Stability of Human Immunodeficiency Virus Type 1 Antibodies in Whole Blood Dried on Filter Paper and Stored under Various Tropical Conditions in Kinshasa, Zaire

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The use of whole-blood spots on filter paper for the detection of antibody to human immunodeficiency virus type 1 (HIV-1) was evaluated during a 20-week period under a variety of storage environments simulating the harsh tropical field conditions in Kinshasa, Zaire. During the first 6 weeks of storage, all replicates of high- and low-titer HIV-1-positive reference samples remained positive by enzyme immunoassay and Western blotting (immunoblotting), and all replicates of HIV-1-negative samples remained negative under all storage conditions. However, hot and humid storage conditions for up to 20 weeks caused a progressive decline in enzyme immunoassay optical density ratio values, which was particularly noticeable in samples with a low HIV-1 antibody titer. Harsh tropical operational conditions did not cause any repeatedly false-positive results during the 20-week storage period. The use of gas-impermeable bags with desiccant for the storage of blood spots on filter paper improved the stability of HIV-1 antibody detection over time and is recommended for the storage of whole-blood spots on filter paper in harsh tropical field settings.

Antibodies against human immunodeficiency virus type 1 (HIV-1) can be detected accurately in whole blood that is collected and dried on filter paper (1, 4, 5). This technique is being used in the United States (3, 6, 9, 11–13) and Great Britain (14) to conduct HIV-1 seroprevalence surveys among childbearing women and has recently been validated in Kinshasa, Zaire (10).

While studies at the Centers for Disease Control (CDC) in Atlanta, Ga., have shown that HIV-1 antibody titers remain stable in specimens that are dried on filter paper and kept for up to 60 days at ambient laboratory temperature when stored in zip-lock closure bags with desiccant, the effect which a tropical environment might have on HIV-1 antibodies in whole blood that is collected and stored for prolonged periods on filter paper has not been investigated (7). The use of whole-blood spots that are collected and dried on filter paper would be particularly practical for sampling under field conditions; expensive and invasive venipuncture would be unnecessary, and blood samples would not require centrifugation and could easily be transported. However, significant time delays can occur between the time of collection and the time of testing in the laboratory; this time delay may expose the blood spots to hot, humid conditions and, possibly, may compromise the HIV-1 test results.

Therefore, we evaluated the stability of HIV-1 antibody detection in whole-blood spots that were collected and dried on filter paper and that were subsequently stored under 14 different, standardized environmental conditions in Kinshasa over a 20-week period.

MATERIALS AND METHODS

Whole-blood specimens used in the study. To prepare reference samples with different HIV-1 antibody concentrations, the following heparinized whole-blood samples were obtained from Zairian individuals (Table 1). S1 specimens were high-titer HIV-1 antibody-positive whole blood from a pool of five HIV-1-seropositive Zairian patients with AIDS. The mean optical density (OD) value by enzyme-linked immunosorbent assay at time zero for these samples was 2.962 (range, 2.838 to 3.082), with a cutoff value of 0.255. S2 specimens were low-titer HIV-1 antibody-positive whole-blood specimens that were obtained by diluting one sample from a Zairian seropositive individual 1:200 with HIV-1 seronegative blood. The mean OD value was 1.008 (range, 0.788 to 1.146). S3 specimens were HIV-1-negative whole blood from a single Zairian blood donor. The mean OD value was 0.054 (range, 0.050 to 0.060). In addition, the following three blood specimens from the CDC Quality Assurance Program (8) prepared in September 1988 were included in the panel. S4 specimens were high-titer HIV-1 antibody-positive quality control (QC) dried blood spots. S5 specimens were low-titer HIV-1 antibody-positive QC dried blood spots. S6 specimens were HIV-1-negative QC dried blood spots.

Preparation of blood spots on filter paper. Blood specimens S1, S2, and S3 were transferred to specimen collection paper (grade 903; Schleicher & Schuell, Keene, N.H.). Each collection card contained five circles each of which was 15 mm in diameter and each of which was completely filled with approximately 75 μl of whole blood. At least 126 cards were prepared for each reference specimen (specimens S1, S2, and S3). The blood spots were dried at ambient laboratory temperature for 48 h prior to initial testing at time zero and prior to storage under the 14 different conditions.

