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Prospective Evaluation of Diagnostic Accuracy of Dried Blood Spots from Finger Prick Samples for Determination of HIV-1 Load with the NucliSENS Easy-Q HIV-1 Version 2.0 Assay in Malawi

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HIV-1 viral load (VL) testing is not widely available in resource-limited settings. The use of finger prick dried blood spot (FP-DBS) samples could remove barriers related to sample collection and transport. Measurement of VL using DBS from EDTA venous blood (VB-DBS) in place of plasma has previously been validated using the NucliSENS Easy-Q HIV-1 v2.0 assay, but information on the accuracy of FP-DBS samples for measuring VL is limited. This prospective study, conducted at Thyolo District Hospital in southern Malawi, compared VL levels measured on FP-DBS samples and plasma using the NucliSENS Easy-Q HIV-1 v2.0 assay. Comparability was assessed by means of agreement and correlation (131 patients with VLs of ≥100 copies/ml), sensitivity, and specificity (612 patients on antiretroviral treatment [ART]). Samples of EDTA venous blood and FP-DBS from 1,009 HIV-infected individuals were collected and prepared in the laboratory. Bland-Altman analysis found good agreement between plasma and FP-DBS VL levels, with a mean difference of −0.35 $\log_{10}$, and 95% limits of agreement from −1.26 to 0.55 $\log_{10}$. FP-DBS had a sensitivity of 88.7% (95% confidence interval [CI], 81.1 to 94.4%) and a specificity of 97.8% (95% CI, 96.1 to 98.9%) using a 1,000-copies/ml cut point and a sensitivity of 83.0% (95% CI, 73.4 to 90.1%) and a specificity of 100% (95% CI, 99.3 to 100%) using a 5,000-copies/ml cut point. This study shows that FP-DBS is an acceptable alternative to plasma for measuring VL using the NucliSENS Easy-Q HIV-1 v2.0. We are conducting a second study to assess the proficiency of health workers at preparing FP-DBS in primary health care clinics.

Quantification of HIV-1 RNA levels (viral load [VL]) in people receiving antiretroviral treatment (ART) is the most sensitive indicator to detect treatment failure (1). Additional benefits of routine VL testing include (i) earlier detection of adherence problems (2), (ii) avoidance of unnecessary switches to second-line antiretroviral drugs (3), (iii) prevention of acquired viral resistance to antiretroviral drugs (4, 5), and (iv) use as a measure in program evaluation (6).

Despite the clinical utility of routine VL monitoring, it is generally not accessible in resource-constrained settings due to a combination of limited capacity for sample transport, a lack of laboratory capacity, and high cost (7–9).

Dried blood spots (DBS) have recently received special attention and are considered an important tool to facilitate scale-up of VL testing in resource-limited settings (10, 11). DBS samples are a practical alternative to plasma for measuring VL in rural settings remote from a laboratory because DBS samples (i) can be obtained from a finger prick, thus doing away with the need for skilled staff to perform phlebotomy and enabling task shifting of sample collection to a lower cadre of health care worker; (ii) have a lower biological risk associated with blood manipulation; (iii) do not require maintenance of the cold chain during transport to the laboratory and can be stored at room temperature without a significant loss of HIV-1 RNA (12); (iv) can be shipped to testing laboratories by regular mail; and (v) can be collected more cheaply than phlebotomy samples when finger prick DBS (FP-DBS) is used (13).

Previous studies have shown acceptable agreement between VL measured on DBS and plasma specimens using the NucliSENS Easy-Q HIV-1 v2.0 (bioMérieux) (14, 15). However, most of the studies have used DBS prepared from EDTA-anticoagulated venous blood, which may restrict sample collection to staff able to perform phlebotomy.

In July 2011, the Malawi Ministry of Health (MOH) released new ART guidelines (16) recommending that VL be measured routinely 6 months and 24 months after starting ART, and thereafter every 2 years. VL monitoring is being introduced gradually, starting in a few pilot districts. Thyolo District was one of the districts chosen to pilot the implementation of VL monitoring.

The purpose of our study was to assess the performance of finger prick DBS (FP-DBS) for measuring VL compared to plasma and venous blood DBS (VB-DBS) specimens.

(Portions of earlier versions of these study results were presented as a poster at the ASLM Conference, December 2013, Cape Town, South Africa, and the 20th Conference on Retroviruses and Opportunistic Infections [CROI], Atlanta, GA, 3 to 6 March 2013).

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MATERIALS AND METHODS

Ethics. The study protocol was approved by the Thyolo District Research committee, and ethics approval was obtained from the Malawi National Health Sciences Research Committee (NHSRC) and the Médecins Sans Frontières (MSF) External Review Board. All participants provided written informed consent to participate in the study.

Study setting. The study took place at Thyolo District Hospital in the MSF-supported VL laboratory that opened in 2011. The laboratory is enrolled in an HIV-1 VL proficiency test program run by the US Centers for Disease Control and Prevention (CDC). Results of the proficiency testing were within the acceptable levels of performance throughout the study period.

Study design. The study was a prospective diagnostic accuracy study. Samples were collected from April 2011 to September 2012.

Participants. All participants were HIV infected. The participants comprised patients requiring VL testing for suspicion of clinical failure or for routine monitoring, according to the criteria stipulated in the Malawian ART guidelines (16), and ART-naive pregnant women enrolling in the prevention of mother-to-child transmission (PMTCT) program. Participants were recruited at Thyolo District Hospital from the ART clinic, the medical ward, and the antenatal clinic. Participants were eligible if they were HIV infected and at least 15 years of age. We did not record the refusal rate.

