JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2004, p. 16–20 Vol. 42, No. 1
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.1.16–20.2004

Simple DNA Extraction Method for Dried Blood Spots and Comparison of Two PCR Assays for Diagnosis of Vertical Human Immunodeficiency Virus Type 1 Transmission in Rwanda

A. Fischer,[1]* C. Lejczak,[1] C. Lambert,[1] J. Servais,[2] N. Makombe,[2] J. Rusine,[2] T. Staub,[3] R. Hemmer,[1,3] F. Schneider,[1,4] J. C. Schmit,[1,3] and V. Arendt[1,3]

Retrovirology Laboratory, CRP-Santé,[1] National Service of Infectious Diseases, Centre Hospitalier de Luxembourg,[3] and Laboratoire National de Santé,[4] Luxembourg, and Treatment and Research AIDS Center, Kigali, Rwanda[2]

Received 4 April 2003/Returned for modification 10 July 2003/Accepted 27 September 2003

Dried blood spots (DBS) on filter paper facilitate the collection, transport, and storage of blood samples for laboratory use. A rapid and simple DNA extraction procedure from DBS was developed and evaluated for the diagnosis of human immunodeficiency virus type 1 (HIV-1) infection in children by an in-house nested-PCR assay on three genome regions and by the Amplicor HIV-1 DNA prototype assay version 1.5 (Roche Molecular Systems). A total of 150 samples from children born to HIV-1-infected mothers were collected in Kigali, Rwanda, in parallel as DBS and as peripheral blood mononuclear cell (PBMC) pellets. The results obtained on DBS by the two PCR assays were compared to the results of nested PCR on PBMCs. Of 150 PBMC samples, 10 were positive, 117 were negative, and 23 were indeterminate for HIV-1 infection. In DNA extracted from filter papers and amplified by using the in-house nested PCR, 9 of these 10 positive samples (90%) were found to be positive, and 1 was found to be indeterminate (only the *pol* region could be amplified). All of the negative samples and all of the 23 indeterminate samples tested negative for HIV-1 infection. When we used the Amplicor DNA test on DBS, all of the 10 PBMC-positive samples were found to be positive and all of the 23 indeterminate samples were found to be negative. Of the PBMC-negative samples, 115 were found to be negative and 2 were found to be indeterminate. We conclude that this simple rapid DNA extraction method on DBS in combination with both detection methods gave a reliable molecular diagnosis of HIV-1 infection in children born to HIV-infected mothers.

Vertical transmission of human immunodeficiency virus type 1 (HIV-1) leads to a high level of infant mortality, especially during the first 2 years of life (19). It is therefore necessary to make an early diagnosis of HIV-1 infection in newborns to initiate therapy of infected infants as early as possible. Serological tests are not useful, since maternal antibodies can be present in children until the age of 18 months (17). Virologic tests such as virus cultures (14) and RNA (20) or DNA PCRs result in an earlier diagnosis. However, virus cultures are time-consuming, require a biosecurity laboratory, and have a poor sensitivity, and data regarding HIV RNA PCR sensitivity are limited. P24 antigen detection is an alternative method to detect the presence of the virus, but the sensitivity is still lower than that of PCR (15).

For these reasons, amplification of the integrated viral genome by PCR has been the preferred method for the diagnosis of HIV infection in children for many years (11). However, this method requires venipuncture in newborns for blood sampling and preparation of lymphocyte pellets, both of which are difficult to perform, particularly in developing country settings.

Dried blood spot (DBS) samples are an interesting alternative for lymphocyte pellets since only a few droplets of blood are required and can be directly collected on a filter paper. Storage and shipment of filter papers is easy since they can be kept at room temperature and DNA has a good stability in dried samples. Finally, DBS have been used for the detection of HIV-1 genome by PCR since 1991 (3–5) with good sensitivity and specificity.

However, previous studies on DNA detection in DBS often applied labor-intensive protocols with multiple extraction steps (6, 7, 9, 16) that cannot easily be performed in the field. A recent study used the DBS directly as a template for PCR, with a sensitivity of 95.4% (2). The extraction steps were therefore simplified, but a commercial washing buffer was still needed. More importantly, only one region of the *pol* gene was amplified.

