JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2007, p. 891–896 Vol. 45, No. 3
0095-1137/07/$08.00+0 doi:10.1128/JCM.01919-06

Use of Dried Spots of Whole Blood, Plasma, and Mother's Milk Collected on Filter Paper for Measurement of Human Immunodeficiency Virus Type 1 Burden[▽]

Workenesh Ayele,[1] Rob Schuurman,[3] Tsehaynesh Messele,[1] Wendelien Dorigo-Zetsma,[4] Yohannes Mengistu,[5] Jaap Goudsmit,[6] William A. Paxton,[7] Michel P. de Baar,[2] and Georgios Pollakis[7]*

Ethio-Netherlands AIDS Research Project, Addis Ababa, Ethiopia[1]; Primagen, Amsterdam, The Netherlands[2]; Department of Virology, University Medical Center Utrecht, Utrecht, The Netherlands[3]; Regional Microbiological and Serological Laboratory, Hospital Hilversum, Hilversum, The Netherlands[4]; Department of Microbiology, Immunology and Parasitology, Faculty of Medicine, Addis Ababa University, Ethiopia[5]; Crucell Holland B.V., Leiden, The Netherlands[6]; and Department of Human Retrovirology, University of Amsterdam, Amsterdam, The Netherlands[7]

Received 15 September 2006/Returned for modification 4 December 2006/Accepted 17 December 2006

We studied the use of dried spots of bodily fluids (plasma, whole blood, and mother's milk) on filter paper as a means of sample collection and storage for human immunodeficiency virus type 1 (HIV-1) viral load testing under stringent field conditions. Plasma placed directly in lysis buffer, which is customarily used for viral load assays, was used for comparison in all our experiments. Utilizing reconstruction experiments, we demonstrate no statistical differences between viral loads determined for plasma and mother's milk spotted on filter paper and those for the same fluids placed directly in lysis buffer. We found that the addition of whole blood directly to lysis buffer was unreliable and could not be considered a feasible option. However, viral load measurements for whole blood spotted onto filter paper correlated with plasma viral load values for both filter spots and lysis buffer (Pearson correlation coefficients, 0.7706 and 0.8155, respectively). In conclusion, dried spots of plasma, whole blood, or mother's milk provide a feasible means for the collection, storage, and shipment of samples for subsequent viral load measurement and monitoring. Virus material spotted and dried on filter paper is a good inexpensive alternative for collecting patient material to monitor the HIV-1 viral load. Measuring the HIV-1 burden from whole blood dried on filter paper provides a suitable alternative for low-technology settings with limited access to refrigeration, as can be found in sub-Saharan Africa.

For patients infected with human immunodeficiency virus (HIV) in resource-limited settings, in recent years there has been increased access to antiretroviral therapy (7), which has led to a growing need to have monitoring systems in place that are capable of assessing the effects of these treatments. Monitoring therapy in the context of many developing nations poses unique challenges. More than 90% of new HIV infections occur in developing countries where there is limited access to equipment for processing specimens, and these countries often also have inadequate storage facilities for preserving sample integrity until testing. In order to overcome the logistical obstacles, innovative approaches to simplifying methods of sample collection from far-to-reach areas have been applied with good results. Spotting and drying blood samples on filter paper for clinical testing has proven to be a highly effective method for sample collection and storage (5, 11). Various types of paper were used in screening for various metabolic disorders in neonates (4, 38). Recently, this method has been applied more widely to the diagnosis of other infectious diseases as well, since this approach is feasible for the collection of large numbers of field specimens (19, 22, 26, 31, 36, 37).

To provide alternative ways to obtain viral load measurements, we developed and evaluated the use of filter papers as a collection and storage medium for HIV-1-infected blood, plasma, and breast milk samples. Analyses were performed in combination with the Primagen Retina Rainbow assay for viral load measurements, which is able to detect all known subtypes and circulating recombinant forms (CRFs) of HIV-1. Dried plasma spot technology is the least expensive way of shipping samples that have been spotted on filter paper and dried. Once samples are dried on filter paper, they remain stable for HIV-1 RNA detection for long periods of time (9, 11), and virus that is spotted is no longer infectious (http://www.cdc.gov/od/ohs/biosfty/driblood.htm), and therefore this approach also provides an inexpensive way to store samples for future analysis.

In addition to dried plasma and mother's milk spots, we tested dried whole-blood spots, as this would be the simplest formulation for sample collection. In addition, we compared the Rainbow assay (13) to the Roche Amplicor 1.5 HIV-1 monitor assay, the most widely used technology in the field.

MATERIALS AND METHODS

Reconstruction experiments for viral load measurements. We evaluated filter paper as a carrier and storage medium for HIV nucleic acids under reconstructed experimental conditions where virus was spiked into a body fluid. The human plasma used was collected as a by-product of the lymphocyte purification protocol, and human milk (surplus) was donated by a breast-feeding mother.

