JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1992, p. 3039–3042
0095-1137/92/123039-04$02.00/0
Vol. 30, No. 12

Stability of Dried Blood Spot Specimens for Detection of Human Immunodeficiency Virus DNA by Polymerase Chain Reaction

SHARON CASSOL,[1*] TERESA SALAS,[2] M. JOHN GILL,[3] MICHAEL MONTPETIT,[2] JAMES RUDNIK,[2] CHEIKH TIDIANE SY,[2] AND MICHAEL V. O'SHAUGHNESSY[1]

The Centre for Excellence in HIV/AIDS, University of British Columbia and St. Paul's Hospital, Vancouver, British Columbia V6Z 1Y6,[1] the Retrovirology Laboratories, Health and Welfare Canada, Ottawa, Ontario K1A 0L2,[2] and the Department of Medicine, University of Calgary, Calgary, Alberta T2N 2T9,[3] Canada

Received 26 March 1992/Accepted 31 August 1992

Blood sampling on filter paper has many advantages for the detection of perinatal human immunodeficiency virus (HIV) infection by the polymerase chain reaction (PCR). However, if the method is to be widely used, an assessment of its performance under field conditions is required. To simulate conditions in the field, 50-μl aliquots of whole blood containing low levels of HIV proviral DNA (4 to 1,024 copies per 100,000 nucleated cells) were spotted onto filter paper; dried; and subjected to heat, humidity, and prolonged storage at room temperature. After exposure, the DNA was recovered and amplified with primers to human leukocyte antigen DQα- and HIV-specific sequences. Treatment at 37°C and 60% humidity for 7 days, storage for 12 weeks at 22°C, and freeze-thawing twice had no adverse effect on PCR reactivity when compared with the results obtained with reference spots stored at −20°C. The lower limits of HIV detection in all tests ranged from 4 to 16 HIV copies per 100,000 cells. Fixation in 70% ethanol improved the amplification of low levels of HIV DNA and reduced biohazard risks. These findings suggest that dried blood spots will provide a powerful new resource for testing for HIV by PCR, especially in remote areas where refrigeration and immediate sample processing are unavailable.

As a result of increased heterosexual transmission, human immunodeficiency virus (HIV)-infected women and children born to HIV-infected women are now the fastest-growing populations of patients with AIDS (4, 5). The World Health Organization estimates that by the year 2000, there will be 15 million to 20 million HIV-infected women and 10 million children with perinatally acquired HIV (32). The majority of these infections, approximately 90%, will occur in developing countries where sample collection and processing are difficult or unavailable (27, 32). Simple and improved diagnostic tests that could be widely applied under difficult field conditions and with minimal training would be highly advantageous. The tests are needed to assess the impact of HIV disease in women and children from different geographical areas over time, predict regional health care needs, plan global strategies, and design clinical and epidemiological interventions.

Whole-blood collection on filter paper provides a simple and powerful approach for monitoring the HIV and AIDS pandemic in women and children. Large numbers of samples can be easily collected in the field and shipped to national reference laboratories under low-level biosafety precautions. The dried blood is biologically stable (up to 30 weeks at 24°C for immunoglobulin G antibody testing) (10, 11), eliminating the need to maintain the sample in the cold. One 50-μl blood spot provides sufficient material for HIV antibody (12), p24 antigen (24), or DNA testing by the polymerase chain reaction (PCR) (2). Dried blood spot (DBS) samples have proven valuable for determining the HIV seroprevalence rates in childbearing women throughout the United States (16, 18) and Canada (17) and, more recently, by using PCR-based technology, for the resolution of the HIV infection status in indeterminate infants born to seropositive mothers (3). Before DBS PCR can be widely used in field studies, however, a full evaluation of its performance under adverse environmental conditions is required. In the study described here, we assessed the stability and PCR reactivities of DBS samples stored under conditions similar to those expected in the field.

MATERIALS AND METHODS

Preparation and treatment of blood spots. Initial studies were designed to determine the effects of heat, humidity, fixation, and prolonged storage on the lower limits of HIV DNA detection in DBS samples. To prepare whole-blood spots with low levels of HIV, 8E5 cells containing a single integrated copy of the HIV type 1 proviral DNA per cell (14) were diluted into uninfected CEM cells to generate a doubling dilution series ranging from 10^6 to 3,906 copies of the HIV genome in a background of 4×10^6 cells per ml. Each 8E5-CEM preparation was further diluted with 19 volumes of seronegative whole blood containing 4,930 leukocytes per μl. Aliquots were soaked onto individual circles of no. 903 Schleicher and Schuell filter paper in 50-μl drops by using a positive displacement pipettor, and the filters were left to dry overnight in a laminar flow hood. In total, 20 sets of 50-μl blood spots containing from 1,024 to 4 HIV DNA copies per 100,000 nucleated cells were prepared. Ten sets were frozen at −20°C for use as reference samples; six sets were stored at 22°C, with one set being taken for analysis after 2, 4, 6, 8, 10, and 12 weeks; one set was heated for 7 days in a 37°C incubator containing a large tray of water to maintain the humidity at 60% (as determined by a direct-reading analog hygrometer); two sets were stored for 12 weeks at 22°C and

