Development and Validation of an Immunoassay for Identification of Recent Human Immunodeficiency Virus Type 1 Infections and Its Use on Dried Serum Spots

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The objective was to develop and to validate an immunoassay to identify recent human immunodeficiency virus type 1 (HIV-1) infections that can be used on dried serum spots (DSS). A single, indirect enzyme-linked immunosorbent assay was developed to quantify antibodies toward four HIV-1 antigens: consensus peptides of the immunodominant epitope of gp41 (IDE), consensus V3 peptides, recombinant integrase, and recombinant p24. The parameters of the logistic regression used to classify the samples were estimated on a training sample (210 serum samples) using resampling techniques to get stable estimates and then applied to a validation sample (761 serum samples). The IDE and V3 peptides were the best able to discriminate between the antibodies present in serum from recently (≤ 6 months) infected individuals and those with long-lasting infection. Combined quantification of antibody binding to these two synthetic antigens allowed us to identify recent infections with an area under the receiver operating characteristic curve of 0.949 and a sensitivity of 88.3%, with a specificity of 97.6% in patients with long-term infection (but not AIDS) and 86.0% in patients suffering from AIDS with a threshold of 0.50 in the validation sample. This simple immunoassay can be used to identify recently HIV-1-infected patients. Its performance is compatible with its use in population-based studies including DSS.

The human immunodeficiency virus (HIV) epidemic is generally assessed by monitoring seroprevalence i.e., the proportion of persons with HIV antibodies (including recently infected people and people who were infected several years previously). To understand recent changes in the HIV epidemic, it is necessary to estimate the incidence, i.e., the number of newly infected subjects in a defined period. A strategy based on a sensitive/less sensitive testing algorithm was recently used to identify serum samples from recently infected individuals (16). This strategy that uses both a sensitive and a less-sensitive enzyme immunoassay (S/LS EIA), also called a detuned assay, was applied to various situations, providing estimates of HIV incidence (12, 14, 16, 18, 24, 33, 35). One of the major drawbacks of this strategy is that the test is an adaptation of a commercial EIA, which poses problems for long-term availability. It is therefore necessary to develop and to validate simple immunoassays that can continuously be used independent of any commercial source. The knowledge of the anti-HIV type 1 (anti-HIV-1) antibody response (5, 8, 20, 38) and recent studies aimed at identifying antigens to distinguish recent infections (27, 30) allowed us to design a candidate assay to assess persons with recent infection. We report the development and the validation of this assay for the identification of recent HIV-1 infections (EIA-RI) and its application to dried blood spots.

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MATERIALS AND METHODS

Antigens. The choice of antigens was dictated by four major criteria. First, the antigens should be recognized by antibodies present in all or virtually all HIV-1-infected patients. Second, they should be highly conserved independent of the HIV-1 clade. Third, they should be easily obtained without drift in their properties. Fourth, antibodies to these antigens should be detected with some delay after seroconversion to make it possible to distinguish early samples from late samples. Three antigens fulfilled these criteria.

(i) **The immunodominant epitope of gp41 (IDE).** Two synthetic peptides, one corresponding to the consensus sequence of all clades (RVLAVERYLKDQQL LGIWGCSGKLICTTAV) and one corresponding to the consensus sequence of clade D (RILAVESTLKDQQLGIWGCSGRHICTTAV), were prepared. The clade D peptide was used because it is the most antigenically divergent due to mutations within the cysteine loop (22). Every infected patient rapidly develops antibodies to this region (4, 25, 27, 30). A low concentration of this mixture should allow the binding of late antibodies that have acquired sufficient avidity and thus quantitative detection.

(ii) **A mixture of five V3 peptides representing consensus sequences of clades A, B, C, D, and CRF01-AE.** The sequences were previously described (6, 31, 32). We did not feel that it was necessary to add other peptides due to the extensive cross-reactivity between the V3 sequences of these clades and other clades (31, 32). Antibodies to these consensus sequences are detectable in most infected people a few months after seroconversion (6, 27, 30).

(iii) **Integrase (IN) p32.** This protein is encoded by the most conserved gene of HIV, and most HIV-1-infected patients develop anti-p32 antibodies. These antibodies are among the last to appear after seroconversion (1, 13, 27). A recombinant integrase produced in *Escherichia coli* was obtained from J. F. Mouscadet (Villejuif, France).

