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MAJOR ARTICLE

Evaluation of Two Techniques for Viral Load Monitoring Using Dried Blood Spot in Routine Practice in Vietnam (French National Agency for AIDS and Hepatitis Research 12338)

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Background. Although it is the best method to detect early therapeutic failure, viral load (VL) monitoring is still not widely available in many resource-limited settings because of difficulties in specimen transfer, personnel shortage, and insufficient laboratory infrastructures. Dried blood spot (DBS) use, which was introduced in the latest World Health Organization recommendations, can overcome these difficulties. This evaluation aimed at validating VL measurement in DBS, in a laboratory without previous DBS experience and in routine testing conditions.

Methods. Human immunodeficiency virus (HIV)-infected adults were observed in a HIV care site in Hanoi, and each patient provided 2 DBS cards with whole blood spots and 2 plasma samples. Viral load was measured in DBS and in plasma using the COBAS Ampliprep/TaqMan and the Abbott RealTime assays. To correctly identify those with VL ≥ 1000 copies/mL, sensitivity and specificity were estimated.

Results. A total of 198 patients were enrolled. With the Roche technique, 51 plasma VL were ≥1000 copies/mL; among these, 28 presented a VL in DBS that was also ≥1000 copies/mL (sensitivity, 54.9; 95% confidence interval [CI], 40.3–68.9). On the other hand, all plasma VL < 1000 copies/mL were also <1000 copies/mL in DBS (specificity, 100; 95% CI, 97.5–100). With the Abbott technique, 45 plasma VL were ≥1000 copies/mL; among these, 42 VL in DBS were also ≥1000 copies/mL (sensitivity, 93.3%; 95% CI, 81.7–98.6); specificity was 94.8 (95% CI, 90.0–97.7).

Conclusions. The Abbott RealTime polymerase chain reaction assay provided adequate VL results in DBS, thus allowing DBS use for VL monitoring.

Keywords. HIV; Viral load; Dried Blood Spots (DBS); virological failure; sensitivity.

In recent years, the number of human immunodeficiency virus (HIV)-infected patients on antiretroviral therapy (ART) has dramatically increased [1]. Early detection of therapeutic failure is a major challenge for the management of HIV patients on ART, to optimize ART efficacy and prevent accumulation of resistance mutations that may compromise future treatment options [2, 3]. In its latest update, the World Health Organization (WHO) encouraged routine viral load (VL) monitoring of patients on ART to identify virological failure (VF) [4]. Efforts have been made to widen access to VL monitoring, although VL monitoring remains scarce in remote areas due to difficulties in specimen transfer, personnel shortages, and insufficient laboratory infrastructures [5].

To overcome these obstacles, one strategy involves point-of-care (POC) machines capable of performing VL measurement in field conditions where infrastructures and highly trained personnel are unavailable [6–8]. Field evaluations of these POC machines have been promising [9, 10], but larger scale validations are required. The advantages of POC machines are that they provide quick results to the patient and have low detection limits. However, equipping each care site may also pose problems due to costs and the need for maintenance and continuous reagent supplies.

A second strategy, introduced into the most recent WHO guidelines [4], makes use of dried blood spots (DBS) as a sampling tool, with the subsequent transfer of DBS to a central laboratory. Some studies have shown that DBS is a useful, efficient tool for monitoring VL in various settings [11, 12], offering a

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practical and easy means of broadening access to VL monitoring for patients observed in remote areas and in level I or II facilities [13]. At the care site level, DBS are easy to perform because they do not require skilled personnel or purchase of new equipment. They are easy to maintain and can be kept at ambient temperature for at least 2 weeks before freezing [14–17]. This capacity facilitates their transfer, which can be conducted through the existing postal network; the DBS are also not contagious. Moreover, at the central laboratory, the technique used for VL monitoring is the same as that used on plasma, and thus it does not require new machines. Dried blood spots also allow monitoring for HIV drug resistance [11, 12, 18]. Disadvantages of DBS are mainly the laboratory manual time imposed by the DBS technique and a higher detection threshold.

