p24 Antigen Rapid Test for Diagnosis of Acute Pediatric HIV Infection

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Abstract: Currently, the majority of HIV-infected infants are found within limited-resource settings, where inadequate screening for HIV due to the lack of access to simple and affordable point-of-care tests impedes implementation of antiretroviral therapy. Here we report development of a low-cost dipstick p24 antigen assay using a visual readout format that can facilitate the diagnosis of HIV for infants in resource-poor conditions. A heat shock methodology was developed to optimize disruption of immune complexes present in the plasma of infected infants. The analytical sensitivity of the assay using recombinant p24 antigen is 50 pg/mL (2 pM) with whole virus detection as low as 42.5k RNA copies per milliliter plasma. In a blinded study comprising 51 archived infant samples from the Women and Infants Transmission Study, our assay demonstrated an overall sensitivity and specificity of 90% and 100%, respectively. In field evaluations of 389 fresh samples from South African infants, a sensitivity of 95% and specificity of 99% was achieved. The assay is simple to perform, requires minimal plasma volume (25 μL), and yields a result in less than 40 minutes making it ideal for implementation in resource-limited settings.

Key Words: carbon nanoparticles, HIV p24 assay, heat shock immune disruption, infant HIV, lateral flow diagnostic

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INTRODUCTION

Approximately 1.5 million infants are born to HIV-infected women each year,[1] majority of whom are not tested for HIV until it is too late for optimal antiretroviral therapy (ART). Without treatment, the mortality rate in HIV-infected infants can be as high as 45% by the first birthday and 59% by the second.[2] Recent studies have demonstrated that early HIV diagnosis and prompt ART intervention can reduce infant mortality by 76% and HIV progression by 75%.[3] Such studies have contributed to a change in treatment guidelines by the World Health Organization to initiate ART therapy in infants as soon as they are diagnosed with HIV.[1]

Worldwide, there is vast disparity in health care provisions for HIV-infected infants in the developed world and those in resource-poor countries. In resource-limited countries, where 90% of the exposed infants are found, several obstacles such as limited screening programs for HIV and the lack of a simple and affordable point-of-care diagnostic currently impede the widespread implementation of ARTs. The current gold standard for HIV testing, DNA polymerase chain reaction (PCR), is not suited for implementation in these settings because of the long turn-around-times and inefficiencies involved in transporting samples to central laboratories and returning results to clinics. These inefficiencies lead to poor follow-up and low turn-outs for testing. Rapid antibody tests make diagnostic results available on the same visit, but cannot be used to diagnose infection as HIV exposed infants can retain maternal antibodies for up to 18 months.[4]

Various studies have highlighted the utility of HIV core p24 antigen detection for adult and pediatric screening,[5,6] prediction of disease progression,[7,8] and monitoring the effectiveness of ART.[9,10] An excellent overview of the work has been presented by Schupbach.[11] However, these studies have been carried out with enzyme-linked immunosorbent assay–based systems which are similar in complexity to PCR techniques in that they are time intensive and require expensive laboratory equipment and technical expertise which are not readily available in most low-income and middle-income countries.

Here, we report the development of a rapid p24 antigen dipstick assay designed to diagnose HIV in infant plasma samples with minimal operator input. The assay uses a visual carbon-based reporter label functionalized with antibodies against HIV p24 antigen, and a heat-shock pretreatment step was incorporated into the assay to disrupt immune complexes. The analytical sensitivity of our assay was determined to be in the single picomolar range in model systems of recombinant p24 antigen and intact HIV virus spiked in human plasma. We subsequently assessed the diagnostic performance of our p24 antigen assay for HIV detection in a set of 51 archived infant samples obtained from the US Women and Infants Transmission Study (WITS) and with 389 target infant

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specimens from a screening laboratory in South Africa. The sensitivity (>90%) and specificity (>99%) measurements resulting from testing infant samples from United States and Sub-Saharan Africa indicates that our prototype assay is suitable for further development to meet infant diagnostic needs in affected countries.