We present here part of a study on vertical transmission of HIV-1 infection in Kigali, Rwanda. Our goals were (i) to develop an easy and rapid method to extract DNA from DBS and (ii) to compare two PCR assays: an in-house nested PCR and Amplicor DNA prototype assay version 1.5, which has already been shown to be sensitive in detecting HIV-1 DNA in whole blood in EDTA and in cell pellets (13).

* Corresponding author. Mailing address: Retrovirology Laboratory, Centre Hospitalier de Luxembourg, Clinique Pédiatrique, 2ème Étage, L-1210 Luxembourg. Phone: (352) 44-11-61-12. Fax: (352) 44-11-61-13. E-mail: fischer.a@retrovirology.lu.

MATERIALS AND METHODS

A total of 150 blood samples were obtained from 139 children born to HIV-1-seropositive mothers in Kigali, Rwanda. Samples were collected as whole blood

on EDTA and as DBS when the children were between 2 and 26 months old. The age distribution of the samples was as following: 71 samples were collected when the children were less than 4 months old, 44 samples were collected when the children were between 5 and 12 months old; and 35 samples were collected when the children were between 13 and 26 months old.

From July 2002, patient follow-up was more efficient, and 11 children could be sampled twice. Nine of these sample pairs were collected the first time at between 2 and 4 months and the second time at between 6 and 8 months; samples from one child were collected at 6 and at 11 months, and the last sample pair was collected when the child was 14 and 18 months old.

Sample preparation. For each sample, four drops of ca. 50 μl of blood were spotted on filter paper (Isocode Card; Schleicher & Schuell, Dassel, Germany) by direct application from a heel stick. The DBS were dried at room temperature, stored in separated plastic bags, and shipped to the Retrovirology Laboratory in Luxembourg for processing.

Positive DBS controls were generated with ACH2 cells (which contain a single integrated copy of HIV-1 HXB2 strain per cell), diluted in HIV-negative EDTA-blood to yield 25, 50, 100, and 500 proviral copies per 50 μl, dropped onto filter papers, and dried at room temperature. Negative DBS controls were obtained by spotting ca. 50 μl of the same HIV-negative whole blood.

Peripheral blood mononuclear cells (PBMCs) were isolated from ca. 1 ml of venous EDTA-blood by density gradient centrifugation (LymphoPrep; AXIS-SHIELD Poc AS, Oslo, Norway), washed in phosphate-buffered saline, and resuspended in 5 ml of medium (88% RPMI 1640, 10% heat-denatured fetal bovine serum, 1% penicillin-streptomycin [10,000 U/ml and 10,000 μg/ml, respectively], and 1% L-glutamine at 200 mM). The cell concentration was determined by cell counting in a Coulter ZF cell counter (Analis, Namur, Belgium). PBMCs were then diluted in medium to a concentration of 10^6 cells/ml and spun to obtain pellets of 10^6 cells. Pellets were stored at −80°C and shipped to Luxembourg for processing.

Positive cell pellet controls were obtained by diluting ACH2 cells in the same medium as the PBMCs to obtain 25 and 50 ACH2 cells in 10^6 uninfected MT4 cells to reproduce the situation in blood, in which most of the cells are uninfected. Negative cell pellet controls consisted in pellets of 10^6 MT4 cells.

DNA extractions. DNA extraction of PBMC pellets and of ACH2 control pellets was performed with the QIAamp mini kit (Westburg, Leusden, The Netherlands), according to the manufacturer's instructions except for the elution, which was performed in 100 μl of elution buffer instead of 200 μl. Samples were immediately used for PCR or stored at −20°C.

