Effect of storage conditions of dried plasma and blood spots on HIV-1 RNA quantification and PCR amplification for drug resistance genotyping

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Objectives: Dried blood spots (DBS) and dried plasma spots (DPS) are easy to collect and store, and have been successfully tested as an alternative to plasma for performing virological analyses. Adequate storage conditions still need to be established and cell-associated proviral DNA in DBS can contribute to the amplified products. We evaluated these two parameters.

Methods: Residual samples from 34 HIV-1-infected patients [mean viral load (VL) = 3.93 $\log_{10}$ copies/mL] were used to prepare DPS and DBS, then stored at 20°C and 37°C. HIV-1 nucleic acids were extracted, with or without DNase treatments, to perform HIV-1 VL quantification and nested RT–PCR to amplify the reverse transcriptase gene (798 bp).

Results: For DBS stored for 3 months at 20°C, VL could be measured for all samples and results were comparable to plasma VL. At 37°C, a slight decrease was observed after 2 and 3 months (0.16 and 0.37 $\log_{10}$ copies/mL mean difference, respectively). For DPS, a significant decrease in VL (0.70 and 1.07 $\log_{10}$ copies/mL after 1 and 2 months, respectively) was seen at 37°C, but not at 20°C. PCR amplifications from DPS were only successful for 50% of samples with an initial VL >10000 copies/mL after 1 month at 20°C. From DBS, PCR amplifications are possible until 3 months for samples with plasma VL >5000 copies/mL. VL and PCR results for DBS treated with DNase are close to results obtained for DPS.

Conclusions: Virological monitoring is still feasible for DBS after 3 months of storage at 37°C when VL is >5000 copies/mL, but DNA contributes largely to the final results.

Keywords: 903 filter paper, drug resistance surveillance, viral load, RNA stability, dried spot conservation, resource-limited countries, DBS, DPS

Introduction

Due to the efforts of national programmes and the support of a wide range of international partners, the number of people receiving antiretroviral therapy (ART) in resource-limited countries has significantly increased, most notably in sub-Saharan Africa and, in order to limit the emergence of drug resistance, HIV treatment should ideally be accompanied by periodic monitoring for viral load (VL) and drug resistance. Whereas plasma is considered optimal for these tests, the dried fluid spot filter paper technology does not require a cold chain and allows shipment with minimal biohazard risks. Dried blood spots (DBS) and dried plasma spots (DPS) seem to yield comparable performance,[1,2] but certain limitations and challenges to their practical use remain, like the lower detection limit, interference of proviral DNA in DBS, stability of DNA and RNA under extreme conditions and long-term storage.[2–7] In order to enable dried spots to become a method of sample collection to monitor patients who receive ART in health structures with limited infrastructure it is necessary to better identify the limitations of this technology. Here we investigated, in more detail, the effect of temperature over time on HIV quantification and drug resistance genotyping on DPS and DBS and sought to understand to what extent proviral DNA from DBS contributes to the results.

Materials and methods

Samples and preparation of DPS and DBS

DPS and DBS were prepared in parallel from residual plasma and whole blood samples of 34 HIV-1-infected patients.[4] Briefly, 50 μL of

blood or plasma was spotted on to 903 filter paper and dried at room temperature for 3 h. Spots were then placed individually in plastic bags and stored in a hermetic box containing silica dessicant for 5 months at −20°C. Various storage conditions were subsequently assessed over time; i.e. storage at 20°C in the laboratory (dry atmosphere) and storage at 37°C in an incubator containing trays of water to maintain a high relative humidity. Dessicants were checked for humidity at regular time intervals and replaced every month and more frequently when necessary.

Nucleic acid extraction from DPS and DBS

In order to improve the lower detection limit, nucleic acids were extracted from four spots using Nuclisens MiniMAG (bioMérieux, Craponne, France). After elution in 4 mL of lysis buffer for 2 h at room temperature, RNA was extracted according to the manufacturer's instructions. Overall we extracted nucleic acid from a total of 347 sample specimens (227 DBS and 120 DPS). DNase treatment was performed on a subset of nucleic acid extracts from DBS using the Turbo DNA-free kit (Ambion/Applied Biosystems, ref. AM1907).