In Vietnam, the latest UNAIDS figures estimated the prevalence of HIV in adults as 0.5% [19]. Antiretroviral therapy coverage was estimated at 67.1% in 2013 [20]. Laboratories able to perform VL monitoring exist solely in large cities such as Hanoi and Ho Chi Minh City. Currently, patients observed in remote areas have no or extremely low access to VL monitoring.

Before implementing VL monitoring using DBS on a wide scale, we evaluated the capacity of molecular testing to identify those with a VL $\geq$ 1000 copies/mL in DBS compared with plasma, in routine testing conditions, using fully automatized polymerase chain reaction (PCR) platforms and manufacturer's DBS protocols from Roche and Abbott, which are currently available in Vietnam.

METHODS

Patients and Samples

The study protocol was approved by the Institutional Review Board from the Hanoi School of Public Health (Hanoi, Vietnam). Only participants providing written informed consent were enrolled, and random anonymous identification numbers were assigned to each patient to guarantee confidentiality.

Adults (>18 years old) observed at the Dong Da outpatient clinic in Hanoi were invited to participate in the study. To obtain a wide range of VL levels, 3 groups of patients were targeted: ART-naive patients, patients on ART for <6 months, and patients on ART for at least 6 months. Patients who consented to participate in the study provided a whole blood sample, and a short questionnaire recorded each participant's gender, age, ART group, and suspicion of treatment failure (based on clinical and immunological criteria). For each participant, the whole blood sample and information form were identified using a randomly attributed identification number. Whole blood samples were transferred the same day to the HIV/acquired immune deficiency syndrome (AIDS) molecular laboratory (National Reference HIV Molecular Laboratory) at the National Institute of Hygiene and Epidemiology (NIHE) in Hanoi.

At the HIV/AIDS molecular laboratory at NIHE, for each patient, a predefined volume (50 µL for use with the Abbott technique and 70 µL for use with the Roche technique) of whole blood was deposited using a calibrated pipette on 2 Munktell TFN cards on each of the 5 precut spots. The DBS cards were left to dry at ambient temperature for 3 hours and were then individually packed in ziplock bags with 2 desiccants. To reproduce the routine practice, DBS were kept for 2 weeks at ambient temperature (maximum delay between DBS collection and reception at the central laboratory) and then placed in a freezer at −20°C until VL measurement (until enough samples were collected to run a compete batch). After the making of the DBS cards, the remaining blood was centrifuged and 2 plasma samples of 2 mL were collected in cryotubes and stored at −80°C until VL measurement.

To ensure independent VL measurements from a same patient, blinded conditions were established by using preidentified stickers with randomly allocated identification numbers for DBS cards and for cryotubes. The correspondence list was created and stored by investigators at Institut Pasteur (Paris, France).

Laboratory Techniques

For each of the following 2 techniques (Roche and Abbott), VL measurements were independently performed in plasma and in DBS. Viral load results in DBS were then compared with the plasma VL (gold standard).

Viral Load Measurement on Plasma

Following manufacturers' protocol (COBAS AmpliPrep TaqMan HIV-1v 2.0 and Abbott Real-time HIV-1), the plasma input volume was 1.1 mL for Roche and 0.8 mL for Abbott. The nucleic acid extraction product was 65 µL for Roche and 70–80 µL for Abbott. For both techniques, the volume used for PCR was 50 µL.

Viral Load Measurement on Dried Blood Spot

In the Roche technique, the new free virus elution (FVE) method [21] was used. One spot of 70 µL was incubated in 1 mL calcium and magnesium-free phosphate-buffered saline ([PBS] 154 nM NaCl, 5.6 nM Na_2HPO_4, 1.1 nM KH_2PO_4, pH 7.4; Corning) in a tube at room temperature for 30 minutes. Using the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test v2.0 (Roche Molecular Systems, Branchburg, NJ), we directly extracted ribonucleic acid (RNA) in the tube containing the elution buffer and the spot specifying the DBS protocol (HI2DFSP96). According to the manufacturer's recommendation for this protocol, a correction factor (+0.3 log copies/mL) was added to each result on DBS [21, 22].