METHODS AND MATERIALS

Lateral Flow Assay Strips

Test strips were assembled as an integrated lateral flow disposable with the conjugate and capture antibody incorporated into the device. Briefly, neutravidin protein (Thermo Fisher Scientific; Rockford, IL) and goat antimouse antibody (Biospacific; Emeryville, CA) were printed onto UniSart nitrocellulose membrane (Sartorius; Bohemia, NY) using a noncontact jetter (Biodot XYZ3050 system; Biodot, Irvine, CA). The membrane was dried and stored with desiccant until ready for assembly. Conjugate probe, consisting of carbon particles labeled with anti-p24 mAb108 and biotinylated anti-p24 mAb115B (below), was sprayed onto sample pad using an airjet dispenser. The membranes were laid onto adhesive polyester backing cards (G&L; San Jose, CA), and dried. Assay cards were assembled by the addition of a glass fiber sample pad (Grade 8964; Ahlstrom Corp, Mt. Holly Springs, PA) on the sample receiving side, and an absorbent pad (Grade 205; Ahlstrom Corp) on the opposite end and a clear laminate (ARcare 7759; Adhesives Research, Glen Rock, PA) over the top of the nitrocellulose. Cards were cut into individual strips of 5 mm width using a guillotine cutter and then stored in foil pouches with desiccant until used.

Assay Conjugate and Biotinylated Antibody

Mouse monoclonal anti-p24 mAb108 (108-394-470; Abbott Diagnostics, Abbott Park, IL) was coupled to carbon black nanoparticles (Evonik Industries Parsippany, NJ) for use as the detector conjugate. Carbon nanoparticles were suspended by sonication at 1 mg/mL, to which 40 μg/mL mAb108 antibody was added and allowed to bind for 2 hours while mixing. After centrifugation, the carbon particles were passivated with 10% BSA and resuspended.

Biotinylated antibody (115B-151-423; Abbott Diagnostics, Abbott Park, IL) targeting a second epitope of p24 was generated and purified precisely as described elsewhere.[12]

Assay Conditions

To perform the dipstick assay, 25 μL plasma specimen was mixed with 75 μL heat shock buffer [0.67% NP-40 and 0.2% sodium dodecyl sulfate (SDS) in phosphate-buffered saline] in 2 mL polypropylene tubes and heated for 4 minutes at 88°C in a water bath. After allowing the sample to cool to room temperature (~25°C), an assay strip was inserted into the tube thereby initiating flow of sample into the capture membrane. After 30 minutes, the assay result was read visually and scanned for quantitation.

Virology Quality Assurance (VQA) HIV virus was obtained from the VQA Laboratory at Rush St. Luke's Medical Center, Chicago, Illinois (contract #NO1-AI-50044). The VQA virus control standard (subtype B) was received as viral culture spiked in normal human plasma at a concentration of 1.5 million copies per milliliter as determined in house by the VQA laboratories.[13] Recombinant p24 protein was provided by Abbott Laboratories (Abbott Park, IL) at a stock concentration of 280 μg/mL. The protein concentration was determined using an Abbott HIV-1 p24 microtiter EIA. Briefly, an Abbott HIV-1 p24 Primary Standard is used to establish a standard curve to calculate p24 concentration. The Abbott p24 Primary Standard is calibrated to the Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS) HIV-1 p24 standard.

Signal Quantitation

The wet assay strips were scanned with a Canon flatbed scanner (model: Canoscan 3000ex) from which semiquantitative measurements of p24 antigen concentration were obtained using a Matlab program. To obtain these measurements, an automated program located the control line for each strip, from where it scanned the area 5 mm upstream reporting the average intensity values in blocks of five pixel rows parallel to the control line. From this intensity profile, it calculated the background subtracted intensity by subtracting the average intensity 1 mm upstream of the test line area. The highest background subtracted intensity located between 1.5 and 5 mm was reported as the signal intensity value in Arbitrary Absorbance Units for that strip.

Specimens

Seronegative Samples

One hudred adult normal frozen plasmas were obtained from Gulf Source Blood Bank (Houston, TX) to determine the assay signal threshold.