For DNA extraction of the DBS, one spot of DBS or of positive or negative DBS control was put into a 1.5-ml Eppendorf tube; the cap was used to detach the spot to avoid cross-contamination. Spots were washed twice in 1 ml of phosphate-buffered saline–0.1% Tween during 10 min at room temperature with shaking. Spots were then transferred to 2-ml screw-caps tubes and 200 μl of Chelex-100 resin (biotechnology grade; Bio-Rad, Nazareth, Belgium) diluted at 5% in H_2O was added. Elution was carried out for 30 min at 60°C, followed by boiling at 100°C for 30 min. The samples were then quick-spun to collect the Chelex in the bottom of the tube, and the supernatant was immediately used for PCR or stored at −20°C. After extraction, DNA from PBMC samples and DBS were processed in the same way.

In-house nested PCR. A total of 10 μl of DNA solution extracted from PBMCs or from DBS was used for nested PCR to amplify parts of the HIV-1 *pol*, *gag*, and *env* genes.

(i) *gag* region. The primers were H1G777 and H1P202 (12) and the cycle protocol on a Perkin-Elmer GeneAmp 9600 thermocycler was 2 min at 94°C and 35 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C, followed by 7 min at 72°C. Then, 2 μl of outer PCR product was used for the inner PCR with the primers H1gag1584 and G17 (12), and the cycle protocol was 1 cycle of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C, and 60 s at 72°C, and then 1 cycle of 7 min at 72°C.

(ii) *env* region. Primers ED5 and ED12 (8) were used for outer PCR, and primers ES7 and ES8 (8) were used for nested PCR. The cycling conditions for both reactions were as follows: 2 min at 94°C, followed by 3 cycles of 60 s each at 94, 55, and 72°C; followed by 32 cycles of 15 s at 94°C, 45 s at 55°C, and 60 s at 72°C, with final extension for 7 min at 72°C.

(iii) *pol* region. Primers A1 and A2 (11) were used, and the cycle protocol was 60 s at 94°C, followed by 35 cycles of 30 s at 94°C, 15 s at 50°C, and 45 s at 72°C, with final extension for 10 min at 72°C. For nested PCR, the primers were A3 and A4 (11), and the cycling conditions were 60 s at 94°C, followed by 25 cycles of 30 s at 94°C, 15 s at 50°C, and 30 s at 72°C, with final extension for 10 min at 72°C.

(iv) Extraction and inhibition control. To test for the presence of PCR inhibitors, the human β-globin (HuBG) gene was amplified in a single PCR assay with the primers PC03 and KM38 (18) under the same conditions as described above for the outer *pol* fragment.

Nested-PCR products were visualized after electrophoresis on an ethidium bromide-stained 2% agarose gel by transillumination at 250 nm. If the β-globin fragment was not present, the sample result was scored as indeterminate for HIV. If the β-globin gene was amplified, results were analyzed as following: if two or more amplifications of the *pol*, *gag*, and *env* genes were positive, the samples were considered HIV positive; if only one region was amplified, the results were reported as indeterminate for HIV; and if none of the three regions were amplified, the samples were considered HIV negative.

Evaluation of the Amplicor DNA prototype assay (v1.5). To compare the prototype test with the in-house PCR, we adapted the Amplicor DNA prototype assay v1.5 (Roche Molecular Diagnostics) to use directly the DNA extracted from DBS: amplification of a 142-bp fragment of the *gag* gene was performed in a 100-μl mixture containing 3.6 μl of internal control DNA diluted to 1/5 in distilled water, 50 μl of DNA extracted from DBS, and 50 μl of a master mix containing dATP, dCTP, dGTP, dUTP, AmpErase, AmpliTaq polymerase, salts, and the biotinylated *gag* primers SK145 and SKCC1B. The cycling conditions were 2 min at 50°C, followed by 5 cycles of 10 s at 95°C, 10 s at 55°C, and 10 s at 72°C, and then followed by 30 cycles of 10 s at 90°C, 10 s at 60°C, and 10 s at 72°C. Amplicons were totally denatured with 100 μl of denaturation solution for 10 min at room temperature.

A 25-μl sample of denatured product was then transferred in a control plate coated with internal control specific probe CP35 to check the presence of PCR inhibitors and in the HIV-1 detection plate coated with HIV-1-specific probe SK102. Colorimetric revelation was performed as indicated by the manufacturer. Appropriate positive and negative controls provided in the kit were added in each assay. ACH2 DBS-positive controls were also tested to evaluate the detection limit of the test.