In order to be eligible for inclusion in the analyses of sensitivity and specificity, participants had to have been taking ART for at least 6 months. In order to have samples with VLs close to cut points used in the sensitivity and specificity analyses, we set a target of recruiting a minimum of 50 participants with a plasma VL in the 100- to 10,000-copies/ml range, but we did not set a target sample size for the number of participants with a plasma VL of <100 copies/ml or >10,000 copies/ml.

In order for samples to be included in the analyses of accuracy, both the plasma sample and the corresponding DBS sample were required to have a VL in the 100 copies/ml to 100,000 copies/ml range. Samples with a VL of <100 copies/ml were excluded because 100 copies/ml was the threshold for quantification of VL in DBS samples, and samples with a VL of >100,000 copies/ml were excluded in order to test samples within a defined range.

Laboratory procedures. (i) Sample collection and storage. Sample collection, processing, and testing were done by three laboratory technologists trained in molecular biology. Each participant provided one 4-ml tube of EDTA-anticoagulated venous blood obtained by phlebotomy and five drops of blood obtained by finger prick. FP-DBS samples were prepared by spotting 50 μl of capillary blood onto each printed circle of Whatman 903 Protein Saver cards (Whatman, Maidstone, Kent, United Kingdom) using a graduated capillary pipette (Microsafe Pastette; Alpha Laboratories, Eastleigh, Hampshire, United Kingdom). VB-DBS samples were prepared by spotting 50 μl of EDTA-anticoagulated venous blood onto each printed circle of Whatman 903 Protein Saver cards using an adjustable automatic pipette. DBS samples were placed in a rack and dried overnight. Each DBS sample was packed individually in a plastic zip-lock bag with two desiccant sachets and a humidity indicator card and stored at room temperature (20 to 25°C) until testing. The remaining EDTA blood was centrifuged for 10 min at 3,000 × *g* within 3 h of specimen collection, and plasma was immediately transferred to a sterile polypropylene tube and stored at −20°C until testing.

(ii) Nucleic acid extraction and amplification. Lysis and RNA extraction of EDTA plasma samples (input volume of 0.5 ml) was performed onboard the NucliSENS EasyMAG (bioMérieux, Craponne, France) according to the manufacturer's instructions in a preamplification room that complied with international standards for a PCR laboratory. DBS samples were lysed off board. Two spots of 50 μl were cut out with a pair of scissors. A new pair of scissors was used for each new specimen and they were decontaminated before reuse with a solution containing 70% ethanol. The two spots were then transferred with the same scissors and incubated in 2 ml of NucliSENS lysis buffer (bioMérieux) in 15-ml tubes on a roller mixer for 30 min at room temperature. RNA extraction from the eluted and lysed blood was subsequently completed using the NucliSENS easyMAG.

Extracted RNA (15 μl eluate) was amplified, detected, and quantified using the NucliSENS Easy-Q HIV-1 v2.0 assay (bioMérieux). Technologists were blind to the VL results of the comparison samples. DBS values were corrected for hematocrit using an estimated population's mean hematocrit of 0.40, assuming a plasma volume in two 50-μl DBS circles of 60 μl.

The assay comprises nucleic acid sequence-based amplification (NASBA) and real-time detection targeting the *gag* gene. The assay is isothermal (41°C) and is therefore strictly RNA specific. It has a linear dynamic range of 18 to 15,000,000 copies/ml when 0.5 ml of plasma is used and a linear dynamic range of 500 to 21,000,000 copies/ml when two DBS spots (0.1 ml of whole blood) are used.

Internal quality control was carried out using a negative control (lysis buffer) and an HIV-1 RNA-positive control (ACCURUN 315; SeraCare Life Sciences) in each run to verify product performance.

(iii) Precision of DBS. To assess repeatability (intra-assay variation) and reproducibility (interassay variation), at least 10 samples of each type (plasma, FP-DBS, and VB-DBS) with a detectable VL were run in duplicate by the same laboratory technologist using the same reagent lot. For repeatability, samples were tested the same day in the same run, and for reproducibility, samples were tested on different days.

Statistical analysis. All analyses were performed using Stata version 11 (StataCorp, College Station, TX). Pairwise comparisons were made of VL measured on finger prick DBS and venous DBS versus plasma. Plasma is considered the reference standard for viral load testing. Two-sample tests of proportions were used to assess the statistical significance of differences in paired VL results. All tests of statistical significance were two-sided with an alpha value of 0.025.

We performed Bland-Altman analyses of $\log_{10}$-transformed VL values to assess agreement between DBS and plasma VL results. The mean bias (difference) was calculated by subtracting the plasma VL value from the DBS VL value. We used linear regression of $\log_{10}$-transformed VL values and calculated Pearson's and Spearman correlation coefficients to assess correlations between DBS and plasma VL results. For the analyses of agreement and correlation, sample pairs were included in the analysis only if both samples had a VL in the 100 copies/ml to 100,000 copies/ml range. Samples with plasma VLs of <100 copies/ml were excluded from the analyses of agreement and correlation because 100 copies/ml was the lowest limit of VL quantification using DBS samples.

