A simple and inexpensive particle agglutination test to distinguish recent from established HIV-1 infection

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Introduction

The ability to classify HIV-1 infections into phases of recent versus established infection is important for the
single-point estimation of incidence in cross-sectional surveys, for assessing the dynamics of the epidemic, and for determining the effectiveness of efforts to reduce HIV transmission in high-risk populations. Currently, sensitive/less sensitive (S/LS) tests are not indicated for patient management (STARHS Workshop, Toronto, 2006). S/LS assays have allowed the estimation of HIV incidence without the time and resources necessary to follow a cohort of high-risk seronegative individuals, and have the ability to discriminate between recent and established infection samples with 83–97% accuracy. S/LS serologic testing strategies have been developed and validated for HIV clade B infections; additionally, one assay has been validated for clades B, E, C, and AD.

Since 1998, a number of S/LS ELISA methods have been developed, and these are based on the concept that antibody titers rise during the first 6 months of infection. Included are the Abbott 3A11 EIA (Abbott Laboratories, Abbott Park, IL), and the Vironostika HIV-1 Microelisa (Dilutional Vironostika (DV), Biomerieux, Durham, NC); rapid HIV tests have also shown encouraging results. Another HIV-1 ELISA (Genetic Systems rLAV EIA, Bio-Rad) also acts as an S/LS assay, but exploits the concept of the changing avidity of HIV antibodies throughout infection.

Currently used S/LS tests have characteristics that limit their use in resource-poor countries. For example, they require relatively complex laboratory instrumentation, which limits their use in settings where stable electricity and sound laboratory infrastructure are lacking. In addition, they require a degree of technical expertise that may be unavailable in a majority of laboratories, and the cost of the test is relatively high.

A particle agglutination (PA) test, the Serodia HIV-1/2 test (Fujirebio, Inc., Tokyo, Japan), is a low-cost, simple-to-perform agglutination assay capable of detecting antibodies to HIV-1 and HIV-2. The test incorporates viral lysate antigens coated on gelatin microbeads (particles) that agglutinate in the presence of specific antibodies.

Because of the widespread use of the Serodia HIV-1/2 PA assay in Asia, Africa, and South America, its technical simplicity, its applicability in laboratories that cannot support ELISA technology, and its low cost, we sought to exploit this assay to develop an S/LS test method that could be used in a much wider variety of testing venues. In this report, we describe the modification of the Serodia HIV-1/2 PA assay to act as a sensitive/less-sensitive assay (PA S/LS) to differentiate recent from established HIV infection.

Methods

Modification of the Serodia HIV-1/2 PA (sensitive PA assay)

The commercially marketed Serodia HIV-1/2 PA assay was first modified for cost savings before being further modified to act as an S/LS test. The modification included diluting the antigen-coated particles 1:68 in PBS, adding 38 μl of this reagent and 8 μl of the test sample diluted 1:10 using Dilsim (Organon Tecknika, Durham, NC) into a microtiter plate. Based on the presence or absence of agglutination, results were interpreted as positive or negative. This modified Serodia HIV-1/2 PA method was considered as the sensitive (PA-S) version of the PA S/LS test.

Validation of the specificity and epidemiologic/analytical sensitivities of the PA-S test

To ensure that the PA-S assay did not lose sensitivity and specificity for correctly determining HIV status as a result of the cost-saving modifications, a total of 990 HIV negative and 200 Western blot confirmed HIV positive samples from the USA were tested to determine the specificity and epidemiologic sensitivity, respectively, of the PA-S test. Samples that produced positive results were repeated using unsensitized (uncoated) particles to rule out reactivity to the gelatin particles themselves; those that exhibited a positive result with the unsensitized particles were considered as producing invalid results and were not included in the analysis. Analytical sensitivity was determined using 83 members from 12 seroconversion panels (Boston Biomedica, Inc., BBI, West Bridgeport, MA), and 13 HIV weak positive samples (as determined by their Western blot profiles). Analytical sensitivity using the panels was determined by comparing the PA-S results with the results from the most sensitive EIAs used to characterize each panel. All samples were tested in a blinded fashion.

