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Evaluation of the Ultrasensitive Human Immunodeficiency Virus Type 1 (HIV-1) p24 Antigen Assay Performed on Dried Blood Spots for Diagnosis of HIV-1 Infection in Infants▽

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The diagnostic accuracy of the modified p24 antigen assay performed on pediatric dried blood spots was evaluated. Samples analyzed within 6 weeks of collection yielded no false-positive results (specificity, 100%) and few false-negative results (sensitivity, 96.5% to 98.3%). Laboratory services with limited resources should assess this option for routine infant diagnosis.

In resource-constrained countries, the human immunodeficiency virus type 1 (HIV-1) diagnostic "gold standard" nucleic acid assays are performed only in a few centralized laboratories. Consequently, most vertically exposed infants do not have access to an early HIV infection diagnosis or appropriate care despite the availability of antiretrovirals.

Several studies show the ultrasensitive HIV-1 p24 antigen (Ag) enzyme-linked immunosorbent assay (ELISA) performed on plasma to be successful for pediatric HIV infection diagnosis, with sensitivities and specificities ranging from 97% to 100% (4, 9, 10, 12, 15, 18, 21). Importantly, it is able to detect viral subtype C (3, 5, 9, 18, 21), which is responsible for the majority of HIV-1 infections in Southern Africa (18, 20, 21). No specialized equipment or training is required to perform the assay. The use of dried whole blood spots (DBS) would further improve accessibility to a HIV infection diagnosis for infants. In a pilot study we adapted the p24 Ag assay for use on DBS on Whatman grade 1 filter paper (13). The sensitivity and specificity of the modified assay were excellent on initial testing (98.8 and 100%, respectively); however, the p24 protein concentration was found to decrease after 6 weeks.

Here we extend the findings of that study by validating the modified p24 Ag ELISA with a larger cohort of children and investigating the performance of the FDA-approved Whatman grade 903 paper (formerly S&S), against Whatman grade 1 filter paper in terms of the performance of the modified assay. Whatman grade 1 paper, which is widely used in laboratories for filtration purposes, was chosen for its affordability and availability in resource-poor countries. We further examined the effect of a desiccant sachet on p24 protein preservation in DBS.

DBS were collected from two populations of children in Johannesburg, South Africa. One group consisted of 6-week-old babies born to HIV-seropositive mothers ($n = 147$; median age, 45 days [range, 20 to 72 days]) being followed up at the Prevention of Mother to Child Transmission Clinic at Coronation Women and Children's Hospital. A group of known HIV-infected children ($n = 99$; median age, 20 months [range, 3 to 72 months]) attending the Harriet Shezi Children's Clinic at the Chris Hani Baragwanath Hospital was included in the study to more accurately test the sensitivity of the p24 Ag assay. Ethics approval was obtained (clearance number M00-01-07; Human Ethics Committee, University of the Witwatersrand), and written informed consent was given by caregivers.

Nucleic acid assays used to determine or monitor the HIV status of the cohort were the HIV DNA PCR (Amplicor HIV-1 DNA version 1.5 assay; Roche Molecular Systems Inc., Branchburg, NJ), HIV RNA PCR (Roche Amplicor HIV-1 Monitor assay, version 1.5), and NASBA EasyQ HIV-1 RNA assay (Nuclisens, bioMérieux, Boxtel, The Netherlands). These results constituted the reference standards against which p24 Ag assay results were compared.

DBS were collected at the same time as venous blood was drawn for routine HIV assay. Blood from 246 children was dried on Whatman grade 1 filter paper and a Whatman grade 903 card (Dassel, Germany). From each child four drops of blood were spotted separately onto the grade 1 paper and at least two complete circles were filled on the 903 card. Filter papers were air dried at ambient temperature for at least 3 h. DBS on each filter paper type from the first 125 children enrolled were stored in individual plastic zip-lock biohazard bags (Stripform Packaging, Capetown, South Africa) at room temperature with no desiccant sachet. DBS from the next 121 children enrolled were stored identically except for the addition of a Minipax desiccant sachet (Multisorb Technologies, Warrington, United Kingdom). Storage at ambient temperature was chosen to simulate likely local conditions. As recommended by CLSI (formerly NCCLS) guidelines (11), DBS that contained insufficient blood (with an area of less than 6 mm), were clotted, or appeared wet or layered were discarded and not entered into the study.

