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Diagnosis of Human Immunodeficiency Virus Type 1 Infection in Infants by Use of Dried Blood Spots and an Ultrasensitive p24 Antigen Assay▽

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We tested 617 dried blood spots (DBS) from human immunodeficiency virus-exposed infants from five countries using an ultrasensitive p24 antigen assay (Up24). The sensitivity was 94.4% (67/71) and the specificity was 100% (431/431) for infants with DBS specimens ≤20 months old; DBS older than 30 months demonstrated only 72.2% sensitivity (39/54) ($P < 0.001$) but displayed 100% specificity (61/61).

Several factors reduce diagnosis of human immunodeficiency virus (HIV) in infancy in resource-limited settings. Simple, rapid antibody assays cannot be used because of the presence of maternal antibodies. As a consequence, viral nucleic acids or proteins must be detected. Nucleic acid assays are technologically complex and subject to contamination and require expensive equipment and highly trained technologists, limiting availability to selected centers of excellence in resource-limited countries (1, 8, 12, 15, 28, 30).

The ultrasensitive p24 antigen assay (Up24), an alternative that overcomes the limitations of antibody assays while using an enzyme-linked immunosorbent assay (ELISA)-based platform, reduces the need for expensive equipment and personnel. It has been used successfully with plasma for over 12 years for diagnosis of HIV infection in infants (9, 11, 13, 18, 19, 26, 29, 32).

Another factor limiting diagnosis of HIV infection in infants is the need for phlebotomy to collect specimens and cold chain maintenance while the specimen is transported to a central laboratory. These have been addressed by adaptation of nucleic acid (2–7, 10, 16, 20, 21, 27, 31), and now Up24 antigen assays, for use with dried blood spots (DBS) (14, 22, 23).

Other investigators have adapted the Up24 assay for use with DBS. Table 1 compares these different extraction methods used for p24 antigen assays with DBS. Li et al. (17), following the manufacturer's instructions for plasma specimens, reported 0% specificity using DBS. Knuchel et al. (14) improved the sensitivity by modifying the elution buffer (25) and increased specificity to 100% by adding a quenching step using hydrogen peroxide after the first wash. Patton et al. (22, 23) improved elution by using the previously described buffer (25) and 0.5% Triton X-100. The reported sensitivity and specificity were 98.3 to 98.8% and 100%, respectively. Our objectives were to develop and optimize a simpler method for HIV infant diagnosis using DBS and reagents found in commercially available Up24 test kits as much as possible and to assess the optimized assay performance.

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DBS obtained from patients in the Dominican Republic ($n = 60$), Malawi ($n = 9$), South Africa ($n = 210$), United States ($n = 264$), and Vietnam ($n = 74$) were collected on Whatman 903 paper (Whatman, Sanford, ME), air dried, stored with a desiccant in individual ziplock bags at room temperature (Dominican Republic, South Africa, and Malawi) or at 4 to 8°C (Vietnam) or at −20°C (United States), and shipped to the laboratory at the University of North Carolina, Chapel Hill (UNC). The infants had been previously tested for HIV type 1 (HIV-1) either by DNA PCR using cell pellets from whole blood using the Roche Amplicor assay, version 1.5 (Branchburg, NJ) for specimens from the United States and South Africa or an in-house HIV DNA assay (Pasteur Institute, Ho Chi Minh City, Vietnam) for specimens from Vietnam. Specimens from Malawi and the Dominican Republic were originally tested as DBS using an HIV RNA assay (NucliSens QT; bioMerieux, Durham, NC). These results were considered the gold standard against which the DBS Up24 antigen results were compared. All infected infants had follow-up specimens to confirm HIV infection.

Most U.S. infants were receiving 6 weeks of zidovudine prophylaxis at the time of sampling. All Malawian infants had received a single dose of nevirapine and 1 week of zidovudine and lamivudine. Some Vietnamese and South African children were beyond infancy and known to be HIV infected. In addition, DBS from South African infants had been tested in a previous Up24 assay study (23). DBS ≥30 months old were evaluated separately.