Wits Infant Samples

Fifty-one blinded samples from infants who participated in the WITS were obtained from Clinical Trials and Surveys Corp (Baltimore, MD). WITS was a prospective cohort study of HIV-infected pregnant women and their children.[14] The plasma specimens were stored at −80°C then thawed on ice before being tested. Viral loads on these samples were performed by Clinical Trials and Surveys Corp using the Roche Amplicor HIV-1 Monitor reverse transcription-PCR.[14]

NHLS Infant Samples

The performance of the assay was evaluated with 389 blinded samples from HIV-exposed infants tested at the National Health Laboratory Service Virology Laboratory, Groote Schuur Hospital, Cape Town, South Africa. Blood samples were collected by heel stick into Microtainers with a scoop (Becton Dickinson; San Diego, CA). All the specimens were from infants younger than 1 year of age, with 88% of the samples being from infants younger than 6 months. Total Nucleic Acid (TNA) PCR (Roche AmpliPrep/COBAS Taqman HIV-1) was performed on 100 μL whole blood precisely as described in the product insert. Residual whole blood was spun down with a centrifuge to obtain plasma fractions. Our p24 assay was performed on these plasma samples.

Analysis

Each specimen was scored by the signal quantitation methodology described above. The signal for each sample's test line was determined in Arbitrary Absorbance Units (AAU) and compared with a predetermined cut-off value to generate a signal to cut off ratio or S/CO. Specimens yielding S/CO ratios equal to or greater than 1.0 were considered HIV positive, and ratios less than 1.0 were considered negative.

RESULTS

To achieve rapid kinetics for antigen detection, we utilized a biotin-neutravidin capture system for the dipstick assay. In this configuration, a 25 μL plasma sample is diluted in a detergent-based buffer and reacted against dried-down monoclonal antibodies specific to 2 unique epitopes of the HIV core p24 antigen on the sample pad of the test strip. One of the antibody pair, mAb115B, is biotinylated, whereas the second, mAb108, is absorbed onto black carbon nanoparticles, forming a sandwich. The sample then flows through the nitrocellulose membrane where capture against a neutravidin test line occurs. In samples containing p24 antigen, a black line is observed at the test line due to capture of antibody–antigen complexes by immobilized neutravidin. Excess carbon conjugate is captured at the control line by immobilized goat antimouse antibody (Fig. 1).

The sensitivity of the assay with the heat shock pretreatment step (below) was evaluated by a model system of recombinant p24 antigen serially diluted in HIV-negative plasma. The wet strips were imaged at 30 minutes after insertion of the assay strip and the absorbance at the test line quantified to provide an objective measure of sensitivity. Using this analysis, the analytical sensitivity of the assay was determined to be 50 pg/mL (Figs. 2A, B). The analytical sensitivity was also determined using live cultured virus. VQA HIV virus[14] was serially diluted into normal plasma and subjected to the assay. The whole virus limit of detection was determined to be 42,500 RNA copies per milliliter (Figs. 2C, D).

In clinical HIV samples, p24 antigen is present within intact whole HIV viruses and free in solution after viral lysis. However, the availability of analyte is limited by low viral titers in addition to the formation of immune complexes after seroconversion. So to disrupt immune complexes due to maternal antibodies, we employed a heat disruption pretreatment step that denatured the immune-complexing antibodies by heating the samples to 88°C for 4 minutes. After allowing the samples to cool to room temperature (~25°C), p24 antigen is available for detection using our assay strips. We empirically tested a range of detergents diluted in phosphate-buffered saline that facilitated heating of plasma to denaturing conditions while precluding the formation of gelled samples (data not shown). Our chosen buffer, consisting of SDS and NP-40, was robust in allowing a heat spike when a plasma sample is diluted 1:3 in the heat shock buffer. To test the efficacy of heat shock immune complex disruption, we examined 1 ng/mL of recombinant p24 antigen spiked in normal plasma in the presence or absence of 10k-fold excess immunocomplexing antibodies and subjected the samples to heat shock (Fig. 3). As expected, we found that immunocomplexing antibodies inhibited the antigen signal completely. However, when heat shocked, the immunocomplexed p24 samples yielded equivalent signals to those lacking immunocomplexing antibodies (Figs. 3A, B).