Samples for calibrating the PA-LS test

Two hundred and seventy-nine samples from 25 HIV-1 clade B seroconverters from Trinidad, and 30 samples from 12 BBI HIV-1 clade B seroconversion panels (total of 309 samples from 37 seroconverters) were used to calibrate the PA-LS test. The Trinidad seroconverters had been previously identified by the presence of p24 antigenemia in the absence of antibody, followed by subsequent seroconversion or through interval seroconversion during a one-year intensive follow-up of high-risk patients with genital ulcer disease. The sampling methods for this study have been previously reported. The approximate date of seroconversion was imputed as the midpoint date between the last antibody negative and first antibody positive bleed, as measured by several antibody EIAs. The selection of the date of seroconversion was aided by the emerging Western blot profiles and, in the instance where no serology results were available, by the date of the appearance of HIV RNA plus 15 days.

Calibration procedure for the PA-LS test

The PA-S test was further modified in order to calibrate it for use as an LS test. Modifications included using the 1:68 dilution of HIV-1 antigen-coated particles, and assessing different sample dilutions, different particle reagent and sample volumes, and different incubation times before reading. The sample dilutions were made in Dilsim rather than the diluent supplied with the test kit, and samples were diluted at 1:10, 1:100, 1:1000, 1:10 000, 1:20 000, 1:40 000, 1:48 000, 1:58 000, 1:68 000, and 1:80 000. The last dilution that produced a positive reaction (≥1+) by the PA-LS test constituted the endpoint dilution (ED), and this was assessed for all members of the 37 panels. The parameters that exhibited the maximum correct classifications of recent and established HIV infection samples as compared with
the known times of infection of the panel members were selected for the PA-LS test procedure.

With the knowledge that each seroconversion panel contributed varying numbers of specimens in this analysis, and that inter-panel readings were not independent, we also selected a single specimen from each panel to analyze in a manner that gave the best continuum of days post-seroconversion, as would be expected in a cross-sectional pool of independent, seropositive specimens of unknown times from infection.

Concordance studies

A comparison study was conducted to determine the concordance of the PA-LS test with the reference Dilutional Vironostika S/LS (DV). A subset (n = 181) of the 279 members of the 25 Trinidad seroconversion panels used to calibrate the PA-LS test was tested by both assays. Concordance was determined at the optimal parameters of the PA-LS test identified during the calibration phase, and at the standardized optical density (SOD) cutoff of 1.0 for the DV test.

Reproducibility studies

To assess the reproducibility of the PA-LS test, a total of 15 archived samples with unknown times of infection were selected; these consisted of five samples clearly identified by the PA-LS assay as being from recently infected persons, five samples clearly identified as being from persons with established infection, and five borderline samples classified as recent (negative) at the sample dilution of 1:40 000, but positive at the 1:20 000 dilution. To determine the reproducibility of results between runs (inter-run reproducibility), each sample was tested in a blinded fashion at dilutions from 1:10 to 1:68 000 over three consecutive days, and results were compared for each corresponding dilution. Additionally, the 15 samples were repeatedly tested 11 times each by the PA-LS to determine the reproducibility of results within a run (intra-run reproducibility). Also, inter-rater reliability was assessed by comparing the agglutination reactions scored independently by two technicians.

Statistical analysis

Receiver operator curve (ROC) analysis, a medical data analysis tool widely utilized to study the effect of varying the threshold on the numerical outcome of a diagnostic test,10 was used to determine the most parsimonious days post-seroconversion cutoff that yielded the greatest sensitivity for correctly classifying recent and established infection samples.

Inter-rater variation was assessed by Chi-square analyses and by computing the kappa statistic (measure of agreement). Reproducibility was assessed by the computation of the coefficients of variation (CV).