We assessed reproducibility and repeatability by calculating the mean, standard deviation (SD), and bias (difference) using $\log_{10}$-transformed VL values and grouping the results by VL values of <3,000 copies/ml and ≥3,000 copies/ml for analysis. Levels of acceptable variability were determined as ≤0.19 standard deviation (SD) and ≤0.3 bias, as described previously (17, 18).

In the sensitivity and specificity analyses, we excluded samples from patients who had been on ART for <6 months because VL testing is used to monitor response to ART. We used cut points of 1,000 copies/ml, 3,000 copies/ml, and 5,000 copies/ml because these cut points are given in WHO guidelines and are the most widely used in clinical decision-making in resource-limited settings.

As we did not test all DBS samples if the VL in the corresponding plasma sample was <1,000 copies/ml, our study data were unable to provide generalizable estimates of positive predictive value (PPV) or negative predictive value (NPV). In order to assist with the clinical interpretation of results, we calculated PPV and NPV using the point estimates of sensitivity and specificity from the analysis of our study data, and assumed a varying prevalence of a VL above the 1,000-copies/ml, 3,000-copies/ml, and 5,000-copies/ml cut points. The prevalence levels were chosen to represent the range likely to be encountered in clinical practice.

PPV was calculated using the formula (sensitivity × prevalence)/(sensitivity × prevalence) + [(1 − specificity) × (1 − prevalence)]. NPV was

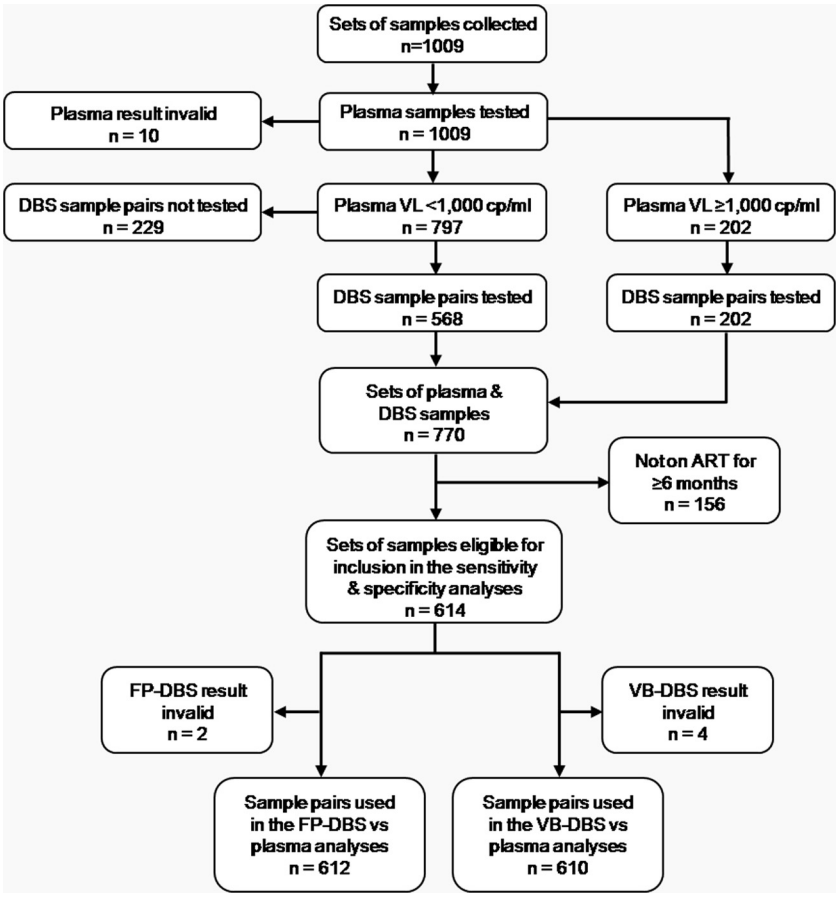

FIG 1 Flow diagram showing the number of participants satisfying the inclusion criteria for the sensitivity and specificity analyses.

calculated using the formula [specificity × (1 − prevalence]/[specificity × (1 − prevalence)] + [(1 − sensitivity) × prevalence)].

RESULTS

Participants. From April 2011 to September 2012, a total of 1,009 participants were recruited into the study. After excluding 10 sets of samples with invalid plasma VL results, and 229 of 797 sets of samples with a plasma VL below 1,000 copies/ml and the corresponding DBS samples not tested in order to overrepresentation of patients with a high viral load, we had 770 participants with all 3 types of sample (plasma, VB-DBS, and FP-DBS) tested. Applying the additional prespecified exclusion criteria resulted in 614 sets of samples being used for the clinical analyses (Fig. 1), and 131 pairs of FP-DBS and plasma samples being used in the agreement analyses (Fig. 2). The 770 participants had a median age of 37 years (interquartile range [IQR], 22 to 52), 65.7% were female, and 57.1% had an undetectable plasma VL. Of those with a detectable plasma VL, the median VL was 9,250 copies/ml (range, 20 to 11,000,000 copies/ml).

Of the 770 patients whose sets of samples were tested, 614 patients (79.7%) had been on ART for 6 months or longer. Of these patients, 2 had an invalid FP-DBS result, and 4 had an invalid VB-DBS result. The 614 patients who had been on ART for 6 months or longer had a median age of 37 years (range, 15 to 74 years), 63.2% were female, and 69.2% had an undetectable VL. Of those with a detectable plasma VL, the median VL was 6,000 copies/ml (range, 20 to 11,000,000 copies/ml). Further details of the participant characteristics are provided in Table 1.

