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Abstract: Accurate methods for estimating HIV incidence from cross-sectional samples would have great utility in prevention research. This report describes recent improvements in cross-sectional methods that significantly improve their accuracy. These improvements are based on the use of multiple biomarkers to identify recent HIV infections. These multiassay algorithms (MAAs) use assays in a hierarchical approach for testing that minimizes the effort and cost of incidence estimation. These MAAs do not require mathematical adjustments for accurate estimation of the incidence rates in study populations in the year before sample collection. MAAs provide a practical, accurate, and cost-effective approach for cross-sectional HIV incidence estimation that can be used for HIV prevention research and global epidemic monitoring.

Key Words: HIV, incidence, cross-sectional, multiassay algorithm

INTRODUCTION

Most HIV prevention research studies rely on HIV incidence as the primary endpoint because of the lack of reliable surrogate endpoints.1,2 This presents challenges for observational studies and clinical trials. Many of these challenges arise from methodological issues with the traditional approach for determining HIV incidence: following cohorts of uninfected persons over time and documenting HIV acquisition.

Cohort studies present challenges in HIV prevention research for several reasons. First, if HIV incidence rates are low, large cohorts are required to accumulate sufficient incident infections for accurate determination of incidence rates. Second, it is often difficult to achieve adequate follow-up among high-risk uninfected persons. Third, longitudinal cohort studies are time consuming, often taking years to complete. Fourth, differential loss of follow-up between study arms can bias estimates of intervention effects. Fifth, study participation may modify HIV infection risk for reasons unrelated to the study intervention. For example, provision of risk-reduction counseling during routine follow-up of a biomedical intervention may decrease HIV incidence by decreasing risk behaviors. The Hawthorne effect may also confound prevention studies, because some behavior changes may be related to learning one’s HIV status or the awareness of being observed, rather than to the intervention under study.3

Many challenges of cohort studies can be addressed by assessing HIV incidence using a single, cross-sectional survey. This approach does not require follow-up of cohorts. In this approach, biological samples are collected in a cross-sectional survey, and biomarkers are used to identify recent HIV infections. In early work, individuals were classified as having recent HIV infection if they were acutely infected (HIV p24 antigen positive and HIV antibody negative).4 A limitation of that approach is that very large samples sizes are needed to identify recent infections because the duration of acute infection is very short.5 Subsequently, the criterion for classification as recently infected was a weak anti-HIV antibody response in HIV-seropositive individuals;5 this was measured using “detuned” serological assays6 or other serologic assays that measure different characteristics of the immune response to HIV infection.7 These efforts have been largely unsuccessful because serologic assays classify some individuals with long-standing infections as recently infected.8,9 Significant progress has been made using combinations of biomarkers eg, multiassay algorithms (MAAs) for cross-sectional incidence estimation.10-13

The objective of this article is to discuss this approach and the role it can play in...
HIV prevention research. We discuss the conceptual and statistical framework of this approach, limitations of some existing assays, and why and how multiple assays can be effectively combined to overcome these limitations.

**USE OF A BIOMARKER APPROACH FOR CROSS-SECTIONAL INCIDENCE ESTIMATION IN HIV PREVENTION RESEARCH**

Here, we discuss several applications of cross-sectional HIV incidence estimation that use biomarkers.

Preparatory or feasibility studies are often performed to estimate HIV incidence rates to determine samples sizes for phase 3 HIV prevention studies. This is challenging, because even small overestimates of HIV incidence rates in sample size calculations can lead to appreciably underpowered studies. Indeed, several HIV prevention trials were stopped early because of lower than expected incidence rates, leading investigators to conclude that the trials would be unable to detect an intervention effect.\(^2\)\(^-\)\(^4\)\(^-\)\(^6\) Furthermore, preparatory cohort studies to estimate incidence in target study populations can take years to complete; in some cases, by the time incidence estimates are obtained, the estimates may be out-of-date (eg, reflecting temporal trends in the epidemic, demographic changes, or ramp-up of HIV treatment programs). Cross-sectional HIV incidence assessments may provide an alternative, rapid approach to facilitate design of phase 3 trials.

