositive individuals are tested for this secondary testing for incidence. If negative specimens (false positives) are included for testing, they will register
as recent HIV-1 infections on the assays and will falsely elevate the HIV-1 incidence. Moreover, compromised specimens as described in previous section should not be used because they may be classified erroneously as recently infected due to decay of HIV antibodies, thus elevating overall incidence.

Conclusions

The importance of HIV-1 incidence cannot be overstated. With availability of simple laboratory tools, HIV-1 incidence can now be measured using cross-sectional specimens, including sentinel surveillance specimens. Many international organizations, agencies, and national governments are allocating significant resources to reduce the spread of HIV epidemic. HIV-1 incidence data can provide point estimates of the current state of HIV-1 transmissions and help target valuable resources in a timely manner. Successful impact of interventions can be confirmed often years earlier by observing a decline in HIV-1 incidence compared to prevalence, thus providing an important tool for monitoring and evaluation. In the context of India, measurement of HIV-1 incidence can be a very valuable addition to the ongoing national surveillance. Extrapolation to national incidence estimates as well as infections averted can be calculated from yearly incidence data and can provide solid support to successful prevention strategies. With improvement in methodologies that can use DBS and serum spot specimens, both HIV-1 prevalence and incidence measurements can be greatly enhanced and should provide a valuable tool to monitor HIV-1 transmissions.

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References


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