Results

Sensitivity and specificity of the modified PA-S assay

With cost savings being one of the major objectives of the development of the PA-LS test, the routine Serodia HIV-1/2 PA assay was initially modified by diluting the antigen to various concentrations, and its sensitivity and specificity were assessed at each dilution. The optimal antigen dilution of the particle reagent at which there was no loss of sensitivity and minimal loss of specificity was determined to be 1:68. Under these conditions, the PA-S correctly identified 200/200 (100% sensitivity) of the HIV-1 Western blot positive samples and 978/990 (98.8% specificity) of the HIV negative specimens. Ten of the 12 HIV negative samples that produced discordant (initially positive) results by the PA-S also produced positive results when re-tested using unsensitized particles; the remaining two samples produced borderline (+/-) results. Therefore, the results from 10 samples were considered invalid, and the actual specificity of the PA-S was then determined to be 99.8%. When assessing the analytical sensitivity using 83 members of 12 BBI seroconversion panels in comparison with the most sensitive ELISA used to characterize the 12 panels, the PA-S test detected infection earlier (by one bleed) in 3/12 panels, later (by one and two bleeds) in two panels, and at the same time in seven panels. Moreover, all of the 13 weak HIV-1 Western blot positive samples were classified as positive by the PA-S.

Performance of the PA-LS assay

Typical reactions by the PA-LS are depicted in Figure 1. Parameters that were selected included a particle reagent volume of 38 μl, a sample volume of 8 μl, and a reading of results time between 6 and 48 hours. Figure 2 shows the sensitivity rates of the PA-LS at three candidate sample dilutions (1:40 000, 1:48 000 and 1:58 000) for recent and established infections. These dilutions were chosen as they yielded the highest rates of sensitivity for correctly classifying known recent and known established seroconversion panel samples. Although the 1:58 000 dilution exhibited the highest sensitivity (98%) for classifying recently infected
samples between 80 and 160 days post-seroconversion, the 1:40 000 dilution gave the highest collective sensitivity for recent and established infection samples corresponding to ≤190 days post-seroconversion. As shown in Table 1, 100/103 sample members known to be from recently infected patients (≤190 days post-seroconversion) were classified correctly by the PA-LS test, thereby yielding a sensitivity for recent infections of 97.1%; three samples were misclassified (specificity for recent infections of 96.6%). Similarly, 199/206 specimens known to be from persons with established infections (>190 days post-seroconversion) were correctly classified by the PA-LS, yielding a sensitivity of 96.6%; seven samples were misclassified (specificity for established infections of 97.1%). One of these seven samples was from an AIDS case, yielding a misclassification rate of 4% among 25 AIDS case samples tested. Figure 3 shows the demarcation point (window period) using the 1:40 000 sample dilution for recent and established infection samples.

Ranges of days post-seroconversion of the samples included in the sensitivity analyses were 0—2709 days (interquartile range (IQR) = 75, 1569). Each panel contributed an average of 8.35 members (range 1—20). Random selection of single specimens from each panel resulted in a near normal distribution of days post-seroconversion with a range of 1—2287, (IQR = 13, 748) (data not shown). Analysis of these independent readings resulted in the correct classification of 18/18 specimens known to be recent (≤190 days) and 19/19 specimens that were known to be established (>190 days) at the 1:40 000 and 1:48 000 dilutions.

Reproducibility studies

Intra-run, inter-run, and inter-rater analyses revealed a 100% agreement of the binary ED readings (agglutination vs. no agglutination) of the samples used for reproducibility when tested in a blinded fashion across each stratum. In addition, all results were reproducible when 1:40 000 dilutions of the 15 samples were tested 11 times in the same run.