Controls were prepared from non-HIV-1-infected whole blood samples. Negative controls consisted of 50-μl spots of whole blood on Whatman 903 paper. Positive-control DBS

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TABLE 1. Comparison of different extraction methods used for p24 antigen assays with DBS[a]

Assay details	Li et al. (17)	Patton et al. (22, 23)	Knuchel et al. (14)	This study
Steps in procedure	1. Elute 50 μl DBS using 0.5% Triton X-100	1. Incubate duplicate 6-mm punches with 25 μl **SNCR buffer**[b] for 10 min at room temp	1. Combine 16-mm DBS plus 0.8 ml of elution buffer (**1 part SNCR buffer,** 1 part water, and 9 parts of 0.5% Triton X-100)	1. Incubate duplicate 6-mm punches with 50 μl **specimen preparation buffer**[c] for 10 min at room temp
	2. Incubate 60 min	2. Add 275 μl of 0.5% Triton X-100	2. Incubate on a shaker/rocker 8 h at room temp	2. Add 0.25 ml of 9 parts of 0.5% Triton X-100 diluted in PBS to 1 part of Tris reagent
	3. Centrifuge 13,600 × *g* for 3 min	3. Incubate overnight at 4°C	3. Boil for 5 min at 100°C	3. Shake for 2 h at room temp or overnight at 4°C
	4. Transfer to a screw-cap cryovial and boil for 5 min at 100°C	4. Boil for 5 min at 100°C	4. Proceed with the Up24 antigen assay protocol using overnight incubation	4. Boil for 5 min at 100°C
	5. Proceed with the standard Up24 antigen assay protocol	5. Proceed with the standard Up24 antigen assay protocol	5. After the first wash, quench with **0.3% H_2O_2 in 50 mM Tris-HCl** for 10 min at 37°C	5. Proceed with the standard Up24 antigen assay protocol
			6. Continue with the standard Up24 antigen assay protocol	
Assay times	~8 h	4 to 5 h on day 1, overnight incubation, 4 h on day 2	~9 h on day 1, overnight incubation, 3 h on day 2	~1 h on day 1, overnight incubation, 4 to 5 h on day 2
Sensitivity (%)	96 if viral load >1,000 copies/ml	98.3–98.8	84	94.4
Specificity (%)	0	100	100	100

[a] Reagents in boldface type were not included in the manufacturer's kit.
[b] **SNCR buffer** consists of 30 mM Tris-HCl (pH 7.2), 450 mM NaCl, 1.5% Triton X-100, 1.5% deoxycholic acid, 0.3% sodium dodecyl sulfate, and 10 mM EDTA.
[c] **Specimen preparation buffer** consists of kit specimen diluent, 10 mM EDTA, and 30 mM Tris-HCl (pH 7.2).

were prepared by adding the PerkinElmer HIV-1 ELISA positive control to normal whole blood to a titer of 100 pg/ml. The control DBS were air dried and stored in ziplock bags at −20°C with a desiccant sachet and a humidity indicator card (Static Control Components, Inc., Sanford, NC).

Two 6-mm disks were punched from the DBS using an ordinary paper puncher cleaned between samples and placed in 1.5-ml screw-cap Sarsted tubes (Newton, NC). The disks were incubated for 10 min at room temperature with 50 μl of a specimen preparation buffer consisting of the PerkinElmer HIV-1 core profile ELISA standard/sample diluent in the ELAST amplification kit NEP116VL (PerkinElmer Life Sciences, Boston, MA) with the addition of 10 mM EDTA and 30 mM Tris-HCl, pH 7.2. The elution buffer consisted of 9 parts of 0.5% Triton X-100 diluted 1:10 in PBS and 1 part (vol/vol) of Tris reagent. The PerkinElmer kit includes both Tris-HCl and Triton X-100. After the addition of 0.25 ml of the elution buffer, the DBS disks were rocked overnight at 4°C and removed from the sample using wooden sticks. The tubes were transferred to a heat block, boiled for 5 min at 100°C, and allowed to cool for 10 min. The Up24 protocol in the ELAST amplification kit was subsequently followed except that instead of using the QuantiKin software to read the assay, an endpoint reading at two wavelengths of 490 and 650 nm was obtained after 30 min of substrate incubation. Four methods of calculating a cutoff were explored. The cutoff selected (mean optical densities of two negative controls plus 0.05) provided the best correlation for the data. Samples with optical densities greater than that number were considered positive. All specimens were tested once. Repeat tests were performed on several specimens; the result was always concordant with the first. UNC testing personnel were blinded as to the infant's infection status.