HIV-1 VL determination and PCR amplifications for genotypic drug resistance testing

HIV-1 viral RNA was quantified by real-time RT–PCR, commercially available as 'Generic HIV Charge Virale' with a detection limit of 300 copies/mL (Biocentric, Bandol, France).[8]

Nested RT–PCR was used, on a subset of 12 of the 34 patients with sufficient residual blood available, to amplify a 798 bp fragment from the reverse transcriptase (RT) region of *pol*, using the ANRS protocol (http://www.hivfrenchresistance.org/) adapted for dried spots.[4] We amplified only the largest fragment RT because it is more sensitive to nucleic acid degradation than protease (350 bp).

Statistical analysis

VLs from plasma, DBS and DPS were compared by a Wilcoxon matched-pairs signed-ranks test (Stata 10.0 software; STATA Corporation, College Station, TX, USA) with undetectable samples considered equal to zero and detectable samples below the detection limit of the assay recorded as cut-off values. A *P* value <0.01 was considered to be significant.

Results

VL from DBS and DPS over time under different temperature conditions

Overall, mean VL from plasma and spots at baseline, i.e. after 5 months of storage at −20°C, were comparable, with a slight overestimation for DBS of 0.19 $\log_{10}$ copies/mL, most likely related to the presence of proviral DNA, and a slight, but not significant, underestimation of 0.30 $\log_{10}$ copies/mL for DPS. Therefore, VLs for the different conditions over time were compared with the initial plasma VL.

After 3 months at 20°C, VL could be measured for all DBS specimens and was still comparable to plasma VL, 3.87 versus 3.93 $\log_{10}$ copies/mL (Table 1). Also, storage of DBS at 37°C had no negative effect on VL measurements after 1 month, but a slight decrease was observed after 2 and 3 months, 0.16 and 0.37 $\log_{10}$ copies/mL, respectively ($P=0.19$ and $P=0.03$, respectively).

VL was stable for DPS after 1 month storage at 20°C and a slight decrease of 0.36 $\log_{10}$ copies/mL was seen after 2 months ($P=0.07$). Although this decrease was not significant, we noted a more dispersed distribution of the individual VL values. In contrast, storage of DPS at 37°C, induced a significant decline in VL, with a mean decrease of 0.70 and 1.07 $\log_{10}$ copies/mL

Table 1. Comparison of HIV-1 VL means from DBS, DBS treated with DNase and DPS stored at 20°C or 37°C for 1, 2 and 3 months with corresponding plasma VL

| Temperature and time (samples) | Mean VL, $\log_{10}$ copies/mL (SD) | | | Mean VL, $\log_{10}$ copies/mL (SD) | | |
	DBS	plasma	*P* value	DPS	plasma	*P* value
20°C						
month 1 (34 DBS; 30 DPS)	4.38 (0.93)	3.93 (1.03)	<0.001	3.90 (1.25)	3.90 (1.07)	0.33
month 2 (34 DBS; 22 DPS)	4.11 (0.87)	3.93 (1.03)	0.12	3.42 (1.46)	3.78 (1.11)	0.07
(22 DBS+DNase)	3.18 (1.43)	3.79 (1.19)	0.001			
month 3 (31 DBS; NA)	3.87 (1.15)	3.93 (1.07)	0.72	NA	NA	NA
37°C						
month 1 (34 DBS; 27 DPS	4.36 (0.55)	3.93 (1.03)	<0.001	3.15 (1.62)	3.85 (1.12)	<0.001
month 2 (34 DBS; 15 DPS)	3.77 (1.07)	3.93 (1.03)	0.19	2.90 (1.56)	3.97 (0.81)	0.001
(22 DBS+DNase)	3.24 (1.20)	3.79 (1.19)	0.001			
month 3 (26 DBS; NA)	3.73 (0.85)	4.10 (0.79)	0.03	NA	NA	NA

NA, not applicable.
The VL values are reported as $\log_{10}$ transformed copy numbers of HIV-1 RNA/mL.
P values were calculated using the Wilcoxon test to compare the various groups with the matched plasma samples (same number of samples compared).
VLs in plasma have been previously determined by the same method, i.e. Generic HIV Charge Virale (Biocentric) from 200 μL of plasma.[4]
The number of DBS and DPS per patient depends on the volume of the residual blood available, and thus explains the different sample numbers over time.