Concordance between the DV and the PA-LS assay

The concordance between the PA-LS assay (at the 1:40 000 dilution) and the DV S/LS assay (at the 1.0 SOD cutoff) using a subset (n = 181) of the seroconversion sample members from

### Table 1

<table>
<thead>
<tr>
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<th>Recent by PA-LS</th>
<th>Established by PA-LS</th>
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<tbody>
<tr>
<td>Recent by known days post-SC (≤190 days post-SC)</td>
<td>100/103 (97.1%)</td>
<td>3/103 (2.9%)</td>
</tr>
<tr>
<td>Established by known days post-SC (&gt;190 days post-SC)</td>
<td>7/206 (3.4%)</td>
<td>199/206 (96.6%)</td>
</tr>
</tbody>
</table>

SC, seroconversion.
Trinidad was found to be 60% for recent infection samples, and 73% for established infection samples. The DV S/LS correctly classified 83% of recent and 54% of established infection samples, compared with 98% of these recent and 93% of established infection samples when testing the same subset by the PA-LS.

**Discussion**

HIV incidence estimations using S/LS testing strategies are easily performed and provide useful information for public health intervention and prevention programs by identifying high-incident populations for targeting. In addition to surveillance, an important potential application of S/LS testing is in clinical settings where knowledge of when a patient was infected can assist healthcare professionals in making important decisions regarding appropriate behavioral intervention, contact tracing, and treatment options. The purpose of this study was to develop an alternative S/LS assay that does not require sophisticated instrumentation and infrastructure to determine recent or established HIV infection. Once the test is fully calibrated and validated, it would be appropriate to evaluate it in cross-sectional surveillance situations before making a conclusion as to the utility of the assay for determining the incidence of HIV in persons at all stages of infection, including those with late stage disease and those on antiretroviral therapy (ART).

The currently available S/LS tests are ELISA methods that require instrumentation and a certain degree of technical expertise; they are also relatively expensive when applied to surveillance activities. In addition, a significant limitation of these tests is their considerable variability (CVs of up to 33%) between runs and between different laboratories. Consequently, there is the need to institute calibrators and software packages for data analysis. Also, the necessity to calculate new control ranges each time a new kit lot is used adds to the rigor of the procedure. In addition to a less than perfect sensitivity for correctly classifying infections, the widely used DV S/LS test requires stable electricity for incubators, plate readers, and washers, expertise for performing the test, maintenance provisions for instruments, and has a relatively high cost. Similarly, the newly marketed BED capture EIA (BED CEIA) involves multiple incubations, requires instrumentation, a freezer for storing some reagents, and even a balance for weighing reagents.

Table 2 provides a comparison of the major parameters of EIA S/LS tests and the PA-LS test.

The utility of rapid HIV S/LS assays has been reported, and these tests exploit the same principle of antibody titer for differentiating recent from established infections. However, the findings of Constantine et al. showed that a rapid test method had a high misclassification rate (17%), although test indices could be improved if coupled with the results of the DV test. In a study by Soroka et al., three rapid HIV tests were evaluated for their ability to identify recent infections by optimizing the sample dilution and comparing test results with samples previously characterized by the Abbott 3A11 S/LS test.

![Figure 3](image-url) Selection of demarcation point (window period) using optimal sensitivity (at 1:40 000 sample dilution) for recent and established infection samples.

Table 2  Comparison of procedures and costs of LS assays previously described in the literature

