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Diminished Human Immunodeficiency Virus Type 1 DNA Yield from Dried Blood Spots after Storage in a Humid Incubator at 37°C Compared to −20°C▽

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Collecting whole blood on filter paper simplifies the processing, transport, and storage of specimens used for the diagnosis of human immunodeficiency virus type 1 (HIV-1) and other tests. Specimens may be collected in tropical or rural areas with minimal facilities for handling specimens. To compare simulated tropical conditions with freezer storage, we examined the stability of HIV-1 DNA in dried blood spots (DBS) stored in humid heat and at −20°C. DBS were created by spotting 50-μl aliquots of whole blood on 903 filter paper. DNA was extracted from DBS at baseline and after 2, 6, or 12 months of storage at −20°C or at 37°C with ~85% humidity. The DNA was tested undiluted or diluted using the Amplicor HIV-1 DNA PCR (Roche), version 1.5. Each reaction was scored positive, negative, or indeterminate based on optical density. Results were compared between storage conditions and over time. A total of 1,832 reactions from 916 DBS were analyzed, including 100 DBS at baseline, 418 stored at −20°C, and 398 stored at 37°C. A chi-square test showed fewer positive reactions for DBS stored at 37°C (55%) than for those stored at −20°C (78%) ($P < 0.0001$). Samples stored at −20°C showed little change in the probability of detection of HIV-1 DNA over time; the odds ratio (OR) was 0.93 after storage for 1 year. Samples stored at 37°C demonstrated a significant change in detection at 1 year (OR, 0.29). We conclude that exposure of DBS to 37°C and high humidity impaired the recovery of HIV-1 DNA from DBS, whereas DNA recovery was preserved when DBS were stored frozen.

An estimated 530,000 children under the age of 15 years were infected with human immunodeficiency virus type 1 (HIV-1) in 2006 (14). The majority reside in the developing world and acquired the infection as infants from their mothers during pregnancy, delivery, or breastfeeding. The high mortality observed for these infants (4, 9) can be substantially reduced by early initiation of antiretroviral therapy (15). However, HIV-1 diagnosis of infants is often delayed, because maternal antibodies transferred to the infant preclude the use of routine serologic assays through the age of 18 months (12) or longer (6). HIV-1 DNA PCR is considered the optimal test for diagnosing HIV-1 infection in infants (10). Although HIV-1 DNA is stable in whole blood samples stored in a refrigerator for up to 10 days (7), testing of whole blood collected on filter paper offers an opportunity to simplify sample collection and the transport of specimens to laboratories for diagnostic testing (1, 11, 13). Dried blood specimens are ideal for the diagnosis and evaluation of HIV-1 infection in infants in resource-limited regions; however, previous reports have shown a decline in the ability to detect HIV-1 antibodies in samples stored under hot, humid conditions (2). Therefore, determining the stability of HIV-1 DNA on filter paper under conditions of high heat and humidity is relevant to the design of practical and reliable methods for the transport and storage of these specimens.

Previous reports have evaluated long-term HIV-1 DNA stability at room temperature in whole blood and detected stability on untreated 903 filter paper lasting up to 15 weeks (5) and on FTA filter paper lasting up to 4 years (Whatman Inc., Florham Park, NJ). FTA filter paper carries proprietary substances to lyse cells, denature proteins, and bind nucleic acids directly to the paper but is more expensive (8). In this study we evaluated the long-term stability of HIV-1 DNA on 903 filter paper stored at two temperatures: 37°C with high humidity, to simulate conditions in tropical and subtropical regions, and −20°C, the temperature of commonly available freezers.

MATERIALS AND METHODS

Study design. Citrate phosphate dextrose-anticoagulated whole blood was obtained from two HIV-1 (clade B)-infected, antiretroviral-naïve donors. Ali-

