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Rapid Method for Screening Dried Blood Samples on Filter Paper for Human Immunodeficiency Virus Type 1 DNA

DANA DEVANGE PANTELEEFF,[1] GRACE JOHN,[2,3] RUTH NDUATI,[3] DOROTHY MBORI-NGACHA,[3] BARBRA RICHARDSON,[4] JOAN KREISS,[2,5] AND JULIE OVERBAUGH[1]*

Departments of Microbiology,[1] Epidemiology,[5] Medicine,[2] and Biostatistics,[4] University of Washington, Seattle, Washington 98195, and Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya[3]

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PCR is a highly sensitive method for the detection of human immunodeficiency virus type 1 (HIV-1) nucleic acids in blood mononuclear cells and plasma. However, blood separation techniques require extensive laboratory support systems and are difficult when a limited volume of blood is available, which is often the case for infants. The use of blood samples stored on filter paper has many advantages for the detection of perinatal HIV-1 infection, but current methods require extraction and purification of target DNA prior to PCR amplification. We report a highly sensitive and rapid method for the extraction and detection of HIV-1 DNA in infant blood samples stored on filter papers. Because this rapid protocol does not involve steps for the removal of potential inhibitors of the PCR, the highest sensitivity is achieved by testing the filter paper lysate in quadruplicate. Assays for HIV-1 DNA were done by using nested PCR techniques that amplify HIV-1 *gag* DNA from blood spot samples on filter paper and from corresponding viably frozen mononuclear cells separated from venous blood samples obtained from 111 infants born to HIV-1-seropositive mothers. PCR results with blood from filter papers showed 100% specificity (95% confidence internal [CI] 93.1 to 100%) and 96% (95% CI, 88.65 to 98.9%) and 88% (95% CI, 79.2 to 94.5%) sensitivity (for quadruplicate and duplicate tests, respectively) compared to PCR results with blood mononuclear cells. Moreover, this method could detect HIV-1 sequences of multiple subtypes.

Detection of human immunodeficiency virus type 1 (HIV-1)-specific nucleic acid by PCR is the method of choice for the diagnosis of HIV-1 infection in situations in which viral antibodies may not yet be present or may be passively acquired. For example, PCR detection is useful for the diagnosis of HIV-1 infection in very young infants born to HIV-1-seropositive mothers because antibody screening cannot discriminate maternal antibodies from the infant's humoral response to the HIV-1 infection (20). Detection of nucleic acid is also useful early in infection in adults, prior to seroconversion. In both circumstances, mononuclear cells separated from whole-blood samples have proven to be sensitive and reliable templates for HIV-1 DNA detection by PCR (21).

Assays for HIV-1 must take into account the genetic variability of HIV-1 isolates. A widely used assay should be able to detect the different clades that are represented in various geographic locations. PCR-based assays have been redefined to detect a range of HIV-1 variants; this is particularly important for areas outside of the United States and Europe, where non-B clades are predominately found. However, the blood separation techniques required to process samples in preparation for PCR are not amenable to all settings, especially those in developing countries, and large volumes of blood must be drawn to ensure an adequate yield of infected cells.

The use of filter papers is an attractive alternative to the use of larger-volume tubes for blood collection and storage for several reasons. Only a few drops of blood are applied to the paper, and this amount can be obtained by a heel stick. Venipuncture of small infants is not always successful, the amount of blood obtained is sometimes insufficient for lymphocyte separation, and mothers of small infants are often more comfortable with a heel stick than venipuncture. Once they have dried, blood samples on filter paper are no longer infectious and can be stored at room temperature, eliminating the need to store and transport whole or separated blood samples in liquid nitrogen. The use of filter papers also provides fewer chances for mislabeling because there are no transfer steps once the blood is applied to the paper. In developing countries, mononuclear cell sample collection, storage, and processing are difficult, expensive, and often unavailable. Thus, since 1973, filter papers have been used to collect blood samples for large field studies of numerous diseases (4, 9, 10, 13).