<table>
<thead>
<tr>
<th>Vironostika LS</th>
<th>BED CEIA</th>
<th>Serodia PA-LS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator</td>
<td>1:20 000</td>
<td>1:101</td>
</tr>
<tr>
<td>Control dilution</td>
<td>1:76</td>
<td>1:101</td>
</tr>
<tr>
<td>Sample dilution</td>
<td>1:20 000</td>
<td>1:101</td>
</tr>
<tr>
<td>Sample incubation</td>
<td>20 min at 37 °C</td>
<td>1 h at 37 °C</td>
</tr>
<tr>
<td>Wash No. 1</td>
<td>4×</td>
<td>4×</td>
</tr>
<tr>
<td>Conjugate incubation</td>
<td>20 min at 37 °C</td>
<td>1 h at 37 °C</td>
</tr>
<tr>
<td>Wash No. 2</td>
<td>4×</td>
<td>4×</td>
</tr>
<tr>
<td>Substrate incubation</td>
<td>10 min at 15–30 °C</td>
<td>15 min at 25 °C</td>
</tr>
<tr>
<td>Stop solution</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>Read results</td>
<td>Immediately</td>
<td>Immediately</td>
</tr>
<tr>
<td>Calculation</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>Interpretation as recent</td>
<td>SOD &lt;1.0</td>
<td>ODN &lt;0.8</td>
</tr>
<tr>
<td>Format</td>
<td>96-well EIA plate</td>
<td>96-well EIA plate</td>
</tr>
<tr>
<td>Incubator, reader, washer</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>No. controls and calibrators/run</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>No. of wells per sample</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Cost per sample</td>
<td>$3 ($1 per well)</td>
<td>$2.75–$11 ($2.75 per well)</td>
</tr>
</tbody>
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EIA, enzyme immunoassay; CEIA, capture EIA; SOD = standardized optical density; ODN = normalized optical density.
LS test. The concordance values of these rapid tests with the Abbott 3A11 S/LS were all greater than 94%. However, the accuracy of these rapid tests for classifying samples was not assessed because samples with known times of infection were not used.

A concern of S/LS EIA and rapid tests is their subtype B-dependent performance, which could significantly limit their use in many parts of the world. This was addressed in the BED-CEIA by using a highly conserved multi-subtype (clades B, E and D) derived branched gp41 peptide in an IgG capture format. Although this S/LS test was validated in locations where different HIV clades exist, concern exists that it may overestimate incidence. Indeed, the Epidemiology Reference Group Secretariat at UNAIDS has recommended in December 2005 that the BED assay not be used for incidence estimates, and called for "exploring alternative laboratory assays".

The Serodia HIV-1/2 particle agglutination assay is widely used in many parts of the world, is simple to perform, and is an inexpensive assay that does not require instrumentation. Also, it incorporates a viral lysate antigen that may be effective in detecting antibodies to non-B clades. Because of these advantages, the test was selected and modified to act as an S/LS assay. The PA-LS test procedure involves only two steps: (1) dilution of samples and antigen, and (2) addition of samples and antigen into reaction wells. The assay does not require any washing steps, preparation of reagents (other than antigen dilution), or multiple incubations; there is only one incubation step conducted at room temperature, and no instruments or equipment are required to perform the PA-LS test. Results are read after 6 hours, but are stable for up to 48 hours, allowing a convenient overnight incubation before interpretation.

The results using the PA-S (sensitive mode) showed that the modifications to the Serodia HIV-1/2 PA test did not result in a loss of sensitivity or specificity; therefore, it could be used for routine HIV testing while offering a significant cost saving (>50 times). However, because false positive results were observed in 12 out of 990 HIV-1 negative samples, it is recommended that laboratories that choose to use the PA-S as the screening test should test all samples with the unsensitized particles, or re-test only those samples that are reactive on the PA-S using unsensitized particles to identify false positive results that are due to non-specific reaction to the gelatin particles. Also, it is important to use only HIV confirmed positive samples when performing S/LS testing because false positive samples may cause an overestimation of recent infection.