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quots (50 μl) of blood were spotted into circles on 903 paper and allowed to dry overnight. The cards with dried blood spots (DBS) were placed in individual plastic Ziploc bags (1.5 mil; Fisher Scientific) with a silica desiccant (MultiSorb Technology, Inc., Buffalo, NY) and then organized into groups of 10 DBS, or a panel, from each donor. A total of 140 DBS were sent at ambient temperature in the winter to each of eight participating laboratories in the United States, and on dry ice to South Africa. DNA was extracted from a DBS panel from each donor within 1 day of arrival at each laboratory. Extracted DNA from two DBS was analyzed undiluted, and DNA from two DBS was diluted 1:2, 1:4, 1:8, or 1:16 prior to analysis in order to simulate specimens with lower HIV-1 DNA concentrations. The extracted DNA from each spot was assayed in duplicate. The remaining panels were divided between −20°C and 37°C with ~85% humidity (except for laboratory A, which used a dry incubator). Each laboratory extracted a total of 70 spots per donor: 10 at baseline and 10 stored at −20°C and at 37°C for 2, 6, and 12 months. The numbers of positive reactions for DBS stored at each temperature were compared in order to assess the effect of temperature and humidity on the recovery of HIV-1 DNA. The proportion of DBS testing positive for HIV-1 under each storage condition was compared across time points to assess changes in HIV-1 DNA recovery over time.

DNA extraction and preparation. DNA was extracted from the entire 50-μl blood spot, after it was cut from the filter paper using a paper punch, by either silica-based (3) or resin-based (Chelex) (16) DNA extraction, as routinely used in each laboratory. Briefly, the silica method immersed all filter paper pieces comprising one entire DBS in a tube with 9 ml of NucliSens lysis buffer (bioMérieux, Durham, NC), rocked the tube at room temperature for 2 h, and then spun it to pellet the paper. The supernatant was transferred to a clean tube, to which 50 μl silica and (in the samples to be analyzed undiluted) 3.3 μl of the internal control (IC) from the Amplicor HIV-1 DNA test, version 1.5 (Roche Molecular Systems, Branchburg, NJ), was added, and the tube was rocked for 10 min. The tube was spun to pellet the silica, and the supernatant was discarded. The silica pellet was washed twice with 1 ml of NucliSens wash buffer (bioMérieux), twice with 1 ml 70% ethanol, and once with 1 ml acetone. The silica pellet was dried for 10 min, eluted in 50 μl of Tris-EDTA buffer, heated to 55°C for 5 min, vortexed, and then heated to 55°C for 5 more min. The silica was then pelleted, and the supernatant containing the nucleic acid was saved.

The resin (Chelex; Bio-Rad, Hercules, CA) extraction began with placing the paper punches from one entire DBS in 1 ml of wash buffer (whole-blood specimen wash solution; Amplicor HIV-1 Monitor test, version 1.5; Roche Molecular Systems, Branchburg, NJ) and rocking for 2 h at room temperature, followed by spinning for 5 min at high speed, after which the red-tinged buffer was removed and discarded. The wash was repeated once, and then 250 μl of Chelex working solution (10% [vol/vol] of Chelex resuspended in DNA dilution buffer [10 mM Tris buffer, pH 8.3, containing 50 mM KCl]) and (in the specimens to be analyzed undiluted) 3.3 μl of the IC were added to the DBS, with frequent mixing to keep the resin in suspension. The samples were heated to 100°C for 1 h with a 10-s vortex after the first 30 min of heating. The tubes were spun for 3 min to pellet the resin, and the supernatant with DNA was removed and stored at −70°C until amplification by PCR.

HIV-1 DNA detection. The Roche Amplicor HIV-1 DNA test, version 1.5, was used to detect HIV-1 DNA extracted from the DBS. All laboratories used kits from the same lot. The DNA extracted from each DBS was spiked with the IC prior to amplification in duplicate, either during DNA elution, for those samples analyzed undiluted, or during the dilution step, for those analyzed at dilute concentrations. The optical densities (OD) for HIV-1 DNA and the IC were read from each well. Each test result was scored as positive when the OD was ≥0.8, negative when the OD was <0.2, indeterminate when the OD was between 0.2 to 0.8, and invalid when a specimen in which HIV-1 was undetectable had an IC OD of <0.2. External controls were also included with each assay.

Statistical methods. Chi-square analysis was used to compare the numbers of positive, indeterminate, and negative test results between storage conditions pooled over laboratories, dilutions, and time points.