In the Abbott technique, following the manufacturer's protocol, 2 spots of 50 µL were placed in a tube with 1.7 mL Abbott Lysis Buffer (*m*Lysis Buffer LN2N77-01). After a 1-hour elution, RNA extraction was performed on m2000sp using 1 mL of the buffer. Amplification and detection were performed on m2000rt using the 1 mL DBS HIV-1 RNA protocol, specifying that the sample originated from DBS.

Statistical Analysis

In accordance with WHO guidelines, VF was defined as a VL ≥ 1000 copies/mL [4]. To conduct a qualitative evaluation of the 2 techniques, sensitivity and specificity were estimated. The sensitivity was the proportion of patients with VL ≥ 1000 copies/mL in DBS among those with plasma VL ≥ 1000 copies/mL. The specificity was the proportion of patients with VL < 1000 copies/mL in DBS among those with plasma VL < 1000 copies/mL. Sensitivity and specificity were also estimated at the thresholds of 3000 and 5000 copies/mL. The concordance coefficient was the proportion of measurements, both above or both below the threshold for plasma and for DBS. Qualitative evaluations were conducted estimating correlations between plasma and DBS VL and through Bland-Altman analysis.

RESULTS

From July to October 2015, 198 patients were enrolled. Most patients were male (145 of 198), and the median age was 37 years. Regarding ART history, 19 (9.6%) were ART naive, 34 (17.2%) were on ART for <6 months, and the remaining 145 (73.2%) were on ART for ≥6 months; among these latter patients, 94 (64.8%) were suspected of therapeutic failure.

The median delay between blood sampling and VL measurement in DBS was 28 (interquartile range [IQR], 18–71) days for the Roche technique and 27 (IQR, 22–70) days for the Abbott technique ($P = .36$).

Plasma Viral Loads

With the Roche technique, 83 (41.9%) plasma samples were undetectable for HIV RNA (<20 copies/mL). In the remaining 115 samples, the median VL level was 601 (IQR, 115–33200) copies/mL. With the Abbott technique, 116 (58.6%) plasma samples were undetectable for HIV RNA (<40 copies/mL). In the remaining 82 samples, the median VL level was 1778 (IQR, 179–43 651) copies/mL. Plasma VL obtained with the 2 techniques were highly correlated ($\rho = .96$, $P < .001$).

At the threshold of 1000 copies/mL, agreement between the 2 techniques was observed in all but 6 VL measurements: these measurements were above 1000 copies/mL with the Roche technique (range, 1220–3250 copies/mL), but were below 1000 copies/mL with the Abbott technique, although above the lower detection limit (range, 80–814 copies/mL).

Evaluation of the Roche Technique

From a strictly qualitative point of view, the correlation between plasma and DBS VL was good ($\rho = .84$, $P < .001$) (Figure 1A), despite underestimation on DBS. This underestimation is also highlighted in the Bland-Altman analysis (mean difference, −0.35 log copies/mL) (Figure 2A).

Of 198 plasma VL, 51 (25.8%) were ≥1000 copies/mL. Among these, 28 VL results were also ≥1000 copies/mL in DBS (sensitivity, 54.9%; 95% confidence interval [CI], 40.3–68.9) (Table 1). Of the 147 plasma VL < 1000 copies/mL, all were also below this threshold in DBS (specificity, 100%; 95% CI, 97.5–100). The concordance coefficient was 88.4% (95% CI, 83.1–92.5).