Storage of dried blood spots. Specimens were stored in four...
TABLE 1. Specimens used for evaluating stability of HIV-1 antibody detection in dried whole blood on filter paper

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Source</th>
<th>HIV antibody</th>
<th>OD values</th>
<th>Cutoff</th>
<th>Bands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>Project SIDA</td>
<td>High titer</td>
<td>2.962</td>
<td>2.838–3.082 (strong)</td>
<td>0.255</td>
</tr>
<tr>
<td>S2</td>
<td>Project SIDA</td>
<td>Low titer</td>
<td>1.008</td>
<td>0.788–1.146</td>
<td>0.255</td>
</tr>
<tr>
<td>S3</td>
<td>Project SIDA</td>
<td>Negative</td>
<td>1.054</td>
<td>0.050–0.060</td>
<td>0.255</td>
</tr>
<tr>
<td>S4</td>
<td>CDC-QA</td>
<td>High titer</td>
<td>1.472</td>
<td>Strong</td>
<td>All'</td>
</tr>
<tr>
<td>S5</td>
<td>CDC-QA</td>
<td>Low titer</td>
<td>0.692</td>
<td>Weak</td>
<td>Subsetf</td>
</tr>
<tr>
<td>S6</td>
<td>CDC-QA</td>
<td>Negative</td>
<td>0.122</td>
<td></td>
<td>None</td>
</tr>
</tbody>
</table>

a p17, p24, p31, gp41, p51, p55, p65, and gp120/160.
b p24, p31, gp41, p66, and gp120/160.
c CDC-QA, CDC Quality Assurance Program for HIV seropositivity screening of dried blood spot specimens; the lot was from September 1988.
d Standard deviation, ±0.230.
e Standard deviation, ±0.143.
f p31, gp41, p51, p65, and gp120/160.
g Standard deviation, ±0.029.

Environments. Environment E1 was a metal cargo container that was directly exposed to the sun in Kinshasa; this environment was expected to provide extreme, maximum values for temperature and relative humidity. Environment E2 was a shaded location provided by a non-air-conditioned house in Kinshasa; this was assumed to approximate the conditions encountered in most laboratories in tropical countries. Environment E3 was a refrigerator at 4°C. Environment E4 was a freezer at −20°C.

Packaging. Packaging C1 was regular paper envelopes. Packaging C2 was locally purchased gas-permeable bags (zip-lock closure type) with desiccant (Multiform Desiccants). Packaging C3 was locally purchased gas-permeable bags (zip-lock closure type) without desiccant. Packaging C4 was gas-impermeable bags (zip-lock closure type) with desiccant. Packaging C4 was used only for environments E1 and E2. Previous stability testing demonstrated no detectable loss in HIV-1 antibody titer by enzyme immunoassay (EIA) or immunoblotting after storage of filter paper specimens in zip-lock closure bags with desiccant at −20 and 4°C for at least 6 months (7).

Time intervals for testing. All specimens were tested initially (time zero). Specimens S1, S2, and S3 were analyzed again at weeks 1, 2, 3, 4, 6, 8, 12, and 20. QC specimens S4, S5, and S6 were analyzed at weeks 2, 4, 8, 12, and 20.

Serologic testing methods. All testing was carried out in the Project SIDA (SIDA is the French acronym for AIDS) laboratory, Kinshasa. For each time point and each storage condition (environment and packaging), six replicates of dried blood specimens S1, S2, and S3 and two replicates of QC specimens S4, S5, and S6 were tested. A paper punch was used to obtain a blood spot of 6.3 mm in diameter from each blood-impregnated circle. Blood spots were then eluted overnight in microtiter wells by using 200 μl of the diluent provided by the manufacturer (LAV EIA; Genetic Systems, Seattle, Wash.). For each time interval and for the 14 storage conditions, two cluates of the six HIV-1-positive replicates (low and high titer) and one cluate of the six HIV-1-negative replicates were tested by Western blot (immunoblot) assay (Miniblotter system; Immuneonics, Cambridge, Mass.). Tago H&L Chain Conjugate (lots M0410903 and M0718901) was used at a 1:300 concentration and an incubation time of 20 min. The criteria of the Centers for Disease Control (2) were followed for the interpretation of the Western blot results; a positive result required the presence of at least two of the following three bands: p24, gp41, and gp120/160.

