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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

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The detection of virus is used to diagnose human immunodeficiency virus type 1 (HIV-1) infection in infants due to the persistence of maternal antibodies for a year or more. An HIV-1 DNA PCR assay with simple specimen collection and processing was developed and evaluated. Whole blood was collected on filter paper that lysed cells and bound the DNA, eliminating specimen centrifugation and extraction procedures. The DNA remained bound to the filter paper during PCR amplification. Assays of copy number standards showed reproducible detection of 5 to 10 copies of HIV-1 in 5 μl of whole blood. The sensitivity of the assay did not decrease after storage of the standards on filter paper for 3 months at room temperature or after incubation at 37 or 45°C for 20 h. The primers used for nested PCR of the HIV-1 *pol* gene amplified templates from a reference panel of multiple HIV-1 subtypes but did not amplify a subtype A or a subtype C virus from children living in Seattle. The assay had a sensitivity of 98.4% and a specificity of 98.3% for testing of 122 specimens from 35 HIV-1-infected and 16 uninfected children and 43 seronegative adults living in Washington. The assay had a sensitivity of 99% and a specificity of 100% for testing of 102 HIV-1-positive (as determined by enzyme immunoassay) Peruvian women and 6 seropositive and 34 seronegative infants. This assay, with adsorption of whole blood to filter paper and no specimen processing, provides a practical, economical, sensitive, and specific method for the diagnosis of HIV-1 subtype B infection in infants.

Approximately 4.1 to 36.7% of infants born to human immunodeficiency virus type 1 (HIV-1)-seropositive mothers become infected with HIV-1 from perinatal transmission (24, 32). Simple, sensitive, specific, and inexpensive assays that could be applied in both developing and developed countries for HIV-1 diagnosis in newborns are needed to determine prognoses, guide therapy, and assess interventions to reduce perinatal HIV-1 infection. Early diagnosis of HIV-1 infection in infants cannot be accomplished with conventional antibody tests due to the persistence of passively transferred maternal antibodies for up to 18 months after birth (29). Detection of HIV-1 DNA by PCR is an established method for determining infection status in children born to HIV-1-seropositive mothers (26) and, when used in combination with dried blood samples collected on filter paper, provides a simple approach for HIV-1 diagnosis in infants.

There are many advantages of the use of DNA PCR with dried blood specimens over PCR with liquid blood specimens. The assays licensed for PCR of HIV-1 DNA use the mononuclear fraction of blood. In contrast, PCR of HIV-1 DNA in dried whole blood on filter paper omits the need to separate the mononuclear cells and thus the need for collection of 1 ml or more of blood and the need for centrifugation. The use of only a minute amount of whole peripheral blood (~80 μl) allows collection of blood by heel prick, requiring less skill and fewer supplies. Once dried, blood samples on filter paper are no longer infectious and, coupled with an absence of glass, reduce the biohazard risk to health care workers (20). Dried blood appears biologically stable (13, 14), and HIV-1 DNA has remarkable stability in dried samples (2), eliminating the need to maintain the specimens at cold temperatures.

The advantages of testing dried blood samples have led several groups to develop protocols for the detection of HIV-1 DNA in blood collected on filter paper. However, most methods in current use include multiple steps of cell lysis, elution, extraction, and purification of the target DNA (1, 5, 6, 25), with the consequent loss of DNA template. Another approach has been direct PCR of a whole-blood cell lysate (10, 21); however, inhibition of PCR can occur with this method.

A simple and efficient method for the detection of HIV-1 in dried whole blood was developed by use of filter paper that lyses cells and binds DNA (FTA card and Gene Guard system; Life Technologies, Rockville, Md.) for the collection and processing of specimens. The filter paper cards have a matrix impregnated with a proprietary formulation that lyses cells while maintaining the integrity of the DNA contained in the specimens. This process eliminates the time-consuming lysis procedures used in other assays. In addition, the DNA remains bound to the filter paper throughout processing and amplification by PCR; therefore, template is not lost by incomplete

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elution of the DNA from the paper. With this filter paper, a sensitive and specific method was developed for the detection of HIV-1 DNA in dried blood samples.