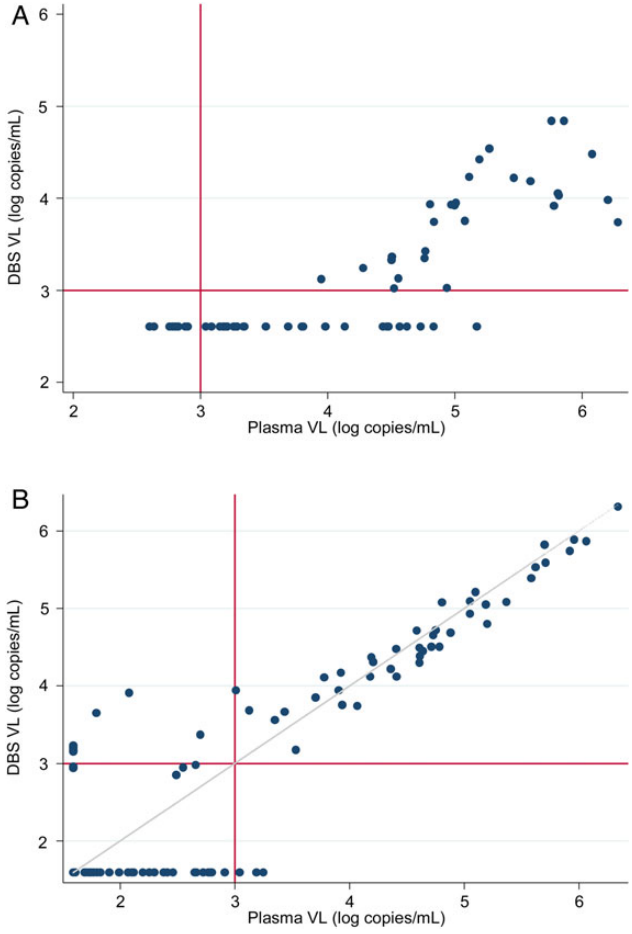

Figure 1. Correlation between viral load (VL) measurements on plasma and on dried blood spots (DBS). (A) Roche technique; (B) Abbott technique.

Although not significant, sensitivity at the threshold of 1000 copies/mL tended to be larger when the delay between DBS collection and VL measurement in DBS was <4 weeks, compared with ≥4 weeks (68.0% vs 42.3%, $P = .06$). However, specificity remained 100%, regardless of the delay. When stratifying analysis by ART duration (<6 or ≥6 months), sensitivity was not modified ($P = .40$).

When VF was defined at the threshold of 3000 and 5000 copies/mL, the sensitivity was 45.2% (95% CI, 29.8–61.3) and 47.5% (95% CI, 31.5–63.9), respectively. The specificity remained at 100%.

Evaluation of the Abbott Technique

A strong correlation was found between VL measured on plasma and on DBS ($\rho = .93$, $P < .001$) (Figure 1B). In the Bland-Altman analysis, the mean difference between DBS and plasma VL measurement was −0.03 log copies/mL (Figure 2B). Concordance was particularly good above the threshold of 3 log copies/mL.

