JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1991, p. 667–671
0095-1137/91/040667-05$02.00/0

Vol. 29, No. 4

Use of Dried Blood Spot Specimens in the Detection of Human Immunodeficiency Virus Type 1 by the Polymerase Chain Reaction

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Received 1 October 1990/Accepted 11 January 1991

Dried blood spots (DBSs) constitute a potentially valuable source of material for human immunodeficiency virus (HIV) serologic and molecular testing. To facilitate molecular testing, we have adapted the polymerase chain reaction (PCR) to the detection of HIV proviral DNA in DBS samples. The method is highly reproducible, with 75 μl of whole dried blood providing sufficient DNA for duplicate testing with three primer sets. By using DBS PCR, 66 of 69 (95.6%) seropositive at-risk individuals tested positive by at least two primer sets and 85 of 85 (100%) low-risk seronegative blood donors tested negative by all three sets of primers. The frequency of HIV DNA detection in seronegative at-risk individuals was low, with only 1 of 58 (1.7%) individuals testing positive. These results show that in a clinical environment, HIV PCR analysis of DBS specimens is specific and sensitive. The method is cost effective and presents a useful alternative to the isolation of HIV from seropositive babies with an undefined infection status.

The technique of collecting capillary blood on filter paper has facilitated large-scale screening efforts by simplifying the shipping of specimens and allowing centralization of laboratory activities (1, 10). Dried blood spots (DBSs) offer economy of sample, require minimal storage facilities, are relatively stable, and pose little biohazard risk. DBS samples have been widely used to screen newborns for metabolic and genetic disorders such as phenylketonuria, galactosemia, hemoglobinopathies, and hypothyroidism (9, 20, 22).

The ease of collecting small amounts of blood onto filter paper has stimulated interest in using DBSs to screen for human immunodeficiency virus (HIV) in newborns. Elution of HIV-specific antibodies from DBSs and testing of the eluates has been useful in determining the seroprevalence of HIV (7, 18, 29), but it does not differentiate between children who are actively infected and children who have passively acquired maternal HIV antibodies. The persistence of maternal antibodies may obscure, or preclude, the serological diagnosis of neonates for up to 18 months (3, 6). Different approaches are needed to identify children who are actively infected with HIV.

The polymerase chain reaction (PCR) technique has shown considerable potential for resolving the infection status of children born to HIV-seropositive mothers (4, 25, 30). Full evaluation of PCR in the perinatal setting, however, has often been hampered by difficulties in obtaining an adequate sample and by the relatively small number of HIV-infected mothers at any one hospital (25). To overcome these problems and facilitate large-scale retrospective and prospective studies, we have established HIV PCR testing of DBS samples. In this report, we describe the development of DBS PCR and its performance evaluation in groups of low-risk blood donors and at-risk homosexual men. To determine the efficacy and clinical applicability of DBS PCR, we have correlated the results of DBS testing with HIV serology and conventional HIV PCR of mononuclear cells (MNCs).

MATERIALS AND METHODS

Study population. Since PCR assays require careful evaluation of sensitivity and specificity, we have performed the initial validation of DBS PCR by using a well-documented study cohort, the Vancouver Lymphadenopathy-AIDS Study (VLAS). VLAS is an ongoing, prospective study that enrolled more than 1,000 homosexual men between November 1982 and December 1987 (28). Since October 1986, follow-up has been on an annual basis. At each visit, the men complete a questionnaire and undergo an examination which includes laboratory testing (CD4 counts, CD4-to-CD8 ratios, serum immunoglobulin G and immunoglobulin A levels, immune complexes, and platelet counts) and disease classification according to Centers for Disease Control criteria. At the time of the study, the Vancouver cohort included 429 seroprevalent (seropositive at the time of enrollment) and 142 seroincident (showing seroconversion after enrollment) subjects. Persons developing AIDS are transferred to the hospital clinic, where they are treated under different protocols.