Interpretation of the results was done as previously described (21). In brief, when the optical density at 450 nm (OD_{450}) in the control plate was <0.2, the presence of PCR inhibitors was detected, and the sample could not be analyzed. When the OD in the control plate was ≥0.2, the sample was considered HIV negative if the OD in the HIV-1 detection plate was <0.2, HIV indeterminate if the OD was ≥0.2 but <0.8, and HIV positive if the OD was ≥0.8. All negative controls should have an OD of <0.2, and positive controls should have an OD of ≥1.5.

TABLE 1. Detection limit of nested PCR on DBS

No. of ACH2 cells/spot[a]	Eluate vol for PCR (μl)	No. of HIV proviral copies/PCR[b]	% Positive
25	10	1.25	75
50	10	2.5	83.3
100	10	5	87
500	10	25	100

[a] Each spot consisted of 50 μl of HIV-negative whole blood spiked with ACH2 cells.

[b] The number of HIV proviral copies per PCR was calculated by the following equation: (number of ACH2 cells per spot/elution volume [200 μl]) × eluate volume used for PCR.

RESULTS

Detection limit of HIV-1 DNA extracted from DBS. To evaluate the detection limit of HIV, PCR was performed on 10 μl of DNA eluate extracted from DBS containing 25, 50, 100, or 500 ACH2 cells in 50 μl of whole blood. With the in-house nested PCR, the 25 ACH2 standard could be detected in 9 of 12 tests (75%), the 50 ACH2 standard could be detected in 15 of 18 tests (83.3%), the 100 ACH2 standard could be detected in 20 of 23 tests (87%), and the 500 ACH2 standard could be detected in 6 of 6 tests (100%). The results are summarized in Table 1. We chose to use a 50 ACH2 and a 100 ACH2 standard as positive controls for each DNA extraction from the DBS. The Amplicor DNA test was able to detect 50 and 100 ACH2 cells/spot in 100% of the tests, corresponding to calculated

TABLE 2. Sensitivity and specificity of nested PCR on DBS[a]

Nested PCR on PBMCs (no. of samples)	Nested PCR on DBS (no. of samples)					
	First test[b]			Repeat[c]		
	+	−	Ind	+	−	Ind
+ (10)	9	0	1	9	0	1
− (117)	0	110	7	0	116	1
Ind (23)	0	23	0	0	23	0

[a] +, HIV-positive result; −, HIV-negative result; Ind, indeterminate result for HIV.
[b] Sensitivity, 90%; specificity, 94%.
[c] Sensitivity, 90%; specificity, 99%.

amounts of 12.5 and 25 HIV proviral copies, respectively, in the 50 μl of eluate used per PCR.

Sensitivity of nested PCR in DBS and PBMCs. Nested PCR was performed on 150 DBS samples, and the results were compared to amplifications obtained in the corresponding PBMC samples.

Among the 150 samples tested in duplicate, the HuBG gene could not be amplified in 23 PBMC samples (15.3%) and in 7 DBS (4.6%). These samples were considered indeterminate for HIV infection. No repeat could be performed on PBMCs since the number of pellets available for each patient was limited. After a new DNA extraction from a second spot, six of seven indeterminate DBS samples tested negative for HIV (and positive for HuBG) and one sample remained indeterminate because of HuBG amplification failure.

Of the 127 PBMC samples in which the β-globin gene could be amplified, 10 were positive (7.8%) and 117 were negative for HIV. Among the 10 positive samples, one was a duplicate sample 2 months after the first sample from one child, so there were nine different children positive for HIV-1 infection.

For the 10 positive samples, HIV provirus was also detected in 9 by nested PCR in DBS. The remaining sample was considered indeterminate since only the *pol* region could be amplified, despite repeated testing. The 23 β-globin-negative PBMC samples were HIV negative on DBS. The initial results and after repeated tests are shown in Table 2.