A recombinant p24 produced in *E. coli* was obtained from F. Mallet (Unité Mixte de Recherche Centre National de la Recherche Scientifique [CNRS]-BioMérieux, Lyon, France). It was used for comparison, as it is theoretically a less relevant antigen. Antibodies to p24 appear early during seroconversion and disappear when immunodeficiency progresses in most patients, except in Africa (3, 21, 27).

EIA-RI. Preliminary studies using sequential serum samples from patients with a known date of infection and serum samples from patients at various stages of

TABLE 1. Description of the serum samples used to evaluate the EIA-RI

Sample type and population	No. in training sample of:		No. in validation sample of:	
	Serum samples	Patients	Serum samples	Patients
Serum				
Seroconverters ≤ 180 days postinfection				
Local panel			94	57
PRIMO cohort	60	24		
Seroconverters > 180 days postinfection				
Local panel			24	10
PRIMO cohort	46	22		
Chronic patients ^a				
Local panel	104	104		
SEROCO/HEMOCO cohorts			500	500
AIDS patients			143	143
Total	210	128	761	700
Dried serum spots				
Seroconverters ≤ 180 days postinfection				
Local panel			92	57
PRIMO cohort	60	24		
Seroconverters > 180 days postinfection				
Local panel			15	10
PRIMO cohort	46	22		
Chronic patients ^a				
Local panel	154	154		
SEROCO/HEMOCO cohorts			250	250
AIDS patients			143	143
Total	260	178	500	450

^a Patients not suffering from AIDS.

the disease allowed us to define assay conditions that might be able to distinguish between recent (≤ 180 days) and established (> 180 days) infection.

Each serum sample was tested in four wells of a polyvinyl microtiter plate (Falcon), each coated with one of the four selected antigens. Wells were coated by incubation for 20 h at 37°C (100 μ l per well) with either an equimolar mixture of the two IDE peptides (0.05 μ g/ml each) or an equimolar mixture of the five V3 peptides (0.2 μ g/ml each) or p24 (1 μ g/ml), all diluted in 0.01 M phosphate-buffered saline (PBS), pH 7.4. Wells were coated with integrase (0.5 μ g/ml diluted in PBS supplemented with 4 M guanidinium thiocyanate) in the same conditions. The wells were washed three times with PBS containing 0.5% Tween 20 (PBS-TW), and the unoccupied sites were saturated with PBS containing 2% newborn calf serum (NBCS) for 45 min at 37°C (200 μ l per well). Serum samples were diluted 1:100 in 0.01 M PBS containing 0.75 M NaCl, 10% NBCS, and 0.05% Tween 20 (PBS-TW-NBCS). Diluted serum (100 μ l) was added to each well, which was incubated for 30 min at 18 to 20°C before being washed five times with PBS-TW. Peroxidase-conjugated goat F(ab)₂ anti-human immunoglobulin (100 μ l of a 1:5,000 dilution in PBS-TW-NBCS; TAGO, Burlingame, CA) was added, and the wells were incubated for 30 min at 18 to 20°C. The wells were washed five times with PBS-TW, and the reaction was revealed by incubation with hydrogen peroxide-*o*-phenylenediamine (H₂O₂-OPD) for 30 min at room temperature (RT). The color development was stopped with 2N H₂SO₄, and the absorbance (*A*) was read at 492 nm. The same pool of sera from 10 unexposed HIV-negative individuals was used as both a negative control (NC) and a calibrator in each run (two replicates). All results are expressed as the sample A/A NC ratio. The same positive control that provided absorbance values in the dynamic range between 0.5 and 1.5 was used to assess the validity of each run (two replicates per antigen). The interrater coefficients of variations were below 15%.