Data coding, processing, and statistical methods. ODs and Western blot banding patterns were recorded for each specimen, along with the corresponding information on storage condition, type of specimen, and time interval. Changes in humidity indicator cards were recorded as follows: 1, circle 30 was pink and circles 40 and 50 were blue; 2, circle 30 was pink; circle 40 was blue-pink, and circle 50 was blue; 3, circles 30 and 40 were pink and circle 50 was blue; 4, circles 30 and 40 were pink and circle 50 was blue-pink; 5, all three circles were pink. For each specimen type, a linear regression model was fit for each of the 14 storage conditions in order to detect changes in the mean OD ratio (sample OD value divided by the cutoff value) over time. In addition, for each pair of storage conditions (i.e., environment and packaging), multiple regression was used to detect differences in the effect of time on the mean OD ratio. Results of Western blot assays were also examined to reveal the changes in patterns over time.

RESULTS

The study was conducted between November 1989 and April 1990 during the rainy season in Kinshasa. The mean temperatures in the metal cargo container were 29°C at 9:00 a.m. and 40°C at 2:00 p.m. The maximum temperature in the metal cargo container was 44°C. The mean temperatures in the non-air-conditioned house were 25°C at 6:00 a.m. and 28°C from noon to 2:00 p.m. The maximum temperature in the non-air-conditioned house was 33°C (noon to 2:00 p.m.). The changes observed in the humidity indicator cards reflected the relative humidities of the micro-environments in which the specimens were stored and are shown in Fig. 1. In both the metal cargo container and the non-air-conditioned house, low-humidity storage conditions were maintained the longest for specimens stored in gas-impermeable bags with desiccant.
STORAGE CONDITIONS

FIG. 1. Changes detected in humidity indicator cards under various storage conditions. Environments and packaging conditions are described in the text. Freshly activated humidity indicator cards showed three blue circles labeled 30, 40, and 50. Humidity turns the circles from blue to pink; increasing levels of humidity are recorded from 1 (circle 30 is pink and circles 40 and 50 are blue) to 5 (all three circles are pink).

During the first 6 weeks of storage under all 14 conditions, all replicates of high- and low-titer HIV-1-positive samples remained EIA and Western blot positive, and all replicates of HIV-1-negative samples remained negative. The blood spots with a high HIV-1 antibody titer remained positive throughout the entire 20-week observation period for all environments and conditions. After 8 weeks of storage, some replicates of the blood spots with low HIV-1 antibody titers (specimen S2) became EIA negative, depending on the environments and conditions (Fig. 2). None of the negative blood spot replicates showed repeatedly positive EIA results after 20 weeks of storage under the 14 different conditions. All the positive (high and low antibody titers) replicates remained Western blot positive, demonstrating the same band patterns throughout the study, and all of the negative specimens remained negative.

Analysis of EIA OD ratios. (i) High-titer HIV-1-positive specimens. The results of the multiple regression analysis failed to demonstrate a decrease over time in the mean OD ratios for high-titer specimen S1 replicates under all storage conditions. Similarly, the mean OD ratios of the high-titer specimen S4 QC replicates except for the specimens stored in the metal cargo container did not change over time. For specimens stored in the metal cargo container, the average ODs decreased significantly, irrespective of the type of packaging.

(ii) Low-titer HIV-1-positive specimens. In the metal cargo container and the non-air-conditioned house, the mean OD ratios of the low-titer HIV-1-positive specimen S2 replicates decreased significantly ($P < 0.001$) over time under all packaging conditions. In contrast, mean OD ratios did not decrease over time for replicates stored in the refrigerator or freezer, irrespective of packaging conditions, indicating that, as expected, the refrigerator and the freezer provide better storage conditions than the metal cargo container or the non-air-conditioned house. No differences in the rate of decrease of the mean OD ratio over time were detected between refrigerated and frozen specimens or between specimens stored in the non-air-conditioned house and those stored in the metal cargo container. Among the QC specimens, only those S5 specimen replicates stored in the metal cargo container and packed in paper envelopes and gas-permeable (zip-lock closure type) plastic bags with desiccant demonstrated a significant ($P < 0.005$) decrease in the mean OD ratio over time.