Because of the ease of sample collection and storage, a number of groups have developed protocols for the detection of HIV-1 DNA that is extracted from dried blood spots on filter papers. These highly sensitive and specific methods involve either long and complicated DNA extraction and/or elution procedures or expensive commercially available biotechnology products (2, 3, 6, 7, 22). In one study, a simplified procedure that did not require extractions or elutions has been shown to be sensitive and specific for the detection of HIV-1 in blood from seropositive adults (12). We have adapted our highly sensitive method of lysing infected cells collected from the genital tract with a dry swab (14) and applied it to the lysis of blood cells that have been collected from infants and dried on filter paper. We show that this method is both sensitive and specific for the detection of HIV-1 DNA in filter paper samples of dried blood from infants whose HIV-1 infection status and the infecting HIV-1 subtypes are known.

MATERIALS AND METHODS

Infected-cell standards were generated with ACH-2 cells, which contain a single integrated copy of HIV-1 proviral DNA per cell (5, 8). ACH-2 cells were diluted in uninfected CEM×174 cells to mimic the situation in blood in which the majority of cells are uninfected. To test for inhibition of the PCR by heme or other inhibitors present in blood but not in cell culture media, dilutions of

* Corresponding author. Mailing address: Department of Microbiology, University of Washington, Box 357242, Seattle, WA 98195. Phone: (206) 543-3146. Fax: (206) 543-8297. E-mail: overbaug@u.washington.edu.

ACH-2 cells in HIV-1-negative blood were also examined. In each case, a range from 50 to 5×10^6 infected cells and a background of 3×10^5 uninfected cells in a volume of 50 μl were adsorbed onto filter paper spots (no. 903; Schleicher and Schuell) and air dried.

Blood samples were obtained from babies enrolled in the Breastfeeding and HIV-1 Transmission Study in Nairobi, Kenya (15). At each clinic visit, enough blood was drawn to allow separation of mononuclear cells, and small amounts of blood from the syringe were applied to filter papers. The larger volume of whole blood was spun, and the mononuclear cell fraction was frozen in a cryovial in liquid nitrogen. The cells were shipped from the University of Nairobi to the University of Washington in liquid nitrogen and were stored in liquid nitrogen until use. For the filter paper samples, individual drops of blood from the syringe, each approximately 50 μl, were spotted onto filter paper, air dried, stored at room temperature (20 to 25°C), transported to the University of Washington, and stored at room temperature (approximately 22°C and not desiccated) until use. The time between filter paper sample collection and PCR analysis ranged from 4 to 36 months.

For both the infected-cell standards and the whole-blood samples from infants, an ethanol-flamed 8-mm hole punch was used to detach the filter paper containing a dried blood spot or a spot of ACH-2 and CEM×174 cells into a 1.7-ml Eppendorf tube, and ethanol-flamed forceps were used to force the disc into the bottom of the tube. One disc of blood obtained from each subject at each visit (approximately 50 μl of blood) was used for testing. One hundred microliters of lysis buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 100 μg of gelatin, 0.45% Tween 20, 0.45% Nonidet P-40, 60 μg of proteinase K per ml) was added to each tube. Lysis was carried out for 90 min at 56°C followed by an incubation at 95°C for 20 min to inactivate the proteinase K. Each sample was spun at $1,000 \times g$ for 7 min to force the filter paper disc and other debris to the bottom of the tube. Supernatants containing lysed samples were either immediately used in PCR or stored at −20°C.

The peripheral blood mononuclear cell (PBMC) samples were thawed and then spun at $1,100 \times g$ at 4°C for 5 min to pellet the cells. The supernatants were removed and the pellets were resuspended in 1.5 ml of phosphate-buffered saline. The samples were then spun again at $1,100 \times g$ at 4°C for 5 min. The supernatants were removed, and the pellets were resuspended in 100 μl of lysis buffer and were incubated at 56°C for 90 min and then at 95°C for 20 min to inactivate the proteinase K.

The amount of lysate tested in a PCR was 1 to 20 μl for all ACH-2 cell standards, 5 μl for filter paper samples of blood from infants, and 2 μl for the PBMC samples. For filter paper samples of blood from infants, the technician performing the PCR analysis was blinded to the results of the subjects' lymphocyte PCR results until all filter paper samples had been tested. The nested PCR protocol used in this study has been described previously (11, 14), and this method has been shown to consistently amplify as little as a single copy of the HIV-1 *gag* gene (14).