* Corresponding author.

then fixed in 70% ethanol (10 min) prior to analysis; and one set was stored at −20°C and subjected to two cycles of overnight thawing at room temperature. After exposure, DNA was eluted from the filter paper, amplified, and compared with the DNA amplified from a set of reference samples processed in parallel. In clinical studies, coded heparinized blood samples collected from 24 HIV-infected volunteers attending the Southern Alberta HIV Clinic at the Foothills Hospital, Calgary, Alberta, Canada, were spotted onto filter paper (50 μl per spot), air dried, and shipped to Ottawa, Ontario, Canada. Upon arrival, the spots (representing a cross-section of patients with AIDS-related complex [ARC] or AIDS and asymptomatic HIV infection) were divided into test and reference sets and subjected to stability testing as described above for 8E5 spots. Negative controls consisted of DBS samples from healthy seronegative blood donors and uninfected CEM cells diluted in donor whole blood.

DNA extraction from blood spots. At the time of analysis, blood was eluted from test and reference samples by incubating each 1.0-cm spot overnight at 56°C in a microcentrifuge tube containing 0.5 ml of digestion buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 50 mM NaCl, 2% sodium dodecyl sulfate, and 0.3 mg of proteinase K per ml). After the addition of 2 μg of carrier *Escherichia coli* tRNA (Boehringer Mannheim, Laval, Quebec, Canada), the elute was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and treated with *n*-butanol to remove the residual phenol. The aqueous phase was transferred to a clean tube, and the DNA was allowed to precipitate for 2 h in 0.2 M ammonium acetate–2 volumes of 100% ethanol onto silica (Basebinder; Applied Biosystems, Foster City, Calif.). DNA was released from the silica by incubation for 3 min at 42°C in 30 μl of sterile water, centrifugation (5 min at 11,000 × *g*), and collection of the DNA-containing supernatant. This elution process was repeated, and the pooled supernatants were transferred to the top portion of an Ultrafree-MC 0.45-μm-pore-size filter unit (catalog no. UFC3 OHV 00; Millipore). After centrifugation (2 min at 11,000 × *g*), the DNA was recovered from the bottom of the filter unit. A detailed outline of sample preparation and DNA extraction is available from the corresponding author on request.

DNA amplification. Aliquots of the supernatant (12.5 μl) were denatured (100°C, 10 min), quenched on ice (4°C, 10 min), reheated to 80°C, and added to preheated (80°C) PCR mixture to give a 50-μl reaction containing 10 mM Tris-HCl; 50 mM KCl; 0.25% Tween 20; 0.25% Nonidet P-40; 2.5 mM $MgCl_2$; 0.2 mM each dATP, dCTP, dGTP, and dTTP; 0.25 pmol of each primer; and 1 U of Amplitaq (Cetus). To investigate the integrity of the DNA after exposure to the various treatments, samples were initially amplified for 30 cycles with primers to human leukocyte antigen DQα (28). HIV-specific amplification was performed for 35 cycles on a programmable thermal cycler (Perkin-Elmer Cetus, Emeryville, Calif.) by using SK145i and SK150 *gag* primers (19) and a thermoprofile of 95°C for 30 s for denaturation; 58°C for 30 s for annealing, and 72°C for 60 s for extension. The 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 SK102 probe. One-third of each hybridization was separated by 10% polyacrylamide gel electrophoresis, and the positive hybridization product was identified by exposure to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) overnight at −70°C. Negative controls (reagents without DNA and uninfected CEM DBS samples) were included in all PCR assays, and recommendations for the prevention of contamination were strictly followed (21).

RESULTS

Quality and yield of DNA. To assess the quality of DNA in blood spots exposed to adverse environmental conditions, the DNA was eluted, extracted, and amplified with primers to human leukocyte antigen DQα. DBS samples subjected to freeze-thawing, storage at 22°C for 1 to 12 weeks, or incubation at 37°C and 60% humidity for 7 days yielded human leukocyte antigen DQα signals comparable to those of reference spots stored at −20°C. The intensity of the signal was strong for all samples, indicating that the DNA was intact and suitable for amplification analysis.