Compared to PBMCs, the overall sensitivity of nested PCR on DBS was 90%, and the specificity after repeat of indeterminate samples was 99%.

Table 3 shows the results according to the children's age at collection time: of the 10 PBMC positive samples, 4 were collected before 4 months of age, 2 were collected at between 4 and 12 months, and 4 were collected at between 12 and 26 months. The sensitivity of nested PCR on DBS was 100% for children less than 4 months old, 100% for children between 4 and 12 months, and 75% for children between 12 and 26 months old.

Evaluation of the Amplicor DNA prototype test. The 150 DBS samples were analyzed with the Amplicor DNA prototype assay version 1.5, and the results were compared to those obtained with nested PCR on PBMCs.

The results obtained are summarized in Table 4. The 23 β-globin-negative PBMC samples were found to be HIV negative and 10 of 10 HIV-positive PBMC samples were found to be positive with the commercial test. Of the 117 HIV-negative PBMC samples, 2 were indeterminate for HIV, 106 were HIV negative, and 9 were detected as HIV-positive with the Amplicor test. After repeated testing, the two indeterminate samples remained indeterminate, with an OD_{450} of between 0.2 and 0.8, and the nine presumed falsely HIV-positive samples were found to be HIV negative. According to these results, the sensitivity of this test was 100%, and the specificity was 98% after repeated testing (Table 4).

TABLE 3. Results of nested PCR on DBS distributed by age at sample collection[a]

Infant age (mo)	Nested PCR on PBMCs (no. of samples)	Nested PCR on DBS (no. of samples)		
		+	−	Ind
≤4[b]	+ (4)	4	0	0
	− (62)	0	61	1
	Ind (5)	0	5	0
>4 to ≤12[c]	+ (2)	2	0	0
	− (31)	0	31	0
	Ind (11)	0	11	0
>12 to ≤26[d]	+ (4)	3	0	1
	− (23)	0	23	0
	Ind (7)	0	7	0

[a] +, HIV-positive result; −, HIV-negative result; Ind, indeterminate result for HIV.
[b] Sensitivity, 100%; specificity, 98%.
[c] Sensitivity, 100%; specificity, 100%.
[d] Sensitivity, 75%; specificity, 100%.

Table 5 summarizes the results by age. HIV infection in four of four children less than 4 months old could be detected by the Amplicor DNA test, so the sensitivity of the test in the first 4 months of life is equivalent to that in the first 26 months of life.

For the 11 children who could be sampled twice at least at a 2-month interval, all of the results obtained by nested PCR and with the Amplicor DNA prototype test were concordant with the first samples.

DISCUSSION

The DBS extraction procedure described here is rapid and easy to perform. Indeed, this procedure requires only two buffers that are easy to prepare and relatively little laboratory material. Therefore, it can be performed under field conditions.

TABLE 4. Sensitivity and specificity of the Amplicor DNA test on DBS[a]

PBMC nested PCR result[a] (no. of samples)	Amplicor DNA test v1.5 on DBS (no. of samples)					
	First test[b]			Repeat[c]		
	+	−	Ind	+	−	Ind
+ (10)	10	0	0	10	0	0
− (117)	9	106	2	0	115	2
Ind (23)	0	23	0	0	23	0

[a] +, HIV-positive result; −, HIV-negative result; Ind, inderminate result for HIV.
[b] Sensitivity, 100%; specificity, 90%.
[c] Sensitivity, 100%; specificity, 98%.

TABLE 5. Results of Amplicor DNA test on DBS distributed by age at sample collection[a]

Infant age (mo)	Nested PCR on PBMCs (no. of samples)	Amplicor DNA test v1.5 on DBS (no. of samples)		
		+	−	Ind
≤4[b]	+ (4)	4	0	0
	− (62)	0	61	1
	Ind (5)	0	5	0
>4 to ≤12[c]	+ (2)	2	0	0
	− (31)	0	30	1
	Ind (11)	0	11	0
>12 to ≤26[d]	+ (4)	4	0	0
	− (23)	0	23	0
	Ind (7)	0	7	0

[a] +, HIV-positive result; −, HIV-negative result; Ind, indeterminate result for HIV.
[b] Sensitivity, 100%; specificity, 98%.
[c] Sensitivity, 100%; specificity, 97%.
[d] Sensitivity, 100%; specificity, 100%.