For samples with discordant results by assays with samples on filter paper and PBMCs, the quality of the DNA in a lysed filter paper sample was assessed. Primers were designed to amplify fragments of the human β-actin gene whose sizes are similar to those of the predicted HIV-1 *gag* and *env* gene products. The smaller, 147-bp fragment was amplified by performing nested PCRs containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% (wt/vol) gelatin, 250 ng of each primer (sequences below), each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 2 U of Perkin-Elmer *Taq* polymerase. For both rounds of amplification, the DNA was first denatured at 94°C for 7 min. This was followed by 30 cycles that included a 1-min denaturation at 94°C, primer annealing for 30 s at 60°C, and product extension at 72°C for 1 min. To optimize the amount of full-length product, a final extension was carried out at 72°C for 6 min. Final PCR products were visualized following electrophoresis on an ethidium bromide-stained 2% agarose gel. The larger, 973-bp fragment was amplified by using PCR conditions similar to those described above but with different primer sets (sequences below) and a slightly different temperature cycling profile. For this fragment, the initial denaturation step was for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 52°C for 1 min, and a 3-min extension at 72°C. An 8-min extension at 72°C followed the 35 cycles. Final PCR products were analyzed on an ethidium bromide-stained 1% agarose gel. β-Actin is expected to be present in every cell, whereas HIV-1 is typically present in less than 1% of PBMCs. Thus, β-actin PCRs were performed with the equivalent of 0.02 μl (2 μl of a 1 to 100 dilution) as the template in the first round 50-μl reaction mixture, and 2 μl of the round one product was used as the template for the second round of PCR. ACH-2 cells served as a positive control for these PCRs.

The 147-bp β-actin fragment primers were as follows: for round 1, ACT3 (GGACCTGAAGCTGCTTCTCTACCGG) and ACT4 (CTCCTTAGAGAGA AGTGGGGTGG); for round 2, ACT1 (TTGCCACTTCCACTGTCGTCAGCC) and ACT2 (GCTTTTAGGATGGCAAGGGACTTCC). The 973-bp β-actin fragment primers were as follows: for round 1, ACT 7 (TAATTCCTCTTCG ACACGATGCAGCG) and ACT4 (CTCCTTAGAGAGAAGTGGGGTGG); for round 2, ACT 5 (GGACCTGAAGCTCGTTCTCTACCGG) and ACT 2 (GCTTTTAGGATGGCAAGGGACTTCC).

TABLE 1. Template volumes and approximate HIV-1 proviral copy number per PCR mixture for ACH-2 cells diluted in HIV-1-negative blood

No. of ACH-2 cells adsorbed onto paper[a]	PCR template vol	Approximate no. of copies per PCR mixture[b]	% Positive of multiple PCRs
50	1	0.5	0
	2	1	27
	5	2.5	40
	10	5	0
	20	10	0
500	1	5	33
	2	10	73
	5	25	100
	10	50	0
	20	100	0
5,000	1	50	100
	2	100	100
	5	250	100
	10	500	100
	20	1,000	0
50,000	1	500	100
	2	1,000	100
	5	2,500	100
	10	5,000	100
	20	10,000	0
500,000	1	5,000	100
	2	10,000	100
	5	25,000	100
	10	50,000	100
	20	100,000	0

[a] The volume of cells on each filter paper sample was 50 μl.

[b] Approximate template copy number per PCR mixture was calculated by the following equation: (number of cells adsorbed onto paper/lysis buffer volume [100 μl]) × PCR template volume.

RESULTS

Amplification of HIV-1 *gag* DNA from ACH-2 cell standards. Filter papers that had known amounts of ACH-2 cells in a background of uninfected CEM×174 human cells were used to examine the ability of the method to detect low numbers of HIV-1-infected cells. HIV-1 *gag* DNA could be detected by PCRs with lysates from filter papers that were predicted to include a single HIV-1 proviral copy in a background of 3×10^5 CEM×174 cells (data not shown).

