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Detection of Human Immunodeficiency Virus Type 1 (HIV-1) in Heel Prick Blood on Filter Paper from Children Born to HIV-1-Seropositive Mothers

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The human immunodeficiency virus type 1 (HIV-1) DNA PCR results of 94 dried blood spot (DBS) samples on filter paper and corresponding venous blood in EDTA obtained from infants born to HIV-1-seropositive mothers were compared. In addition, the results of HIV-1 DNA PCR on DBS and the HIV-1 RNA PCR from plasma of 70 paired samples were compared. A 100% specificity and a 95% sensitivity for HIV-1 DNA PCR on DBS compared with results for venous blood were observed for the 94 paired samples. The results of the DBS HIV-1 DNA PCR and HIV-1 RNA PCR of 70 corresponding plasma samples correlated perfectly (100%). The DBS HIV-1 DNA PCR method proved reliable for HIV-1 detection.

Early diagnosis of human immunodeficiency virus type 1 (HIV-1) infection in infants born to HIV-1-seropositive mothers has proved difficult with conventional antibody tests. Such infants often harbor maternal antibodies transferred transplacentally for up to 15 months (14). Virus culture, regarded as a standard, is slow, expensive, and biohazardous for routine HIV-1 diagnosis (3, 5, 12–14). Antigen testing is insensitive (10), and virus culture and PCR on venous blood require large quantities of blood (5, 13). Recently, PCR on dried blood spot (DBS) samples collected on filter paper for HIV-1 detection was claimed to be as sensitive and specific as other conventional methods (2, 17, 18). However, these studies used a long and complicated DNA extraction procedure. In the present study, we have simplified the existing DBS PCR detection methods (2, 18) and evaluated this method in parallel with the conventional HIV-1 DNA PCR on venous blood and the reverse transcription PCR on plasma from children.

This study was part of a cohort study meant to determine the maternal risk factors for mother-to-child transmission of HIV-1 in Kenya (15). One hundred and twenty-five paired blood samples were collected between 1991 and 1992 at different time points from 101 children aged 9 days to 3 years. Table 1 shows the age groups in detail. These 101 children were born to 12 seronegative and 88 HIV-1-seropositive Kenyan mothers. Samples were collected in parallel as DBS (each was 1 drop of blood) on filter paper (DOH-1514; Schleicher and Schuell 903) by a heel prick, as whole blood in EDTA (1.5 ml), and as plasma (0.5 ml). Of the 125 paired samples, 39 were matched DBS, venous blood, and plasma, 94 (39 + 55) were paired DBS and venous blood, and 70 (39 + 31) were paired DBS and plasma (Table 2). From 36 infants under 3 months of age, DBS samples were collected every 2 months for a 6-month period.

Positive DBS controls consisted of 8E5 LAV cells (containing a single integrated copy of HIV-1 per cell) diluted to have 2.5 and 25 proviral copies per pair of 50-μl samples of negative whole blood in EDTA, dropped on filter paper, and dried at room temperature. In PCR, each pair of 38-μl samples of the 70-μl filter paper eluate theoretically contained 1 and 10 proviral copies of DNA per PCR. The negative control was the same negative whole blood without 8E5 cells. The DBS samples were dried at room temperature for 5 h, sealed in separate plastic bags for 1 month at room temperature, and shipped to Antwerp, Belgium, for processing. The DBS was perforated into a 2-ml screw-cap tube, and the perforator was disinfected, flamed, and cooled after each sample. One milliliter of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 1% Triton X-100) was added to each tube, rotated at 60 rpm for 15 min at room temperature, and microcentrifuged at 100 × *g* for 1 min, and the hemoglobin-tinged supernatant was aspirated. This step was repeated three times. The treated filters were stored at −20°C or processed immediately. To lyse the residual peripheral blood mononuclear cells on the processed filter paper, 50 μl of 1.5× PCR buffer (Tween 20 and Nonidet P-40 [Sigma], both 0.68%, 100 μg of proteinase K [Boehringer-Mannheim] per ml, 40 mM Tris-HCl [pH 8.3], and 3.75 mM $MgCl_2$) was added to each tube. The tubes were vortexed for 1 min, incubated in a water bath at 52°C for 1 h, vortexed for 1 min, and boiled for 30 min. The tubes were immediately snap cooled in ice water, the contents were transferred into pipette tips adapted in 1.5-ml tubes, and the eluate was collected at the bottom of the tubes after centrifugation at 50 × *g* for 5 min. The eluates were stored at −20°C or used immediately for PCR.

Crude lymphocyte lysates from venous blood were obtained as described elsewhere (8, 9). RNA was extracted from 100 μl of plasma by the method of Boom et al. (1) and reverse transcribed to cDNA as described elsewhere (4, 16).

Primers amplifying a part of the *pol* and *env* region previously described (9) were used in a nested PCR. An aliquot corresponding to 1.5 × 10^5 cells of each lysate and 5 μl of cDNA samples were used for PCR in a 50-μl reaction mixture (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each deoxynucleoside triphosphate, 1.25 U of *Taq* DNA polymerase [Perkin-Elmer, Zaventem, Belgium] and

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TABLE 1. HIV-1 PCR results of corresponding venous blood and DBS samples and plasma and DBS samples according to age groups

Age (mo)	No. tested (VB[a] and DBS)	No. positive by:		No. tested (plasma and DBS)	No. positive by:	
		VB	DBS		Plasma	DBS
0–1	29	5	5	16	3	3
>1–2	15	3	2	13	5	5
>2–3	7	2	2	6	2	2
>3–6	16	3	3	17	3	3
>6–12	9	1	1	8	1	1
>12	18	4	4	10	3	3
Total	94	18	17	70	17	17

[a] VB, venous blood.