The cross-sectional approach is especially useful in community-randomized trials of structural interventions. This approach was recently used to evaluate the primary endpoint of a large, multinational HIV prevention trial, NIMH Project Accept (HPTN 043).\(^7\) Project Accept evaluated the impact of interventions aimed at increasing the uptake of voluntary counseling and testing, changing community norms, and increasing social support for persons with HIV infection.\(^8\) A longitudinal cohort study was not suitable to evaluate the impact of the intervention, because HIV testing was part of the study intervention package.

The cross-sectional biomarker approach has also been applied to epidemiological studies of risk factors for HIV acquisition. An early application of this approach was a case-control study of acute HIV infection that used p24 antigen to identify incident HIV infections in HIV-seronegative patients.\(^9\) In later work, the BED capture immunoassay (BED-CEIA) was used to evaluate risk factors for HIV acquisition in a nationally representative, population-based survey.\(^1\) However, the inability of the BED-CEIA and other serologic incidence assays to accurately distinguish recently occurring from long-standing infections limits their utility. Development of accurate methods for identifying recent infection has important applications to epidemiologic studies of risk factors for HIV acquisition. The methods could also have important roles for identification of sexual or other networks with active disease transmission to target prevention efforts.\(^10\)

Finally, cross-sectional incidence estimates could be used to evaluate local and national time trends in HIV incidence. Nationally representative surveys of HIV prevalence, such as the Demographic and Health Surveys, have been conducted in more than 30 countries.\(^21\) Assessment of HIV incidence in samples collected in those surveys (eg, using a MAA) would incur marginal additional cost and could yield direct estimates of HIV incidence. Furthermore, comparisons of cross-sectional incidence in serial surveys could assess changes in HIV incidence. This approach could circumvent problems with other approaches, such as inferring incidence from changes in HIV prevalence over time, because that approach is known to be very sensitive to assumptions about mortality and migration.\(^22\)

**CONCEPTUAL AND STATISTICAL FRAMEWORK**

The cross-sectional incidence approach is based on classification algorithms that use assays to classify individuals into 1 of 2 states: either MAA positive or MAA negative. An objective of these algorithms is for individuals classified as MAA positive to have shorter durations of infection than individuals classified as MAA negative. The duration of time that persons are classified as MAA positive depends on the specific algorithm that is used. Furthermore, for a given algorithm, the duration of time that individuals are classified as MAA positive varies from person to person. The mean (or average) duration of time individuals are classified as MAA positive for a given algorithm is called \(\mu\) or the mean window period. From reference samples with known (interval-censored) durations of infection, one can estimate \(\mu\). One can also estimate the distribution of durations individuals are MAA positive for a given algorithm, that is, \(\psi(t)\) is the proportion of individuals infected for \(t\) days who are classified MAA positive.

A fundamental equation in epidemiology describes the relationship between prevalence, incidence, and duration and provides the basis for how to estimate incidence from cross-sectional samples using biomarkers.\(^23\) The estimate of the HIV incidence rate in a population from a representative cross-sectional sample of persons from that population is

\[
I = \frac{w}{n \mu} \tag{1}
\]

where \(w\) is the number of individuals classified MAA positive and \(n\) is the number of individuals who are HIV seronegative.\(^4\) Confidence intervals for the incidence rate that account for uncertainty in \(\mu\) are obtained using procedures described in previous reports.\(^1\)\(^-\)\(^2\)\(^4\)\(^-\)\(^5\)