Sensitivity and specificity of DBS. The sensitivities and specificities of VLs measured on DBS samples relative to plasma are shown in Table 2. The sensitivities of FP-DBS were 88.7%, 84.9%, and 83.0% at 1,000-, 3,000-, and 5,000-copies/ml cut points, respectively. The specificities of FP-DBS were 97.8%, 99.8%, and 100% at 1,000-, 3,000-, and 5,000-copies/ml cut points, respectively. Similar results were obtained for VB-DBS samples. There were no statistically significant differences in sensitivities or specificities of FP-DBS and VB-DBS specimens at either of the VL cut points ($P = 0.52$ for sensitivity and $P = 0.54$ for specificity at the 1,000-copies/ml cut point, and $P = 0.29$ for sensitivity and $P = 0.30$ for specificity at the 5,000-copies/ml cut point).

PPV and NPV of DBS. Using a cut point of 1,000 copies/ml, the estimated PPV of DBS ranged from 65.5% if 5% had a true VL of ≥1,000 copies/ml to 92.3% if 25% had a true VL of ≥1,000 copies/ml. Using a cut point of 3,000 copies/ml, the PPV of DBS ranged from 92.0% if 5% had a true VL of ≥1,000 copies/ml to 98.2% if 25% had a true VL of ≥1,000 copies/ml. Using a cut point of 5,000 copies/ml, the PPV of DBS ranged from 97.8% if 5% had a true VL of ≥5,000 copies/ml to 99.5% if 25% had a true VL of ≥5,000 ml. The NPV was >96% at all VL cut points for all the assumed proportions of patients with a true VL above the cut point (Table 3). In general, the PPV was directly affected by the

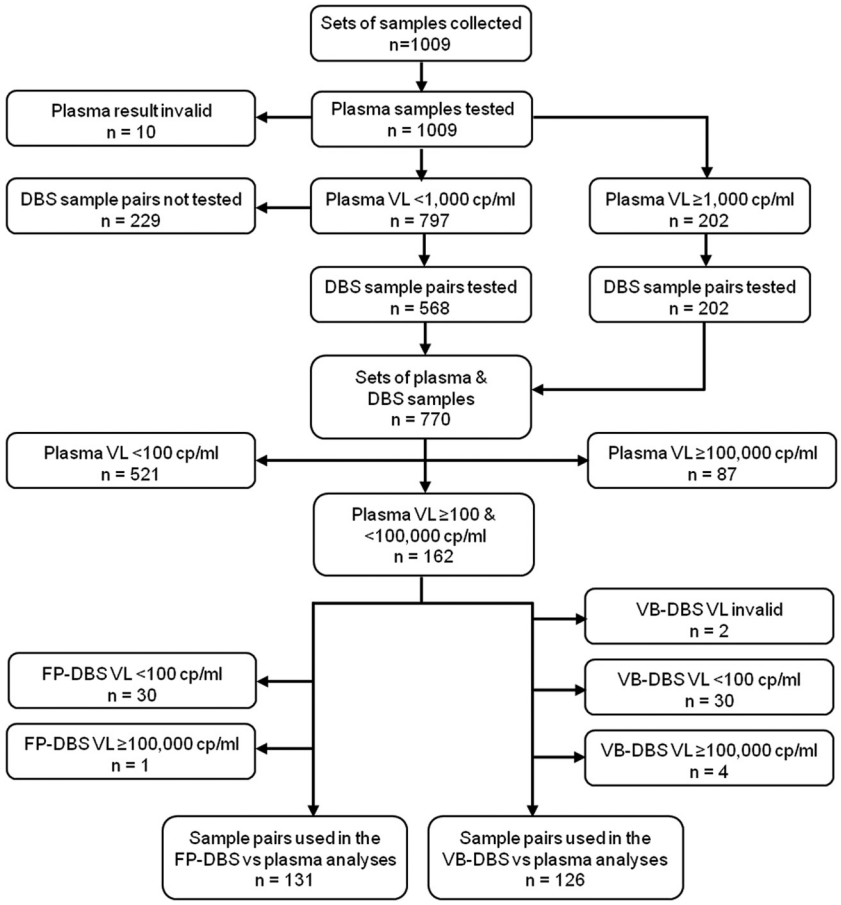

FIG 2 Flow diagram showing the number of participants satisfying the inclusion criteria for the agreement and correlation analyses.

prevalence of virological failure, and using DBS with a 5,000-copies/ml cut point resulted in a higher PPV than using DBS with 1,000- and 3,000-copies/ml cut points.

Plasma viral load levels and corresponding detection rates in DBS samples. Table 4 shows the proportion of DBS samples according to the plasma viral load level. In general, the proportion of DBS samples with a detectable VL increased progressively with increasing plasma VL levels. Only 53.2% and 47.8% of FP-DBS and VB-DBS generated detectable VL results when the plasma VL was <1,000 copies/ml.

Among the undetectable plasma viral loads, 14.2% and 13.3% of the FP-DBS and VB-DBS generated quantifiable VL results (range, 165 to 3,940 copies/ml; median, 333 copies/ml) and only 1.5% and 2.8% of FP-DBS and VB-DBS had VLs of ≥1,000 copies/ml.