Of 617 DBS from HIV-exposed infants assessed, 324 were presumed to be subtype B and came from the United States and Dominican Republic; 219 from Malawi and South Africa were presumed to be subtype C, and 74 from Vietnam were presumed to be subtype A/E. DBS that were ≤20 months old were evaluated with the optimized assay and compared to their respective gold standard (Table 2). Among the 431 true negative specimens, there were no false-positive results (100% specificity). The sensitivity of the assay increased from 50% within the first week of life to 80% for infants 1 to 6 weeks old to ≥95% for infants and children >6 weeks old. Both false-negative results in the youngest age group were from Malawian infants receiving zidovudine-lamivudine-nevirapine for prevention of mother-to-child transmission. The false-negative specimen in the 1- to 6-week age group was from a 3-week-old U.S. infant receiving zidovudine prophylaxis. The false-negative result for the 6- to 26-week age group was from a 14-week-old infant from the Dominican Republic who was not receiving prophylaxis. Patton et al. (22, 23) never tested neonates in the

TABLE 2. Sensitivity and specificity of the Up24 antigen assay with DBS by age of the infant and age of DBS

Age of infant	DBS <20 mo old				DBS >30 mo old			
	Sensitivity (%)[a]	Specificity (%)[b]	PPV (%)[c]	NPV (%)[d]	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
<7 days	50 (2/4)	100 (80/80)	100	97.6	Not tested	Not tested	Not tested	Not tested
1–6 wks	80 (4/5)	100 (95/95)	100	98.9	100 (3/3)	100 (10/10)	100	100
>6–26 wks	95 (21/22)	100 (192/192)	100	99.5	87.5 (7/8)	100 (51/51)	100	98.1
>26 wks–72 wks	100 (24/24)	100 (63/63)	100	100	72.7 (8/11)	Not tested		
>72 wks	100 (16/16)	100 (1/1)	100	100	65.6 (21/32)	Not tested		
Total	94.4 (67/71)	100 (431/431)	100	99.1	72.2 (39/54)	100 (61/61)	100	80.3

[a] The values in parentheses are the number of specimens with a positive result/total number of specimens tested.
[b] The values in parentheses are the number of specimens with a negative result/total number of specimens tested.
[c] PPV, positive predictive value.
[d] NPV, negative predictive value.

first week of life (the youngest was 20 days old). If the age group within the first week of life is excluded, the sensitivity is 97% (65/67).

Among 115 DBS from South Africa that had been tested previously and then stored at room temperature for ≥30 months (23), there was a trend toward decreasing sensitivity as the age of the infant increased, although it was not significant ($P = 0.12$); the sensitivity was 100% (3/3) for DBS from infants >1 and <6 weeks old, while it was 66% (21/32) for infants of ≥72 weeks of age (Table 2). The specificity was 100% across all age groups. Some older infants and children may have been on antiretroviral treatment at the time of collection, but no treatment data were collected.

Our aim was to simplify the procedure and maximize the use of the reagents already in the manufacturer's kit. The final optimized procedure used a specimen preparation buffer that contained only two reagents not found in the kit—10 mM EDTA and 30 mM Tris-HCl. We have now tested this procedure on 617 DBS from HIV-exposed infected and uninfected infants and children. The assay demonstrated 94.4% sensitivity for diagnosis of HIV infection with presumed HIV-1 subtypes B, C, and AE ($n = 502$) using DBS ≤20 months old versus 72.2% in older specimens ($P < 0.001$), achieving 100% specificity without complicating the procedure with a quenching step.

This is the largest series of infants <7 days of age tested using the Up24 assay using DBS. Although the sensitivity was only 50% (2/4), the specificity was 100% (80/80). Others have suggested that p24 assays might be less specific in very young infants (24). However, we previously tested plasma samples with the Up24 assay and observed similar specificity in infants 0 to 7 days of age (1 of 109 [99%]) (11). The optimal time to use the Up24 antigen assay for infant diagnosis requires further investigation, especially as prevention of mother-to-child transmission programs in low-resource countries move to more-complex and longer postdelivery antiretroviral regimens.

The lengths of storage time and temperatures varied, depending on the original collection site (country), but did not appear to affect assay sensitivity when specimens were tested within 20 months of collection, in contrast to data suggesting that DBS should be tested within 6 weeks of collection if Whatman no. 1 filter paper was used (22). When Whatman 903 was employed (as done here), little loss of sensitivity was observed at 12 weeks. In most cases, samples are tested within a few weeks of collection. DBS from infants 6 to 26 weeks of age demonstrated 94.5% sensitivity and 100% specificity, regardless of the length of room temperature storage (up to 20 months); 72% of DBS stored more than 30 months at room temperature still had detectable antigen using the optimized Up24 assay.

Our results confirm that infant HIV infection can be accurately diagnosed using DBS and an optimized Up24 antigen assay, with lower cost and less complexity.

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