(iii) HIV-1-negative specimens. Average OD ratios of specimen S3 and HIV-1-negative QC specimen S6 stored in the metal cargo container in paper envelopes or in gas-permeable (zip-lock closure type) plastic bags with or without desiccant decreased over time. In contrast, changes in OD ratios were not observed for specimens stored in gas-impermeable bags with desiccant in metal cargo containers. OD ratios of specimens stored in the non-air-conditioned house, refrigerator, and freezer did not change over time, regardless of the packaging conditions.

DISCUSSION

Detection of HIV-1 antibody in whole-blood spots from Zairian individuals with high HIV-1 antibody titers that were collected on filter paper and stored over a 20-week time period was not affected by environmental conditions. In Kinshasa, we observed that the sera of most HIV-1-infected individuals have high HIV-1 antibody titers. However, during seroconversion, for example, it is important that low antibody levels be detected accurately. Storage conditions can alter the OD ratio values of blood spots with a low HIV-1 antibody titer, with ratios waning over time, particularly in specimens stored under the hottest conditions (metal cargo container). The use of gas-impermeable bags with desiccant for storage of blood spots on filter paper sustained a better microenvironment for the samples and, thus, improved the stability of HIV-1 antibody detection over time. However, the most important observation of this study was that the whole-blood spots that are collected and dried on filter paper can be stored under tropical conditions for 6 weeks without changing the categorical result (positive or negative) of the HIV-1 antibody screening and confirmation tests. It is also reassuring that no reproducible false-positive results were
observed after 20 weeks of storage of whole-blood samples under poor conditions.

In this study, we evaluated some of the harshest operational conditions to which dried blood spots may be subjected. Therefore, our findings should be applicable to a wide range of specimen collection and storage conditions. Although collecting, drying, and storing of whole blood on filter paper simplifies sample collection, the conduct of careful and precise laboratory procedures by trained personnel in a well-equipped laboratory with an efficient quality assurance program in place is still required. If local facilities are not adequately equipped, filter paper specimens could be sent to an appropriate laboratory within the time limits identified in this report.

In conclusion, whenever it is difficult to obtain and properly store sera for HIV-1 antibody detection, which is a common situation in developing countries, collection of whole-blood spots on filter paper is an excellent alternative method.

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Letter to the Editor

Stability of Human Immunodeficiency Virus Type 1 Antibodies in Whole Blood-Impregnated Filter Papers under Various Tropical Conditions

The deliberate exposure of whole blood specimens with high and low human immunodeficiency virus type 1 (HIV-1) antibody titers to an adverse tropical environment by Behets et al. (2) was associated with a progressive decline in enzyme immunoassay optical density ratios. This, along with the simultaneous exposure of quality control sera with high and low HIV-1 titers to 14 different standardized environmental conditions during the rainy season in Kinshasa, Zaire, was a remarkable attempt to simulate the harsh environmental rigors prevailing in developing countries. The exercise lasted 20 weeks and involved regular monitoring of the microenvironment of the blood samples for humidity changes by employing innovative microenvironment cards. Storage in gas-impermeable bags with a desiccant produced maximum stability for HIV-1 antibody detection and was recommended for sample storage in harsh environments.

Before universal acceptance of the recommendations of this outstanding study (2), it would be essential to carry out identical investigations in other seasons at Kinshasa itself and also to carry the metal cargo container in peripheral areas in Zaire. The metal cargo containers could be transported by the routine modes of transportation and stored in remote areas in shaded or even unshaded locations in non-air-conditioned houses. The retrieved whole blood preparations and quality control sera could be tested in Kinshasa to ascertain whether there were any remarkable differences in declines in enzyme immunoassay optical density values or whether there were any repeatedly false-positive results attributable to storage in peripheral areas in different seasons. Furthermore, it might be worthwhile to carry out similar experiments in regions where ambient temperatures would be less than 10°C. Such situations are not that infrequent in developing countries, where facilities in which room temperature is maintained around 20°C are not available.