Sera. We tested 971 serum samples from untreated patients (Table 1). Of these, 224 serial specimens came from 81 seroconverters from either a local panel ($n = 118$) or the multicenter French Agence Nationale de Recherche sur le Sida (ANRS) PRIMO cohort ($n = 106$) identified during primary infection or soon after seroconversion (11). Subjects presenting during or soon after primary infection, whether it was symptomatic or not, were enrolled in this cohort. Recent infection was confirmed by an incomplete Western blot pattern (absence of anti-p68 and anti-p34), a positive p24 antigenemia or detectable plasma HIV RNA with a negative or weakly reactive enzyme-linked immunosorbent assay (ELISA) test, or an interval of less than 6 months between a negative and a positive ELISA test. The date of infection was estimated as the date of symptom onset minus 15 days, the date of incomplete Western blotting minus 1 month, or the midpoint between a negative and a positive ELISA test. The remaining 747 serum samples came from chronically infected patients from either a local panel or the multicenter French ANRS SEROCO/HEMOCO cohorts (9): 604 without AIDS and 143 suffering from clinical AIDS. The serotype or genotype was not determined for every patient. However, the patients were infected by subtype B variants in the large majority when it was known.

An additional set of serum samples from the PRIMO cohort was used to evaluate the influence of treatment on the results of the EIA-RI. This set of samples consisted of 385 serial serum samples from 78 patients whose treatment (highly active antiretroviral therapy [HAART]) was initiated at the time the primary infection or seroconversion was diagnosed: 219 samples were collected within 180 days of diagnosis, and 166 samples were collected thereafter.

Statistical analysis. The serum samples were divided into a training sample and a validation sample to allow an external validation of the parameters estimated in the training sample. The objective of the statistical analysis was to classify the samples in two groups: those corresponding to a recent infection (≤ 180 days) and those not corresponding to a recent infection. We used logistic regression for this purpose, which gives the probability *P* of being classified as a nonrecent seroconverter depending on the combination of antigens considered. When the probability *P* was > 0.50 , the serum sample was classified as a nonrecent seroconverter, whereas when *P* was ≤ 0.50 , the serum sample was classified as a recent seroconverter. Two techniques were used to handle the dependency of the observations because some patients had several measures taken over time in either the training or the validation sample. First, we used a bootstrap resampling procedure, which provides robust estimates (34): sera from the training sample were randomly sampled with replacement 1,000 times and the logistic regression parameters estimated. The final parameters were the means of the 1,000 estimates. Second, we used generalized estimating equation (GEE) logistic models accounting for the dependency between multiple samples of a given patient (23). A block diagonal correlation matrix was used to specify the nature of the correlation among repeat observations within the patient. We used an exchangeable correlation structure in which the correlation between observations made on a given patient at different times was assumed to be constant. The sensitivity (the ability to classify a patient infected with HIV in the past 180 days as a recent infection) and specificity (the ability to classify a patient infected with HIV for more than 180 days as having a nonrecent infection) were then calculated in the validation sample by calculating how many recent seroconverters were classified as such and how many nonrecent seroconverters, chronically infected patients, or patients suffering from AIDS were classified as such according to the logistic model results. Thresholds other than 0.50 were also explored, from 0.00 to 1.00, using receiver operating characteristic (ROC) curves.

All possible combinations of the levels of antibodies against the four selected antigens were tested. The best models were selected on the basis of the area under the ROC curves obtained on the validation sample. The final threshold was chosen on the basis of the sensitivity and specificity levels obtained on the validation sample with the retained combination of antigens in order to get the best sensitivity given an overall specificity higher than 95%.

Adaptation of the EIA-RI to dried blood spots. To make it possible to apply this assay to blood, serum, or plasma collected on filter paper, we evaluated also the EIA-RI using dried serum spots (DSS). Seven hundred sixty serum samples from untreated HIV-1-infected patients were used: 213 serial specimens from 81 seroconverters and 547 samples from patients with chronic infection, 143 of whom were suffering from AIDS. Most of them were those described above, but there were a few different samples due to lack of availability (Table 1). Filter paper was spotted with 20 μ l of serum and then allowed to dry overnight at RT. Disks were punched out of the DSS (diameter, 6 mm), placed in 500 μ l of PBS-TW-NBCS buffer, and then incubated at RT for 1 h in an ultrasonic cleaner. The eluted serum was directly placed in the wells of the microplates (100 μ l per well) and subjected to the EIA-RI. Only the two antigens identified as being the most relevant, IDE and V3 (see Results), were tested. Statistical analysis was performed as described above with the parameters estimated on the training

FIG. 1. Distribution of the sample A/A NC ratios for each antigen and each population. The horizontal lines represent the 10th, 25th, 50th, (median), 75th, and 90th percentiles of the ratios. Training sample (TS) ≤ 180 , sera collected within 180 days of primary infection ($n = 60$); TS > 180 , sera collected more than 180 days after primary infection ($n = 150$). Validation sample (VS) ≤ 180 , sera from seroconverters collected within 180 days of primary infection ($n = 94$); VS > 180 , sera from seroconverters collected more than 180 days after primary infection ($n = 24$); VS chronic, sera from patients with established infection not suffering from AIDS ($n = 500$); VS AIDS, sera from patients suffering from AIDS ($n = 143$).

sample with a bootstrap resampling procedure and then applied to the validation sample.