MATERIALS AND METHODS

Blood specimens obtained from two populations were used to evaluate the sensitivity and specificity of the assay. The populations were HIV-1-infected children, HIV-1-exposed uninfected children, and adult laboratory workers in Washington State and HIV-1-seropositive women and their infants monitored in Lima, Peru. After venipuncture of the laboratory workers, a few drops of blood were immediately applied to the filter paper (FTA card; catalog no. 10786-010; Life Technologies); the remainder was used for an enzyme immunoassay (EIA) to evaluate HIV-1 status. Three or four drops of blood from perinatally HIV-1-exposed, non-breast-fed uninfected children and from HIV-1-infected children and women were applied to FTA cards during blood draws at routine clinic visits. All HIV-1-exposed children had two or more tests for HIV-1 after 6 weeks of age, including PCR for HIV-1 DNA and RNA and, if more than 18 months old, an HIV-1 EIA. HIV-1 infection was diagnosed in infants and children who had two or more positive tests on different dates. Infants were diagnosed as non-HIV-1 infected if they had two or more specimens from separate dates after 6 weeks of age that tested negative for HIV-1 and no positive tests for virus or if they had a negative HIV-1 EIA result after 18 months of age.

The peripheral blood applied to the center of each FTA card was allowed to dry completely before storage at room temperature in a plastic bag with silica desiccant. A 3-mm hole punch was used to punch two discs of filter paper from the center of the blood spot; the discs were then transferred with forceps to separate 0.2-ml PCR tubes (ISC Bio Express, Bountiful, Utah). The hole punch was cleaned between each sample by repeatedly punching through clean filter paper for five to seven punches, as recommended by the FTA card manufacturer.

To determine the sensitivity of the assay, HIV-1 copy number standards were prepared by mixing a known number of 8E5 cells, which contain one copy of HIV-1 DNA per cell (15), with anticoagulated fresh whole blood from an HIV-1-seronegative individual. Drops of these standards, ranging from 20 to 0.2 copies/μl of blood, were individually spotted on filter paper, air dried, and stored at room temperature until use.

Each 3-mm-diameter filter paper disc was washed in the PCR tube three times with 200 μl of FTA Purification Reagent (Life Technologies) and twice with Tris-EDTA buffer. The filter paper punches were allowed to air dry in the same tube and were subsequently used in PCR amplification. Nested PCR amplification of the HIV-1 *pol* gene was carried out with primers RT1 and RT2 as outer primers and RT3 and RT4 as inner primers as previously described (12). DNA from 8E5 cells diluted to 10 copies of HIV-1 per μg of uninfected genomic DNA (equivalent to 150,000 cells) was used to control for the sensitivity of the PCR. To control for the presence of PCR inhibitors, filter paper specimens were subsequently tested for the human β-globin gene by a single round of PCR with primers KM38 and Fluo-GH120 (7). The β-globin gene PCR was carried out on additional punches of filter paper processed as described above with the same PCR mixture and cycling conditions as those used for the amplification of the HIV-1 *pol* gene. Ten microliters of each PCR mixture was electrophoresed on a 1.5% agarose gel. The 665-bp fragment containing the gene encoding amino acids 17 to 237 of the HIV-1 reverse transcriptase (RT) and the 350-bp fragment of the human β-globin gene amplified were visualized by ethidium bromide staining. A sample was labeled positive if one or two of the duplicates showed the 665-bp band, negative if neither duplicate had the RT band but had a positive β-globin reaction, and indeterminate if neither RT nor β-globin was amplified.

The HIV-1 proviral DNA copy number of selected patient samples was determined by limiting-dilution PCR of lysed peripheral blood mononuclear cells (PBMC). Briefly, cryopreserved PBMC were lysed (34) to a concentration of 150,000 cells/10 μl of buffer and were used to prepare serial dilutions. Five nested PCRs were done for each dilution as previously described (12). A dilution resulting in one positive PCR out of five was considered to have one copy of HIV-1. The number of HIV-1 copies/10^6 PBMC was calculated with the following equation: (66.7 × dilution factor × number of positive reactions)/(total number of reactions × volume of DNA template).