HIV PCR reactivity. To assess the stability of HIV DNA, attention was focused on the lower limits of HIV detection. Blood spots containing a known low copy number of HIV (8E5 cells) and a constant number of erythrocytes and leukocytes were subjected to various treatments and processed in parallel with a set of identical reference spots stored under optimal conditions (frozen desiccated at −20°C). Treatment at 37°C and 60% humidity for 7 days, storage for up to 12 weeks at 22°C, and freeze-thawing caused no reduction in HIV PCR reactivity when compared with the reactivities of the signals generated by the reference samples. The lower limits of HIV detection in all tests ranged from 4 to 16 HIV DNA copies per 100,000 nucleated cells. Occasionally, a sample at or near the limits of detection failed to amplify, irrespective of storage or treatment conditions. Duplicate and repeat testing usually generated the expected PCR product, suggesting variability at the extreme limits of HIV detection. This variability could be overcome by fixing the DBSs in 70% ethanol for 10 min. As shown in Fig. 1, ethanol fixation enhanced and stabilized HIV DNA detection, especially when the virus was present in low copy numbers. Ethanol fixation after storage for 12 weeks at 22°C gave the same enhancing effect as immediate fixation of fresh blood spots did. Uninfected CEM cells and blood donor spots were consistently PCR negative.

Reactivities of clinical samples. To validate the stability of clinical DBS samples, sets of 10 identical spots were prepared from a cross-section of seropositive patients with ARC (n = 5), AIDS (n = 9), and asymptomatic HIV infection (n = 10). Five sets of reference spots were stored at −20°C; four sets of test spots were stored at 22°C for 3, 6, 10, or 15 weeks; and one set was subjected to freeze-thawing. Again, storage for up to 15 weeks at 22°C (Table 1) and freeze-thawing had no deleterious effect on our ability to detect HIV DNA relative to our ability to detect DNA in the reference samples. Strong PCR signals were generated by all test extracts, including those from the 10 patients with asymptomatic infection and high CD4 counts (average, 329.7 cells per mm^3; range, 182 to 748 cells per mm^3) and the 9 patients with advanced AIDS and significantly lower CD4 counts (average, 29.8 cells per mm^3; range, 5 to 84 cells per mm^3).

DISCUSSION

PCR is increasingly being used for the diagnosis of HIV infection in the perinatal period when other assays are insensitive or noninformative (7–9, 20, 26, 30). In studies conducted in six different laboratories, PCR correctly identified 31 of 36 HIV-infected infants who were less than 12 weeks of age (7). The standard source of DNA in all of those studies was fresh lymphocytes separated on Ficoll-Hy-

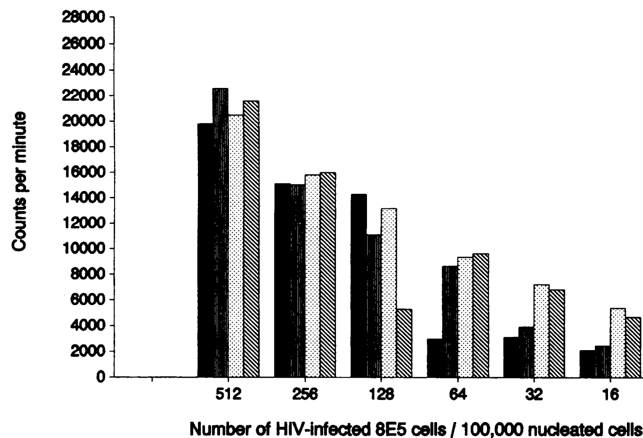

FIG. 1. Effect of fixation on low levels of HIV DNA (mean values). Doubling dilutions of 8E5 cells containing a single copy of integrated HIV DNA were adjusted to a constant cell count and added to whole blood from a seronegative blood donor. Aliquots, applied to filter paper, were stored at 22 or −20°C for 12 weeks. At the time of analysis, two sets of spots from each storage condition were fixed in 70% ethanol, and the DNA was extracted and amplified with HIV *gag* primers in parallel with unfixed spots. After solution hybridization with the ^{32}P-labeled probe SK102 and separation by polyacrylamide gel electrophoresis, the ^{32}P-labeled *gag* product was excised from the gel and counted. ■, unfixed, −20°C; ▥, unfixed, 22°C; ▤, ethanol fixed, −20°C; ▧, ethanol fixed, 22°C.

paque. Since the HIV and AIDS pandemic occurs primarily in less developed regions of the globe, there is an increased need to develop simple and more convenient methods for storing field samples until they can be returned to a laboratory for analysis.