We used a panel of virus isolates, comprising all subtypes and circulating recombinant forms 1 and 2 (CRF01/CRF02). The isolates were quantified by

* Corresponding author. Mailing address: Human Retrovirology, Academic Medical Center, Room K3-113B, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Phone: 31205668572. Fax: 31205669064. E-mail: g.pollakis@amc.uva.nl.

▽ Published ahead of print on 24 January 2007.

using several methods, including electron microscopy (17). Each virus was diluted in the body fluid in use and at the desired concentrations, ranging from 100 to 1,000,000 virus particles per milliliter. Subsequently 200 μl of the fluid spiked with the virus was dissolved in lysis buffer (L6) as required for RNA isolation by the method of Boom and colleagues (8) or laid on Schleicher & Schuell 903 filter paper (Keene, NH) in four spots of 50 μl each. All samples spotted on paper were air dried for 30 min and then stored, sealed in a bag with desiccant, for 24 h to several weeks at room temperature.

Subjects and samples. Validation of the method using air-dried filter paper for clinical samples was performed for two sets of blood-derived material collected from HIV-1-infected individuals.

A set of 103 plasma samples was collected between January 2000 and December 2002 from 103 HIV-1-infected patients visiting the outpatient clinic of the University Medical Center of Utrecht University in The Netherlands. Immediately upon arrival, Portions of samples were analyzed for HIV-1 viral RNA levels by using the Amplicor 1.5 assay, whereas the remainders of the samples were stored at −80°C until further use. These samples encompassed a range of viral loads from >6 $\log_{10}$ copies RNA per ml to values below the detection level of the Amplicor assay, which means <50 copies RNA per ml. Of the 103 plasma samples, 19 were proven to be of a non-B subtype.

A set of 35 samples collected in Ethiopia in 2003 were from HIV-1-seropositive individuals enrolled in an Ethio-Netherlands AIDS Research Project cohort of factory workers. The cohort was formed to study the natural history of HIV-1 infection in Ethiopia and has been described elsewhere (34, 35). Blood was collected using EDTA anticoagulant as required for routine viral load determination using the NucliSENS HIV-1 QT assay (bioMérieux, Boxtel, The Netherlands). Blood samples from three healthy seronegative cohort participants were included as controls. For the purpose of the study, each of these samples was prepared four different ways, and all four preparations were compared in our analysis. Whole blood or plasma (200 μl) was aliquoted in lysis buffer and stored immediately at −80°C until processing. Additionally, same-volume amounts of whole blood and plasma were spotted on filter paper in four spots of 50 μl each.

Viral load determination. Viral RNA from all samples was assayed by the Primagen Retina Rainbow assay, performed according to the instructions of the manufacturer. In short, 5 μl of purified nucleic acid from a total elution volume of 50 μl was assayed by mixing it with the amplification reagents. After the addition of the enzymes, the samples were placed in a fluorimeter with a thermostat for 90 min. After the run, the results were analyzed and reported as the number of copies per milliliter or "<500 copies per ml" if the sample was positive but not quantifiable or "not detectable" if no signal was detected.

The method for isolating virus RNA from HIV-1-containing fluids that were dissolved in lysis buffer was previously described (8). For the filter paper spots, there was an additional elution process after punching of the plasma spots of the paper. All four spots were incubated for at least 2 h or overnight at room temperature in 3 ml lysis buffer following the removal of the papers from the lysis buffer and further processing according to standard protocols for the extraction of nucleic acids using a silica-based isolation method by Boom and colleagues (8). Purified RNA was dissolved in a total elution volume of 50 μl.

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version 4; GraphPad Software, Inc., San Diego, CA) and SPSS for Windows (version 11.5; SPSS. Inc., Chicago, IL). Correlations were substantiated with the Pearson statistical test, and group values were compared with the Wilcoxon matched-pairs signed-ranks test unless otherwise stated. The significance level was set at a P value of <0.05 in all cases. For the purposes of analysis, samples which were negative or positive but below the limit of detection of the assay were reported at the cutoff value (2.7 $\log_{10}$/ml).

RESULTS

Reconstruction experiments. By adding cultured virus particles to uninfected human plasma or mother's milk, we reconstructed samples with known RNA copy contents (17) prior to quantification by the Rainbow assay. We used viral isolates that had previously been quantified by electron microscopy to reconstruct samples with virus concentrations spanning a range of 2 to 6 $\log_{10}$. The virus-spiked samples were subsequently processed in two different ways, either directly dissolved in lysis buffer or laid as spots on blotting paper. Figure 1 shows the correlation of the viral RNA measurements generated by the two formulations (virus in plasma or in milk subsequently dissolved in lysis buffer or spot dried on paper). Regardless of whether human plasma or mother's milk was used as a carrier prior to the RNA isolation, there was a high correlation between the two formulations: the Pearson correlation coefficients were 0.91 for the plasma set processed directly in lysis buffer or via dried spot and 0.93 for the mother's milk set (P was <0.001 for both). A good linear correlation was found between the values we generated with the Rainbow Retina assay and the theoretical values according to the electron microscopy quantification (Pearson correlation coefficient, 0.82; $P < 0.001$ [data not shown]). Furthermore, when we compared the results from the paper blot formulations for plasma and milk (Pearson correlation coefficient, 0.99; $P = 0.001$) a good linearity across the 5-log range ($r^2 = 0.98$) was observed (data not shown).