The PA-LS test was successfully calibrated as an S/LS test strategy, as evidenced by its excellent performance when testing 37 seroconversion panels consisting of 309 samples with known times of infection. The test correctly identified 97.1% and 96.6% of samples from persons with recent and established infections, respectively. Although a preliminary analysis of this data set showed that 1/25 (4%) of AIDS samples were misclassified as being recent, which is similar to the 4.3% rate shown in studies with the BED assay, future studies with a larger sample size of AIDS patients would provide a more definitive misclassification rate. This near-perfect performance occurred with a selected window period of 190 days, not much different from that of other S/LS assays that have window periods ranging from 133 to 170 days. We selected this window period to yield the best collective performance with all samples. Surprisingly, a poor concordance was observed between the performances of the DV S/LS test and the PA-LS using a subset of 25 seroconversion panels. This was evidently because of the poor accuracy of the DV S/LS test, where only 83% of recent and 54% of established infection samples were correctly classified. The reason for this poor performance is unknown. When single specimens were selected from each panel (n = 37) and analyzed by the PA-LS as independent points that might mirror a true cross-sectional testing situation, the PA-LS correctly classified 100% of all specimens (recent and established). The optimal predictive value of the PA-LS to correctly detect known recent infections (≤190 days) was 99% at the 1:20,000 dilution; however, 10.7% of known recent samples were misclassified (data not shown).

To determine the PA-LS test’s ability to accurately discriminate samples close to the 190-day window period, the analysis also included four specimens that were around the 190 days post-seroconversion cutoff. With respect to the reproducibility of test results, the PA-LS exhibited 100% reproducibility, thereby suggesting no need for repeat testing. In addition, the interpretation of results was consistent when read by different operators.

There are three disadvantages of the PA-LS test, however. First, the reagents do require refrigeration, which might limit its use in some laboratories without stable electricity. Second, as with most S/LS assays, the test requires a number of dilutions to arrive at the 1:40,000 sample dilution, a maneuver that requires good pipetting technique. Third, the viral lysate antigen is derived from HIV clade B and the test has not been validated with sera from non-B clades. To address issues related to quality assurance and quality control when performing the PA-LS test, it may be necessary to include a calibrator or other means to ensure that the test is performing as expected with all users and in all testing venues. Such needs are usually determined and incorporated by a manufacturer at the time of commercialization to ensure that all users attain a high degree of confidence in the performance of the test. The inclusion of a calibrator or quality control check would verify that users, whether in a centralized or more remote testing laboratory, are performing the test correctly.

The most attractive feature of the PA-LS is its extremely low cost. To understand the potential cost-savings that could be achieved using the PA-LS test, we determined, as an example, the cost involved in the measurement of HIV incidence by testing archived samples previously collected in a typical sentinel surveillance study. Assuming a sample size of 50,000 HIV positive samples collected from a survey, and an estimated 20% rate of recent infection, the BED test (at $2.75 per test, and triplicate testing of samples initially classified as being from recent infection) would cost $220,000, and the DV (at $1 per test, and triplicate testing of all samples) would cost $150,000. However the PA-LS test (at $0.10 per test, including the cost of the Dilsim sample diluent, and only single testing of samples) would cost only $5,000. This cost-saving (about 30—44-fold) using the PA-LS will offer a more affordable means to measure and monitor incidence, identify recently infected persons, and to evaluate the efficiency of intervention programs. Moreover, laboratory professionals in most parts of the world will not have difficulty adopting the PA-LS test since most are already
familiar with the routine Serodia HIV-1/2 PA test, which is identical to the PA-LS test except for the initial dilution step.

In summary, we have developed a simple serologic assay that can be used for incidence estimates and identifying persons who have been recently infected with HIV. The test is simple to perform, applicable for resource-limited countries because of its low cost and lack of required instrumentation, and its format is familiar to laboratories throughout the world. These attributes address the major limitations of currently used S/LS assays; that is, the ability of the PA-LS assay to be performed in laboratories that cannot support EIA technology because of infrastructure or technical expertise requirements. In conclusion, the attractive features of the PA-LS, along with its near-perfect performance for differentiating recent from established HIV infection, pose the PA-LS test to be a practical choice for health organizations and remote local laboratories, especially in less developed nations where limited financial and material resources have to be wisely and systematically allocated to implement HIV prevention and treatment programs.

Conflict of interest: No conflict of interest to declare.

References