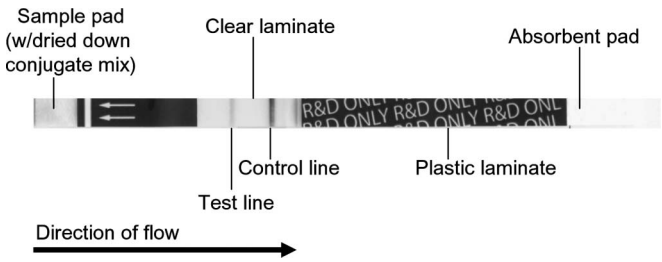

FIGURE 1. Lateral flow p24 assay. Antibody–antigen complexes form as the reaction rehydrates conjugate probe in the sample pad which then flow downstream the assay strip by capillary action and are captured by the neutravidin capture (test line) immobilized on the nitrocellulose membrane. Excess mouse mAb108 carbon conjugate is captured by goat antimouse antibody (control line) immobilized downstream of the test line.

To interpret images of the assay's test line, we first had to determine a quantitative threshold signal to cut-off (S/Co) value. Thus 100 normal plasma samples were tested with the assay to estimate the signal intensity and variation associated with HIV-negative specimen. From these data, an arbitrary absorbance cut-off value of 5.7 was assigned (the mean HIV-negative signal of 2.4 plus 3 standard deviations of 1.1). This cut-off value was then used to assign S/Co values to subsequent samples to uniformly assess the result of a particular sample tested.

As a preliminary assessment of our pediatric HIV assay with the heat shock pretreatment step, a set of the archived samples received from US-derived WITS[13] were subjected to analysis (Table 1). Twenty-five microliter of plasma was tested from a total of 51 blinded infant samples. Like before, the assay strips were imaged and quantified at 30 minutes. Images of the weakly positive WITS samples from Table 1 are shown in Figure 4A. Using the quantitative metric, the assay correctly identified 17 positive samples of 19, giving an 89.5% sensitivity [95% confidence interval (CI): 69% to 97%] and 100% specificity in all 32 negative samples (95% CI: 89% to 100%). Absorbance values for the p24-positive samples ranged from 11.9 to 156 arbitrary absorbance units (median 122 AAU), and those for p24 negative samples ranged from 1.2 to 5.0 arbitrary absorbance units (median 2.7 AAU). The performance of the assay was satisfactory given that the 2 positive samples that were later unblinded as false negatives had viral loads of 9.3k and 35.2k RNA copies per milliliter, both of which were lower than the viral threshold established in Figure 1. The assay was, however, able to pick up 4 samples with viral loads lower than our limit of detection, possibly due to the presence of large concentrations of extraviral p24 liberated from lysed viruses. To demonstrate our test's feasibility as a visually scored assay, all of the WITS samples were scored visually by a naive observer (26-year-old male) at 30 minutes. The identification of positives and negatives was identical to the quantitative method.