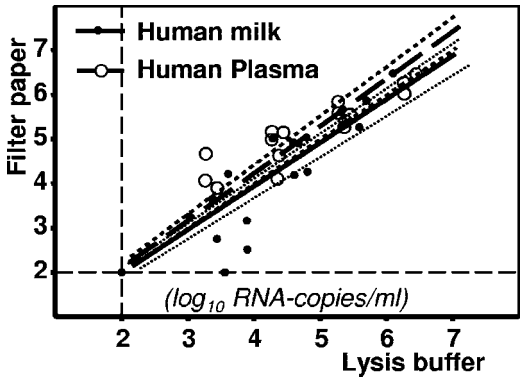

FIG. 1. Correlation between results for filter spots and fluids placed in lysis buffer. Log_{10} measurement of the HIV-1 RNA content in plasma or milk as assessed by the LTR-based, nucleic acid sequence-based amplification (Retina-Rainbow) assay. The virus-containing fluid was dissolved directly in lysis buffer or was initially spotted on filter paper (four spots of 50 μl each) prior to RNA isolation and quantification. The values for the spotted samples are plotted against the values for the corresponding samples dissolved directly in lysis buffer. The linear correlation between the two formulations, together with the 95% confidence interval, is shown. The broken line with the open circles is human plasma-containing virus from a panel of cultured isolates, encompassing subtypes A, B, and CRF01. The solid line with the closed circles is human milk-containing virus from the same panel of cultured isolates.

These results provide evidence that the isolation and quantification of viral RNA from samples collected on blotting paper is a viable alternative to the traditional freezing method for the transportation of clinical samples. This is an important incentive for limited-resource settings, especially under the consideration that monitoring will be a key factor in curtailing the HIV-1 pandemic.

Evaluation of the viral RNA measurements with subtype B viruses. A total of 84 patient samples from The Netherlands, all infected with a subtype B virus, were selected for the comparison of the two formulations, plasma in lysis buffer and plasma on paper. These samples had previously been analyzed with the Amplicor 1.5 assay, and the viral load values ranged from undetectable to $\log_{10}$ 6.5. The RNA copy number values generated by the Retina Rainbow assay for both plasma in lysis buffer and plasma on paper were compared (Fig. 2A). The correlation was high, with a Pearson correlation coefficient of 0.96 ($P < 0.001$).

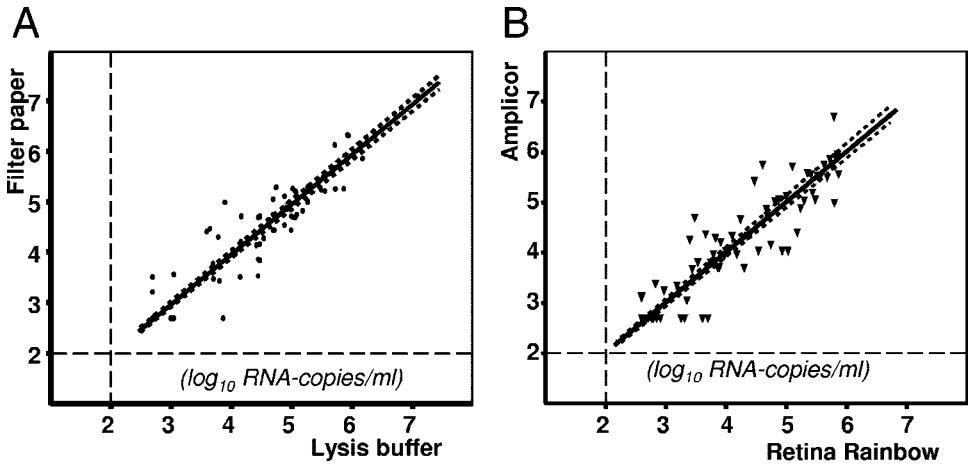

FIG. 2. (A) HIV-1 subtype B-infected patient sera assessed by the Retina-Rainbow (LTR-based nucleic acid sequence-based amplification) assay. Two hundred milliliters of the sera was dissolved in lysis buffer or dried as spots (four spots of 50 μl each) on filter paper prior to RNA isolation and quantification. The $\log_{10}$ RNA level values for the spotted sample were plotted against the corresponding value for the sample dissolved in lysis buffer. The solid lines represent the linear correlation between the two formulations, and the dotted lines represent the 95% confidence interval. (B) Log_{10} RNA level values for the panel of patient samples as measured by the Retina-Rainbow assay (*x* axis) plotted against the $\log_{10}$ RNA values as determined by the Amplicor 1.5 assay.