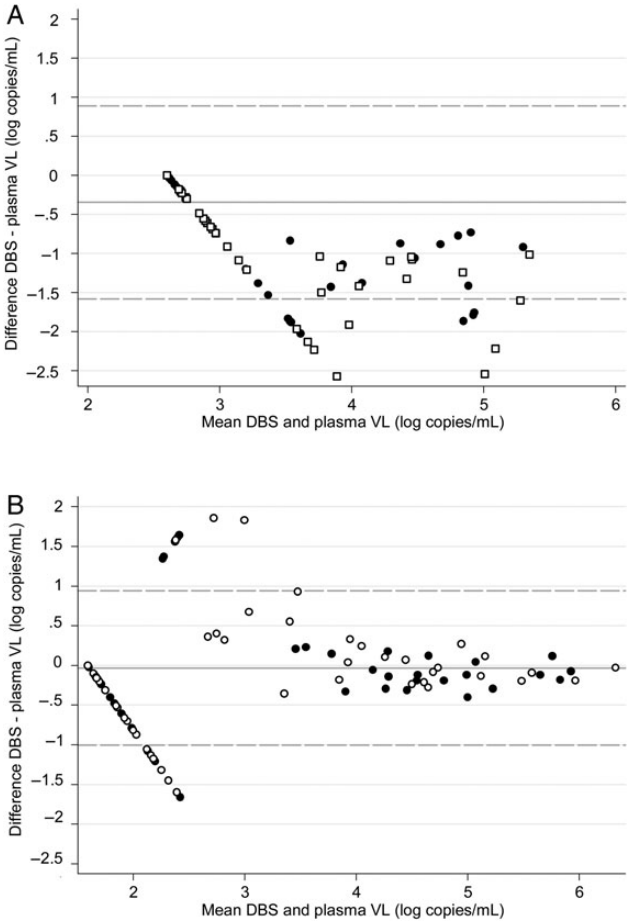

Figure 2. Bland-Altman analysis of agreement between dried blood spots (DBS) and plasma viral loads (VL). (A) Roche technique; (B) Abbott technique. The horizontal lines represent the mean (solid line) and the ± 1.96 standard deviations (dashed lines). Empty square: on antiretroviral therapy (ART) for <6 months; full circles: on ART for ≥6 months.

Of 198 plasma VL, 45 (22.7%) were ≥1000 copies/mL. Among these, 42 were also ≥1000 copies/mL in DBS (sensitivity, 93.3%; 95% CI, 81.7–98.6) (Table 2). Of the 153 plasma VL < 1000 copies/mL, 145 VL were also <1000 copies/mL in DBS (specificity, 94.8%; 95% CI, 90.0–97.7). The concordance coefficient between plasma and DBS results was 94.4% (95% CI, 90.3–97.2).

Three patients with a plasma VL ≥ 1000 copies/mL, ranging from 1096 to 1543 copies/mL, were undetectable when VL was measured in DBS. In these patients, the delay between blood collection and VL measurement in DBS were 17, 22, and 70 days; it was not significantly different from the delay observed in other patients with a plasma VL ≥ 1000 copies/mL ($P = .80$). Of note, with the Roche technique, these patients also presented intermediate plasma VL results (range, 1630–1920 copies/mL) and undetectable HIV RNA in DBS.

Eight patients with plasma VL < 1000 copies/mL presented a VL ≥ 1000 copies/mL in DBS. Four were on ART for more than 6 months and had undetectable plasma VL but moderately high VL in DBS (1427 to 1708 copies/mL). The other 4 were on ART for <6 months and had low plasma VL (1 with plasma VL < 40 copies/mL, otherwise 62 to 502 copies/mL); of note, these 4 patients had detectable plasma VL with the Roche technique. The corresponding VL in DBS ranged from 1480 to 8128 copies/mL.

Table 1. Sensitivity and Specificity of the Techniques on DBS Compared With Plasma, Roche Technique

Threshold-Defining Failure	VL > Threshold on Plasma	VL ≤ Threshold on Plasma
1000 copies/mL		
VL > threshold on DBS	28	0
VL ≤ threshold on DBS	23	147
Total	51	147
	Se, 54.9 (95% CI, 40.3–68.9)	Sp, 100 95% CI, 97.5–100)
3000 copies/mL		
VL > threshold on DBS	19	0
VL ≤ threshold on DBS	23	156
Total	42	156
	Se, 45.2 (95% CI, 29.8–61.3)	Sp, 100 (95% CI, 97.7–100)
5000 copies/mL		
VL > threshold on DBS	19	0
VL ≤ threshold on DBS	21	158
Total	40	158
	Se, 47.5 (95% CI, 31.5–63.8)	Sp, 100 (95% CI, 97.7–100)

Abbreviations: CI, confidence interval; DBS, dried blood spots; Se, sensitivity; Sp, specificity; VL, viral load.