In general, Equation 1 is not estimating incidence at the time of sample collection but rather at a time before the collection of samples. The question of how far back in time is answered by the concept of the shadow, which is denoted by \(\psi\).\(^26\) Equation 1 is estimating HIV incidence \(\psi\) days before collection of the samples.\(^26\)\(^-\)\(^27\) The shadow can be calculated from the curve \(\psi(t)\) using numerical integration as described in previous reports.\(^1\)\(^-\)\(^2\)\(^7\)

Generally, it is preferable for MAAs to have large mean window periods (\(\mu\) and also small shadows (\(\psi\)). This is preferable because incidence estimates will have smaller standard errors (and variances) if \(\mu\) is large and will also be more current and therefore potentially less biased if \(\psi\) is small. Although the mean window period (\(\mu\)) and shadow (\(\psi\)) are
distinct numbers, they tend to be positively correlated; this presents the classic statistical tradeoff between bias and variance (Fig. 1 in Brookmeyer, Konikoff, Laeyendecker, et al.12). The question of how to choose optimal MAAs to address this tradeoff is discussed in the article by Brookmeyer, Konikoff, Laeyendecker, et al., which describes an approach to identify algorithms that maximize the mean window period subject to the constraint that the shadow is not too large (eg, <1 year).

HIV prevention interventions in a phase 3 trial can be compared by the ratio of their incidence rates [ie, the rate ratio (RR)], which is obtained by taking the ratio of Equation 1 for the 2 groups. In the special case where \( \mu \) is the same for the 2 groups, the RR for group 1 relative to group 2 is

\[
RR = \frac{\mu_1 n_1}{\mu_2 n_2}
\]

We see that the mean window period, \( \mu \), cancels out in Equation 2 and, therefore, is not required in the calculation. Two points about Equation 2 should be emphasized. First, the equation is estimating the incidence RR for days before sample collection and not on the date of sample collection. This point is important if there is a ramp-up period for an intervention to achieve its maximal effectiveness. Second, the assumption that the mean window periods (\( \mu \)) cancel out in Equation 2 may not hold for all interventions. For example, if an intervention increases uptake of antiretroviral therapy (ART) services, \( \mu \) may become larger if the MAA is based solely on serologic assays, because the performance of those assays is impacted by viral suppression. MAAs that include assays for viral load or antiretroviral drug exposure can account for this effect by classifying individuals with low viral loads and those on antiretroviral drugs as MAA negative. Therefore, use of Equation 2 may be justifiable in some settings using certain testing algorithms.

An important question is how to optimize testing algorithms to maximize the statistical power for detecting an effect in comparative trials. The choice of which assay or MAA to use involves balancing the bias-variance tradeoff. The primary endpoint of NIMH Project Accept (HPTN 043), a large community-randomized trial, was based on use of a MAA that was optimized to maximize the power for detecting an intervention effect.58

LIMITATIONS OF CURRENT SEROLOGICAL ASSAYS

Although reliable methods exist for identifying acute HIV infections, this approach is only useful for surveying very large populations that have high incidence rates because the acute phase of infection is very short.4,5 Serologic incidence assays that have much longer mean window periods have been developed that measure different characteristics of the anti-HIV antibody response (eg, antibody avidity or the proportion of IgG specific for HIV antigens; for review, see articles by Murphy and Parry7 and Guy, Gold, Calleja, et al.8). Some serologic incidence assays are based on modifying commercial assays developed for HIV diagnosis (eg, Abbott detuned assay,6 Vironostika less sensitive assay,29 AxSYM HIV1/2gO avidity,30 BioRad avidity assay31), whereas others have been based on noncommercial (in-house) assays (eg, V3 IDE assay32, Luminex assay33). The BED-CEIA29 and the limited antigen avidity enzyme immunoassay (Lag-Avidity EIA)35,36 are the only assays specifically manufactured for HIV incidence testing.