Specimen preparation. Heparin-anticoagulated peripheral blood specimens (2 ml) were drawn from 128 homosexual men enrolled in the VLAS. Measured aliquots (25 μl) of each whole-blood sample were spotted onto precut 1.0-cm circles of no. 903 Schleicher & Schuell newborn screening paper. Control spots were prepared from the peripheral blood of 85 healthy HIV-seronegative blood donors. Blood spots were stored at −20°C until required for analysis.

The remaining (unspotted) portion of each sample (1.8 ml) was separated into plasma and MNC fractions by centrifugation on Ficoll-Hypaque. The plasma was analyzed for antibodies to HIV. MNCs were adjusted to 6×10^6 cells per

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ml in lysis buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Tween 20, 0.5% Nonidet P-40) and digested with 60 μg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) per ml for 60 min at 56°C. The proteinase K was inactivated (95°C for 10 min), and a 12.5-μl aliquot of each lysate (equivalent to 75,000 MNCs) was amplified by using modified HIV PCR protocols (17, 23).

HIV serology. The antibody status of each patient (donor) was determined by enzyme immunoassay using a Vironostika anti-HTLV-III kit (Organon Teknika Inc., Scarborough, Ontario, Canada) and/or Recombigen-HIV EIA plus GAG kit (Cambridge BioScience Corp., Worcester, Mass.). Positive enzyme immunoassays (EIAs) were confirmed by HIV Western immunoblot (DuPont Co., Wilmington, Del.).

DNA extraction from blood spots. After optimization of DNA extraction and precipitation conditions, the following protocol was routinely used. Blood was eluted from the filter paper 1 week to 6 months after spotting by incubating each 1.0-cm circle in 0.5 ml of digestion buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 50 mM NaCl, 2% sodium dodecyl sulfate [SDS], 0.3 μg of proteinase K per ml) for 1 h at 56°C. The blood recovered from five identical spots was pooled and transferred to a 15-ml polypropylene tube (Sarstedt, St. Laurent, Quebec, Canada). Five micrograms of carrier *Escherichia coli* tRNA (Boehringer Mannheim, Laval, Quebec, Canada) was added, and the sample was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was transferred to a clean tube and treated with *n*-butanol to remove traces of phenol-chloroform. After the addition of another 5.0 μg of tRNA, the aqueous phase was divided into aliquots in five microfuge tubes. The DNA was precipitated overnight at room temperature in 2.5 M ammonium acetate and 2 volumes of 100% ethanol and recovered by centrifugation in a microfuge for 30 min at 11,000 × *g*. DNA pellets were washed in 70% ethanol, air dried, and reconstituted in 25 μl of sterile H_2O.

DNA amplification. Amplification was performed for 30 cycles on an automated DNA thermocycler (Perkin-Elmer-Cetus, Emeryville, Calif.). The extracted DNA was denatured (100°C, 5 min; 4°C, 5 min) and then reheated to 72°C before being added to preheated (72°C) PCR mix to give a final reaction of 50 mM KCl–10 mM Tris HCl (pH 8.3)–2.5 mM $MgCl_2$–0.2 mM each deoxynucleoside triphosphate–0.25% Tween-20–0.25% Nonidet P-40–0.25 pmol of each primer–1 U of Amplitaq (Cetus) and an amount of DNA equivalent to 10,000 leukocytes. Comparative testing of MNC lysates was performed in the same manner, except that each reaction contained 75,000 cells.

To determine whether the DNA was suitable for amplification, samples were initially amplified with primers (GH26 and GH27; Synthetic Genetics, San Diego, Calif.) (27) to a conserved region of the human lymphocyte antigen (HLA)-DQ alpha locus. The thermoprofile was 95°C, 30 s; 58°C, 30 s; and 72°C, 60 s. Amplified product (10 μl) was then denatured (95°C, 5 min) and hybridized (15 min, 56°C) with 250,000 cpm of ^{32}P-end-labeled GH64 (16). One-third of each reaction mixture was separated by 10% polyacrylamide gel electrophoresis (PAGE), and the positive hybridization product was identified by exposure to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) for 4 to 18 h at −70°C. Generation of an amplified HLA-DQ alpha product provided a measure of the reproducibility, quality, and yield of each DNA extraction.