TABLE 1. Successful amplification of HIV type 1 sequences from clinical DBSs stored at 22 and −20°C for 15 weeks

Patient no.	Clinical stage	CD4 count (mm^{-3})	PCR reactivity at[a]: 22°C	PCR reactivity at[a]: −20°C
073	ARC	58	1+	±
117	ARC	32	2+	2+
209	ARC	168	3+	4+
355	ARC	252	4+	4+
387	ARC	104	4+	4+
024	AIDS	84	4+	4+
059	AIDS	5	2+	2+
071	AIDS	59	2+	3+
127	AIDS	16	4+	4+
176	AIDS	8	2+	1+
221	AIDS	8	2+	2+
222	AIDS	15	3+	2+
326	AIDS	32	3+	3+
500	AIDS	42	3+	3+
020	Asymptomatic	350	4+	4+
041	Asymptomatic	748	4+	4+
343	Asymptomatic	288	4+	4+
385	Asymptomatic	234	3+	4+
396	Asymptomatic	220	4+	4+
440	Asymptomatic	336	4+	4+
520	Asymptomatic	224	4+	4+
525	Asymptomatic	187	2+	2+
543	Asymptomatic	182	2+	1+
544	Asymptomatic	528	4+	4+

[a] The signals on the radioautographs are defined as follows: ±, weak; 1+, moderate; 2+, moderate to strong; 3+, strong; and 4+, very strongly visible.

We and others (2, 3, 6) have recently shown that DBSs collected on newborn screening blotters provide suitable material for HIV PCR analysis. PCR testing of stored DBS samples (up to 18 months at −20°C) was found to be as sensitive as conventional PCR performed on fresh lysed lymphocytes. In one study (3), HIV DNA was detected in 100% of 25 infected children with subsequently proven HIV infection, including 12 asymptomatic infants who were under 10 weeks of age. The advantages of the DBS PCR approach include ease of sample collection, storage, and handling and removal of stringent restrictions on transit time and sample size. The same sample is sufficient for p24 antigen and HIV antibody testing.

In the study described here, we examined the stability of samples on filter paper stored under conditions similar to those encountered in the field. DBS samples placed at room temperature (22°C) for 1 to 15 weeks or at 37°C and 60% relative humidity for 7 days gave results comparable to those of blood spots dried overnight at room temperature and stored under optimal conditions (desiccated at −20°C). Similarly, two cycles of freeze-thawing, a condition which might occur during shipping, caused no appreciable reduction in the strength of PCR signals. These findings are consistent with those of other studies showing the stability of human genomic sequences in dried blood. McCabe et al. (23) reported that genomic DNA in blood spots is stable for over 4 months, and Gill et al. (15) have shown the stability of dried blood on cotton for up to 4 years. In other studies, DNA recovered from 13-year-old blood smears provided sufficient high-quality DNA to permit molecular analysis of hematological malignancies (13), and DNA from a 17-year-old blood spot was successfully used to determine carrier status for cystic fibrosis (31). Although the DNA was partially degraded in some of those studies, the DNA sequence of interest was still detectable because it was originally present at a high copy number (i.e., one to two copies per cell). Low-abundance HIV sequences would be expected to degrade sooner. In our studies, 4 to 16 copies of HIV DNA were still detectable after 15 weeks of storage at 22°C. Moreover, weak signals at the lower limits of detection could be enhanced by ethanol fixation, indicating that HIV DNA was still present in an intact form. The variation in signals seen with low-copy-number sequences may reflect the presence of inhibitors rather than DNA degradation. McCabe (22) found that methanol fixation improved the success of β-globulin amplification and attributed this effect to the inactivation of inhibitors. PCR inhibitors are frequently found in archival samples of soft tissues (25). Further studies of ethanol fixation are warranted since it not only inactivates inhibitors but also reduces potential biohazard risks and may permit longer storage in the field. Smith et al. (29) reported that DNA recovered from 6-year-old, ethanol-fixed tissues was indistinguishable from DNA of fresh samples on the basis of purity, restriction endonuclease patterns, and the ability to specifically hybridize with probes. Recent studies have shown the superiority of ethanol or methanol over other commonly used fixatives (Bouin's, Zenker's, formalin) when attempting to amplify genetic material by PCR. Ethanol fixation may also open the way for RNA PCR studies for assessment of viral replication (1).

The sensitivity of DBS PCR and the inherent stability of DBS samples demonstrated in this study suggest that DBS PCR will prove valuable for large-scale epidemiological studies and perinatal screening programs in populations that are culturally or geographically isolated. Widespread use as a screening assay, however, will require further refinements, including standardization and optimization, automation, and

the development of universal (or population-specific) primers that will recognize most, if not all, HIV strains in a given population.

ACKNOWLEDGMENT

This study was supported by Health and Welfare Canada.

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