Since the viral load values measured by the Roche Amplicor 1.5 HIV RNA assay were available to us, we compared them with the results we generated with the Retina Rainbow assay (Fig. 2B). The Amplicor assay has a sensitivity of approximately 50 copies per ml, and the Retina Rainbow assay in its standard format has a sensitivity of 500 copies per ml. This indicates that values of between 50 and 500 copies per ml are unlikely to be detected with the Retina Rainbow assay. The samples that were part of the comparison studies were selected based on the Amplicor results. With this method, there was a one-sided bias in the lower range in favor of the Amplicor assay since all samples were positive with the Amplicor assay and likely negative with the Rainbow assay.

Evaluation of the viral RNA measurements with non-subtype B viruses. Since the vast majority of HIV-1 isolates worldwide encompasses subtypes other than B (non-B), with subtype C being the most prevalent, we wanted to verify that non-B HIV-1 isolates could be detected equally as well as subtype B plasma isolates spotted on paper. For this purpose, we analyzed a set of 19 plasma samples from The Netherlands. The samples were collected from individuals infected with non-B HIV-1 viruses (CRF01 AE, CRF AG, D, and C, including one CRF06-cpx and one K), together with a set of 19 plasma samples collected from HIV-1 subtype C-infected individuals in Ethiopia. The subtype C isolates were detected with a correlation similar to that of the subtype B isolates (Fig. 3).

These samples were analyzed by the Rainbow assay, dissolved directly in lysis buffer, or laid as four 50-μl spots on blotting paper. The correlation between the two formulations was similar to that for the subtype B isolates, with a Pearson correlation coefficient of 0.9164 and a linearity r^2 value of 0.8398.

Viral load measurements for whole-blood dried spots. The purpose of this study was to show that there are simpler, low-cost ways of monitoring HIV-1 viral load measurements. We therefore wanted to study whether whole blood collected from a finger prick, for example, would be feasible for viral load measurements since it is less expensive and laborious than separating plasma from whole-blood specimens. When viral RNA was isolated from whole blood dissolved directly in lysis buffer, the viral load values generated by the Rainbow assay were not reproducible and correlated poorly with values obtained from the plasma from the same donor in lysis buffer. Also, the whole-blood values were systematically lower than those of the plasma samples (data not shown). This indicates that there are factors in whole blood that inhibit efficient isolation and/or amplification of the viral genetic material. Collecting whole blood in lysis buffer would therefore not be a valid method for sample collection for subsequent viral load measurement. Nevertheless, we found that measurements for whole blood after it had been spotted and dried on filter paper correlated well with those for plasma in lysis buffer or plasma spotted on paper (Fig. 4A). The Pearson correlation coeffi-

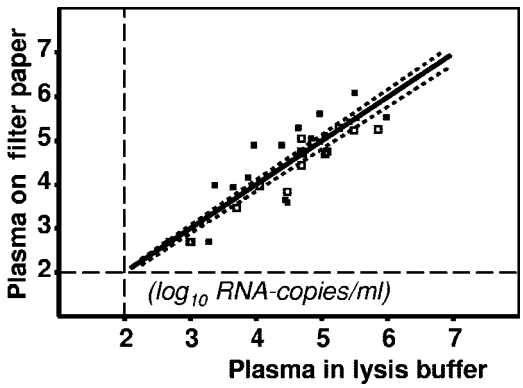

FIG. 3. Sera infected with the HIV-1 non-B subtype was assessed by the Retina Rainbow assay as described in the legend for Fig. 2A. The closed symbols represent 19 subjects sampled in The Netherlands infected with HIV-1 subtypes A, C, D, K, CRF01, CRF02, and CRF06. The open symbols represent 19 HIV-1 subtype C-infected subjects sampled in Ethiopia.