To exclude false PCR results, DNA samples were amplified with three different sets of HIV primers: SK68i/SK69i2 (with SK70 probe) for envelope and SK38i/SK39 (with SK19 probe) and SK145i/SK150 (with SK102 probe) for the *gag* region of the HIV type 1 (HIV-1). The SK19, SK39, SK70, SK102, and SK150 primers and probes have been used extensively for HIV type 1 PCR analysis (16, 23). SK39i and SK145i were identical to SK39 and SK145 (16), except that a T nucleotide was deleted from the 3′ end of each primer. SK68i and SK69i2 have been described in detail elsewhere (2). The sequences of these primers were as follows: SK68i, 5′-TTCTTIGGAGCAGCIGGAAGCACIAT GG-3′, and SK69i2, 5′-TT(G/A)ATGCCCCAGACIGTIAGT TICAACA-3′. Amplification, hybridization, and detection of *gag* sequences were performed as previously described for human HLA-DQ alpha. Envelope PCR was performed by using a thermoprofile of 95°C, 60 s; 55°C, 40 s; and 72°C, 40 s and a hybridization reaction containing 150 mM NaCl. Titrated standards, containing 6 to 50 copies of HIV proviral DNA, were included in all amplifications to measure sensitivity and intertest variability. These standards were prepared by diluting 8E5 cells, containing one copy of HIV type 1 per cell (8), into uninfected cells isolated from seronegative blood donors. In addition to 8E5 standards (positive controls), each run had a negative reagent control (PCR mix without DNA) and samples of uninfected DNA extracted simultaneously with clinical blood spots.

To avoid contamination, DNA extractions and preamplification reactions were set up in a biosafety cabinet that was free of PCR-amplified product. Additional precautions included the use of single-aliquot reagents and designated positive displacement pipettes. Sample handling was minimized, and great care was taken when the caps of microfuge tubes were opened. To prevent cross-contamination at the blood spot level, the blood was applied to precut circles of filter paper and each sample was stored individually in separate zip-lock plastic bags. In cases in which the circles were not precut, each DBS sample was excised with a new pair of scissors (or scissors depurinated in 1.0 N HCl). Samples were considered positive only if they gave positively hybridizing fragments of the expected length with at least two primer-probe combinations.

RESULTS

Initial investigations were designed to determine the optimal conditions for DNA extraction and recovery from blood spot samples. The method described above reproducibly gave yields of approximately 0.8 μg of DNA from the dried equivalent of 75.0 μl of whole blood. The DNA was intact and suitable for PCR analysis as shown by the consistent successful amplification of human HLA-DQ alpha sequences from both control (blood donor) and clinical (VLAS) extracts. One-sixth of each extract (corresponding to 12.5 μl of whole blood) provided sufficient DNA to routinely generate strong HLA-DQ alpha signals when amplified for 30 cycles and analyzed by oligomer solution hybridization (Fig. 1).

The sensitivity of the method for HIV detection was assessed in reconstruction experiments. Uninfected control cells (isolated from an HIV-seronegative blood donor) were spiked with limiting dilutions of 8E5 cells containing a single copy of integrated HIV proviral DNA per cell. Twenty-five microliters of each dilution was spotted onto filter paper and used for DNA extraction and amplification with SK68i/SK69i2 and SK145i/SK150. Using this reconstruction system, we were able to detect amounts as small as six molecules of HIV DNA per PCR (Fig. 2, lane 10).