Future investigations on the field stability of HIV-1 antibodies in which warehouse temperature, humidity, and air velocity are monitored would provide invaluable guidance to ensure field stability of vaccines and to eliminate any decline in the potency of meticulously calibrated antibody reference preparations (1). The therapeutic failures of antivenom preparations against Russell viper venom (Viper russelli pulchella) to clear antigenemia in 19 of the 20 patients in the interior region of Sri Lanka bitten by Russell vipers are alarming (5). The performance of a freeze-dried measles vaccine in immunization centers during the 1990s has been dismal. The inadvertent use of vaccine batches with potencies below 10² 50% tissue culture infectious doses was associated with a mere 26% seroconversion (4). A search is on for a live polio virus vaccine that is stable at 45°C which would withstand field rigors (3). Extended investigations are essential to define the prevailing harsh environmental hazards in tropical countries. Development of environmentally stable immunodiagnostic tests for HIV (6) and therapeutic or prophylactic vaccines, as well as determination of ideal conditions for carriage of specimens from the field, should be undertaken, and the outstanding studies of Behets et al. (2) should be extended in other geographic locations in the near future.

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Author’s Reply

We thank Dr. Arya for his interest in our study regarding the stability of blood specimens stored on filter papers under various tropical conditions. While it would have been interesting to have extended our studies for a longer period of time and at different temperatures in Zaire, these experiments are no longer possible with the unfortunate closure of Projek SIDA due to recent political difficulties. Fortunately, extensive stability studies have been performed by the Centers for Disease Control (CDC) as part of a quality assurance program for the use of dried blood spots (DBS) on filter papers for a variety of tests used in newborn screening. These tests have measured the stability of protein and nonprotein analytes, thyroid-stimulating hormones, thyroxin, phenylalanine, galactose, progesterone, hemoglobin, and HIV-specific antibodies. In these studies, blood spots on sheets of filter paper are dried routinely for 24 h at ambient temperature and then placed in plastic zip-closure bags with low gas permeability containing desiccant packages and humidity indicator cards (2). The DBS materials are stored at –70°C, and the humidity is maintained below 30%. Bags with low permeability to gas are important for maintaining active desiccant. The source of filter paper is important, and only paper certified for whole-blood collection must be used. We have found that these conditions are ideal for long-term storage of DBS for all analytes that have been studied.
Humidity is the primary contributor to instability of analytes in DBS.

Results from stability studies indicate that under a variety of heat and humidity conditions, HIV antibodies in DBS are reasonably stable. Under a variety of climate conditions and seasons around the world (45 states and 17 countries), we have not detected any DBS stability problems with DBS in plastic bags containing desiccant during these “round-robin” tests of transportation conditions. These round-robin tests involved double-package distribution to a given location in the world and return of one package to the CDC. All return samples were tested in the same assays to minimize analytical variance. We do not recommend use of metal cargo containers for transport of DBS. Wood or heavy cardboard would retain and conduct less heat and result in less potential for compromise of DBS. If airtight containers with desiccant cannot be used, the best shipping storage conditions are paper containers that acclimate quickly. Containers that sweat with change in temperature must be avoided unless desiccant is used. Finally, storage containers with DBS (with or without desiccant) brought from low to ambient temperatures must be allowed to acclimate before being opened.

Environmental control stability studies are carried out both as accelerated and as longitudinal studies at the CDC. DBS are stored at multiple temperatures, either in zip-closure bags with desiccant or unprotected. A reduction in HIV antibody titer of approximately 15% is seen within 30 days of storage in ambient laboratory conditions (unprotected); at higher temperatures this loss in titer is enhanced (2). Samples stored in controlled humidity at the same ambient conditions were stable for up to 190 days. Ongoing longitudinal stability studies have demonstrated that HIV antibodies in DBS stored under our controlled conditions of humidity and at a low temperature are stable for at least 56 months. DBS samples with significant temperature-induced loss in titer (55°C for 160 days) were examined by quantitative assessment of HIV-specific bands on Western immunoblots, and it was determined that the loss in titer appeared to be proportional across all HIV-specific bands.

For stability testing, a dilution series that extends from an optical density of 2.0 to 0.1 and that contains all Western blot HIV-specific bands is preferred. This aspect is important in testing the stability of HIV antibody, because HIV-positive samples frequently have very high OD values and cannot be tested because of loss in titer. For 4 years the CDC has monitored a DBS dilution series prepared and stored 5 years ago for stability by enzyme immunoassay and Western blot testing and has found no significant change in response after storage under the above-described conditions and after transportation to laboratories across the United States.

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