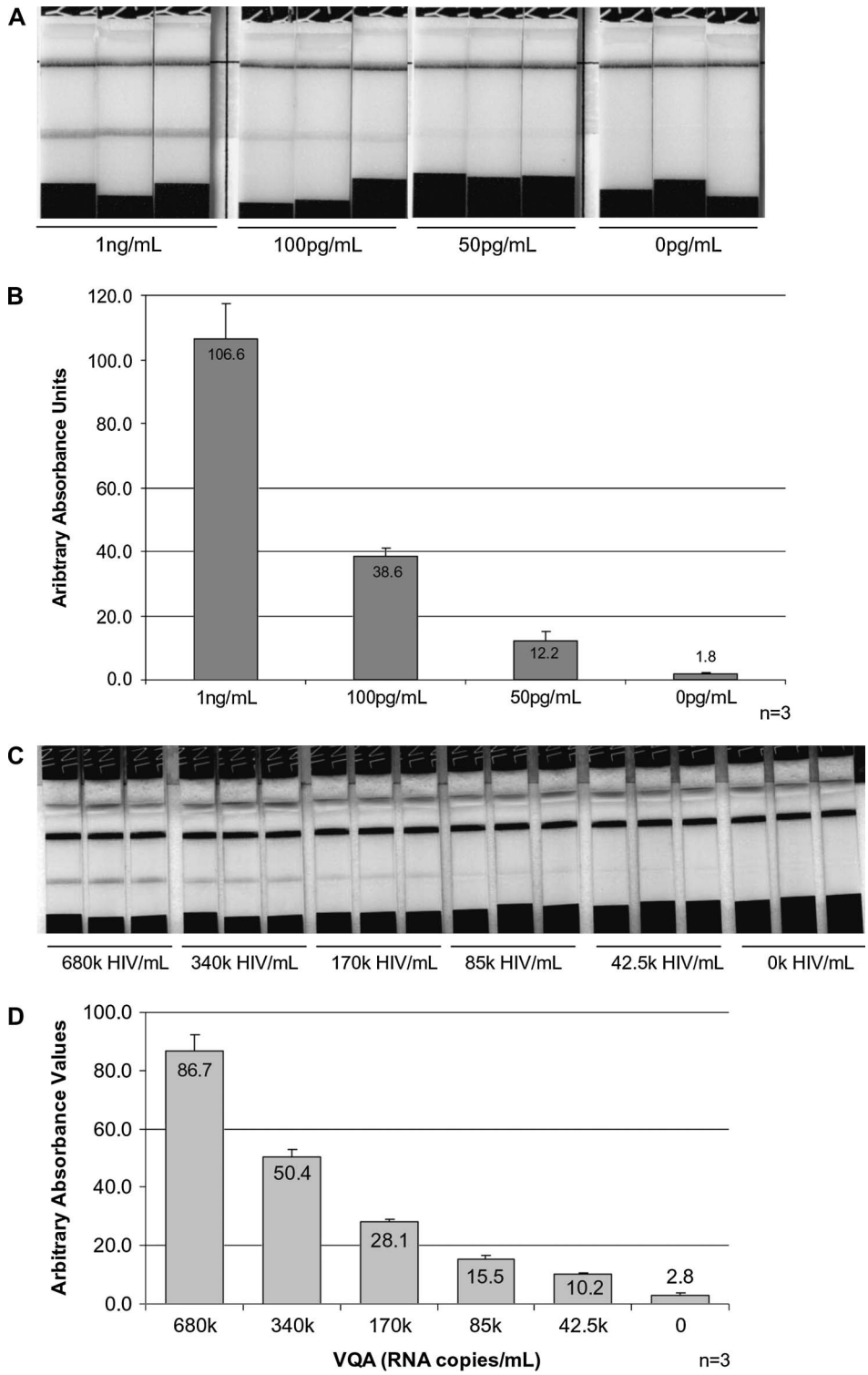

FIGURE 2. p24 assay limit of detection. A, Dose response of recombinant p24 protein diluted in plasma applied to the test strips in Figure 1. B, Quantitation of the dose response in (A). C, Dose response of serially diluted VQA HIV virus in normal plasma. D, Quantitation of the dose response in (C). The assay could distinguish 42,500 RNA copies/mL from the background.

With this encouraging data, we next evaluated the performance of the assay with field specimens from a target patient population by testing samples collected via heel stick that came through an African screening laboratory within a 3-week period. A total of 389 fresh blinded infant samples at the National Health Laboratory Service Virology Laboratory, Groote Schuur Hospital, Cape Town, South Africa, were tested. The quantified assay results were compared with those from TNA PCR performed on the same samples (Roche AmpliPrep/COBAS Taqman HIV-1) to determine the sensitivity and specificity of our assay. Here again, the assay strips were imaged and quantified at 30 minutes and images of the