To examine the effect of blood, which is known to inhibit *Taq* polymerase (1), known amounts of ACH-2 cells were mixed with HIV-1-negative blood and applied to filter papers. Various amounts of lysate were examined for each dilution of ACH-2 cells such that the predicted proviral copy number in a PCR mixture ranged from less than 1 up to 100,000. The results of this analysis are presented in Table 1. In general, we noted an increase in sensitivity with an increase in copy number until the lysate volume added to a PCR mixture exceeded 5 or 10 μl. For example, 1 copy could be detected in 27% of tests, 10 copies could be detected in 73% of tests, and 25 or more copies could be detected in 100% of tests with lysate volumes of 5 μl or less. However, with higher lysate volumes (10 to 20 μl), an inhibitory effect was sometimes observed, especially when low copy numbers were present in high lysate volumes. For example, while 10 proviral copies were detected in 73% of the tests when the 10 copies were added in a 2-μl volume, 10 copies were never detected when 20 μl of lysate was added to the PCR mixture. In addition, 50 copies were de-

TABLE 2. Sensitivity and specificity of filter paper PCR with respect to corresponding PBMC data[a]

Lymphocyte PCR results	No. of samples with the following filter paper PCR results:			
	Two tests with 5 μl[b]		Four tests with 5 μl[c]	
	+	−	+	−
+	61	8	66	3
−	0	42	0	42

[a] +, positive result; −, negative result.
[b] Sensitivity, 88% (95% CI, 79.2% to 94.5%); specificity, 100% (95% CI, 93.1% to 100%).
[c] Sensitivity, 96% (95% CI, 88.6% to 98.9%); specificity, 100% (95% CI, 93.1% to 100%).

tected 100% of the time when the 50 copies were present in 1 μl of lysate but were never detected when 50 copies were added in a 10-μl volume.

Amplification of HIV-1 *gag* DNA from clinical specimens. We first tested the PBMC samples from the group of infants by a standard method for the amplification of HIV-1 *gag* DNA. Previous studies have shown that the sensitivity and specificity of this method are 98 and 89%, respectively (14). The samples analyzed were taken from 111 infants of HIV-1-infected mothers. The infants' HIV-1 infection status had been determined by analysis of sequential PBMC samples. For an infant to be considered HIV-1 positive, at least two consecutive PBMC samples had to test positive for HIV-1 *gag* sequences by DNA PCR. The PCR results for 111 filter paper samples containing blood from the same group of infants were analyzed and compared to their corresponding PBMC PCR results with samples obtained at the same clinic visit. When the filter paper samples were tested by duplicate PCRs with the same dried blood spot, we observed a sensitivity of 88% (95% confidence interval [CI], 79.2 to 94.5%) and a specificity of 100% (95% CI, 93.1 to 100%) with respect to the corresponding PBMC PCR results. When the dried blood spots were tested in quadruplicate, the sensitivity was increased to 96% (95% CI, 88.6 to 98.9%) and the specificity remained 100% (95% CI, 93.1 to 100%) (Table 2). In addition to the diagnostic PCRs performed with 5 μl of lysate, larger volumes of 10 and 20 μl of lysate were tested to examine the effects of blood present in the clinical specimens. Four lysates of filter paper samples from infants that each tested positive for HIV-1 *gag* DNA with 5 μl of lysate were tested in duplicate with 10 and 20 μl of lysate. One test with 10 μl of lysate was faintly positive, but all other PCRs were negative (data not shown). For the three clinical samples that were stored on filter paper and that had discordant results compared to the results for the corresponding lymphocyte sample, PCR was performed for detection of a housekeeping gene (β-actin) to assess the quality of DNA in the lysate. To best mimic the HIV-1 *gag* PCR, β-actin primers were designed to amplify a fragment similar in size to the HIV-1 *gag* PCR product. In addition, only a fraction (1%) of the amount of template used for the HIV-1 *gag* PCR was used for the β-actin PCR to compensate for the difference in copy number. The small β-actin fragment could be detected in all three samples with discordant results.