In the clinical setting, DBS PCR was found to be both sensitive and specific and there was a strong correlation

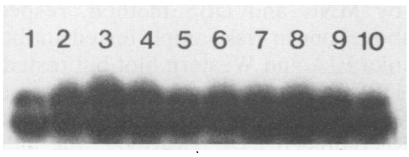

FIG. 1. Representative autoradiograph (4-h exposure) showing the results of HLA-DQ alpha PCR amplification to measure the yield and integrity of DNA extracted from clinical DBS specimens. The generation of strong hybridization signals (of approximately equal intensity) in all samples indicated that the extraction protocol was highly reproducible and yielded large amounts of high-quality DNA.

between DBS PCR positivity and serological evidence of HIV infection (Fig. 3A and B). All 85 individuals in the low-risk control group tested DBS PCR negative, indicating that within the bounds of this investigation, the DBS method was 100% specific and laboratory contamination did not occur. In the at-risk seropositive population, 66 of 69 individuals tested positive with at least two primer sets, giving an analytical sensitivity of 95.6% (Table 1). Among the three remaining individuals, one was indeterminant (positive by one primer set only) and two were negative. One of the DBS negatives was also MNC PCR negative; the second negative and the indeterminant were weakly MNC PCR positive. All three discordant DBS samples gave strong positive amplification for HLA-DQ alpha, suggesting that the failure to detect HIV DNA was probably due to low levels of virus

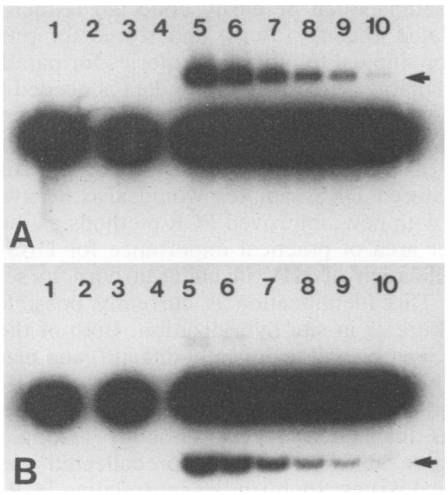

FIG. 2. Representative autoradiographs (7-h exposure) showing results of a quantification experiment. Cells from uninfected blood donors were spiked with various numbers of 8E5 cells (containing one copy of HIV DNA per cell) and applied to filter paper. DNA was then extracted, amplified for 30 cycles with SK68i/SK69i2, and analyzed by solution hybridization with a ^{32}P-SK70 probe. (A) Arrow indicates the position of the 158-nucleotide product hybridized to the SK70 envelope probe. The intensity of the hybridization signal was directly proportional to HIV DNA copy number. Lane 1, Seronegative donor control; lane 3, reagent control; lanes 5 through 10, decreasing amounts of HIV DNA (200, 100, 50, 25, 12, and 6 molecules of HIV DNA, respectively). The lower band represents unhybridized 5'-end-labeled probe. (B) The samples described above were restricted with *Hae*III endonuclease prior to electrophoresis. The cleavage of a 10-nucleotide fragment (arrow) from the probe-product hybrid confirms that the hybrid contained a *Hae*III restriction site. This site is characteristic of SK68/SK69 envelope products (23).