Correlation and agreement between DBS and plasma viral load levels. The linear regression analysis of FP-DBS VL versus plasma VL ($n = 130$) found a slope of 0.75, an intercept of 0.63, an R^2 value of 0.66, and a Spearman's rho value of 0.82 ($P < 0.05$) (Fig. 3A). The corresponding Bland-Altman analysis found a negative mean (±SD) difference (bias) of −0.35 (±0.46) and 95% limits of agreement of −1.26 to 0.56 $\log_{10}$ (Fig. 3B), with 61.5% of the paired VL results within 0.5 $\log_{10}$ and 91.5% within 1.0 $\log_{10}$.

The linear regression analysis of VB-DBS VL versus plasma VL ($n = 126$), found a slope of 0.82, an intercept of 0.49, an R^2 value of 0.67, and a Spearman's rho value of 0.81 ($P < 0.05$) (Fig. 3C). The corresponding Bland-Altman analysis showed a negative mean (±SD) difference (bias) of −0.22 ± 0.46 and 95% limits of agreement of −1.13 to 0.69 $\log_{10}$ (Fig. 3D), with 71.4% of the paired VL results within 0.5 $\log_{10}$ and 93.7% within 1.0 $\log_{10}$.

The linear regression analysis of FP-DBS versus VB-DBS ($n = 158$), found a slope of 0.85, an intercept of 0.44, an R^2 value of 0.77, and a Spearman's rho value of 0.89 ($P < 0.05$) (Fig. 3E). The corresponding Bland-Altman analysis showed a negative mean (±SD) difference (bias) of −0.14 ± 0.35 and 95% limits of agreement of −0.90 to 0.62 log10 (Fig. 3F).

Precision of VL measurement in DBS samples. For VL values of ≥3,000 copies/ml, FP-DBS and VB-DBS samples had SD, bias, and intra-assay variability within acceptable limits, according to the predetermined criteria (Table 5). Similarly, for VL values of <3,000 copies/ml, FP-DBS and VB-DBS samples had an SD and bias within acceptable limits. We did not assess intra-assay variabilities of FP-DBS samples of <3,000 copies/ml due to the difficulty obtaining samples within this range and performing repeat testing on the same day.

DISCUSSION

VL testing is recommended by the WHO as the best diagnostic tool to monitor patients on ART because of its clinical superiority for assessing response to ART and adherence counseling interventions, and for the early detection of treatment failure relative to immunological and clinical criteria (1). Following WHO recom-

TABLE 1 Clinical and demographic characteristics of study participants

Characteristic	All patients with DBS samples tested ($n = 770$) (%)	Patients on ART for ≥6 mo ($n = 614$) (%)
Female	65.7	63.2
Age		
15–25 yr	8.1	5.9
25–35 yr	33.4	29.9
35–45 yr	32.2	35.0
≥45 yr	26.2	29.1
On ART for ≥6 mo	79.7	100.0
Place of recruitment		
HIV clinic	70.4	77.9
Hospital ward	9.6	9.8
Antenatal clinic	20.0	12.4
Reason for VL testing		
Routine	58.4	73.3
Targeted	21.3	26.7
Study (ART naive)	20.3	0.0
Plasma VL		
Undetectable	57.1	69.2
Detectable, not quantifiable	2.6	2.9
20–999 copies/ml	14.0	10.6
1,000–4,999 copies/ml	4.2	2.9
5,000–9,999 copies/ml	2.1	1.1
10,000–99,999 copies/ml	8.7	5.5
≥100,000 copies/ml	11.3	7.7

TABLE 3 Positive predictive values and negative predictive values of DBS according to the prevalence of elevated viral loads in the referent samples

Viral load cut point (copies/ml)	Sensitivity (%)	Specificity (%)	Plasma viral load ≥ cut point (%)	PPV (%)	NPV (%)
1,000	90.1	97.5	5.0	65.5	99.5
			10.0	80.0	98.9
			15.0	86.4	98.2
			20.0	90.0	97.5
			25.0	92.3	96.7
3,000	87.6	99.6	5.0	92.0	99.3
			10.0	96.1	98.6
			15.0	97.5	97.9
			20.0	98.2	97.0
5,000	85.8	99.9	5.0	97.8	99.1
			10.0	98.9	98.1
			15.0	99.3	97.1
			20.0	99.5	95.9

mendations, several African countries with generalized HIV epidemics have recently introduced policies advocating routine VL monitoring of patients on ART, but implementation of these policies has been hampered by the difficulty of obtaining and transporting plasma samples from remote clinics to viral load laboratories (19). The operational advantages of using DBS instead of plasma to simplify sample collection and transport makes DBS a key tool to scaling-up VL testing in resource-limited settings.

In this study, we found that FP-DBS and VB-DBS had comparable diagnostic accuracies, but FP-DBS has the advantage over VB-DBS that it can be prepared without requiring phlebotomy, thus giving an opportunity to task-shift sample collection to a lower cadre of health care worker.

Previous studies have assessed the diagnostic accuracy of DBS for VL testing, but most studies used DBS samples prepared from EDTA whole blood (10, 11) and did not assess the diagnostic accuracy of viral load measured on FP-DBS samples. One published study that evaluated FP-DBS for VL measurement using the NASBA technique had a small sample size ($n = 51$) and included only ART-naive patients in the study (20), and another study evaluated VL measured on heel-prick DBS samples of infants using the Amplicor assay (21). Both studies found a strong correlation between VL measured on DBS samples and plasma, but as neither study assessed the accuracy of VL measured on capillary DBS samples of patients on ART, they do not provide information on the suitability of FP-DBS for VL monitoring under conditions of routine use.