Serologic incidence assays are relatively inexpensive and simple to use. However, if only serological assays are used in a MAA, some individuals remain MAA positive for long periods, whereas others who become MAA negative may subsequently revert to being MAA positive because of changes in their antibody responses to HIV infection (eg, because of viral suppression or advanced HIV disease).29,37,40 For these reasons, the distribution of durations that individuals are classified as MAA positive using only serological assays have long right tails.41 When serologic assays are used exclusively for incidence testing, 2 factors that commonly cause individuals with long-standing infection to be classified as MAA positive are viral suppression (natural or induced by ART) and advanced HIV disease.29,37–40 The performance of serologic incidence assays is also impacted by HIV subtype.29,42,43 A systematic evaluation of currently available assays used for cross-sectional HIV incidence estimation is being performed by the Consortium for the Evaluation and Performance of HIV Incidence Assays (CEPHA).44

USE OF MAAs FOR CROSS-SECTIONAL INCIDENCE ESTIMATION

The use of multiple assays in combination can increase the accuracy of cross-sectional incidence estimates. The cost of this approach can be reduced by using hierarchical, step-wise testing algorithms (MAAs). In each step, a single assay is used to refine the classification of samples that were provisionally classified as MAA positive at the prior step(s) (Fig. 1). If logistically feasible, assays can be performed in order of cost, with less-expensive, high-throughput assays in the initial steps and more-expensive or labor-intensive assays performed in the later steps.

A MAA for HIV incidence estimation in subtype B epidemics has been developed that uses 2 serologic assays (BED-CEIA measured as a normalized optical density, OD-n) and the BioRad avidity assay [measured as a avidity index] and 2 nonserologic biomarkers (CD4 cell count and HIV viral load) as illustrated in Figure 1 (MAA #1).10 This MAA was validated using >2200 validation samples from >1000 individuals with known duration of HIV infection (range <6 months to >8 years).10,12 The mean window period for this MAA was initially determined to be 141 days [95% confidence interval, (CI): 94 to 150].10 The performance of this MAA was evaluated in 3 longitudinal cohort studies,10,11,45 The cross-sectional estimates of incidence were determined by testing samples collected at the end of the follow-up period of the cohort study. In this manner, the incidence estimates obtained using cross-sectional testing could be compared with those observed in the cohorts based on documentation of HIV acquisition. The MAA and cohort incidence estimates were very similar (Fig. 2). In a subsequent study, the same validation data were reanalyzed using
FIGURE 1. MAAs for cross-sectional HIV incidence estimation in subtype B epidemics. MAAs were developed that combine serologic markers (BED-CEIA and an avidity assay) with nonserologic biomarkers (HIV viral load, with or without CD4 cell count). In each MAA, assays are performed using a hierarchical approach, with an optimal cutoff defined for each assay. CD4 cell count cutoffs are expressed as cells/mm$^3$; BED-CEIA cutoffs are expressed as normalized optical density; avidity results are expressed as avidity index values (%); HIV viral load cutoffs are expressed as HIV RNA copies/mL. For each MAA, samples that meet the criteria for all assays are classified as MAA positive. Three MAAs are shown. MAA #1 is a 4-assay MAA described in the articles by Laeyendecker, Brookmeyer, Cousins, et al.$^{10,12}$ and Brookmeyer, Konikoff, Laeyendecker, et al.$^{12}$ which was used to estimate incidence in 3 clinical studies (Fig. 2). MAA #2 is an alternate 4-assay MAA described in the article by Brookmeyer, Konikoff, Laeyendecker, et al.$^{12}$ which maximizes the mean window period subject to the shadow being less than 1 year. MAA #3 is the 3-assay MAA described in the article by Brookmeyer, Konikoff, Laeyendecker, et al.$^{12}$ which does not require CD4 cell count data and maximizes the mean window period subject to the shadow being <1 year. The mean window periods and shadows for all 3 of these MAAs were determined in a previous study$^{12}$; the 95% confidence intervals for each mean window period and shadow are shown in parentheses.