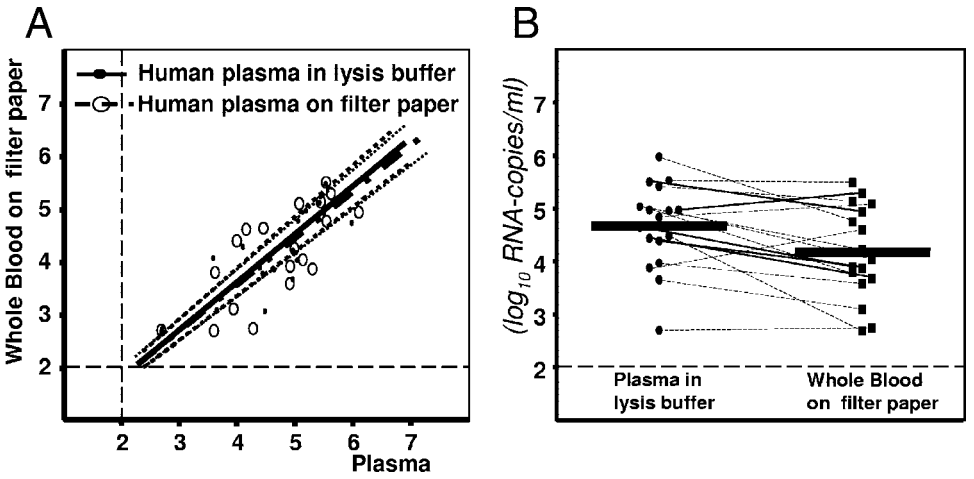

FIG. 4. Assessment of whole blood collected as dried spots on filter paper for the monitoring of HIV-1 viral burden. (A) The $\log_{10}$ RNA values derived from 200 μl blood spotted on filter paper were plotted against the $\log_{10}$ RNA values derived from 200 μl plasma dissolved in lysis buffer (solid line/closed circles) or against the $\log_{10}$ RNA values derived from 200 μl plasma spotted on filter paper (broken line/open circles). (B) A pairwise comparison of the $\log_{10}$ RNA values from whole blood spotted on paper to the $\log_{10}$ RNA values from plasma dissolved in lysis buffer, which is the standard formulation.

cients were 0.7706 for plasma in lysis buffer and 0.8155 for plasma spotted on paper. Despite the fact that we analyzed a set of only 17 samples, we found no significant difference between the values for the samples and the values generated from plasma placed directly in lysis buffer (Fig. 4B). The values generated for the whole-blood spots tended to be slightly lower than those for the plasma samples, but we did not take into account the relative volume differences due to the high red blood cell content of whole blood, which adjustments could be made for.

DISCUSSION

The increased access to life-saving antiretroviral drugs has raised hope for many AIDS patients in resource-limited countries but also raises challenges for appropriate monitoring. The determination of viral endpoints remains the gold standard by which current clinical trials assess HIV treatment efficacy (21, 28). Besides, viral load measurement has an established value in predicting clinical progression to disease (14, 24, 25, 33), in monitoring response to antiretroviral therapy (20, 33), and in assessing the risk of vertical transmission by HIV-seropositive mothers to newborns (16, 27). However, for many areas in the developing world, the prohibitive costs of carrying out viral RNA measurements is limiting and has mandated the search for suitable alternatives validated against this standard (18, 23). The costs associated with carrying out HIV viral load testing in developing nations can be partially decreased by the development of suitable technologies allowing simpler methods of sample collection and preservation. Samples collected in this way may be subsequently transported to centralized testing facilities without the risk of specimen deterioration and without the need for expensive shipping facilities, including those necessary for dry-ice shipping.

The detection of HIV-1 DNA (11, 30) or RNA (3, 10, 36) by using dried blood specimens collected on filter paper has previously been reported. Apart from its use for viral load testing, the dried blood spot (DBS)/dried plasma spot technique of sample collection has been utilized for other purposes, including, due to the small volumes required, conducting studies on perinatal HIV transmission (6, 16). In addition, molecular epidemiology studies for genotyping HIV-1 have also exploited the technique (12, 32).

Recently the DBS technology was extended further to provide a simple method for $CD4^+$ cell enumeration feasible to undertake in the field (29). Using a combined viral load and CD4 measurement for one sample of dried whole blood, such as that from a heel or finger prick, would be optimal for monitoring patients on antiretroviral programs in resource-limited settings like Ethiopia. The current investigation suggests that whole blood spotted on filter paper could be a way of monitoring high viral loads or dramatic therapy failures. Plasma spotted onto filter paper provided a better estimation of HIV-1 viral load in a patient than did plasma in lysis buffer as a reference. Logistically this presents the added step of requiring blood collection equipment for the separation of plasma from whole blood, which may not always be readily available in remote areas. If the feasibility of using dried whole-blood spots for CD4 cell enumeration is demonstrated satisfactorily in Ethiopia, whole-blood spotting onto filter paper immediately after collection could be followed by the spotting of plasma for the viral load testing aspect.

Previously it was shown that the porphyrin moiety of heme from contaminating erythrocytes inhibits nucleic acid amplification by PCR (1), but the blotting of blood on paper could lead to irreversible absorption of inhibiting factors. In our study, while whole blood placed directly in lysis buffer remains unreliable, with blood spotted on paper, a substantial amount of inhibiting factors seemed to be retained on the filter paper during the process of RNA extraction, providing a good readout.

The dried blood and plasma spots used in this study were maintained at room temperature (22 to 24°C) at the site of sample processing, with no perceptible loss of viral RNA com-

pared to that with the standard approach of sample collection, processing of plasma, and storing at −80°C in lysis buffer until analysis. The data were in agreement with findings by other investigators who report remarkable stability of RNA under extreme climate or prolonged storage conditions (9, 15). We found that the correlation was highest with the plasma spot/plasma in lysis buffer comparison, which was also consistent with a high correlation reported by other investigators (10).

