Dried Blood Spots Perform Well in Viral Load Monitoring of Patients Who Receive Antiretroviral Treatment in Rural Tanzania

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Background. Monitoring of antiretroviral treatment (ART) with human immunodeficiency virus (HIV) viral loads, as recommended in industrialized countries, is rarely available in resource-limited settings because of the high costs and stringent requirements for storage and transport of plasma. Dried blood spots (DBS) can be an alternative to plasma, but the use of DBS has not been assessed under field conditions in rural Africa. The present study investigates the performance of DBS in HIV viral load monitoring of patients who received ART in rural Tanzania.

Patients and Methods. From November 2007 through June 2008, parallel plasma and DBS specimens were obtained from patients who received ART at Haydom Lutheran Hospital in rural Tanzania. DBS specimens were stored at tropical room temperature for 3 weeks before testing with the NucliSENS EasyQ HIV-1 v1.2 assay. Results obtained with DBS were compared with results obtained with use of a gold-standard plasma assay.

Results. Ninety-eight plasma-DBS pairs were compared, and plasma viral loads ranged from <40 to >1,000,000 copies/mL. The correlation between plasma and DBS viral load was strong ($R^2 = 0.75$). The mean difference ($\pm$ standard deviation) was 0.04 ± 0.57 $\log_{10}$ copies/mL, and only 8 samples showed >1 $\log_{10}$ copies/mL difference. HIV type 1 RNA was detected in 7%, 60%, and 100% of DBS specimens with corresponding plasma viral loads of 40–999, 1000–2999, and $\geqslant$3000 copies/mL, respectively.

Conclusions. DBS, in combination with the NucliSENS EasyQ HIV-1 v1.2 asay, performed well in monitoring HIV viral loads in patients who received ART in rural Tanzania, although the sensitivity was reduced when viral burden was low. The use of DBS can simplify virological monitoring in resource-limited settings.

Access to antiretroviral treatment (ART) of human immunodeficiency virus (HIV) infection and AIDS has improved substantially over the past several years in resource-limited settings. By the end of 2007, 3 million people were receiving ART in low- and middle-income countries, which is a >10-fold increase since 2002 [1]. This massive public health operation has been the result of determined political leadership combined with the large-scale production of generic low-cost antiretroviral drugs. Early reports from resource-limited settings have described high levels of adherence to therapy [2] and short-term virological efficacy rates similar to those observed in industrialized countries [3].

Scaling-up of ART has, however, not been accompanied by a similar strengthening of laboratory capacity. In high-income countries, it is standard of care to monitor patients who receive ART by means of HIV viral load measurements and genotypic resistance testing to determine when treatment has failed and when a switch to second-line therapy is needed [4, 5]. These tests require highly specialized and well-equipped laboratories, as well as stringent procedures for storage and

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Table 1. Plasma Viral Load and Corresponding HIV Type 1 RNA Detection Rates in Dried Blood Spots (DBS) among 98 Patients who Received Antiretroviral Treatment at Haydom Lutheran Hospital, Tanzania

Plasma viral load	No. of samples	Detectable in DBS[a]	Detection rate, % (95% CI)
<LLD	33[b]	2	6 (1–20)
40–999 copies/mL	28	2	7 (1–24)
1000–2999 copies/mL	10	6	60 (26–88)
3000–9999 copies/mL	7	7	100 (59–100)
⩾10,000 copies/mL	20	20	100 (83–100)

NOTE. CI, confidence interval; LLD, lower limit of detection.

[a] Any detectable HIV-1 RNA.

[b] <40 copies/mL in 1 patient, <400 copies/mL in 32 patients.

shipment of plasma. In resource-limited settings in which there is limited access to viral load measurements, the World Health Organization (WHO) recommends that treatment failure be assessed by clinical signs or CD4 cell counts [6]. However, clinical symptoms and decreases in CD4 cell counts have limited sensitivity and specificity for the detection of virological failure [7, 8]. Consequently, patients with adequate virological suppression risk being incorrectly classified as having experienced treatment failure and undergoing a premature switch to costly and complex second-line therapy. Furthermore, patients with actual treatment failure risk not being detected, and prolonged exposure to a failing ART regimen leads to the accumulation of drug-resistance mutations and thus jeopardizes future treatment options [9].