To analyze changes in the recovery of DNA over time, each OD was dichotomized as positive (OD, ≥0.80) or negative (OD, <0.80). Generalized estimating equations (GEE) were then employed to model the probability of a positive result as a function of time in storage, donor, laboratory, and dilution, expressed as the actual dilution multiplier (1, 0.5, 0.25, etc.). Data from the two storage conditions were analyzed separately, with the baseline data included in both analyses. The interaction between laboratory and storage time was also included in order to examine variation among the laboratories. The parameter estimate for the effect of storage time in each laboratory was derived from the fitted GEE model with the interaction term. Odds ratios (ORs) for a positive result after 1 year of storage compared to baseline were calculated as e^{365b}, where b is the parameter for storage time in the GEE model (i.e., the rate of change in the probability of a positive result) and e is the base of natural logarithms.

TABLE 1. Comparison of pooled Amplicor HIV-1 DNA test results from all laboratories between DBS samples from HIV-1-infected donors stored at −20°C or 37°C for 2, 6, or 12 months

Time	Storage temp	No. of samples with the indicated result/total samples (%)[a]		
		Positive (OD, >0.8)	Indeterminate (OD, 0.2–0.8)	Negative (OD, <0.2)
Baseline	No storage	164/200 (82)	0/200 (0)	36/200 (18)
2 mo	−20°C	210/280 (75)	1/280 (0)	69/280 (25)
	37°C	123/280 (44)	6/280 (2)	151/280 (54)
6 mo	−20°C	218/278 (78)	3/278 (1)	57/278 (21)
	37°C	127/280 (45)	5/280 (2)	148/280 (53)
12 mo	−20°C	223/278 (80)	2/278 (1)	53/278 (19)
	37°C	140/236 (59)	6/236 (3)	90/236 (38)
Total	−20°C	651/836 (78)	6/836 (1)	179/836 (21)
	37°C	439/796 (55)	17/796 (2)	340/796 (43)

[a] Total percentages are rounded to the nearest whole number.

RESULTS

Specimens analyzed. A total of 1,056 DBS were evaluated in duplicate tests (n = 2,112) for detection of HIV-1 DNA. Our analysis was conducted on results from 1,832 reactions from 916 DBS, including 100 DBS evaluated upon receipt at the laboratories, 418 DBS stored at −20°C, and 398 DBS stored at 37°C. Data excluded from analysis include baseline data from laboratory A and all data from laboratory B. Baseline data from laboratory H were not received due to problems with the assay, and no data were collected in laboratory D for DBS stored for 12 months at 37°C, because all tests at 6 months were negative. Six laboratories used the resin (Chelex) DNA extraction method (laboratories A, C, E, F, G, and H), and one (laboratory D) used the silica (Boom) method. All internal and external controls performed appropriately in the assays analyzed.

Comparison of HIV-1 DNA detection after different storage conditions. Of 200 PCR (Amplicor) tests of 100 DBS at baseline, 164 (82%) were positive, none (0%) were indeterminate, and 36 (18%) were negative. Combining data from subsequent time points, 836 PCRs on DBS stored at −20°C resulted in 651 (78%) positive test results, 6 (0.8%) indeterminate results, and 179 (21.4%) negative results. The 796 PCRs on DBS stored at 37°C resulted in 439 (55%) positive, 17 (2.1%) indeterminate, and 340 (42.7%) negative test results (Table 1). A chi-square comparison of positive results between samples stored at −20°C and 37°C showed a significantly lower rate of positive results for samples stored at 37°C ($P < 0.0001$).

When results were grouped by the level of dilution and storage conditions, HIV-1 DNA was detected in the undiluted specimens stored at −20°C in 156/164 (97%) tests compared to 128/156 (82%) tests for those stored at 37°C (Table 2). The detection of HIV-1 DNA decreased progressively with the dilution of the extracted DNA, but detection was consistently higher in samples stored at −20°C than in those stored at 37°C.

TABLE 2. Comparison of Amplicor HIV-1 DNA PCR results for DBS samples from known HIV-1-infected donors stored at −20°C versus 37°C with ~85% humidity[a]

Storage temp and result	No. (%) of isolates with the indicated result under the indicated storage conditions				
	Undiluted	Diluted:			
		1:2	1:4	1:8	1:16
−20°C					
Positive (OD, >0.8)	165 (96)	162 (92)	141 (80)	131 (74)	70 (42)
Indeterminate (OD, 0.2–0.8)	0 (0)	1 (1)	2 (1)	1 (1)	2 (1)
Negative (OD, <0.2)	7 (4)	5 (7)	33 (19)	44 (25)	102 (58)
Total	172	176	176	176	168
37°C					
Positive (OD, >0.8)	128 (78)	121 (72)	84 (50)	63 (38.5)	43 (27)
Indeterminate (OD, 0.2–0.8)	10 (6)	3 (2)	2 (1)	1 (0.5)	1 (0.5)
Negative (OD, <0.2)	26 (16)	44 (26)	82 (49)	104 (62)	124 (74)
Total	164	168	168	168	160

[a] DNA was extracted from the dried blood spot samples after storage for 2, 6, or 12 months (pooled in the table). At each time point, DNAs from two DBS were assayed undiluted, while DNAs from the other eight samples were diluted prior to the assay.