FIGURE 2. Comparison of cross-sectional incidence estimates and incidence observed from longitudinal follow-up of HIV-uninfected cohorts in 3 clinical studies performed in the United States. HIV incidence was evaluated in 3 clinical studies: the HIV Prevention Trials Network (HPTN) 064 study,$^{53}$ the HIV Network for Prevention Trials (HIVNET) 001 Vaccine Preparedness study,$^{26}$ and the HPTN 061 study.$^{55}$ Annual HIV incidence was determined in each study using 2 methods: longitudinal follow-up of HIV-uninfected individuals (filled symbols) and cross-sectional analysis using a MAA (MAA 1 shown in Fig. 1; open symbols).$^{10,11,44}$ Note that the mean window period of MAA 1 is based on the analysis reported in Laeyendecker, Brookmeyer, Cousins, et al.$^{11}$ using midpoint imputation for seroconversion times; a reanalysis using multiple imputations is reported in Brookmeyer, Konikoff, Laeyendecker, et al.$^{12}$

multiple imputations to address uncertainty from interval-censored infection times.$^{12}$ Based on that analysis for this MAA, the mean window period was 123 days and the shadow was 146 days.$^{12}$ In the same study, the validation data were used to identify an optimal MAA by searching through 11,340 MAAs with different assay cutoffs; the goal was to find the MAA that had the longest mean window period ($\mu$) with the constraint that the shadow ($\phi$) was <1 year.$^{12}$ The optimal 4-assay MAA had a mean window period of 159 days and a shadow of 184 days (Fig. 1, MAA #2). The study also evaluated MAAs that included 3 assays (the BED-CEIA, the avidity assay and viral load, but not CD4 cell count); those MAAs offer advantages in settings where CD4 cell count data cannot be obtained. The optimal 3-assay MAA had a mean window period of 101 days and a shadow of 194 days (Fig. 1, MAA #3). The 3- and 4-assay MAAs described above (MAAs #1–3 in Fig. 2) did not classify any of 845 samples from individuals who were infected >5 years as MAA positive; the sample set included 512 samples from individuals who were infected >8 years.$^{8}$

The MAA used to determine the primary endpoint of NIMH Project Accept (HPTN 043) was identified by analyzing the performance of MAAs for incidence estimation in a Southern African setting using a set of >5000 subtype A and C validation samples from >3400 individuals with known durations of infection (range 1 month to >10 years)$^{56}$ The goal of that study was to select the MAA that provided the highest power for detecting a reduction in incidence in intervention communities in the Project Accept trial.$^{18}$ The performance of 403 candidate MAAs was evaluated in 3 simulated epidemic scenarios (emerging, stable, and waning epidemics). Those analyses identified the following optimal MAA for this application: BED-CEIA <1.2 OD-n and avidity index <90% and CD4 cell count >200 cells/mm$^3$ and viral load >400 copies/mL.$^{28}$

In the MAAs described above, viral load testing is used to identify individuals with long-term infection who have low
BED-CEIA and avidity assay results because of viral suppression. The precision of MAAs may be further enhanced by also including a criteria of “no ART,” because ART per se may serve as a supplemental, surrogate marker of nonrecent infection. If ART is used as a component of an MAA, direct detection of antiretroviral drugs may be more reliable than self-report of antiretroviral drug use. The recent development of a low-cost, high-throughput, multidrug screening assay makes this kind of screening feasible. This antiretroviral drug-screening assay was used in the Project Accept trial as the final step for classifying individuals as MAA positive. It is important to note that some antiretroviral drugs may not be detected in samples because of their short half-lives. Furthermore, because some antiretroviral drugs are used for HIV prevention, some individuals may be classified as MAA negative based on antiretroviral drug detection even though their duration of infection is short (eg, in recently infected women receiving nevirapine for prevention of mother-to-child HIV transmission). In addition, antiretroviral drug testing will not identify elite controllers with long-standing infection, who may be classified as MAA positive based on results from serologic assays; viral load assays are needed to identify those individuals.