Dried blood spots (DBS) have been used for >40 years to screen for metabolic disorders in neonates [10]. The main advantage of DBS over plasma is that DBS can be stored at ambient temperatures for weeks without degradation of RNA [11], thus allowing transport of blood specimens from peripheral clinics to a central laboratory. Previous studies have shown that DBS can be used to reliably measure HIV viral load [11–15]; however, these studies have been performed under standardized conditions in modern laboratories in North America and Europe and may not reflect real-life application in resource-limited settings. In the present study, which was performed under field conditions in rural Tanzania, we used DBS to measure viral load in patients who received ART, and we compared the results with those obtained using a gold-standard plasma assay. If proven to be reliable, this method has the potential to markedly simplify viral load monitoring in peripheral, resource-limited settings.

PATIENTS AND METHODS

Study setting and participants. From November 2007 through June 2008, a cross-sectional virological survey was performed among HIV-infected individuals who received ART at Haydom Lutheran Hospital in northern Tanzania. The HIV program in Haydom has been described in detail elsewhere [16]. All patients who had started ART >6 months before and attended the clinic during the survey period were considered to be eligible for the study. Plasma was obtained to measure HIV viral load, and parallel DBS specimens were prepared to compare results with those obtained from plasma. The study was approved by the relevant authorities in Norway (Regional Committee for Medical Research Ethics) and Tanzania (National Institute for Medical Research), and all patients gave written consent to participate in the study.

Preparation and testing of plasma. Blood samples were collected on plasma preparation tubes by venous puncture and centrifuged within 3 h. Plasma was immediately transferred to sterile plastic tubes and was stored at −20°C for a median of 18 days (range, 1–129 days). The manufacturer's instructions were followed with regard to sample collection and transport. HIV viral load was measured at Muhimbili National Hospital (Dar es Salaam, Tanzania) using the Cobas TaqMan 48 Analyzer (Roche Diagnostics) with a lower limit of detection of 40 copies/mL. However, because of equipment malfunction, 36 of 98 plasma samples included in this study were analyzed with the Cobas Amplicor HIV-1 Monitor, version 1.5 (Roche Diagnostics), with a lower limit of detection of 400 copies/mL. Thirty-two of the 33 samples with undetectable viremia were among these samples, because the samples with negative results were selected towards the end of the study period.

Preparation of DBS. DBS were prepared in parallel with plasma from the same blood specimen. An unknown volume of whole blood from a plasma preparation tube was spotted onto a Whatman 903 filter paper to completely fill the circles. The filter papers were left to air-dry overnight and stored at −20°C in plastic ziplock dispensing bags (purchased locally) with a silica desiccant (Elcon-Broker) for a median of 106 days (range, 0–203 days). Before shipment to the reference laboratory, desiccants were replaced with new ones, after allowing the DBS to equilibrate at room temperature for a minimum of 1 h. During transport, DBS were exposed to tropical room temperature for 20 days. After arrival at Hospital Carlos III (Madrid, Spain), samples were refrozen at −20°C until processing 1 month later. Humidity indicators were not used, but none of the DBS showed evidence of humidity on visual inspection.

Extraction of RNA from DBS. To elute blood from the filter papers, 2 circles of dried blood were cut with scissors and placed in 9 mL of NucliSENS lysis buffer (BioMérieux) for 2 h with gentle rotation. Filter papers were then removed from the tube and the solution containing the eluted material from the DBS was further processed.

Nucleic acid isolation was performed using the Boom method, which is a silica-based technology [17]. Extraction was performed manually using the NucliSENS Isolation Kit (BioMérieux), according to manufacturer's instructions.

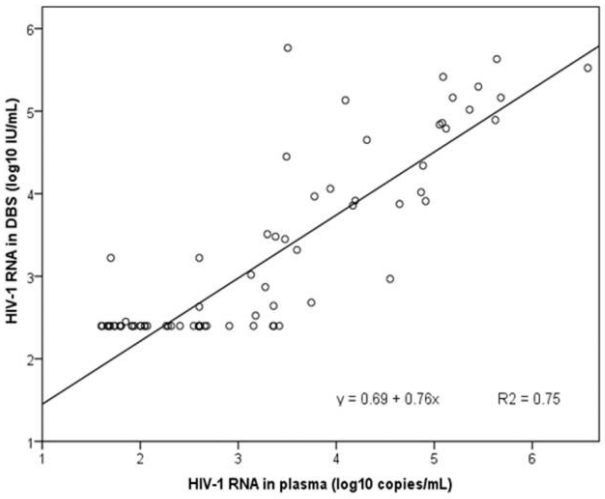

Figure 1. Regression analysis of HIV type 1 (HIV-1) RNA levels in 98 paired plasma and dried blood spot (DBS) specimens from patients who received antiretroviral therapy at Haydom Lutheran Hospital, Tanzania

Viral load quantification was performed using the NucliSENS EasyQ HIV-1 assay, version 1.2 (BioMérieux), which comprises nucleic acid sequence–based amplification (NASBA) and real-time detection using molecular beacons targeting the *gag* gene. This assay is an isothermal transcription-based amplification system designed specifically for RNA detection [18–20]. It has a linear dynamic range of 50–3,000,000 IU/mL when 1 mL of plasma is used [21].