The detection limit of the nested PCR on DBS was determined to be 25 ACH2 cells in 50 μl of whole blood, which were detected in 75% of the tests. Since this corresponds to a value of 1.25 proviral copy in 10 μl of eluate used for the PCRs, we do not expect to improve this limit, unless by an increase in the volume of eluate used for each PCR.

The number of indeterminate samples probably due to the presence of PCR inhibitors was significantly higher (P value $<$ 0.01 [chi-square test]) in the nested PCR on PBMCs (15.3%) than in the nested PCR or in the Amplicor test on DBS (4.6 and 0%, respectively). These results suggest that lymphocyte pellets are probably difficult to prepare in the field, and we suppose that the presence of the PCR inhibitors could be due to hemoglobin remaining in the pellets. The DBS avoid the separation of lymphocytes; moreover, the elution with Chelex-100 should eliminate the potential PCR inhibitors, since Chelex is a cation-chelating resin: the positively charged ions are captured by the resin, whereas the DNA, negatively charged, remains free in the solution.

The sensitivities of the Amplicor DNA test and of the nested PCR on DBS were similar (100 and 90%, respectively), but nested PCR is theoretically more stringent since three different regions of the HIV-1 genome are amplified, whereas the commercial test amplifies only one region. Moreover, the specificity of the nested PCR is significantly better than the specificity of the Amplicor DNA test, which presented a number of false-positive results (7.1%, $P < 0.01$ [chi-square test]) in the first run, even if all false-positive results could be corrected after a repeated test. Nested PCR on DBS presented no false-positive samples but did present a number of indeterminate results in the first run, probably because of the presence of PCR inhibitors, as suggested by the failure to amplify the β-globin gene. All of these samples were HIV negative in a repeated test. Compared to nested PCR, which is time-consuming and requires good technical experience in PCR, the commercial test is rapid, requires less equipment, and is easy to perform.

The nested-PCR primers were able to amplify fragments of 175, 700, and 460 bp from the *pol*, *env*, and *gag* regions, respectively, confirming that DNA fragments from different sizes can be amplified from DBS. These fragments could potentially be used for other applications, such as sequencing.

Previous results have shown that most of the patients in Rwanda are infected with HIV-1 subtype A (1). Therefore, one can speculate that both the nested PCR and the Amplicor DNA test could be used for HIV-1 detection in regions where non-B subtypes are predominant.

In conclusion, the DNA extraction method from DBS developed here is rapid, is easy to perform, and permits reliable diagnosis of HIV-1 infection when combined with nested PCR or with the Amplicor DNA prototype assay. Both PCR methods have a sensitivity superior to 90% and a high specificity (>98%) and seem to be able to detect patients infected with non-B subtypes. Moreover, no difference of sensitivity was observed in the assays in children up to 4 months old compared to assays with children up to 26 months. The most ideal time to perform HIV-1 diagnosis would be the neonatal period when both mother and child are still in the hospital, but even the DNA PCR on PBMCs has a sensitivity of only 38% at 48 h of life. The sensitivity then increases rapidly to 93% at 2 weeks of age (10). In any case, children also have to be tested when they are between 2 and 4 weeks old.

The choice of one test instead of the other depends on the setting in which it is to be used: if screening of a large number of children is needed in a short time in difficult laboratory conditions, the Amplicor test is preferable, but nested PCR might be better in studies involving a reduced number of samples and laboratory technicians experienced in PCR.

Further evaluation of this test is currently ongoing in Kigali in 1-month-old children to evaluate the sensitivity of the test in younger children. Moreover, the entire procedure (sample collection, DNA extraction, and PCR) is now being performed in Kigali to confirm that it can be done under field conditions.