Detection of HIV-1 DNA over time within the same storage condition. The interaction between storage time and laboratory was statistically significant for storage at −20°C (P = 0.011), suggesting that some laboratories had higher rates of HIV-1 detection than others. The parameter estimates for laboratories D and G departed substantially from those for the other laboratories (data not shown). The interaction remained statistically significant when either laboratory D or laboratory G was excluded from the analysis (with the exclusion of laboratory D, P = 0.0204; with the exclusion of laboratory G, P = 0.0267) but not when both were excluded (P = 0.0869), suggesting that these two laboratories had consistently lower rates of DNA detection. No loss of HIV-1 DNA was detected in the other five laboratories in a model without the interaction term (P = 0.29).

After 1 year of storage at −20°C, the recovery of DNA in two laboratories (D and G) differed from the changes in the majority of laboratories, but the rates of loss in DNA detection in laboratories D and G did not differ from each other (P = 0.55). A slightly positive parameter estimate for storage time, in a model for data from these two laboratories, indicates an increasing probability of DNA detection over time (P = 0.0062). Inspection of the data showed that the positive slopes in the two laboratories were attributable to a low percentage of positive results at one of the first two time points in both laboratories rather than to an overall trend in the data (Fig. 1A). The low percentages of positive results in these two subsets of the data accounted for the difference in the changes in recovery of HIV-1 DNA between these two laboratories and the other laboratories. Specifically, the laboratory-time interaction is no longer statistically significant if the baseline data from laboratory G and the data from the first follow-up time point for laboratory D are excluded (P = 0.1080). Importantly, if the two subsets of data in question are excluded and the interaction term is deleted from the model, then no loss of HIV-1 DNA is detected in DBS stored at −20°C (P = 0.81), and the 1-year OR for a positive result is 0.93.

The interaction between storage time and laboratory was also statistically significant for detection of HIV-1 DNA after the storage of DBS at 37°C ($P < 0.0001$). The parameter estimates for the effect of storage time on detection indicate a much-decreased rate of HIV-1 DNA detection in both laboratories D and H. Without these two, no interaction between storage time and laboratory was detected (P = 0.1149). Im-

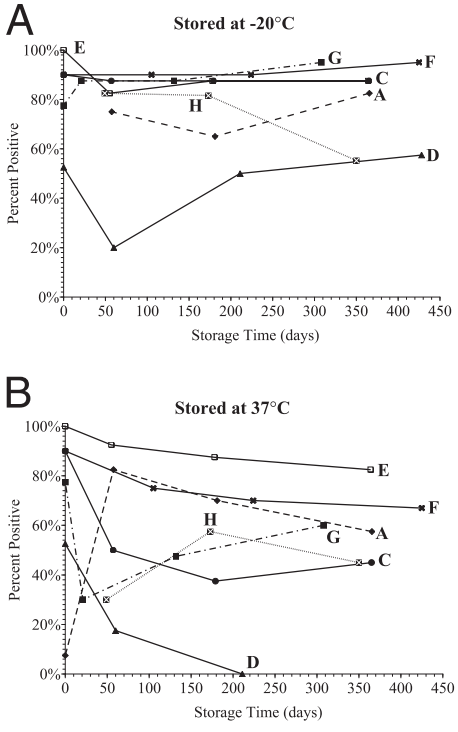

FIG. 1. Percentage of known HIV-1 positive dried blood spot samples testing positive with the Amplicor HIV-1 DNA test in each laboratory (designated by curves A to H) over time. Samples were tested at baseline and after storage at −20°C (A) or 37°C (B) at four time points (2 months, 6 months, and 12 months).

portantly, in the five remaining laboratories, a model without the laboratory interaction term indicated a 1-year OR for detecting HIV-1 DNA of 0.29 ($P < 0.0001$) (Fig. 1B).