Viral diversity assays are also being explored for use in HIV incidence testing, either alone or in combination with other assays. The rationale is based on the premise that viral diversity increases with duration of infection and may serve as a biomarker that is independent of serologic biomarkers. A high-resolution melting (HRM) diversity assay has been developed that quantifies the genetic diversity in HIV without sequencing. This assay is relatively inexpensive, easy to perform, and provides quantitative measures of diversity that are highly correlated with diversity measures obtained by deep sequencing. Our recent studies suggest that the HRM diversity assay may offer an alternative to CD4 cell count in MAAs without compromising their performance.

**DISCUSSION**

HIV incidence is a critical outcome in HIV prevention research. Accurate methods for cross-sectional HIV incidence estimation may facilitate HIV prevention research, particularly when evaluating population-level interventions for HIV prevention. Other intermediate outcomes (eg, self-reported high-risk behaviors, frequency of HIV testing, proportion of eligible HIV-infected persons on ART) have been used as alternative endpoints for assessing the impact of prevention interventions and have provided insights into why some prevention interventions are not effective. However, changes in intermediate endpoints do not necessarily predict changes in HIV incidence. Ultimately, the question that HIV prevention research must address is whether or not an intervention decreases incidence. The cross-sectional biomarker approach for HIV incidence estimation helps address a number of challenges in incidence determination. The main advantage of this approach is that incidence can be determined from samples collected in a single cross-sectional survey, without requiring longitudinal follow-up of infected individuals.

Recent improvements in methods for cross-sectional incidence estimation make the approach feasible. To date, no single serologic assay has been developed that can accurately estimate incidence. To overcome these limitations, attention has now turned to using multiple biomarkers in combination. Recently, MAAs have been identified where the probability of being classified as MAA positive converges to zero within several years of infection. MAAs that use multiple biomarkers in combination are essentially binary decision trees; results from each assay are used to decide whether or not to test a sample with the next assay in the algorithm. This hierarchical approach to testing reduces costs. Recent research has demonstrated that the multiple biomarker (MAA) approach is extremely powerful and corrects the deficiencies of individual biomarkers.

Statistical methods have been developed for assessing the accuracy of MAAs and for identifying optimal MAAs by calculating mean window periods, shadows, and statistical power for comparative trials. These methods require large validation sample sets from individuals with a broad range of known (interval-censored) durations of infection. The validation sample sets should represent the full range of durations of infection and include infected persons with the relevant HIV subtypes, with viral suppression, and with advanced HIV disease. Theoretically, the MAAs that we have identified should be capable of estimating incidence with minimal bias regardless of the stage of the epidemic (eg, mature vs. rapidly changing). The MAAs that we identified characterized individuals with either viral suppression or advanced HIV disease as MAA negative. Therefore, the mean window period and the shadow of the MAAs that we identified should not be dependent on epidemic stage. Figure 2 shows that one of these MAAs performs well in 3 very different epidemic settings. Further validation of MAAs in diverse settings is warranted. Although the MAAs should yield unbiased estimates of incidence regardless of the epidemic setting, the samples sizes needed to obtain accurate incidence estimates will vary by setting. In general, the sample sizes needed to obtain precise incidence estimates in cross-sectional surveys are greater in lower incidence settings than in higher incidence settings, which is also the case for longitudinal cohort studies.

MAAs have been successfully developed for use in settings with both subtype B and subtype A and C epidemics. These MAAs do not require any further mathematical adjustments for accurate estimation of the incidence rates. Work is now ongoing to identify optimal MAAs for other HIV subtypes and for other applications. A robust MAA has been developed that does not include the CD4 cell count. We conclude that the cross-sectional biomarker approach using MAAs is a practical, accurate, and cost-effective approach for HIV incidence estimation that can be used for HIV prevention research and global epidemic monitoring.

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