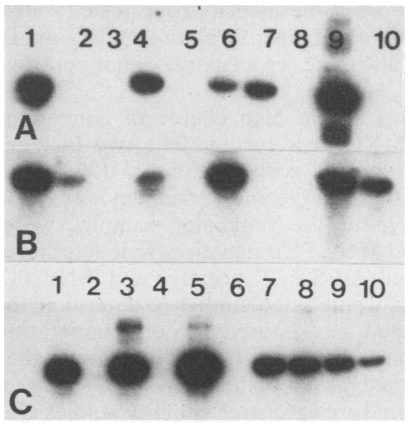

FIG. 3. Autoradiographs (7-h exposure) showing results of HIV PCR amplification from DBSs (A and B) and crude MNC lysates (C). (A) HIV *gag* sequences amplified with SK145i/SK150 and detected with ^{32}P-SK102. Lanes 1, 4, 6, 7, and 9, Amplification products from HIV-seropositive homosexual men; lanes 2, 3, 5, 8, and 10, products from HIV-seronegative homosexual men. (B) HIV envelope sequences amplified with SK68i/SK69i2 and detected with ^{32}P-SK70. Lanes 1, 2, 4, 6, 9, and 10, Samples from seropositive homosexual men; lanes 3, 5, 7, and 8, samples from seronegative homosexual men. (C) Cell lysates amplified with SK68i/SK69i2 and detected with ^{32}P-SK70. Amplification products were from seropositive (lanes 1, 3, and 5) and seronegative (lanes 2, 4, and 6) homosexual men. Lanes 7, 8, 9, and 10, Standards containing 50, 25, 12, and 6 copies of HIV DNA per 150,000 MNCs, respectively.

rather than inadequate recovery of DNA. The frequency of DNA positivity (presumed HIV infection) within the at-risk seronegative population was low, with only 1 of 58 (1.7%) individuals testing DBS PCR positive. One sample (not included in Table 1) was found to be serologically indeterminant (i.e., positive by Recombigen but negative by Vironostika EIA and Western blot). This sample was strongly DBS (and MNC) PCR positive for all three primer combinations, suggesting that it may represent an early seroconversion. Follow-up studies to resolve the infection status of this individual are planned.

A close relationship was also observed between MNC PCR positivity and positive serology (Fig. 3C). By using MNC PCR, 64 of 69 (92.7%) antibody-positive individuals tested positive by at least two primer sets, 4 of 69 (5.8%) were indeterminant (positive by one primer set), and 1 of 69 (1.4%) was negative. The lower sensitivity of the MNC PCR assay was unexpected and may reflect the presence of erythrocyte contaminants in MNC preparations. Fifty-eight out of 58 (100%) individuals within the at-risk seronegative population tested MNC PCR negative. As described above,

TABLE 1. Comparison of concurrent DBS PCR and HIV antibody results in 85 low-risk blood donors and 127 at-risk homosexual men

HIV antibody result	n^a	No. (%) of PCR results	
		Positive	Negative
Positive	69	66 (95.6)	3 (4.3)
Negative	143	1 (0.7)	142 (99.3)

[a] All 69 individuals in the seropositive group were homosexual men. The seronegative group consisted of 58 homosexual men and 85 blood donor controls.

one sample from the at-risk group tested indeterminant by serology but tested positive by both MNC and DBS PCR. Of 85 individuals in the seronegative donor control group, 100% were MNC PCR negative.

There was also a high degree of concordance between DBS and MNC PCR results. The two PCR methods gave concordant results with 206 of 213 (96.7%) samples; 144 samples were negative and 62 samples were positive by both assays. Five of the remaining samples were DBS PCR positive and MNC PCR negative (or indeterminant), and two samples were DBS PCR negative and MNC PCR positive.

Recently, we initiated studies to determine the stability of DNA in blood spots stored at room temperature. We found no significant change in DNA yields or in the amounts of HIV-amplified product after 2 weeks of storage at 20°C (data not shown). More extensive stability studies are in progress.

DISCUSSION

We have developed a sensitive and specific method for the amplification of HIV DNA from small amounts of dried blood collected on filter paper. The procedure involves DNA extraction (15, 26) and precipitation in the presence of ammonium acetate and carrier tRNA. Using DNA extracted from 12.5 μl of dried whole blood and optimized PCR capable of detecting as few as six HIV molecules, we were able to identify HIV sequences in 66 of 69 (95.6%) seropositive individuals, none of whom had an AIDS-defining illness. Since 12.5 μl of blood contains approximately 10,000 MNCs, this indicates that the viral load in most seropositives, including patients with asymptomatic infection and normal CD4 counts, must be greater than six HIV DNA molecules per 10,000 MNCs. Other investigators, using PCR to measure viral load in AIDS patients, have reported levels as high as 56,000 to 89,000 copies of HIV DNA per μg of DNA (or 1 HIV molecule for every 2 to 3 MNCs) (5). These estimates are significantly higher than those determined by endpoint dilution culture. Ho et al. (12), using the culture approach, estimated that only 1 in 50,000 MNCs harbors HIV in asymptomatic individuals, and that this titer rises to 1 in 400 MNCs in patients with AIDS-related complex and AIDS. It should be noted, however, that the relationship between the number of cells carrying latent DNA (as determined by PCR) and the percentage of cells that are actively expressing virus (determined by culture) has not been established and that the presence of viral DNA may not correlate with viral activity.