The specificity of the primers used in our assay for the amplification of the HIV-1 *pol* gene region in non-subtype B viruses was tested by PCR amplification of a panel of plasmids containing cloned nearly full-length reference HIV-1 genomes (19).

TABLE 1. Sensitivity of HIV-1 detection with filter paper specimens containing copy number standards

No. of HIV-1 copies/ punch	No. of positive tests/ total no. of tests	% Positive reactions
1	6/20	30
5	9/14	64
10	16/18	88
50	4/4	100
100	6/6	100

RESULTS

Evaluation of the sensitivity of HIV-1 *pol* gene amplification from dried blood using known copy number standards. The sensitivity of the assay is shown in Table 1. One copy of HIV-1 was detected in 30% of the single-copy standards; assuming a normal distribution of 8E5 cells in the specimen, this result suggests sensitive detection to one copy of HIV-1. As expected, HIV-1 DNA amplification became more frequent when the input of virus was increased. Amplification was detected in 88 and 100% of samples estimated to contain 10 and 50 copies of HIV-1 per sample, respectively. PCRs with larger filter paper discs (6-mm diameter) or more than two discs per 100-μl reaction volume showed inhibition of amplification, probably due to an increase in the amount of inhibitors from the blood that remained bound to the paper. The sensitivity of the assay was unchanged when the standard dilution series was tested after storage for over 3 months at room temperature or after incubation for 20 h at 37 or 45°C.

Sensitivity and specificity of HIV-1 *pol* amplification from clinical specimens collected in Washington. Sixty-five dried blood specimens from HIV-1-infected children 9 months to 15 years old and 58 HIV-1-seronegative controls, including 16 infant specimens submitted as "unknowns," were tested by PCR amplification of two filter paper punches taken from the same blood spot. HIV-1 *pol* was amplified from 62 of the 65 specimens from HIV-1-infected children tested. The three in which the amplification of HIV-1 sequences failed did not have inhibitors of PCR, as the human β-globin gene was amplified from these specimens. PBMC from one of these three specimens amplified well with the RT1-RT2 and RT3-RT4 primers, but only when a large number of cells was tested (1 μg of DNA, equivalent to 150,000 cells). By limiting-dilution PCR, it was determined that the HIV-1 DNA copy number in this specimen was very low, 31 copies/10^6 PBMC.

PBMC from the other two children who had a false-negative result in the filter paper assay did not amplify *pol*; however, HIV-1 *env* was amplified well with outer primers ED31-BH2 and inner primers ES7-ES8 (8). Sequencing and heteroduplex mobility assay analysis (8) of these *env* templates indicated that one child was infected with HIV-1 subtype A and the other was infected with subtype C. With a different set of primers (outer pair: GA1 [5′-GGGAAGTGACATAGCAGGAACTACTAG] and RTE2 [5′-TTAACAAACTCCCACTCAGGAATCCAGGTG]; inner pair: GA2 [5′-ACTCTAAGAGCCGAGCAAGCTTCACAG] and RTE3 [5′-GTGGCTATTTTTTGCACTGCCTCTG]), a fragment extending from *gag* to the 3′ end of *pol* was amplified from the subtype C virus. Sequence analysis of this sample showed numerous mutations in the regions corresponding

TABLE 2. Sensitivity and specificity of HIV-1 PCR for clinical specimens collected on filter paper[a]

HIV-1 infection status	No. of samples with the following filter paper PCR result:	
	Positive	Negative
Positive	62	1
Negative	1	57

[a] Sensitivity: 98.4% (95% CI, 91.45 to 99.96%); specificity, 98.3% (95% CI, 91.06 to 99.96%).

to primers RT1 and RT3. The *pol* region of the subtype A PBMC sample was not amplified by any of multiple combinations of primers.

Only 1 of the 58 specimens from HIV-1-seronegative individuals tested positive, with amplification detected in one of the two duplicates. The intensity of the band corresponding to the amplified HIV-1 *pol* fragment in this sample was low compared to that in the other positive samples. This specimen tested negative when a new set of duplicate punches from this blood spot was analyzed.