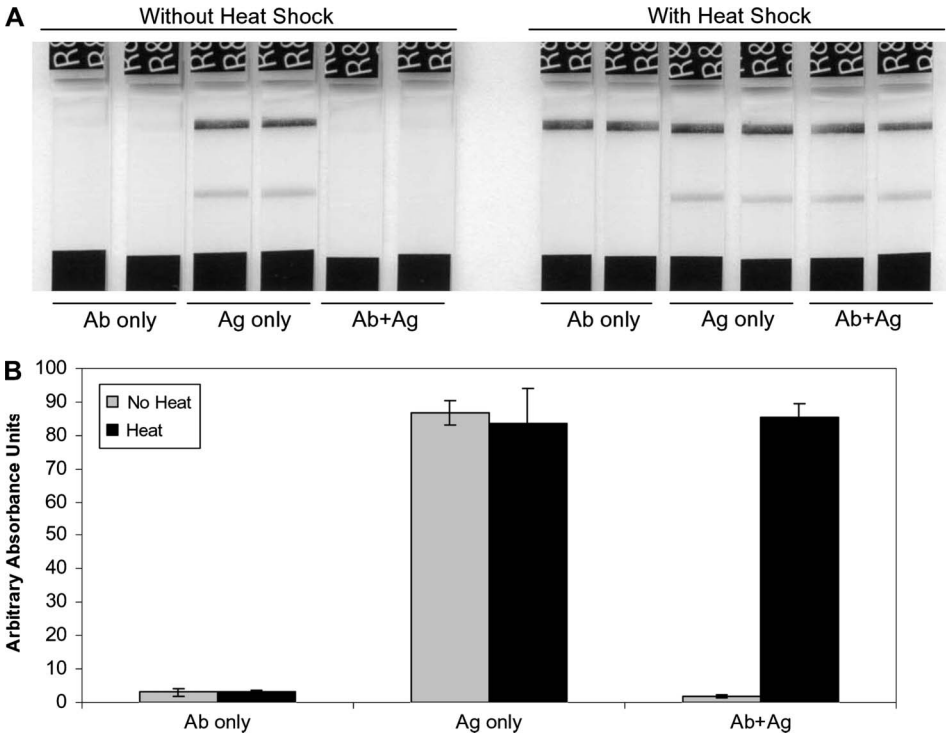

FIGURE 3. Evaluation of heat shock methodology. Samples were prepared containing 1ng/mL p24 antigen with or without 10k-fold competing mouse monoclonal antibodies (mAb108 & mAb115B) against p24. A, Image of test strips of reactions with only competing antibodies (Ab), only p24 antigen (Ag) or with p24 antigen and competing antibodies (Ag/Ab) in the absence or presence of heat shock. Note that antigen-specific signal could only be measured after heating. The very faint appearance of the control lines in the unheated Ab and Ab/Ag samples is due to presence of the competing mouse antibodies added to the reactions. The denaturation of the conformational epitope recognized by the anti-mouse IgG of the control line following heat shock restored control line appearance in those samples. B, Quantitation (n = 2) of the test strips shown in (A).

weakly positive samples from Table 2 are shown in Figure 4B; 6.8% (24 of 389) of the tested infant population were HIV positive by TNA PCR. Our rapid test achieved a sensitivity of 95.8% (95% CI: 88% to 99%) and specificity of 99.4% (95% CI: 98% to 100%) as shown in Table 2. The 1 HIV DNA–positive sample that we missed in our testing was from a 4-month-old infant. It is worth noting that 5 additional samples that were tested gelled when heated in our heat shock reaction. These samples (1% of total specimens) were regarded as a nonresult and were accordingly excluded from sensitivity/specificity determinations. The cause of this gelling is at this point unclear, although initial investigations by our lab indicates that it could be due to elevated levels of immunoglobulins and lipids in the samples (data not shown).

TABLE 1. Evaluation of the Assay With Archived WITS Samples

HIV+ Sample	Infant Age	Viral Load	p24 Assay Result		
			Absorbance	S/Co	Result
1	1 Week	35,205	4.8	0.8	Negative
2	1 Month	2,042,124	33.3	5.8	Positive
3	2 Months	963,098	135.8	23.8	Positive
4	4 Months	204,000	146.6	25.7	Positive
5	4 Months	945,000	65.8	11.5	Positive
6	4 Months	1,793,561	117.3	20.6	Positive
7	6 Months	166,318	143.8	25.2	Positive
8	9 Months	281,000	143.9	25.2	Positive
9	12 Months	9321	3.1	0.6	Negative
10	12 Months	78,438	136.0	23.9	Positive
11	12 Months	84,200	105.7	18.5	Positive
12	12 Months	85,003	122.4	21.5	Positive
13	12 Months	85,003	87.7	15.4	Positive
14	12 Months	236,000	156.3	27.4	Positive
15	12 Months	502,000	134.6	23.6	Positive
16	15 Months	15,400	151.9	26.6	Positive
17	18 Months	9040	113.5	19.9	Positive
18	18 Months	34,000	11.9	2.1	Positive
19	18 Months	36,028	24.5	4.3	Positive