0.4 μM each primer). A first round of 35 amplification cycles was done with the outer primers and was followed by a second round of 25 cycles with 1 μl of first-round product with inner primers. Cycling conditions for both rounds were 1 min each at 94, 50, and 72°C in a thermal cycler (Biozyme). In the last cycle, the extension was prolonged to 7 min. The β_2-microglobulin gene was amplified (to verify the quality of the DNA extraction) in a single PCR assay for 35 cycles under the same conditions as described above but with a primer concentration of 0.2 μM each. Nested PCR for DBS samples was done as described above but was refined as follows: included in the first amplification round were 5 pmol of each outer primer and 5 and 38 μl of sample and control eluates, respectively. The nested round included 30 pmol of inner primer and 2 μl of sample from the first amplification round. Eight microliters of the amplified products was electrophoresed in a 2% agarose gel and stained with ethidium bromide, and the DNA was visualized by transillumination at 245 nm. A sample was considered positive when it showed amplification with both *pol* and *env* primers, but when both of these primers could not amplify it whereas the β_2-microglobulin primers did, it was considered negative.

The nested PCR used detected one proviral DNA molecule per 50 μl of blood on filter paper. For a total of 20 control paired specimens containing 1 and 10 proviral HIV-1 copies, a positive PCR result was obtained with 14 (70%) (95% confidence interval [CI], 50 to 90%) and all (100%) of the specimens, respectively. All 12 infants born to seronegative mothers were negative with both primers (*pol* and *env*). All DBS and venous blood samples negative with *pol* and *env* primers were amplified by β_2-microglobulin primers, indicating that DNA was preserved and that the polymerase activity was not inhibited. PCR results of the 39 matched venous blood, DBS, and plasma samples correlated perfectly; 9 were positive by all methods (Table 2). Of the 94 paired DBS and venous blood samples, 17 and 18 paired samples were positive, respectively (Table 2), resulting in a 95% (95% CI, 85 to 100%) sensitivity and a 100% specificity of the HIV-1 DNA PCR on DBS with the venous blood samples as the reference [calculated as sensitivity = (number of PCR HIV-1 DNA DBS positives × 100)/number of PCR HIV-1 DNA venous blood positives = (17 × 100)/18 and specificity = (number of PCR HIV-1 DNA DBS negatives × 100)/number of PCR HIV-1 DNA venous blood negatives = (76 × 100)/76]. Both *pol* and *env* sequences were amplified in 17 paired DBS and plasma specimens of 70 paired samples tested (Table 2), indicating a 100% sensitivity and specificity of HIV-1 DNA PCR on DBS when results on plasma are considered as reference [calculated as sensitivity = (number of PCR HIV-1 DNA DBS positives × 100)/number of PCR HIV-1 cDNA plasma positives = (17 × 100)/17 and specificity = (number of PCR HIV-1 DNA DBS negatives × 100)/number of PCR HIV-1 cDNA plasma negatives = (53 × 100)/53]. Totals of 5 of 29 venous blood samples and 3 of 16 plasma samples with corresponding DBS samples from infants under 1 month of age were positive (Table 1). Of 36 infants under 3 months of age with consecutive DBS samples collected and tested every 2 months, only 8 (22.2%) were positive at initial collection. Subsequent follow-up samples from these eight infants were positive throughout the 6-month follow-up period. All 28 infants negative at initial collection of the DBS samples were negative on subsequent testings of their follow-up samples.

TABLE 2. HIV-1 PCR results of corresponding venous blood (DNA), DBS (DNA), and plasma (RNA) samples of children born to HIV-1-seronegative and -seropositive mothers

No. tested	No. positive by:		No. positive by:	
	VB[a]	DBS	Plasma	DBS
55	9	8	ND[c]	ND
39[b]	9	9	9	9
31	ND	ND	8	8
Total	18	17	17	17

[a] VB, venous blood.
[b] Corresponding venous blood, DBS, and plasma samples.
[c] ND, not done.

Blood collected on filter paper as DBS samples proved reliable for HIV-1 detection. This method of collection is easier, requiring a smaller amount of blood (about 50 μl) from a heel prick, compared with 1 ml of blood by venipuncture. As observed in this study and by others (2), DBS samples, unlike venous blood or plasma, can be stored at room temperature and transported in a less stringent way to laboratories with facilities to do PCR, thus also permitting epidemiological studies. Unlike other methods, in which the total volume of the eluate is used (17, 18), we are using only 5 μl of the 70 μl of eluate for PCR, which permits repeated testing of the same sample. The DNA extraction time was reduced to 1 h, still with maintenance of a high sensitivity and specificity. HIV-1 infection could be detected in infants less than 1 month old (Table 1), thus indicating the high sensitivity of the DBS PCR method. We observed a 95% (95% CI, 85 to 100%) sensitivity and 100% specificity with respect to PCR on corresponding venous blood samples. One sample was discordant despite retesting (positive on venous blood and negative on DBS), but mislabeling can not be excluded. PCR product carryover is also known to be a potential factor leading to discrepant results. It was not possible to trace the infant to evaluate the discordance.

These results as well as the consistent sensitivity and specificity observed with consecutive specimens from 36 infants monitored over time confirm previous findings (2, 17, 18) that DBS samples collected on filter paper are suitable for HIV-1 detection. In comparison with reverse transcriptase PCR on the 70 paired plasma samples, a 100% correlation was observed. Studies done by others (6, 7, 11) have shown reverse transcriptase PCR on plasma for HIV-1 detection in asymptomatic adults and children to be less sensitive than DNA PCR. In our study, we had no information on the clinical status and the CD4 counts of the children.

In conclusion, the DBS on filter paper appears to be a reliable source for HIV-1 detection by PCR. This method is

easier for sample collection and shipment and permits centralization of laboratory tests.

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