Amount of plasma in DBS. According to the manufacturer, each circle of a Whatman 903 card holds 75–80 μL of whole blood [22]. Two circles of dried blood were used per viral load analysis, and the contribution of hematocrit was corrected for by use of the mean hematocrit in the general population (40%, according to a recent survey in Haydom) [23]. Thus, the amount of plasma in 2 circles of dried blood was estimated by the equation: $2 \times 80\ \mu\text{L} \times 0.60 = 96\ \mu\text{L}$.

Statistics. First, DBS and plasma HIV type 1 (HIV-1) RNA levels were compared by linear regression analysis on $\log_{10}$-transformed data. To describe agreement between plasma and DBS results, the analysis described by Bland and Altman was used [24]. For the purposes of analysis, all negative DBS results were plotted at 250 IU/mL (the lowest positive measurement was at 280 IU/mL), whereas negative plasma results were plotted at the detection limit of the assay (40 and 400 copies/mL for the TaqMan and Amplicor, respectively). Subsequently, we assessed the ability of DBS to identify patients in need of second-line ART, using a threshold of $\geqslant$5000 copies/mL to define major treatment failure warranting the switch to second-line ART in resource-limited settings [6]. Receiver operating characteristic curves were used to identify the optimal corresponding DBS threshold. Positive predictive value and negative predictive value were calculated assuming 10% major virological failure, an approximation from studies conducted in other resource-limited settings [25, 26]. Data were analyzed using SPSS software, version 16.0 for Windows (SPSS), except 95% confidence intervals for proportions, which were calculated with NCSS software, version 2007 (NCSS).

RESULTS

Patient characteristics. Plasma viral loads were obtained from 233 patients, with results ranging from <40 to >1,000,000 copies/mL. Corresponding DBS from 98 patients were sent for analysis; 65 of these patients had detectable viremia and 33 had undetectable viremia in plasma.

Of the 98 patients included in the study, 72 were women, and the median age was 32 years (range, 2–68 years). Median time since ART initiation was 24 months (range, 6–53 months). The initial ART regimen was stavudine-lamivudine-nevirapine in 55 patients, stavudine-lamivudine-efavirenz in 17, zidovudine-lamivudine-nevirapine in 24, and zidovudine-lamivudine-efavirenz in 2 patients. Five patients had chosen to discontinue ART at the time of the survey.

Sensitivity of DBS in samples with low-level viremia. Plasma viral load levels are given in table 1, together with the corresponding detection rates in DBS. Sensitivity of DBS was reduced in samples with low-level viremia. In samples with plasma viral loads of 40–999 copies/mL, only 2 (7%) of 28 had detectable HIV-1 RNA in DBS, whereas 6 (60%) of 10 with

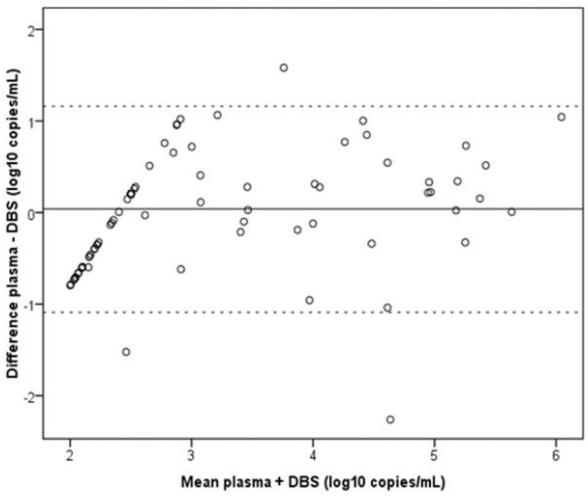

Figure 2. Bland and Altman analysis of agreement between HIV type 1 (HIV-1) RNA levels in 98 paired plasma and dried blood spot (DBS) specimens. The horizontal lines represent the mean difference *(continuous line)* and $\pm$1.96 standard deviations *(dotted lines).*

Table 2. Concordance between Dried Blood Spots (DBS) and a Gold-Standard Plasma Assay in Diagnosing Major Virological Failure

Viral load determined with DBS	No. of patients, by viral load determined by plasma						
	⩾5000 copies/mL	<5000 copies/mL	All	Sensitivity	Specificity	PPV	NPV
⩾5000 IU/mL	21	2	23	0.91	…	0.79[a]	…
<5000 IU/mL	2	73	75	…	0.97	…	0.99[a]
All	23	75	98	…	…	…	…

NOTE. Major virological failure was defined as a viral load ⩾5000 copies/mL. NPV, negative predictive value; PPV, positive predictive value.