In our hands, the blood spot method gave fewer false-negatives (antibody positive, PCR negative, or indeterminant) than standard PCR performed on MNC lysates. This was particularly surprising, since the lysates contained the equivalent of 75,000 cells, which is approximately 7.5 times more cells than were used for DBS amplification. The higher efficiency of the DBS method may be due to the extraction and complete removal of erythrocyte contaminants, such as the porphyrin moiety of heme, which is known to be a strong inhibitor of PCRs (11). MNC preparations are frequently contaminated with erythrocytes.

The results of both PCR assays were highly concordant with serological evidence of HIV infection. HIV DNA was identified in most seropositive individuals by both DBS and MNC PCR. Possible reasons for false-negative results include an extremely low viral titer, the presence of inhibitors, and/or genetic variation. All antibody-negative control samples were PCR negative, and the frequency of HIV DNA positivity among at-risk seronegative persons was low (0.0 and 1.7% by MNC and DBS method, respectively). As discussed above, one at-risk sample tested antibody negative by Vironostika EIA and Western blot but tested positive by MNC and DBS PCR. This sample may represent an early seroconversion, since HIV antibodies were ultimately detected by Recombigen EIA. However, the individual who tested DBS PCR positive but negative by all other assays probably represents a false-positive DBS result. False-positives can arise from specimen contamination, carryover of PCR product from one amplification tube to the next, or nonspecific amplification of genomic sequences. These discrepancies can usually be resolved by repeat testing of a fresh specimen. Overall, our results are consistent with those of other recent studies that have shown a strong correlation between the results of serological and PCR testing and a low frequency of HIV infection in seronegative high-risk persons (14, 21, 31). Some investigators, however, have failed to find a clear correlation between HIV serology and PCR reactivity, and several laboratories have reported a significant number of HIV infections among at-risk seronegative persons by PCR (19, 24) and lymphocyte culture (13). These discrepancies need to be resolved, since they have important implications for blood donor screening and the early diagnosis and confirmation of HIV infection.

The ability to use DBSs for both antibody and PCR analysis provides a new resource for resolving these issues. Blood spot sampling opens the way for large-scale population studies in which PCR results can be correlated with the individuals' clinical and serological status. We have used DBS sampling for intravenous drug users, since the samples can be conveniently collected in the street. Large numbers of DBS samples can be easily collected (either locally or from isolated endemic areas) and stored for retrospective analysis or shipped to other laboratories for parallel testing. An accumulation of corroborative data is needed to confirm the diagnostic and prognostic value of PCR and to provide information concerning the duration of the latency period between HIV exposure and the onset of a serological response. Stored DBS samples would also be available for retesting with new, improved PCR methods as they evolve.

Another area of practical importance for DBS testing is the identification of HIV in children born to seropositive mothers. This identification is currently possible only by tissue culture or in situ hybridization. Both of these procedures are lengthy and technically difficult, and many laboratories do not have the appropriate facilities for HIV culture. We and others have already collected large numbers of DBS specimens for maternal HIV antibody testing. Although retrospective analysis of these precollected spots would raise ethical issues, such studies, correlating PCR positivity with HIV serology and known clinical outcome, could rapidly yield information about the frequency and mode of vertical HIV transmission from infected mothers to their offspring. DBS sampling would also facilitate the follow-up of neonates and could lead to a better understanding of the natural history of HIV infection in young children.

ACKNOWLEDGMENTS

We thank Sue Ann Blakely, Marilyn Davis, Margaret Naylor, and Michael Finkelstein for their excellent research assistance.

This study was supported by Health and Welfare Canada.

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