DBS standards, controls, and blanks were prepared as described previously (13) using the stock standard provided with the kit (HIV-1 p24 ELISA; Perkin-Elmer Life Sciences, Boston, MA) diluted to give an effective range from 10,000 pg/ml to 4.9 pg/ml. Equal volumes of diluted standards were mixed

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TABLE 1. Performance of the p24 Ag assay on all DBS after short- and long-term storage

Assay[a]	No. of children		% Sensitivity[e]	% Specificity[e]	% Prevalence	LR	
	Total	With false-negative results				Positive	Negative
First							
Grade 1 paper							
Without desiccant	124[b,c]	2	96.5 (87.4, 99.7)	100 (93.7, 100)	44.4	∞	0.0351
With desiccant	121[d]	2	96.6 (87.6, 99.7)	100 (93.3, 100)	46.3	∞	0.0345
903 card							
Without desiccant	125[b]	2	96.6 (87.6, 99.7)	100 (93.7, 100)	44.8	∞	0.0345
With desiccant	121[d]	1	98.3 (89.3, 99.9)	100 (93.3, 100)	46.3	∞	0.0175
Second							
Grade 1 paper							
Without desiccant	124[b,c]	3	94.8 (85.3, 98.8)	100 (93.7, 100)	44.4	∞	0.0517
With desiccant	121[d]	7	88.9 (78.8, 94.8)	100 (93.3, 100)	46.3	∞	0.1111
903 card							0.082
Without desiccant	125[b]	5	91.8 (81.8, 96.8)	100 (93.7, 100)	44.8	∞	
With desiccant	121[d]	1	98.3 (89.8, 99.9)	100 (93.3, 100)	46.3	∞	0.0175

[a] The first assay was performed within 6 weeks of sample collection, and the second assay was performed 6 weeks after the first.
[b] DBS from the first 125 children enrolled.
[c] No DBS on grade 1 paper from one child.
[d] DBS from the last 121 children enrolled.
[e] Values in parentheses are 95% confidence intervals.

with washed semipacked red blood cells (RBC) from a consenting HIV-negative individual to give a standard range of 5,000 pg/ml to 2.45 pg/ml. High (1,000 pg/ml) and low (50 pg/ml) DBS controls were also prepared from the stock standard. The blank DBS consisted of washed RBC mixed with sterile phosphate-buffered saline. Use of this DBS blank eliminated the high background noise encountered by others measuring p24 Ag in DBS using a liquid blank (7, 8).

DBS were incubated with a virus-lysing buffer (30 mM Tris-HCl [pH 7.2], 450 mM NaCl, 1.5% Triton X-100, 1.5% deoxycholic acid [sodium salt], 0.3% sodium dodecyl sulfate, 10 mM EDTA) (17) prior to performing the assay on duplicate 6-mm discs from blanks, standards, controls, and samples DBS according to the manufacturer's instructions and as described elsewhere (2). These DBS blanks, standards, controls, and samples were used instead of liquid blanks, standards, controls, and plasma samples in the assay. Two DBS that eluted poorly as indicated by the lack of transfer of material from the DBS to the elution buffer, which remained clear, were discarded because previous work indicated that these spots give falsely low optical densities (data not shown). Signal amplification was achieved with the ELAST amplification system (Perkin-Elmer Life Sciences, Boston, MA). Negative control DBS were made from the kit negative control mixed with RBC and used to calculate the reactivity baseline (mean absorption of four negative control DBS plus 3 standard deviations) (16). The assay was performed on all DBS within 6 weeks of collection (short-term storage) and repeated 6 weeks after the first assay (long-term storage).

The goodness of fit of the standard curves generated from DBS standards on Whatman grade 1 and 903 filter paper was evaluated from the coefficient of determination (r^2), defined as the square of the correlation between the predicted and observed values.

Assay reliability was determined for both filter paper types in both storage conditions from the interassay variability (coefficient of variance [CV] = standard deviation/mean) using values for the high and low DBS controls from eight assays. The diagnostic accuracy of the assay was evaluated from the sensitivity (the probability that the assay result is a true positive) and the specificity (the probability that the assay result is a true negative) using the molecular virological assay results as the reference values.

Because the overall prevalence was manipulated, positive and negative likelihood ratios (LRs) rather than predictive values were used to evaluate the validity of the p24 Ag assay on all DBS to diagnose HIV infection. A positive LR (sensitivity/1 − specificity) of greater than 10 strongly suggests that a positive result is indicative of the presence of a condition. A negative LR (1 − sensitivity/specificity) of less than 0.1 virtually rules out the presence of the condition.

The study cohort comprised 246 children with a median age of 1.5 months (range, 1.0 to 72 months). Fourteen of the 147 vertically exposed babies were found to be HIV infected by a molecular reference assay, giving a prevalence rate of 9.5%. Inclusion of DBS from 99 children with a known HIV infection diagnosis gave an overall prevalence of 45.95%.

The DBS standard curves consistently showed a good fit, with r^2 values from 0.996 to 0.999 for the grade 1 DBS standard curve and from 0.995 to 0.999 for the 903 DBS standard curve (data not shown). These values compare favorably with those for liquid standards (13). The detection range of the p24 Ag ELISA on DBS was 2.45 pg/ml to 5000 pg/ml.