Table 2. Sensitivity and Specificity of the Techniques on DBS Compared With Plasma, Abbott Technique

Threshold-defining failure	Plasma VL > Threshold	Plasma VL ≤ Threshold
1000 copies/mL		
VL > threshold in DBS	42	8
VL ≤ threshold in DBS	3	145
Total	45	153
	Se, 93.3 (95% CI, 81.7–98.6)	Sp, 94.8 (95% CI, 90.0–97.7)
3000 copies/mL		
VL > threshold in DBS	37	6
VL ≤ threshold in DBS	1	154
Total	38	160
	Se, 97.4 (95% CI, 86.2–99.9)	Sp, 96.3 (95% CI, 92.0–98.6)
5000 copies/mL		
VL > threshold in DBS	37	2
VL ≤ threshold in DBS	0	159
Total	37	161
	Se, 100 (95% CI, 90.5–100)	Sp, 98.8 (95% CI, 95.6–99.8)

Abbreviations: CI, confidence interval; DBS, dried blood spots; Se, sensitivity; Sp, specificity; VL, viral load.

At the threshold of 1000 copies/mL, sensitivity and specificity were not different when VL in DBS was measured <4 or ≥4 weeks after DBS collection ($P = .68$ and $P = .63$, respectively). When stratifying analysis by ART duration (<6 or ≥6 months), sensitivity was not modified ($P = .99$) and specificity tended to be improved (88.2% vs 96.6%, $P = .07$).

At the threshold of 3000 copies/mL, sensitivity was 97.4% (95% CI, 86.2–99.9) and specificity was 96.3% (95% CI, 92.0–98.6). At the threshold of 5000 copies/mL, sensitivity was 100% (95% CI, 90.5–100) and specificity was 98.8% (95% CI, 95.6–99.8).

DISCUSSION

From a clinical viewpoint, we conducted a qualitative evaluation because decisions regarding ART management are taken essentially based on VL measurements above or below a specific threshold. At the threshold of 1000 copies/mL, sensitivity (ability to identify patients in VF) was high with the Abbott technique (93.3%) and resembled findings of previous reports [18, 23]. On the other hand, the sensitivity was low (54.9%) with the Roche technique.

The sensitivity of the Abbott technique was high, and only 3 patients with a plasma VL result ≥1000 copies/mL were undetectable in DBS. It is of interest to note that, for these 3 patients, the same result occurred with the Roche technique. The delay between DBS collection and VL measurement did not seem to account for this result, because it was not different from the delay observed in the other patients. In these 3 patients, the plasma VL level was low (1096–1776 copies/mL). Degradation of RNA may have occurred because of bad conservation conditions of the DBS cards and thus may explain this result. An incapacity to identify patients failing on ART is a serious problem. Nevertheless, such patients would likely be identified as failing at their next virological evaluation, even though it means that identifying this failure would be delayed.

The sensitivity observed with the Roche technique was much lower than in previous evaluations [21, 24]. The new elution (FVE) protocol used in the present study has been the subject of a single previous evaluation [21] in which the authors stated that PBS use during the elution phase offers the advantage of reducing the overquantification of HIV VL in DBS by limiting proviral deoxyribonucleic acid (DNA) amplification, thus explaining the good specificity. The authors also acknowledged that it may also underestimate HIV RNA quantification in DBS. This result could explain the reduced sensitivity observed. The present evaluation was conducted with field-collected samples, in routine practice and in a laboratory not dedicated to research. Such factors could also explain the difference observed. In the evaluation by Andreotti et al [24], the sensitivity was also higher than that observed in the present study. However, extraction was performed with the NucliSENS miniMAG system (Biomerieux, Marcy l'Etoile, France), because their first attempt with the Roche COBAS TaqMan assay led to a significant underestimation. Despite this, VL on DBS was still underestimating plasma VL. The lower sensitivity we observed could also be explained by the use of Munktell TFN cards, whereas the other 2 studies used Whatman 903 DBS cards [25].