DISCUSSION

PCR and enzyme-linked immunosorbent assay–based assays currently used for the detection of acute HIV-1 infection in infants do not provide cost-effective results in most resource-limited settings. They must be run in centralized laboratories where reliable electricity, refrigeration, and trained clinicians are available. Samples originating from all parts of a country thus have to be transported refrigerated or as dry blood spots to the few centralized laboratories to perform testing. Because of this impediment, the time between blood draw and the report of results to the patient can be as long as 3 months.[15] Furthermore, our own studies suggest that the operational sensitivity (ie, percentage of positive results reported to patients) of dry blood spots DNA PCR may be as low as 50% due to sample loss or loss of the patient to follow-up.[16,17] A point-of-care assay would eliminate these problems as the assay would be conducted at the sample collection site, and the results conveniently provided within the same patient visit.

To address the issues outlined above, we have developed a rapid dipstick p24 antigen assay for the diagnosis of acute

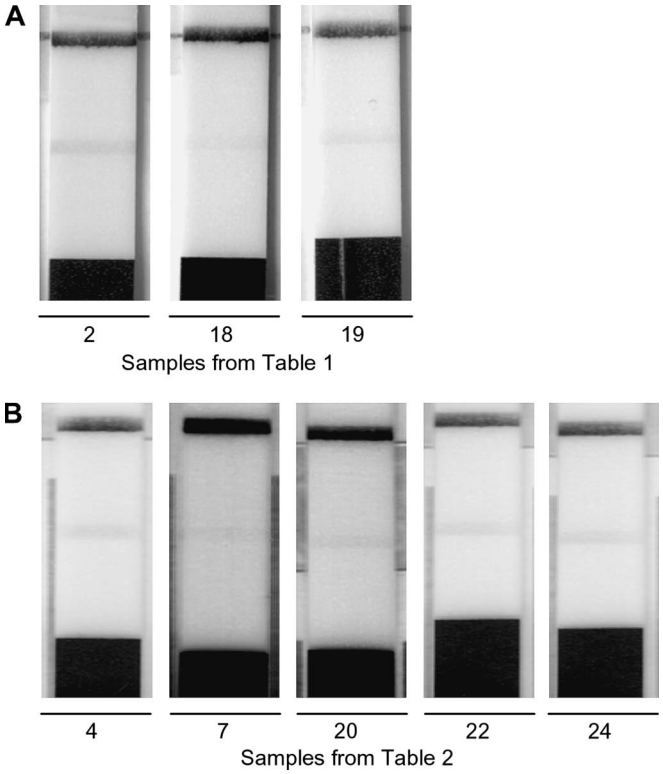

FIGURE 4. HIV-positive clinical samples. A, 25μL of starting infant plasma was mixed with buffer and heated for analysis with the assay strip. As examples of discrimination at the visual limit of detection, images of weakly positive (A) WITS samples from Table 1 (#'s 2, 18 & 19) and (B) of African samples from Table 2 (#'s 4, 7, 20, 22, and 24) are shown.

HIV infection in infants. The assay comprises 4 simple user steps. A 25 μL plasma sample is added to a prefilled tube containing 75 μL of heat shock buffer. The tube is heated for 4 minutes at 88°C and allowed to cool passively to room temperature. An assay strip consisting of antibody conjugates is then inserted into the tube to initiate a reaction. The test and control lines can then be read for diagnosis after 30 minutes, with strong positives appearing in as few as 5 minutes. Given the small reaction volume of the assay, efforts are underway in developing a prototype heating/cooling unit that is battery powered and portable for point-of-care use even in the most remote settings and a prototype plasma separation device for use with whole blood collected from a heel stick.