Amplification of larger DNA fragments. The *gag* DNA target of the diagnostic PCR is only 142 bp in length, whereas amplification of HIV-1 for genetic analysis typically focuses on larger sequences to detect informative differences. To determine if the DNA from filter papers could be used to amplify larger fragments, PCR was also performed with primers designed to amplify a 1.2-kb fragment of the envelope gene and primers designed to amplify a 0.9-kb fragment from Kenyan samples (16, 17, 19). Again, β-actin primers designed to amplify a similarly sized fragment were tested in parallel with seven (four HIV-1 positive and three HIV-1 negative) recently lysed samples. Results were negative for all attempts at the amplification of fragments of each size, suggesting that larger fragments of DNA may be difficult to amplify from the dried blood sample, at least when crude lysates are used.

Sensitivity for detection of various subtypes of HIV-1. Multiple clades of HIV-1 are found in Africa, and multiple clades were found in the group of subjects under study here. For example, clade A, C, and D HIV-1 subtypes, as well as occasional outlier variants, have been detected in the women enrolled in the breastfeeding transmission study in Kenya (16, 18). Previous studies demonstrated that our method of detecting HIV-1 *gag* DNA in blood mononuclear cells in Kenyan individuals was highly sensitive compared to the sensitivities of serological assays (14), suggesting that a range of HIV-1 subtypes can be detected by PCR under the conditions outlined above. The high sensitivity and high specificity of the filter paper assay versus those of the mononuclear cell PCR assay suggest that the filter paper PCR method is equally sensitive for the detection of viral sequences representing different HIV-1 subtypes. Subtype data were available for 99 of the corresponding samples from the mothers of the infants whose samples were examined in the present study, and 73 were found to contain clade A viruses, 20 contained clade D viruses, and 6 contained clade C viruses (16). Samples from the infants of mothers infected with each of the three clades tested positive by the PCR assay with filter paper samples (Table 3). Subtype A was detected with a sensitivity of 95% (95% CI, 83 to 99%), subtype D was detected with a sensitivity of 100% (95% CI, 75 to 100%), and subtype C was detected with a sensitivity of 75% (95% CI, 22 to 99%). The difference in sensitivity between the detection of clade C and that of clades A and D is not statistically significant. However, with only four HIV-1-positive infants of mothers infected with the clade C virus, there is not enough power to detect a statistically significant difference.

DISCUSSION

This report describes a rapid, highly sensitive method for the detection of HIV-1 DNA from blood samples stored on filter papers. We observed an overall sensitivity of 96% (95% CI,

TABLE 3. Distribution of HIV-1 subtypes and sensitivity and specificity of assay with respect to clade

Clade	No. of mothers	No. of babies with positive PBMCs	No. of babies with positive filter papers	Sensitivity by clade (%)	No. of babies with negative PBMCs	No. of babies with negative filter papers	Specificity by clade (%)
A	73	43	42	98	30	30	100
D	20	15	15	100	5	5	100
C	6	4	3	75	2	2	100

88.6 to 98.9%) and a specificity of 100% (95% CI, 93.1 to 100%) compared with the PCR results for the corresponding PBMC samples. Optimal conditions were achieved by maximizing the number of template copies in each PCR mixture while minimizing the effects of blood-related inhibition. The result was that fewer than three copies of HIV-1 DNA could be detected 40% of the time when 5 μl of lysate was used. If the concentration of infected cells is 5- to 10-fold higher, a template volume of 5 μl yields a sensitivity of 100%.

Our data suggest that the filter paper method described here, which was optimized with a clade B HIV-1-infected cell standard (ACH-2 cells), is also applicable to samples from individuals infected with various subtypes of HIV-1. Clades A, D, and C of HIV-1 are represented in our study population (16, 18). HIV-1 DNA was detected in infants infected with each of these subtypes of HIV-1 with a high sensitivity and a high specificity.

In summary, the method described here is highly sensitive and specific and can detect a range of HIV-1 subtypes. Of particular relevance to studies in developing countries, the collection, processing, and storage of samples are straightforward and are thus amenable to use in field settings. This method is inexpensive, rapid, and simple and does not require the use of organic solvents or extraction procedures. The collection of the sample is also simplified, because the method described here requires a smaller amount of blood (about 50 μl) that can be obtained from a heel prick, whereas 1 ml or more of blood is required for the lymphocyte separation technique and the blood must be collected by venipuncture. Lastly, sample processing time is reduced, while high sensitivity and specificity are maintained.

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