The difference in performance between the 2 techniques was unexpected. It should not be explained by condition of DBS conservation, because they were all maintained the same way. Furthermore, it could not be explained by the duration of conservation of the DBS cards, because the delay of VL measurement on DBS did not differ between the 2 techniques. Of note, sensitivity tended to decrease with the Roche technique when the delay between DBS collection and VL measurements on DBS was more than 4 weeks. However, even when this delay was less than 4 weeks, the sensitivity was only 68%. We found no evidence of an impact of this delay for the Abbott technique. The 2 techniques also differed in terms of quantity of blood used (1 spot of 70 µL for the Roche technique, 2 spots of 50 µL each for the Abbott technique) and in terms of elution procedure, which could potentially explain the sensitivity difference between the techniques.

Specificity evaluated the ability to identify patients with VL < 1000 copies/mL. The specificity was 100% using the Roche technique and slightly lower using the Abbott technique (94.8%).

With the Abbott technique, lack of detection of the virological success (VL in DBS ≥ 1000 copies/mL, whereas plasma VL < 1000 copies/mL) was likely due to proviral DNA and intracellular RNA contaminations [26, 27]. Eight patients with plasma VL < 1000 copies/mL presented VL ≥ 1000 copies/mL in DBS. It is interesting to note that 4 of these patients were on ART for <6 months. These patients may still present a large viral reservoir, although the circulating virus was reduced by ART, and the VL result in DBS could be due to amplification of proviral DNA. In routine practice, only patients with at least 6 months on ART would be evaluated, thus reducing the risk of false identification of failure. It must also be noted that, following current WHO guidelines [1] applied in Vietnam, patients with a VL ≥ 1000 copies/mL undergo counseling to improve ART adherence; and a new VL measurement at least 1 month later is conducted. Thus, the probability of having 2 consecutive (separated by 1 to 3 months with strengthening of adherence) false-positive VLs (VL ≥ 1000 copies/mL) is low, making a small default in specificity less worrisome, despite a small additional cost because of unnecessary VL retesting of a small proportion of patients.

For the Roche technique, Wu et al [21] suggest that the use of PBS in the elution protocol prevents proviral DNA amplification.

A systematic review of the use of DBS for VL monitoring showed that VL results on DBS, apart from the VL technique

itself, depend on punch size, method for punching, elution buffer used, and sample volume after extraction [28]. To limit the impact of these interventions, procedures were standardized and followed manufacturers' recommendations. We used cards with precut spots to reduce the time needed to cut the spots and the risk of contamination with the use of a punch. Moreover, we minimized the disadvantages of the manual time imposed by the DBS technique by using as many automatized procedures as possible.

This study is one of the few studies conducted under "real-life" conditions. We sought to reproduce routine conditions using field-collected samples and to reproduce the delay between DBS collection and VL measurement, by maintaining the DBS cards at ambient temperature for 2 weeks before freezing. Therefore, all procedures used in this study can be implemented on a larger scale.

It is now recognized that use of DBS is the most feasible way to provide VL monitoring to patients observed in decentralized areas [29–31]. At the laboratory level, manual laboratory time can be greatly reduced with new elution protocols and automatization, but manufacturers have to integrate official guidelines for VL measurements using DBS to their manuals. It is important to note that machines used are those used for VL measurement on plasma and thus new equipment is not required. Moreover, DBS also allow monitoring HIV drug resistance in patients in VF [11, 12, 18].

CONCLUSIONS

Following current guidelines, clinical decisions regarding ART maintenance or modification were made based on VL $\geq$ 1000 copies/mL, rendering sensitivity as the most important performance criterion. High sensitivity is important to identify those patients for whom ART is failing. In our setting, the use of Abbott technique for VL measurement in DBS performed well. Moreover, when we followed the WHO suggestions for VL measurement on DBS and adopted the threshold defining failure as 3000 or 5000 copies/mL, the sensitivity and specificity of the Abbott technique reached approximately 100%.

In Vietnam, VL monitoring can now be implemented using the Abbott platform, centralizing the DBS for VL measurement at the molecular laboratory of NIHE.

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