DISCUSSION

In this study, recovery of HIV-1 DNA appeared stable over 1 year when 903 filter paper was frozen at −20°C and appeared diminished when the filter paper was stored at 37°C with high (~85%) humidity regardless of whether it was stored for 2, 6, or 12 months, suggesting that optimal storage may be in a freezer at −20°C. Our study design did not evaluate whether the decrease in HIV-1 DNA detection was due primarily to heat or to humidity. However, a detrimental effect of heat is suggested, because DBS stored dry (without humidity) at 37°C in laboratory A had diminished detection of HIV-1 DNA, similar to that of DBS stored in humid incubators.

Laboratories differed in their proficiency at detecting HIV-1 DNA in DBS. Differences in the familiarity of the technical staff with DNA extraction from DBS, or variability in storage conditions, could contribute to the inconsistent results between laboratories. Two of the laboratories with interactions between change in DNA detection and time demonstrated increased detection of HIV-1 DNA in DBS stored at −20°C over the course of the study, suggesting that technical skills improved over time. The profound decrease in HIV-1 DNA detection in DBS stored at 37°C in laboratory D was unexpected. The only factors unique to this laboratory were the fact that mold grew on the DBS stored in the incubator and the use of silica extraction. The growth of mold and the fact that gas permeability differs across plastic bags suggests that humidity indicators should be used and that when humidity increases within the bags, the desiccants should be changed.

Our study design did not allow us to determine the mechanism leading to a decrease in HIV-1 DNA detection in DBS stored at 37°C, or the time frame at which this change occurred. The heat and/or humidity could have led to degradation of the DNA or to fixation of the DNA to the paper. Several laboratories noted impaired lysis of the red blood cells on the 903 paper stored at 37°C, such that the red color could not be washed off the paper. We suspect that heat and/or humidity fixed the DNA to the filter paper, making it resistant to extraction.

Decreased detection of HIV-1 DNA in DBS stored at 37°C was apparent after 2 months in most laboratories. DBS were not evaluated at earlier time points to assess whether the effects of heat and humidity were instantaneous. Also, the variability in the detection of HIV-1 DNA across laboratories did not allow us to evaluate if effects from heat and humidity were additive over time.

HIV-1 DNA detection was studied on only one type of filter paper: Whatman 903 (previously SS 903) filter paper. This paper was chosen because it is inexpensive, is readily available worldwide, and can be used to measure a number of analytes. Several other types of filter paper are available, including FTA Classic and FTA Elute (formerly Isocode; Schleicher & Schuell, Keene, NH). These papers are treated with compounds to facilitate the binding of nucleic acids and are not suitable for the detection of antibodies, proteins, or other analytes. The principal advantage of these treated filter papers is elimination of the rather tedious specimen processing to extract DNA from the paper. Rather, lysed red blood cells are washed from the filter paper, and a 3-mm punch of the paper with entrapped nucleic acids is then submitted to PCR. The small sample input minimizes PCR inhibitors, and technical variability is reduced by the limited processing of DBS.

Collection of blood on DBS for HIV-1 diagnostic or laboratory testing is less costly at the site because it involves less labor, fewer supplies, and less equipment than venipuncture and the processing of whole blood in tubes. Storage of DBS requires less space and fewer supplies than cryovials with peripheral blood mononuclear cells and plasma. However, the significant drop in HIV-1 DNA detection that we observed from DBS stored on Whatman 903 paper at 37°C suggests that cooler, drier conditions are needed to optimally preserve HIV-1 DNA. The stability of HIV-1 DNA on DBS stored at 37°C in high humidity was previously evaluated (5) and was shown not to affect the recovery of HIV-1 DNA from DBS. An important difference between the two studies is the use of a more rigorous phenol-chloroform extraction. This extraction may have overcome the adverse effects of heat and/or humidity on the DNA in DBS, and if so, provides circumstantial evidence supporting the hypothesis that heat and/or humidity can fix the DNA to the DBS.

Our study suggests that for optimal recovery of HIV-1 DNA from Whatman 903 paper, DBS should be stored frozen. Further studies comparing preservation strategies using various filter papers and extraction methods could be worthwhile, since preserving specimens outside of the freezer and transporting them at ambient temperatures is generally more convenient and always less expensive.

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