ACKNOWLEDGMENTS

This study was supported by the Centre de Recherche Public Santé and the Fondation Recherche sur le SIDA (Luxembourg) and Roche Molecular Systems, which provided the prototypes assays.

REFERENCES

1. **Bachmann, M. H., E. L. Delwart, E. G. Shpaer, P. Lingenfelter, R. Singal, and J. I. Mullins.** 1994. Rapid genetic characterization of HIV type 1 strains from four World Health Organization-sponsored vaccine evaluation sites using a heteroduplex mobility assay: W.H.O. isolation and characterization. AIDS Res. Hum. Retrovir. **10:**1345–1353.
2. **Beck, I., K. D. Drennan, A. J. Melvin, K. M. Mohan, A. M. Herz, J. Alarcón, J. Piscoya, C. Velαzquez, and L. M. Frenkel.** 2001. Simple, sensitive, and specific detection of human immunodeficiency virus type 1 subtype B DNA in dried blood samples for diagnosis in infants in the field. J. Clin. Microbiol. **39:**29–33.
3. **Cassol, S. A., N. Laponite, T. Salas, C. Hankins, M. Arella, M. Fauvel, G. Delage, M. Boucher, J. Samson, J. Charest, M. L. Montpetit, and M. V. O'Shaughnessy.** 1992. Diagnosis of vertical HIV-1 transmission using the polymerase chain reaction and dried blood spot specimens. J. Acquir. Immune Defic. Syndr. **5:**113–119.
4. **Cassol S. A., B. G. Weniger, P. G. Babu, M. O. Salminen, X. Zheng, M. Thet Htoon, A. Delaney, M. O'Shaughnessy, and C. Y. Ou.** 1996. Detection of HIV type 1 env subtypes A, B, C, and E in Asia using dried blood spots: a new surveillance tool for molecular epidemiology. AIDS Res. Hum. Retrovir. **12**:1435–1441.
5. **Cassol, S. A., S. Read, B. G. Weniger, P. Gomez, N. Lapointe, C. Y. Ou, and P. G. Babu.** 1996. Dried blood spots collected on filter papers: an international resource for the diagnosis and genetic characterization of human immunodeficiency virus type-1. Mem. Inst. Oswaldo Cruz **91:**351–358.
6. **Comeau, A. M., H. W. Hsu, M. Schwerzler, G. Mushinsky, E. Walter, L. Hofman, and G. F. Grady.** 1993. Identifying human immunodeficiency virus