We used the long-terminal repeat (LTR)-based Retina Rainbow viral load assay for our study since this assay was previously shown to consistently detect all subtypes of HIV-1, even those that were underdetected using other commercially available tests (13). In addition, it has a format adaptable for use with either dried blood or dried plasma specimens. Parallel testing of plasma samples in our study by using the more established Amplicor assay, together with the Retina Rainbow test, showed that the results of the two assays correlated well. In addition, the comparability of the Retina Rainbow assay with the NucliSENS assay was similarly good (data not shown), with both being reported to efficiently detect HIV-1 subtype C (2, 13), the dominant subtype both worldwide and in our Ethiopian cohort.

Providing an easy way of collecting, storing, and shipping samples can aid in the monitoring of circulating strains of virus in different parts of the globe, usefully contributing to the monitoring of vaccine efforts and therapy distribution to curtail the pandemic. From the present study, it can be concluded that this technology may be used meaningfully in resource-limited settings for the collection of specimens from populations not readily served by laboratories. This technology is feasible to carry out under field conditions without access to refrigeration or electrical power supply, although the results presented here suggest that HIV viral load testing from samples of whole blood as dried spots, in combination with the Retina Rainbow assay, requires somewhat further optimization. DBS/dried plasma spot technology offers the advantages of a stable environment for the analysis and ease of sample collection and shipment with minimal biohazard risks, thereby providing a highly suitable and affordable alternative to the common practice and one that could be exploited for multiple purposes.

ACKNOWLEDGMENTS

This study is part of the Ethio-Netherlands AIDS Research Project (ENARP), a collaborative effort of the Ethiopian Health and Nutrition Research Institute (EHNRI) in Addis Ababa, Ethiopia, the Amsterdam Municipal Health Service (GG/GD), the Department of Human Retrovirology of the Academic Medical Center (University of Amsterdam), and the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (CLB).

ENARP is financially supported by The Netherlands Ministry of Foreign Affairs and the Ethiopian Ministry of Health (MOH) as a bilateral project. This work was partly funded through a grant from the Elizabeth Glaser Pediatric AIDS Foundation (27-PG-51269).