Excluding the two patients for whom *pol* was not amplified with the RT1-RT2 and RT3-RT4 primers, the assay detected HIV-1 in the small amounts of blood tested at a sensitivity of 98.4% and a specificity of 98.3% compared to HIV-1 DNA PCR of lysed PBMC (Table 2).

Use of dried blood specimens for HIV-1 diagnosis of infants in Lima, Peru. As part of an ongoing project to determine the rate of perinatal HIV-1 transmission in Peru, blood from a finger or heel stick collected on filter paper from 128 children born to HIV-1-seropositive mothers in Lima, Peru, was mailed to Seattle, Wash., for HIV-1 DNA PCR, with an HIV-1 EIA being done later, after 17 months of age. There was 100% concordance between the 40 specimens assayed by both PCR and EIA; 6 children tested positive for HIV-1 infection, and the remaining 34 tested negative.

Of 102 HIV-1-seropositive mothers tested, 101 had HIV-1 *pol* DNA detected by PCR of the filter paper. The sensitivity and specificity of the assay when the test results from the mothers and infants were combined were 99.1% (95% confidence interval [CI], 94.94 to 99.98%) and 100% (95% CI, 89.72 to 100%), respectively, compared to the EIA.

Detection of multiple subtypes of HIV-1 DNA. Primers RT1-RT2 and RT3-RT4 efficiently amplified the *pol* region in non-B subtypes of HIV-1 in a panel of samples consisting of 10 different HIV-1 reference sequences cloned in plasmids (19), including HIV-1 subtypes A, B, C, D, F, G, and H, as well as some recombinants of these subtypes.

DISCUSSION

The assay for the detection of HIV-1 *pol* DNA described here is simple to perform, sensitive, and specific. The simplicity of the assay is derived from the use of whole blood collected on a filter paper that lyses cells and binds DNA. Thus, the centrifugation and lysis procedures generally used prior to HIV-1 DNA PCR of liquid blood samples (12) are not required. In addition, due to the properties of the filter paper used, the DNA elution and extraction steps necessary in other procedures that used dried blood samples (1, 5, 10, 21, 25) are also eliminated. The DNA present in the blood spot remains bound to the filter paper, while proteins and other cellular contaminants are degraded and later washed away. The filter paper with the immobilized DNA is then used directly for nested PCR amplification of the HIV-1 *pol* gene. An added benefit is that there is no need to keep the specimen cool or frozen. This assay is relatively inexpensive, with supplies and reagents for the test costing $9.00 per patient when a batch of 10 specimens is assayed in duplicate, including negative and positive control specimens and β-globin amplification to control for the inhibition of PCR.

When tested with clinical specimens, the HIV-1 DNA PCR of whole blood collected on filter paper had a sensitivity of 98.4% compared to DNA PCR performed with 150,000 lysed PBMC. The lower limit of sensitivity of this assay did not allow the detection of HIV-1 DNA in the rare individual with a very low viral load; however, this assay was sufficiently sensitive to detect HIV-1 DNA in all untreated infants and young children in Washington and Peru. Each 3-mm-diameter filter paper disc contained approximately 5 μl of whole blood which assuming a median lymphocyte count of 5×10^6 cells/ml of blood in children less than 2 years old (9), would contain approximately 25,000 PBMC. In two punches of filter paper, an estimated 50,000 cells were included in each assay. The only specimen with a false-negative PCR result for whole blood collected on filter paper amplified well with our RT primers when the equivalent of 150,000 cells was tested. By limiting-dilution PCR, it was found that this girl had only 31 copies of proviral DNA/10^6 cells; thus, it is understandable that her specimen fell under the limit of detection of the assay. She was diagnosed as HIV-1 seropositive at 15 years of age, having been infected through adult-type behaviors. In spite of no antiretroviral therapy, her HIV-1 PBMC cultures have been negative and her plasma viral load has remained under 400 copies/ml.