RESULTS

EIA-RI. The panels of sera from HIV-1-infected patients with recent infection (≤ 180 days) or established infection (> 180 days) were used to determine which antigens permit the best separation of the two groups. The results obtained with the training and validation samples were very similar (Fig. 1). Median ratios for antibodies to IDE and V3 were more than 20-fold higher for sera from chronically infected patients than for sera from recently infected patients (45.74 versus 1.75 and 84.37 versus 3.63 for IDE and V3, respectively, in the training sample). They were more than 10-fold higher for antibodies to IN (25.24 versus 2.07) and similar for antibodies to p24 (39.76 versus 43.00). The distribution of the ratios indicates that the most discriminatory antigens were IDE and V3, as the ratios for earlier and later samples overlapped only slightly (Fig. 1). Conversely, a large degree of overlap was observed for p24. Intermediate results were obtained with IN. Sera from AIDS patients gave lower ratios for each antigen than those from

chronically infected patients not yet suffering from AIDS, generating more overlap between AIDS patients and recently infected patients.

Table 2 shows the sensitivities and specificities calculated based on the classification obtained using the corresponding bootstrap logistic regression model for each combination of antigens in both the training and the validation samples for a threshold of 0.50. It indicates that the most discriminatory antigen was IDE alone or in combination with V3. In the validation sample, the sensitivity of the IDE/V3 combination for recent infection was 88.3%. Twelve of the 500 serum samples from patients with chronic infection and 20 of the 143 serum samples from AIDS patients were misclassified, leading to specificities of 97.6% and 86.0%, respectively. Both classifications, the one obtained with bootstrap and the other obtained with GEE, gave similar results, indicating that repeat samples from the same individuals did not introduce any measurable bias. The two ROC curves show the good discriminatory power of this combination to allow a correct classification (both areas under the curve were evaluated as 0.949). As an example, the IDE/V3 combination is shown in Fig. 2. The

TABLE 2. Area under the ROC curve and sensitivity and specificity for each antigen or combination of antigens on the validation sample when using a threshold of 0.5

Antigen or combination	Validation sample		Validation sample parameter (with threshold of 0.50)			
	Area under the ROC curve	SE	Sensitivity at ≤ 180 days	Specificity at > 180 days	Specificity	
					Chronic patients	AIDS patients
IDE	0.946	0.015	88.3	100	95.6	86.7
V3	0.936	0.016	83.0	95.8	96.0	80.4
IN	0.831	0.021	62.8	87.5	88.2	75.5
p24	0.380	0.021	0	100	68.0	97.2
IDE + V3	0.949	0.014	88.3	100	97.6	86.0
IDE + IN	0.945	0.014	85.1	100	95.4	81.1
IDE + p24	0.944	0.015	88.3	100	95.4	94.4
V3 + IN	0.938	0.016	86.2	100	96.2	80.4
V3 + p24	0.937	0.017	81.9	100	96.8	93.0
IN + p24	0.778	0.022	26.6	100	88.0	95.1
IDE + V3 + IN	0.949	0.014	86.2	100	97.2	84.6
IDE + V3 + p24	0.952	0.015	84.0	100	97.6	96.5
IDE + IN + p24	0.938	0.015	80.9	100	96.2	94.4
V3 + IN + p24	0.931	0.017	78.7	100	96.0	95.1
All four	0.943	0.015	79.8	100	97.0	95.1

^a Specificity values are given for the following various populations: seroconverters > 180 days postinfection, chronically infected patients not suffering from AIDS, and AIDS patients. Sensitivity was estimated on serum samples from seroconverters ≤ 180 days following the diagnosis of primary infection and/or seroconversion. We used logistic regression, which gives the probability P of being classified as a nonrecent seroconverter depending on the combination of antigens considered. When the probability P was greater than the threshold, the serum sample was classified as a nonrecent seroconverter, whereas when P was less than or equal to the threshold, the serum sample was classified as a recent seroconverter. Boldface values correspond to the best combination of one, two, or three antigens.

threshold of 0.50 was leading to the highest sensitivity for a specificity greater than 95% and was therefore appropriate. A higher specificity could be obtained in patients suffering from AIDS (96.5%) with a p24/IDE/V3 combination (Table 2).