By incorporating a pretreatment step to disrupt immune complexes, the interference due to maternal anti-p24 antibodies is overcome. The heat shock buffer used for the pretreatment step was formulated to allow efficient recovery of heat-treated p24 antigen, although minimizing loss of sensitivity due to dilution. The buffer consists of simple detergents such as SDS and NP-40 to prevent coagulation of plasma proteins during heating. Other heat shock buffer formulations have been demonstrated by Schupbach,[18,19] though these formulations require sample dilutions into large diluent buffer volumes (up to 500 μL) and constituted of reagents not compatible with our assay (data not shown).

TABLE 2. Evaluation of the Assay With South African Infant Samples

		HIV PCR Result		p24 Assay Result		
HIV+ Sample	Infant Age	Ct	Result	Absorbance	S/Co	Result
1	34 Days	21.1	Positive	111.0	19.5	Positive
2	43 Days	19.6	Positive	121.2	21.3	Positive
3	43 Days	23.5	Positive	90.9	16.0	Positive
4	43 Days	26.5	Positive	31.9	5.6	Positive
5	44 Days	20.9	Positive	115.6	20.3	Positive
6	44 Days	20.3	Positive	117.5	20.6	Positive
7	46 Days	27.1	Positive	8.1	1.4	Positive
8	49 Days	18.9	Positive	104.4	18.3	Positive
9	51 Days	22.8	Positive	110.5	19.4	Positive
10	64 Days	23.6	Positive	79.3	13.9	Positive
11	67 Days	20.2	Positive	109.6	19.2	Positive
12	71 Days	35.4	Positive	98.9	17.4	Positive
13	81 Days	23.8	Positive	113.8	20.0	Positive
14	91 Days	24.8	Positive	117.3	20.6	Positive
15	93 Days	21	Positive	99.1	17.4	Positive
16	93 Days	23.9	Positive	45.8	8.0	Positive
17	93 Days	22.3	Positive	111.5	19.6	Positive
18	4 Months	30.7	Positive	3.7	0.6	Negative
19	5 Months	21.4	Positive	100.1	17.6	Positive
20	7 Months	25.6	Positive	11.1	2.0	Positive
21	9 Months	18.7	Positive	118.4	20.8	Positive
22	9 Months	30.4	Positive	27.5	4.8	Positive
23	13 Months	26.8	Positive	110.9	19.5	Positive
24	14 Months	26.9	Positive	9.6	1.7	Positive

The analytical sensitivity of the dipstick p24 antigen assay coupled with heat shock pretreatment was determined to be 50 pg/mL (2 pM) in a matrix of recombinant p24 antigen spiked into normal plasma. The diagnostic performance of the p24 antigen assay was evaluated with 51 archived infant samples obtained from the US WITS study. In these samples from infants aged younger than 18 months, the assay performed with a sensitivity of 89.5% and specificity of 100%. These promising results indicated that we could achieve similar if not better performance with fresh infant samples in a clinical setting from African patients. To this end, further diagnostic evaluation with the dipstick assay yielded comparable performance when 24 HIV-positive and 365 HIV-negative samples were tested from HIV-exposed infants younger than 1 year of age. Sensitivity was determined at 95.8% and specificity at 99.4% in this population.

There is widespread agreement by health care providers that a simple inexpensive point-of-care assay for HIV diagnosis would increase the number of infants initiated onto treatment due to improved identification of infected individuals. According to a recent decision analysis model,[20] a pediatric test with moderate clinical performance such as 90% sensitivity and specificity can greatly benefit at-risk populations in terms of total adjusted life years saved assuming only a 5% ART availability.

In conclusion, we have demonstrated that the rapid p24 antigen assay exhibits good accuracy among samples from the

target population in sub-Saharan Africa. The rapid p24 antigen assay is designed to be amenable as a point-of-care test performed by clinic staff in approximately 40 minutes. The assay procedure is similar to that of widely used HIV antibody rapid tests, with the addition of a pretreatment step to disrupt immune complexes. The rapid p24 antigen assay would enable critical health impact in the pediatric HIV population in resource-limited settings that currently lack point-of-care diagnostics.

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