Although in our study DBS samples were corrected assuming a hematocrit of 40%, the agreement analysis found that DBS VL levels were consistently lower than plasma VL levels. This may have been partly due to the incomplete elution of the blood from the filter paper. Other studies using NASBA platforms have also found DBS VL levels consistently lower than plasma VL levels (-0.31 $\log_{10}$ [22], -0.44 $\log_{10}$ [23], and -0.36 $\log_{10}$ [14]). The underquantification of VL measurement on DBS specimens could be improved by introducing a correction factor to account for the loss of blood during elution (23).

The finding of a lower probability of VL detection in DBS samples if the plasma VL was ≤1,000 copies/ml, and a lower sensitivity of DBS with plasma VLs in the lower range, is consistent with some other studies (15, 22, 24), but contrasts with another reported study (14) with higher VL detection probabilities in DBS specimens (92% with a VL of 600 copies/ml and 96% with a VL of 850 copies/ml). In this last study, however, DBS samples were prepared from HIV-1 RNA-spiked EDTA blood, which could explain the higher detection rates obtained.

The PPV of DBS is affected by the prevalence of an elevated VL

TABLE 2 Sensitivities and specificities of FP-DBS and VB-DBS for measuring viral loads at different cut points

Comparison	n	Data (% [95% CI]) for viral load cut points (copies/ml) of: 1,000 Sensitivity	1,000 Specificity	3,000 Sensitivity	3,000 Specificity	5,000 Sensitivity	5,000 Specificity
FP-DBS vs plasma	612	88.7 (81.1–94.0)	97.8 (96.1–98.9)	84.9 (76.0–91.5)	99.8 (98.9–100)	83.0 (73.4–90.1)	100 (99.3–100)
VB-DBS vs plasma	610	91.4 (84.4–96.0)	97.2 (95.4–98.5)	90.2 (82.2–95.4)	99.4 (98.3–99.9)	88.5 (79.9–94.3)	99.8 (98.9–100)
FP-DBS vs VB-DBS	608	83.6 (75.4–90.0)	97.6 (95.8–98.7)	90.7 (82.5–95.9)	99.6 (98.6–100)	92.3 (84.0–97.1)	99.8 (99.0–100)

TABLE 4 Proportions of DBS samples with a detectable viral load according to plasma viral load level

Plasma characteristic	FP-DBS detectable		VB-DBS detectable	
	No. detected/no. tested	% (95% CI)	No. detected/no. tested	% (95% CI)
Target not detected	62/438	14.2 (10.9–17.4)	58/437	13.3 (10.2–16.8)
<20 copies/ml	1/20	5.0 (0.1–24.9)	3/20	15.0 (3.2–37.9)
20–99 copies/ml	14/61	23.0 (13.2–35.5)	10/61	16.4 (8.2–28.1)
100–999 copies/ml	25/47	53.2 (38.1–67.9)	22/46	47.8 (32.9–63.1)
1,000–4,999 copies/ml	27/32	84.4 (67.2–94.7)	27/32	84.4 (67.2–94.7)
5,000–9,999 copies/ml	15/16	93.8 (69.8–99.8)	15/15	100.0 (78.2–100)
10,000–99,999 copies/ml	65/67	97.0 (89.6–99.6)	66/67	98.5 (92.0–100)
≥100,000 copies/ml	86/87	98.9 (93.8–100)	86/87	98.9 (93.8–100)
All samples	295/768	38.4 (35.0–42.0)	287/765	37.5 (34.1–41.0)

and the clinical cut point used, with PPV being greater the higher the proportion of samples with VL over the cut point. As PPV is directly affected by the prevalence of virological failure, using DBS with a 5,000-copies/ml cut point will result in a higher PPV. This needs to be considered at the time of implementing DBS, for example, the prevalence of an elevated VL, and the VL levels are likely to be higher if targeted VL testing is done because of suspected treatment failure than if VL testing is done for monitoring purposes. A systematic review of VL in patients in sub-Saharan African ART programs (6) found similar prevalences of virological failure (median, 14% at a 1,000-copies/ml cut point and 10% at a 5,000-copies/ml cut point) as those found in our study.

It has been shown, however, that drug resistance can develop in patients with a sustained VL of >1,000 copies/ml (25). Although current WHO guidelines recommend using a threshold of 1,000 copies/ml to define virological failure, they recommend the use of a cut point of 5,000 copies/ml for programs testing VL in DBS samples (1). The results of this study support this recommendation.

We found that about 13% of DBS samples generated detectable VLs (>100 copies/ml) when the corresponding plasma sample was undetectable (Table 2). Ninety-two percent of these detectable samples, however, showed a low-level viremia (<1,000 copies/ml) and, therefore, would have a negligible impact in clinical decision-making when using a cut point of ≥1,000 copies/ml. Other studies that have used the NASBA technology have also reported false-positive rates of 6% (15), 8% (26), and 8.5% (14). This might be attributed to the contribution of intracellular RNA present in white blood cells in whole blood. Another explanation could be that there was cross-contamination during testing; however, we think this is unlikely because steps to mitigate cross-contamination were taken during the manual excision of filter paper, such as the disinfection of each pair of scissors for every new specimen; in addition, all negative controls run during viral load testing were also negative. DBS-to-DBS carryover has been reported to be minimal in previous studies using either manual or automated cutting approaches (27, 28). Perforated filter paper is now commercially available and eliminates the need for manual cutting with scissors and therefore greatly reduces the likelihood of cross-contamination. In another study performed by us (unpublished data) we used perforated filter paper (Munktell TFN; Lasec SA PTY, Ltd.) for viral load testing with the NucliSENS assay, and the percentage of false-positive results compared to samples with an undetectable viral load in plasma remained similar to this study (11%), which suggests that intracellular RNA may be the cause of false positives rather than DBS cross-contamination.