infection at birth: application of polymerase chain reaction to Guthrie cards. J. Pediatr. **123:**252–258.
7. **Comeau, A. M., J. Pitt, G. V. Hillyer, S. Landesman, J. Bremer, B. H. Chang, J. Lew, J. Moye, G. F. Grady, and K. McIntosh.** 1996. Early detection of human immunodeficiency virus on dried blood spot specimens: sensitivity across serial specimens. J. Pediatr. **129:**111–118.
8. **Delwart, E. L., E. G. Sphaer, J. Louwagie, F. E. McCutchan, M. Grez, H. Rübsamen-Waigmann, and J. I. Mullins.** 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 genes. Science **262:**1257–1261.
9. **DeVange Panteleeff, D., G. John, R. Nduati, D. Mbori-Ngacha, B. Richardson, J. Freiss, and J. Overbaugh.** 1999. Rapid method for screening dried blood samples on filter paper for human immunodeficiency virus type 1 DNA. J. Clin. Microbiol. **37:**350–353.
10. **Dunn, D. T., C. D. Brandt, A. Krivine, S. A. Cassol, P. Roques, V. Borkowsky, A. De Rossi, E. Denamur, A. Ehrnst, and C. Loveday.** 1995. The sensitivity of HIV-1 DNA polymerase chain reaction in the neonatal period and the relative contributions of intra-uterin and intra-partum transmission. AIDS **9:**F7–F11.
11. **Fransen, K., P. Zhong, H. De Beenhouwer, G. Carpels, M. Peeters, J. Louwagie, W. Janssens, P. Piot, and G. van der Groen.** 1994. Design and evaluation of new, highly sensitive and specific primers for polymerase chain reaction detection of HIV-1-infected primary lymphocytes. Mol. Cell. Probes **8:**317–322.
12. **Heyndrickx L., W. Janssens, L. Zekeng, R. Musonda, S. Anagonou, G. Van der Auwera, S. Coppens, K. Vereecken, K. De Witte, R. Van Rampelbergh, M. Kahindo, L. Morison, F. E. McCutchan, J. K. Carr, J. Albert, M. Essex, J. Goudsmit, B. Asjö, M. Salminen, A. Buvé, Study group on heterogeneity of HIV epidemics in african cities, and G. Van der Groen.** 2000. Simplified strategy for detection of recombinant human immunodeficiency virus type 1 group M isolates by gag/env heteroduplex mobility assay. J. Virol. **74**:363–370.
13. **Jackson, B. J., J. S. Parsons, L. S. Nichols, N. Knoble, S. Kennedy, and E. M. Piwowar.** 1997. Detection of human immunodeficiency virus type 1 (HIV-1) antibody by Western blotting and HIV-1 DNA by PCR in patients with AIDS. J. Clin. Microbiol. **35:**1118–1121.
14. **McIntosh, K., J. Pitt, D. Brambilla, S. Carroll, C. Diaz, E. Handelsman, J. Moye, and K. Rich.** 1994. Blood culture in the first 6 months of life for the diagnosis of vertically transmitted human immunodeficiency virus infection. J. Infec. Dis. **170:**996–1000.
15. **Nesheim, S., F. Lee, M. L. Kalish, C. Y. Ou, M. Sawyer, S. Clark, L. Meadows, V. Grimes, R. J. Simonds, and A. Nahmias.** 1997. Diagnosis of perinatal human immunodeficiency virus infection by polymerase chain reaction and p24 antigen detection after immune complex dissociation in an urban community hospital. J. Infec. Dis. **175:**1333–1336.
16. **Nyambi, P., K. Fransen, H. De Beenhouwer, E. N. Chomba, M. Temmerman, J. O. Ndinya-Achola, P. Piot, and G. Van Der Groen.** 1994. Detection of human immunodeficiency virus type 1 (HIV-1) in heel prick blood on filter paper from children born to HIV-1-seropositive mothers. J. Clin. Microbiol. **32**:2858–2860.
17. **Rakusan, T. A., R. H. Parrott, and J. L. Sever.** 1991. Limitations in the laboratory diagnosis of vertically acquired HIV infection. J. Acquir. Immune Defic. Syndr. **4:**116–121.
18. **Saiki, R. K., C. A. Chang, C. H. Levenson, T. C. Warren, C. D. Boehm, H. H. Kazazian, Jr., and H. A. Erlich.** 1988. Diagnosis of sickle cell anemia and beta-thalassemia with enzymatically amplified DNA and non-radioactive allele-specific oligonucleotide probes. N. Engl. J. Med. **319:**537–541.
19. **Spira R., P. Lepage, P. Msellati, P. Van de Perre, V. Leroy, A. Simonon, E. Karita, and F. Dabis.** 1999. Natural history of human immunodeficiency virus type 1 infection in children: a five-year prospective study in Rwanda. Pediatrics **104:**e56. [Online.]
20. **Steketee, R. W., E. J. Abrams, D. M. Thea, T. M. Brown, G. Lambert, S. Orloff, J. Weedon, M. Bamji, E. E. Schoenbaum, J. Rapier, and M. L. Kalish.** 1997. Early detection of perinatal human immunodeficiency virus (HIV) type 1 infection using HIV RNA amplification and detection. J. Infect. Dis. **175:**707–711.
21. **Zijenah, L. S., J. Humphrey, K. Nathoo, L. Malaba, P. Zvandasara, A. Mahomva, P. Iliff, and M. T. Mbizvo.** 1999. Evaluation of the prototype Roche DNA amplification kit incorporating the new SSK145 and SKCC1B primers in detection of human immunodeficiency virus type 1 DNA in Zimbabwe. J. Clin. Microbiol. **37:**3569–3571.