Based on these results, we decided that it was possible to apply the IDE/V3 assay in surveys in which the AIDS status was known independently. In this case, the probability (P) of being classified as a nonrecent seroconverter was given by the equation

$$P = \exp(-1.1433 + 0.06079 \cdot ide.r + 0.02977 \cdot V3.r) / [1 + \exp(-1.1433 + 0.06079 \cdot ide.r + 0.02977 \cdot V3.r)]$$

where *ide.r* and *V3.r* are the sample A/A NC ratios in the IDE and V3 wells, respectively. This formula was applied to the 761 serum samples from the validation sample. The distribution of the probability of being classified as a nonrecent seroconverter in the different population groups revealed a clear discrimination between sera collected within the first 6 months of infection and those collected later, even though there was some overlap between a few samples (Fig. 3A).

EIA-RI in treated patients. The EIA-RI was applied to 385 sequential serum samples from 78 patients treated by HAART at the time of early diagnosis. Most of the 166 samples collected more than 180 days after diagnosis of primary infection

FIG. 2. ROC curves showing the similarity of results obtained with the bootstrap resampling procedure (circles) and the GEE logistic model (squares) for the IDE/V3 combination at the 0.5 threshold. The arrow indicates the results for a threshold of 0.50. Other thresholds are not shown for clarity. The hatched curve is an example of the result that would be obtained with an assay without discriminating power.

FIG. 3. Distribution of the probability (P) of being classified as a nonrecent seroconverter obtained with the EIA-RI on the validation sample using the serum samples (A) or the dried serum spots (B); and distribution of the probability (P) with the EIA-RI applied to sera collected within (≤ 180) or beyond (> 180) 180 days of primary infection from seroconverters who received early HAART (C). $Sc \leq 180$, sera from seroconverters collected within 180 days of primary infection; $Sc > 180$, sera from seroconverters collected more than 180 days post-primary infection; chronic, sera from patients with established infection not suffering from AIDS; AIDS, sera from patients suffering from AIDS.

had a probability below the 0.5 cutoff (Fig. 3C) and therefore would be misclassified as recently infected, whereas all the late samples from untreated seroconverters were clearly above this cutoff (Fig. 3A).

EIA-RI on dried serum spots. Each DSS was subjected to the EIA-RI with IDE and V3 after elution. The corresponding logistic regression equation is given as follows, with slightly different parameter values:

$$P = \exp(-2.11234 + 0.15367 \cdot ide.r + 0.01242 \cdot V3.r) / [1 + \exp(-2.11234 + 0.15367 \cdot ide.r + 0.01242 \cdot V3.r)]$$

This formula was applied to the 500 DDS in the validation sample. The distribution of the probability of being classified as a nonrecent seroconverter in the different population groups of the validation sample showed a clear discrimination between sera collected within the first 6 months of infection and those collected later, as found for the sera (Fig. 3B).

Twelve of the 92 DSS collected within 180 days of primary infection were misclassified, giving a sensitivity of 87.0%. None of the 15 late serum samples from seroconverters were misclassified (100% specificity), whereas 5 of the 250 chronic patients and 13 of the 143 AIDS patients were misclassified, giving specificities of 98.0% and 90.9%, respectively.

DISCUSSION

The recently developed S/LS EIA strategy to distinguish recently infected people from those with established infection is an innovative and simple approach to estimate the proportion of recent HIV infections in cross-sectional studies. Most previous approaches were based on commercially available assays in a detuned format. Another approach is to calculate the avidity index of antibodies; however, this also requires commercially available assays (36, 37). The use of commercial