Among the HIV-1-infected children for whom HIV-1 *pol* could be amplified from the dried blood samples, 53 of 62 had a positive result in PCR of both duplicate filter paper punches and 9 had DNA amplification in only one of the duplicates. In each case in which only one of the two filter paper duplicates showed amplification, the patient had either a low CD4 count ($<400/mm^3$) or a low HIV-1 DNA copy number (<200 copies/10^6 PBMC). In addition, it is worth noting that the majority of the HIV-1-infected children in Washington whose specimens were used to evaluate the sensitivity of the assay were receiving highly active antiretroviral therapy, and they ranged in age from 9 months to 15 years.

The limit of detection of our assay was calculated to be 40 copies of HIV-1/10^6 PBMC, based on reliable amplification of single copies of HIV-1. HIV-1 DNA copy number varies greatly but appears to be higher in children. Twelve of 33,000 copies/10^6 PBMC have been reported in adults (4, 28, 30), compared to 50 to more than 300,000 copies/10^6 PBMC in children (27, 33). Moreover, younger children, 2 years old or less, have significantly higher cell-associated HIV-1 DNA loads (mean, 75.4 ± 104.3 copies/10^3 PBMC) than children more than 5 years old (mean, 13 ± 17.8/10^3 PBMC), regardless of disease status (33). Assuming that healthy children 2 years old and younger have approximately 5 to 6 million PBMC/ml of blood (9) and that each 3-mm filter paper punch holds 5 μl of blood, it is likely that this assay would detect HIV-1 DNA in all

infected children less than 2 years old, as was seen for the Peruvian EIA-positive infants. Exceptions would include children with highly mutated viruses, some non-subtype B strains, low lymphocyte counts, and very low proviral loads (rare).

The RT1-RT2 and RT3-RT4 primers used in this assay did not amplify HIV-1 *pol* in two children, one infected with subtype A virus and a second infected with subtype C virus. These primers have successfully amplified *pol* from hundreds of individuals, mostly residents of the United States and Peru and presumably infected with subtype B virus (11, 12, 18, 22, 23; unpublished data), and all the viruses in the panel of diverse subtypes (19). Alignments of 86 HIV-1 sequences from the Los Alamos National Laboratory Database, representing all the different subtypes, showed few base changes in the regions complementary to primers RT1-RT2 and RT3-RT4. These mismatches were located several bases away from the 3′ end of the primers and therefore should not have had a great effect on the efficiency of amplification from the target template. However, the genetic diversity of *pol* may not be accurately reflected in the Los Alamos database. Others have reported that primers for the region of *pol* encoding RT may be insensitive for some non-B subtypes (31). The sequence of the *gag-pol* fragment from the child with subtype C virus in our study identified multiple mutations at the site of primers RT1 and RT3, indicating that they were not optimal for detection of that child's virus. Fortunately, the simple format of this assay allows for optimization of primers for the prevalent HIV-1 subtype or the use of primers for regions known to be conserved across subtypes (3, 10, 16).

The assay had specificities of 98.3% when evaluated with specimens from seronegative children and adults living in Washington and 100% with specimens from Peruvian seronegative infants. The sample with the single false-positive test result showed amplification in one of the two duplicates tested, and the intensity of the DNA band was weak compared to that in the true-positive samples or the PCR-positive controls. Because this sample tested negative when the assay was repeated using a new set of duplicate punches taken from the same blood spot, the weak positive result may have resulted from carryover contamination during PCR amplification. Measures to prevent PCR contamination as well as repeat testing of discordant duplicate filter paper samples may enhance the specificity of the assay. The testing of a second specimen collected on a different date to confirm all positive tests also should be routine to avoid the misdiagnosis of HIV-1 infection due to mislabeling of specimens and errors during laboratory tests (17).

In conclusion, this assay, using HIV-1 DNA PCR of whole blood collected on filter paper binding DNA, offers a simple, sensitive, and specific test appropriate for the diagnosis of HIV-1 subtype B infection of infants. The small amount of blood required, the ease of collection, storage, and transport of samples, and the low cost of the test make this assay ideal for HIV-1 testing of infants in the field or where resources are limited.

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