[a] Based on 10% prevalence of major virological failure.

plasma viral loads of 1000–2999 copies/mL were HIV-1 RNA detectable in DBS. All 27 samples with plasma viral loads ⩾3000 copies/mL had detectable HIV-1 RNA in DBS.

Two (6%) of 33 samples with undetectable viremia in plasma had HIV-1 RNA detectable in DBS (430 and 1700 IU/mL). These 2 plasma samples were tested with the less sensitive Amplicor assay, and 1 sample actually had detectable HIV-1 RNA, although it was below the dynamic range of the assay.

Correlation between DBS and plasma viral load results. All plasma-DBS pairs were included in a linear regression model. There was a strong correlation between HIV-1 RNA levels in plasma and DBS, with a slope of 0.76 ($P < .001$), an intercept of 0.69, and an R^2 value of 0.75 (figure 1). The corresponding Bland and Altman plot illustrated the agreement between plasma and DBS (figure 2). HIV-1 RNA levels were, on average, slightly higher in plasma than in DBS; the mean difference ($\pm$ standard deviation [SD]) was 0.04 ± 0.57 $\log_{10}$ copies/mL, and 97% of results were within 1.96 SDs (-1.09 to $+1.16$ $\log_{10}$ copies/mL). Eight samples yielded >1 $\log_{10}$ copies/mL difference between plasma and DBS values.

The linear regression analysis was repeated after excluding samples with undetectable viremia. The correlation for 35 plasma-DBS pairs detectable by both assays was still strong, with a slope of 0.67 ($P < .001$), an intercept of 1.26, and an R^2 value of 0.54. HIV-1 RNA levels were, on average, 0.13 ± 0.76 $\log_{10}$ copies/mL higher in plasma than in DBS.

Detection of treatment failure. With plasma viral load results as gold standard, the ability of DBS to detect major virological failure (⩾5000 copies/mL) was assessed using receiver operating characteristic curves. Area under the curve was 0.97, indicating that DBS had strong diagnostic properties. The optimal DBS threshold appeared to be at 5000 IU/mL, giving a sensitivity, specificity, positive predictive value, and negative predictive value of 0.91, 0.97, 0.79 and 0.99, respectively. Ninety-four of 98 plasma/DBS pairs (96%) showed concordant results (table 2).

DISCUSSION

We found a strong correlation between viral load results in plasma and DBS among patients who received ART in rural Tanzania. Previously, laboratory studies from North America and Europe have found that HIV-1 RNA levels in plasma and DBS correlate well [11–15]. These studies, however, were performed under research conditions in well-equipped laboratories and their findings might not be generalizable to basic resource–limited clinics. Only a few studies on the use of DBS for viral load monitoring have been performed in sub-Saharan Africa. A study of patients who received ART in Kampala, Uganda, found that DBS was associated with a large number of false-positive viral load results using the TaqMan assay [27]. On the contrary, a recent publication from Dakar, Senegal, reported an excellent correlation between viral load levels determined in plasma and in DBS, with no significant degradation after 15 days of storage at 37°C, with use of the NucliSENS EasyQ assay [28]. Our study is the first to report results from rural Africa, the setting where DBS can be of most use. Additional studies should be performed to evaluate the performance of DBS in other rural resource-limited settings.

In clinical practice, viral load is measured to detect treatment failure and to assess the need for second-line treatment. Guidelines for North America and Europe define treatment failure as any sustained detectable viremia [4, 5]. However, because standard genotypic-resistance assays have low success rates in specimens with viral loads <500 copies/mL [29], many clinicians prefer to delay treatment modification until the viral load exceeds this level and a resistance profile is available. In resource-limited settings, where the selection of second-line antiretroviral drugs is scarce, the WHO recommends that first-line treatment be conserved as long as the viral load does not exceed 5000–10,000 copies/mL, because the risk of clinical progression is limited when the viral load is below this level [6]. In our study, DBS had reduced sensitivity to detect HIV-1 RNA when viral burden was low (<3000 copies/mL), which has also

been observed by others [11, 14]. However, using a threshold of 5000 copies/mL to define major virological failure, DBS showed high sensitivity, specificity, and predictive values. DBS, therefore, although not sufficiently sensitive to be used with the North American and European guidelines, would be a reliable tool in combination with the WHO guidelines to decide who needs regimen switching in resource-limited settings. Thereby, patients with treatment failure would be detected before developing severe symptoms, and premature changes to second-line regimens would be avoided.