assays may lead to the interruption of incidence surveillance studies due to the cessation of production of the EIA by the manufacturers. This happened with the Abbott 3A11 assay (16). Therefore, our goal was to develop a simple assay based on a homogeneous and permanent source of antigen(s) to guarantee its continuous availability on a long-term basis. Our assay was developed and validated using a very large panel of highly documented serum samples. We used a robust statistical analysis to estimate the parameters, as advised when developing prognostic scores (34) and both a training and an independent validation sample to ensure that our results were robust and generalizable (26). Moreover, we checked that repeat samples from the same individuals did not introduce any bias by using a GEE logistic model that takes account of the dependency between the visits of a given patient. Initial studies of the S/LS EIA strategy using a Markov model estimated that the mean time between seroconversion with the two assays was 129 days (16). Our approach was different, translating the question into a classification problem. We aimed to develop a specific and sensitive procedure based on a fixed 180-day period to distinguish between recent and established infection.

Our results indicate that synthetic peptides including sequences of the IDE are excellent candidates for being included in a test for recent HIV infection. This was already reported by Parekh et al. (30). However, we found that the discriminatory power of the assay could be slightly improved by combining the quantitative detection of antibodies to a mixture of V3 peptides with that of antibodies to IDE. This combination gave the best sensitivity (88.3%) and the best specificity in patients with established infection not yet suffering from AIDS (97.6%). As already reported, sera from patients with end-stage AIDS were more frequently misdiagnosed as being from recently infected subjects (16). The quantitative detection of antibodies to p24/IDE/V3 improved the specificity of the EIA-RI in AIDS patients. However, we decided to focus on the assay that used only IDE and V3 for three reasons. First, the individual antibody response to p24 is highly variable, particularly in African patients who develop high anti-24 antibody titers that rarely disappear even during end-stage AIDS in contrast to what is commonly observed in Western countries (3, 21). Second, it is much easier to work with an assay based on chemically produced oligopeptides than recombinant proteins in the long term. Third, one of the major purposes of this assay was its application to the recently implemented mandatory system of notification of new HIV diagnosis in France. As this system requires the simultaneous collection of clinical and epidemiological data, including information on AIDS clinical status, patients with end-stage disease are identified even if the EIA-RI mistakenly identified them as being recently infected. In this application, it is important to evaluate the impact of the performance of the assay on the positive (PPV) and negative (NPV) predictive values. For instance, if the proportion of recent infection is 10%, the PPV can be estimated as 83.7% and the NPV as 98.5%; if the prevalence is 40%, the PPV and NPV are 96.8% and 91.9% respectively. Thus, although this strategy may be useful for modeling incident infections in populations, it has limitations and must not be interpreted at the individual level.

The sensitivity and specificity of the EIA-RI are in the same range as those of the assays based on commercially available

reagents. For instance, the sensitivity and the specificity in the identification of recent HIV infections were 88.2% and 86.9%, respectively, for the avidity assay (36, 37). However, blinded comparisons of the different assays to identify recent HIV-1 infections in panels of well-characterized serum samples would be very useful in the future. Recent studies demonstrated that the ability of the S/LS EIA strategy to detect recent seroconversions differs between different HIV-1 subtypes (15, 28, 39). Our evaluation was done with serum samples from patients residing in France, most of who were infected by subtype B variants. Although we tried to overcome the difficulty linked to the HIV-1 diversity by selecting antigens representative of the various subtypes, additional studies in populations exposed to non-B subtypes are necessary to validate the EIA-RI.

HIV-1 seroconversion is delayed when HAART is started during the acute phase of primary infection and/or as soon as early seroconversion is diagnosed (2, 19). Not surprisingly, the analysis of the performance of the EIA-RI in 78 patients who were treated early after primary infection indicated that the assay is not applicable to treated patients, at least when HAART is initiated at the time of primary infection or early seroconversion. To our knowledge, this limitation has not been reported yet, but other S/LS EIA strategies are likely to face the same problem.

It is essential to monitor the incidence of HIV infection in different populations around the world to assess the epidemic dynamics and to propose adapted preventive measures (29). The testing of blood collected on filter papers has been proven to be a very convenient way to simplify collection, transportation, and conservation. Dried blood spots have been used worldwide for seroepidemiological studies and also to detect or to quantify HIV genomic sequences (7, 10, 17). We show here that the EIA-RI is equally accurate with DSS and with crude serum or plasma. This is the first time that an assay able to identify recent infections has been validated on samples collected on filter paper. This is particularly relevant for large field studies and in settings where collection, centrifugation, storage, and shipment are difficult.

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