Our study found that both types of DBS samples gave reproducible results at both low and high VLs, with SDs of <0.15 $\log_{10}$. This is consistent with findings from other studies. One study found an intra-assay standard deviation (SD) variation of 0.15 $\log_{10}$ when VL was measured on plasma samples using NASBA technology (29), and another study (30) found a mean SD of 0.12 $\log_{10}$.

In our study, the finding of a specificity of >96% for both types of DBS samples at both cut points is also consistent with findings from previous studies using DBS with the NASBA technology. One study found a specificity of 95.8% (23) and another study found a specificity of 98.1% (26), both using a 1,000-copies/ml cut point to determine an elevated VL. With the NASBA technology, the complete amplification reaction is performed at the predefined temperature of 41°C (isothermal amplification); at this temperature, double-stranded DNA cannot be amplified, making the assay almost strictly RNA specific. In contrast, studies that have tested VL on DBS samples using reverse transcription–quantitative real-time PCR (RT-qPCR) technology have found specificity levels of 77.8% (31), 58.3% (23), and 73.5% (32). RT-PCR methods amplify total nucleic acids (RNA and DNA) from peripheral blood mononuclear cells (PBMCs) leading to false-positive results mainly due to the contribution of proviral DNA. The lower specificity of VL measured on DBS with RT-PCR assays relative to NASBA assays has important clinical implications, as falsely elevated VL results can lead to clinical mismanagement, specifically misdiagnosing patients as having virological failure and making unnecessary switches to second-line ART.

This study has several strengths. It was carried out in the context of a rural district laboratory in Malawi and, therefore, assessed the use of DBS samples for VL testing under routine field conditions. The large sample size relative to other studies allowed more precise estimates of sensitivity and specificity. The exclusion of ART-naive participants and participants who had been on ART for less than 6 months from sensitivity and specificity analyses make our estimates applicable to the type of patients in resource-limited settings who require undergoing VL testing in accordance with WHO guidelines. The NucliSENS Easy-Q HIV-1 v2.0 is one of the most frequently used of the commercially available VL assays, and unlike RT-PCR assays, it is RNA specific due to its isothermal amplification, making it a suitable platform for the appli-