Interassay CVs for the low (50 pg/ml) and high (1,000 pg/ml) Whatman grade 1 DBS controls were 4.6% (range, 47.7 to 55.0 pg/ml) and 10.3% (range, 842 to 1,145.4 pg/ml), respectively. For the low and high DBS controls on 903 cards, the CVs were 19.5% (range, 28.1 to 45.9) and 12.6% (range, 1,643.6 to

TABLE 2. Performance of the first p24 assay on DBS from 6-week-old infants and from children between 6 weeks and 6 years of age

Assay material	6-wk-old infants					Children >6 wk and <6 yr				
	No. of children			Positive predictive value (%)[d]	Negative predictive value (%)[d]	No. of children			Positive predictive value (%)[d]	Negative predictive value (%)[d]
	Total	With false-positive results	With false-negative results			Total	With false-positive results	With false-negative results		
Grade 1 paper										
Without desiccant	75	0	1[a]	100 (64.6, 100)	98.6 (92.2, 99.7)	49	0	1[c]	100 (92.7, 100)	0 (0, 79.3)
With desiccant	72	0	0	100 (64.6, 100)	100 (94.4, 100)	49	0	2[a,c]	100 (92.7, 100)	0 (0, 65.8)
903 card										
Without desiccant	75	0	2[a,b]	100 (64.6, 100)	97.1 (90.2, 99.2)	50	0	0	100 (92.9, 100)	
With desiccant	72	0	0	100 (64.6, 100)	100 (94.4, 100)	49	0	1[a]	100 (92.7, 100)	0 (0, 79.3)

[a] False-negative p24 result on DBS on both filter paper types from the same child.
[b] False-negative result on DBS on 903 card but not on grade 1 paper from the same child.
[c] False-negative result on DBS on grade 1 paper but not on 903 card from the same child.
[d] Values in parentheses are 95% confidence intervals.

2,304.5), respectively. These values are comparable to the CVs determined previously for DBS and support the hypothesis that the volume of blood in each 6-mm punched disc is relatively constant, thereby abolishing the need to apply a known volume of blood to the filter paper (13).

The results for the p24 assay (n = 982) on initial testing (short-term storage) and again after long-term storage are shown in Table 1. With no false-positive results, the p24 assay yielded a specificity of 100% regardless of the filter paper type or storage conditions. This agrees with specificities for the assay on plasma from other non-B subtypes (3, 4, 5, 9, 12, 18, 21) and with our and others' previous findings on DBS (7, 13). The sensitivities of the assay for each filter paper type and storage condition range from 96.5% for grade 1 paper stored without a desiccant to 98.3% for DBS on 903 cards stored with a desiccant sachet (Table 1). With some overlap in the confidence intervals, the performance of grade 1 paper could be considered acceptable in circumstances where 903 paper is not available. In the older group of children, 12 were receiving highly active antiretroviral therapy and had viral loads ranging from 40,000 copies/ml to 1,570 copies/ml. With the exception of one viral load of 11,000 copies/ml that was not detected by the assay on either filter paper type, all the remainder were positively identified. All false-negative results were confirmed by reassaying.

When tested 6 weeks after the first assay, the sensitivity fell for all experimental conditions except for Whatman 903 paper stored with a desiccant, where a sensitivity of 98.3% was maintained (Table 1), indicating that the addition of a desiccant conserves p24 protein in DBS on 903 paper over time at ambient temperature. Although there are no data on p24 protein stability after storage for longer than 2 weeks (8), HIV-1 antibody titers have been shown to be stable in DBS stored in a zip-lock bag with a desiccant for up to 8 weeks at ambient temperature (1, 6). The sensitivity of the assay for grade 1 paper stored with a desiccant fell from 96.6% in the first assay to 88.9% in the second. This may reflect the fact that unlike Whatman 903 paper, Whatman grade 1 filter paper is not manufactured and standardized for DBS collection.

Positive LRs for the p24 Ag assay on all short- and long-term-stored DBS were infinity. With the exception of grade 1 paper stored long term with a desiccant (negative LR = 0.1111), negative LRs of less than 0.1 were found for all DBS stored short and long term. These results strongly support the validity of the p24 assay on DBS to detect HIV even after long-term storage. When the data were sorted by age group, no false-positive p24 results were found in this study, as noted earlier (Table 2). For the group of 6-week-old infants, no false-negative results occurred on either filter paper type stored with a desiccant, underscoring the utility of the modified p24 assay on DBS for early HIV infection diagnosis. There was little difference between the performances of grade 1 and 903 paper with regard to false-negative results (Table 2).

Blood collection on filter paper is particularly suitable for infants and children (19), with no requirement for the venesection skills that are largely unavailable outside major urban centers in developing countries. In this study, DBS were prepared from venous blood. Although the use of DBS prepared from capillary blood obtained by heel or finger stick has been validated for pediatric diagnosis by HIV-1 DNA PCR (14), it has not yet been validated for the p24 assay.

In the two studies completed by us, the modified ultrasensitive p24 Ag ELISA has been performed on a total of 387 DBS from children, 240 of which were from 6-week-old infants. In line with this study, there were no false-negative results in the group of 6-week-old babies in the first study. These results indicate that the assay on DBS is a reliable, affordable alternative to virological nucleic acid assays for early pediatric HIV infection diagnosis. It is important for the methodology to be validated by other laboratory services in countries that elect to use the ultrasensitive p24 Ag assay on DBS for routine infant diagnosis.

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