An important question when DBS are used to quantify HIV-1 RNA is whether proviral DNA can contribute to the end product. DBS consist of whole blood, and cell-associated HIV-1 DNA from peripheral blood mononuclear cells will be present in the nucleic acid sample [18]. This can explain why some studies, including the study from Uganda mentioned previously, have obtained false-positive results with DBS using a reverse-transcriptase polymerase chain reaction–based assay [27]. The NASBA method, however, is an isothermal amplification (41°C) that specifically amplifies single-stranded RNA by the use of T7 RNA polymerase. In the absence of heat denaturation at 95°C, double-stranded DNA cannot participate in the amplification process [18–20]. NASBA technology, therefore, is particularly suitable when dried whole blood spots are used instead of cell-free plasma for HIV-1 RNA quantification.

In the present study, we assessed "real-life" performance of DBS in the field: an unknown volume of blood was spotted onto filter papers, storage was in plastic dispensing bags bought locally, and DBS were exposed to tropical room temperature for 20 days and 2 freeze-thaw cycles. Some inaccuracy was introduced by not using quantitative micropipettes, but the manufacturer guarantees that each circle of a Whatman 903 card holds 75–80 μL of blood when saturated [22]. Furthermore, using the population's mean hematocrit instead of individual hematocrit correction also reduced the precision; however, previous studies have found that the amount of plasma in each spot differs by <10% when hematocrit is 30%–50% [10]. Our priority was to use a method simple enough to be feasible under basic field conditions, and further work is warranted to validate simple and user-friendly standard operating procedures for the use of DBS in HIV molecular diagnostics.

Storage of DBS has previously been studied in detail. Fiscus et al [12] found some loss of HIV-1 RNA with storage over a 28-day period, whereas a recent study from Thailand found that viral loads frequently decreased >0.5 $\log_{10}$ in samples stored for >3 months at room temperature or at 37°C [30]. However, in a multicenter study from North America, HIV-1 RNA was stable in DBS stored at room temperature and at −70°C for at least 1 year [11]. In our study, DBS were stored at −20°C before shipment to the laboratory, which would often not be feasible in rural clinics. Nevertheless, subsequent storage at tropical room temperature for 20 days did not seem to affect DBS quality. For clinical monitoring of patients on ART, monthly shipment of DBS to a reference laboratory would probably be appropriate, both to avoid degradation of HIV-1 RNA and to assure timely switch to second-line ART in case of treatment failure.

There were some weaknesses of this study. First, the sample size was limited, and a larger study would have given more exact results. However, our aim was to assess the feasibility and reliability of DBS in a field setting, as other studies have already shown good results under standardized conditions. Second, we used different assays for plasma and DBS viral loads. This was based on availability and might have affected the results. Comparative studies have found good agreement between the Amplicor and NucliSENS EasyQ assays, although NucliSENS EasyQ, on average, gave lower results than did the Amplicor assay [21]. This may have contributed to the loss of sensitivity of DBS in our study. Third, the linear regression model included 33 plasma values and 61 DBS values that were below the lower limit of detection, which might have affected the regression line. However, the strong correlation between plasma and DBS results remained after excluding these samples, although the exact values for the slope and intercept differed from those in the initial model. Finally, the lower limit of detection was set at 250 IU/mL for the NucliSENS EasyQ assay and 400 copies/mL for the Amplicor assay, which might have influenced the mean difference between the assays, but the contribution of this was limited.

To summarize, DBS, in combination with the NucliSENS EasyQ HIV-1 assay, performed well, compared with plasma, in monitoring viral loads in patients who received ART in rural Tanzania. Although sensitivity was reduced in patients with low-level viremia, DBS were sufficiently sensitive to identify patients in need of switching to second-line ART according to the current WHO recommendations. The use of DBS can simplify virological monitoring in resource-limited settings, where logistical constraints often preclude storage and transport of plasma. A monitoring strategy based on DBS, with fixed viral load thresholds to define treatment success and failure, would markedly improve patient care in remote clinics and is currently the only viable option in such settings.

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Potential conflicts of interest. All authors: no conflicts.

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