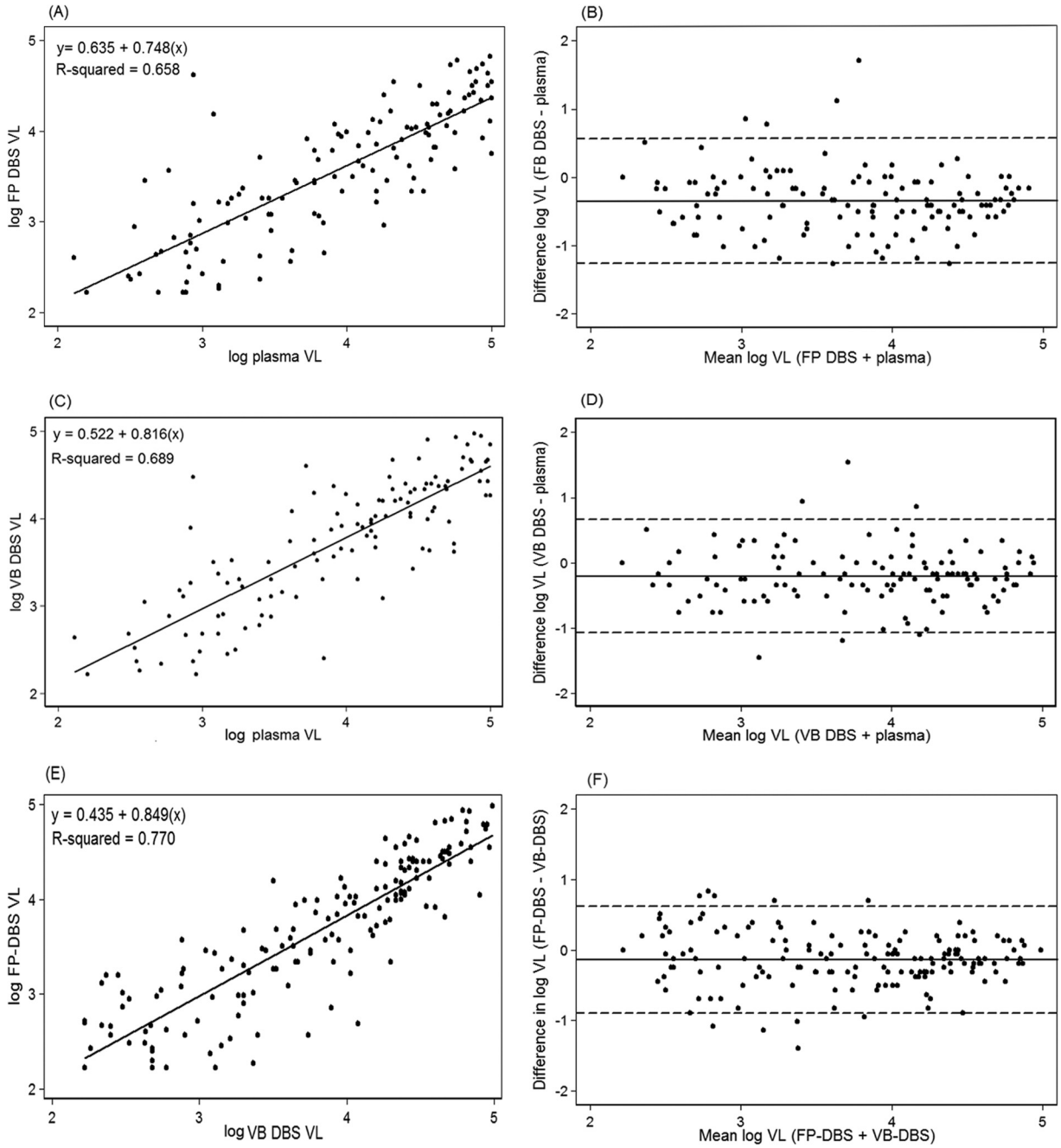

FIG 3 Comparison of HIV-1 viral loads in DBS and plasma. All values are expressed in $\log_{10}$ copies/ml. (A) Correlation of finger prick DBS (FP-DBS) (*y* axis) and plasma (*x* axis) in 130 paired samples. DBS values were corrected for hematocrit (40%) ($R^2 = 0.63$, Spearman rho = 0.82). (B) Corresponding scatter plot of the mean of the differences between FP-DBS and plasma (difference [mean ± SD], −0.35 [continuous line] ± 0.46; 95% limits of agreement [dotted lines], −1.26 to 0.56). (C) Correlation between venous blood DBS (VB-DBS) (*y* axis) and plasma (*x* axis) in 126 paired samples. DBS values were corrected for hematocrit (40%) ($R^2 = 0.67$, Spearman's rho = 0.81). (D). Corresponding scatter plot of the mean of the differences between VB-DBS and plasma (difference [mean ± SD], −0.22 ± 0.46; 95% limits of agreement, −1.13 to 0.69 $\log_{10}$). (E) Correlation of finger prick DBS (FP-DBS) (*y* axis) and venous blood DBS (VB-DBS) (*x* axis) in 158 paired samples. DBS values were corrected for hematocrit (40%) ($R^2 = 0.77$, Spearman rho = 0.89). (F) Corresponding scatter plot of the mean of the differences between FP-DBS and VB-DBS (difference [mean ± SD], −0.14 ± 0.35; 95% limits of agreement, −0.90 to 0.62 $\log_{10}$).

cation of DBS (10). The most frequently used non-NASBA platform (Roche) is unsuitable for VL testing using whole blood samples because the presence of cellular nucleic acids inflates the VL levels measured to a clinically significant degree, making accuracy and precision poor when the VL is moderately elevated (<3,000 copies/ml).

The lower sensitivity of testing DBS samples instead of plasma samples using the NucliSENS platform is an acceptable tradeoff, given the benefits of enabling access to VL testing among patients who would not otherwise have access to testing. Nonetheless, efforts are needed to improve the accuracy of both for NASBA and non-NASBA platforms for measuring VL on DBS samples to op-

TABLE 5 Precision of viral load measurements on duplicate DBS samples

Viral load (copies/ml)	Sample type	Intra-assay variation (log copies/ml)			Interassay variation (log copies/ml)		
		Sample size (*n*)	SD	Bias	Sample size (*n*)	SD	Bias
≤3,000	FP-DBS				10	0.19	−0.08
	VB-DBS	2	0.07	0.10	7	0.09	−0.06
≥3,000	FP-DBS	9	0.11	0.03	22	0.13	0.10
	VB-DBS	10	0.07	0.09	22	0.10	0.08

timize the utility of DBS samples for VL testing, particularly in remote populations in resource-limited settings.

The study also has some limitations. The number of DBS samples used to assess intra-assay variation at low VLs was small, and so future studies should confirm our findings regarding intra-assay variability. Another study found low precision (high intra-assay variability) of DBS samples with VLs in the lower range (<3,000 copies/ml) using the Roche Cobas TaqMan v2.0 assay (33). Diagnostic tests should be evaluated under conditions of intended use. We did not assess performance of FP-DBS prepared by nonlaboratory staff in a clinic setting. We are currently conducting a second study to assess the proficiency of lay health care workers at preparing DBS samples in primary health care clinics.

The use of DBS samples for VL monitoring needs to be coupled with strategies such as pooled sample testing to further reduce the price of VL testing and to support full implementation of VL monitoring in resource-constrained settings (9, 34). Another recent study that was also conducted in the Thyolo District using the same VL laboratory (35) found that FP-DBS in combination with pooling is feasible, produces accurate results, and can reduce the cost of testing substantially, with cost savings varying depending on the prevalence of a raised VL in the patient population and the VL cut point used.

Taken together, the results of this study add to the growing evidence that DBS is an acceptable alternative to plasma for VL testing in rural, resource-limited settings, and that the use of FP-DBS has the potential to increase access to VL testing by further decentralizing sample collection and task-shifting sample collection to a lower cadre of health care workers in clinics distant from a laboratory.

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We declare that we have no conflicts of interest.

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