REFERENCES

1. **Akane, A., K. Matsubara, H. Nakamura, S. Takahashi, and K. Kimura.** 1994. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J. Forensic Sci. **39:**362–372.
2. **Alaeus, A., K. Lidman, A. Sonnerborg, and J. Albert.** 1997. Subtype-specific problems with quantification of plasma HIV-1 RNA. AIDS **11:**859–865.
3. **Alvarez-Muñoz, M. T., S. Zaragoza-Rodriguez, O. Rojas-Montes, G. Palacios-Saucedo, G. Vazquez-Rosales, A. Gomez-Delgado, J. Torres, and O. Munoz.** 2005. High correlation of human immunodeficiency virus type-1 viral load measured in dried-blood spot samples and in plasma under different storage conditions. Arch. Med. Res. **36:**382–386.
4. **Beckers, C., C. Cornette, B. Francois, and A. Bouckaert.** 1979. Screening for neonatal hypothyroidism by thyroxine and thyrotrophin radioimmunoassays using dried blood samples on filter paper. Clin. Endocrinol. **10:**567–573.
5. **Behets, F., M. Kashamuka, M. Pappaioanou, T. A. Green, R. W. Ryder, V. Batter, J. R. George, W. H. Hannon, and T. C. Quinn.** 1992. Stability of human immunodeficiency virus type 1 antibodies in whole blood dried on filter paper and stored under various tropical conditions in Kinshasa, Zaire. J. Clin. Microbiol. **30:**1179–1182.
6. **Biggar, R. J., W. Miley, P. Miotti, T. E. Taha, A. Butcher, J. Spadoro, and D. Waters.** 1997. Blood collection on filter paper: a practical approach to sample collection for studies of perinatal HIV transmission. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **14:**368–373.
7. **Boerma, J. T., K. A. Stanecki, M. L. Newell, C. Luo, M. Beusenberg, G. P. Garnett, K. Little, J. G. Calleja, S. Crowley, J. Y. Kim, E. Zaniewski, N. Walker, J. Stover, and P. D. Ghys.** 2006. Monitoring the scale-up of antiretroviral therapy programmes: methods to estimate coverage. Bull. W. H. O. **84:**145–150.
8. **Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
9. **Brambilla, D., C. Jennings, G. Aldrovandi, J. Bremer, A. M. Comeau, S. A. Cassol, R. Dickover, J. B. Jackson, J. Pitt, J. L. Sullivan, A. Butcher, L. Grosso, P. Reichelderfer, and S. A. Fiscus.** 2003. Multicenter evaluation of use of dried blood and plasma spot specimens in quantitative assays for human immunodeficiency virus RNA: measurement, precision, and RNA stability. J. Clin. Microbiol. **41:**1888–1893.
10. **Cassol, S., M. J. Gill, R. Pilon, M. Cormier, R. F. Voigt, B. Willoughby, and J. Forbes.** 1997. Quantification of human immunodeficiency virus type 1 RNA from dried plasma spots collected on filter paper. J. Clin. Microbiol. **35:**2795–2801.
11. **Cassol, S., T. Salas, M. J. Gill, M. Montpetit, J. Rudnik, C. T. Sy, and M. V. O'Shaughnessy.** 1992. Stability of dried blood spot specimens for detection of human immunodeficiency virus DNA by polymerase chain reaction. J. Clin. Microbiol. **30:**3039–3042.
12. **Cassol, S., B. G. Weniger, P. G. Babu, M. O. Salminen, X. Zheng, M. T. Htoon, A. Delaney, M. O'Shaughnessy, and C. Y. Ou.** 1996. Detection of HIV type 1 env subtypes A, B, C, and E in Asia using dried blood spots: a new surveillance tool for molecular epidemiology. AIDS Res. Hum. Retrovir. **12:**1435–1441.
13. **de Baar, M. P., M. W. van Dooren, E. de Rooij, M. Bakker, B. van Gemen, J. Goudsmit, and A. de Ronde.** 2001. Single rapid real-time monitored isothermal RNA amplification assay for quantification of human immunodeficiency virus type 1 isolates from groups M, N, and O. J. Clin. Microbiol. **39:**1378–1384.
14. **de Wolf, F., I. Spijkerman, P. T. Schellekens, M. Langendam, C. Kuiken, M. Bakker, M. Roos, R. Coutinho, F. Miedema, and J. Goudsmit.** 1997. AIDS prognosis based on HIV-1 RNA, $CD4^+$ T-cell count and function: markers with reciprocal predictive value over time after seroconversion. AIDS **11:**1799–1806.
15. **Fiscus, S. A., D. Brambilla, L. Grosso, J. Schock, and M. Cronin.** 1998. Quantitation of human immunodeficiency virus type 1 RNA in plasma by using blood dried on filter paper. J. Clin. Microbiol. **36:**258–260.
16. **Garcia, P. M., L. A. Kalish, J. Pitt, H. Minkoff, T. C. Quinn, S. K. Burchett, J. Kornegay, B. Jackson, J. Moye, C. Hanson, C. Zorrilla, and J. F. Lew, et al.** 1999. Maternal levels of plasma human immunodeficiency virus type 1 RNA and the risk of perinatal transmission. N. Engl. J. Med. **341:**394–402.
17. **Jagodzinski, L. L., D. L. Wiggins, J. L. McManis, S. Emery, J. Overbaugh, M. Robb, S. Bodrug, and N. L. Michael.** 2000. Use of calibrated viral load standards for group M subtypes of human immunodeficiency virus type 1 to assess the performance of viral RNA quantitation tests. J. Clin. Microbiol. **38:**1247–1249.
18. **Jennings, C., S. A. Fiscus, S. M. Crowe, A. D. Danilovic, R. J. Morack, S. Scianna, A. Cachafeiro, D. J. Brambilla, J. Schupbach, W. Stevens, R. Respess, O. E. Varnier, G. E. Corrigan, J. S. Gronowitz, M. A. Ussery, and J. W. Bremer.** 2005. Comparison of two human immunodeficiency virus (HIV) RNA surrogate assays to the standard HIV RNA assay. J. Clin. Microbiol. **43:**5950–5956.
19. **Jourbert, J. J., J. B. Dewar, J. Weinberg, M. De Beer, J. S. Parker, and A. D. Steele.** 2003. A cost-effective particle agglutination assay to detect viral antibodies in dried blood spots—a simple solution to HIV and HCV screening. Cent. Afr. J. Med. **49:**127–130.
20. **Kappes, J. C., M. S. Saag, G. M. Shaw, B. H. Hahn, P. Chopra, S. Chen, E. A. Emini, R. McFarland, L. C. Yang, and M. Piatak, Jr.** 1995. Assessment of antiretroviral therapy by plasma viral load testing: standard and ICD HIV-1 p24 antigen and viral RNA (QC-PCR) assays compared. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **10:**139–149.
21. **Marschner, I. C., A. C. Collier, R. W. Coombs, R. T. D'Aquila, V. DeGruttola, M. A. Fischl, S. M. Hammer, M. D. Hughes, V. A. Johnson, D. A. Katzenstein, D. D. Richman, L. M. Smeaton, S. A. Spector, and M. S. Saag.** 1998. Use of

changes in plasma levels of human immunodeficiency virus type 1 RNA to assess the clinical benefit of antiretroviral therapy. J. Infect. Dis. **177:**40–47.
22. **McCarron, B., R. Fox, K. Wilson, S. Cameron, J. McMenamin, G. McGregor, A. Pithie, and D. Goldberg.** 1999. Hepatitis C antibody detection in dried blood spots. J. Viral Hepat. **6:**453–456.
23. **Mekonnen, Y., N. H. Dukers, E. Sanders, W. Dorigo, D. Wolday, A. Schaap, R. B. Geskus, R. A. Coutinho, and A. Fontanet.** 2003. Simple markers for initiating antiretroviral therapy among HIV-infected Ethiopians. Ethiop. Med. J. **41**(Suppl. 1)**:**61–65.
24. **Mellors, J. W., L. A. Kingsley, C. R. Rinaldo, Jr., J. A. Todd, B. S. Hoo, R. P. Kokka, and P. Gupta.** 1995. Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. Ann. Intern. Med. **122:**573–579.
25. **Mellors, J. W., C. R. Rinaldo, Jr., P. Gupta, R. M. White, J. A. Todd, and L. A. Kingsley.** 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science **272:**1167–1170.
26. **Mendy, M., G. D. Kirk, M. van der Sande, A. Jeng-Barry, O. A. Lesi, P. Hainaut, O. Sam, S. McConkey, and H. Whittle.** 2005. Hepatitis B surface antigenaemia and alpha-foetoprotein detection from dried blood spots: applications to field-based studies and to clinical care in hepatitis B virus endemic areas. J. Viral Hepat. **12:**642–647.
27. **Mofenson, L. M., J. S. Lambert, E. R. Stiehm, J. Bethel, W. A. Meyer III, J. Whitehouse, J. Moye, Jr., P. Reichelderfer, D. R. Harris, M. G. Fowler, B. J. Mathieson, and G. J. Nemo for the Pediatric AIDS Clinical Trials Group Study 185 Team.** 1999. Risk factors for perinatal transmission of human immunodeficiency virus type 1 in women treated with zidovudine. N. Engl. J. Med. **341:**385–393.
28. **Murray, J. S., M. R. Elashoff, L. C. Iacono-Connors, T. A. Cvetkovich, and K. A. Struble.** 1999. The use of plasma HIV RNA as a study endpoint in efficacy trials of antiretroviral drugs. AIDS **13:**797–804.
29. **Mwaba, P., S. Cassol, R. Pilon, C. Chintu, M. Janes, A. Nunn, and A. Zumla.** 2003. Use of dried whole blood spots to measure $CD4^+$ lymphocyte counts in HIV-1-infected patients. Lancet **362:**1459–1460.
30. **Panteleeff, D. D., G. John, R. Nduati, D. Mbori-Ngacha, B. Richardson, J. Kreiss, and J. Overbaugh.** 1999. Rapid method for screening dried blood samples on filter paper for human immunodeficiency virus type 1 DNA. J. Clin. Microbiol. **37:**350–353.
31. **Parker, S. P., M. B. Taylor, A. E. Ades, W. D. Cubitt, and C. Peckham.** 1995. Use of dried blood spots for the detection and confirmation of HTLV-I specific antibodies for epidemiological purposes. J. Clin. Pathol. **48:**904–907.
32. **Plantier, J. C., R. Dachraoui, V. Lemee, M. Gueudin, F. Borsa-Lebas, F. Caron, and F. Simon.** 2005. HIV-1 resistance genotyping on dried serum spots. AIDS **19:**391–397.
33. **Saag, M. S., M. Holodniy, D. R. Kuritzkes, W. A. O'Brien, R. Coombs, M. E. Poscher, D. M. Jacobsen, G. M. Shaw, D. D. Richman, and P. A. Volberding.** 1996. HIV viral load markers in clinical practice. Nat. Med. **2:**625–629.
34. **Sahlu, T., A. Fontanet, T. Rinke de Wit, T. Messele, R. Doorly, H. Yeneneh, P. Bindels, and R. Coutinho.** 1998. Identification of a site for a cohort study on natural history of HIV infection in Ethiopia. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **17:**149–155.
35. **Sahlu, T., E. Kassa, T. Agonafer, A. Tsegaye, T. R. De Wit, H. Gebremariam, R. Doorly, I. Spijkerman, H. Yeneneh, R. A. Coutinho, and A. L. Fontanet.** 1999. Sexual behaviours, perception of risk of HIV infection, and factors associated with attending HIV post-test counselling in Ethiopia. AIDS **13:** 1263–1272.
36. **Sherman, G. G., G. Stevens, S. A. Jones, P. Horsfield, and W. S. Stevens.** 2005. Dried blood spots improve access to HIV diagnosis and care for infants in low-resource settings. J. Acquir. Immune Defic. Syndr. **38:**615–617.
37. **Stark, K., C. Warnecke, V. Brinkmann, H. R. Gelderblom, U. Bienzle, and G. Pauli.** 1993. Sensitivity of HIV antibody detection in saliva. Med. Microbiol. Immunol. **182:**147–151.
38. **Thomas, C.** 2005. The use and control of heel prick blood samples. Med. Law **24:**259–277.