($P<0.001$ and $P=0.001$, respectively), after 1 and 2 months, respectively.

VLs for DBS are always higher than for DPS and we investigated whether this was due to the contribution of proviral DNA by treating a subset of nucleic acid extracts with DNase. Compared with plasma, mean VL for DBS decreased significantly ($P=0.001$) after DNase treatment and was close to values observed for DPS; a decline of 0.61 and 0.55 $\log_{10}$ copies/mL was observed after 2 months at 20°C and 37°C, respectively.

PCR amplifications of RT for genotypic drug resistance testing from DBS and DPS over time under different temperature conditions

At baseline, the RT gene could be amplified for all DBS and for 10 of 12 DPS specimens (Table 2). After 1 and 2 months at 20°C, the RT fragment could be amplified in 11/12 DBS and for 10/11 DBS after 3 months. In contrast, at 37°C, the PCR efficiency for DBS decreased over time from 10/12 to 7/12 and 8/10 after 1, 2 and 3 months, respectively. The PCR efficiency for DPS was significantly lower than for DBS: after 1 month, the RT gene could only be amplified in 4/12 and 0/11 samples for DPS kept at 20°C or 37°C, respectively, and, after 2 months, no samples were amplified from DPS when kept at 37°C and only 1/8 at 20°C.

Globally, PCR efficiencies seem to be correlated with higher VL; all DPS specimens with an initial VL <10000 copies/mL were negative after 1 month of storage at 20°C whereas for DBS, PCR amplifications were only negative after 3 months of storage at 20°C or 37°C for samples with plasma VL <5000 copies/mL.

After DNase treatment of nucleic extracts obtained from DBS, PCR efficiencies were comparable to those observed for the corresponding DPS samples.

Discussion

One of the crucial points for the use of dried fluid spots for VL quantification and drug resistance genotyping in the field is the stability of the nucleic acids, DNA and especially RNA, over time. Dried spots can be kept for long periods when refrigerated or frozen in hermetic bags with dessicant,[2] but exposure to high temperatures for extended periods seems to be associated with degradation of nucleic acids. Few studies have reported on long-term storage or testing under field temperature conditions.[2,5,9,10] Moreover, contradictory results have been reported and often only limited numbers of samples were tested either for VL or genotyping.

In this report we studied the impact of temperature on long-term storage in parallel for DBS and DPS and for both VL and genotyping. Overall we confirm the negative effect of temperature, but RNA decline has a slower impact on VL measurements than on PCR amplifications and we observe better results for DBS than DPS over time. More precisely, for DPS, VL and PCR amplifications (798 bp) are still comparable to those of plasma after 1 month at 37°C when plasma VL is >5000 and >10000 copies/mL, respectively. For DBS specimens, HIV-1

Table 2. Efficiency of HIV-1 amplification of RT fragments from DBS, DBS treated with DNase and DPS stored at 20°C or 37°C for 0 (M0), 1 (M1), 2 (M2) and 3 (M3) months

			DBS										DPS				
			20°C					37°C						20°C		37°C	
			M1		M2			M1		M2				M1	M2	M1	M2
Plasma VL	Plasma[a]	M0[b]	no DNase	with DNase	no DNase	with DNase	M3	no DNase	with DNase	no DNase	with DNase	M3	M0[b]	M1	M2	M1	M2
3.14	+	+	−	NA	+	NA	−	−	NA	−	NA	−	−	−	−	−	−
3.56	+	+	+	NA	+	NA	NA	−	NA	−	NA	NA	+	−	−	−	−
3.70	+	+	+	NA	−	NA	+	+	NA	+	NA	+	+	−	−	−	−
3.79	+	+	+	NA	+	NA	+	+	NA	−	NA	NA	−	−	−	−	NA
4.08	+	+	+	+	+	−	+	+	−	+	−	+	+	−	NA	−	NA
4.10	+	+	+	−	+	−	+	+	−	−	−	+	+	−	−	−	−
4.23	+	+	+	−	+	−	+	+	−	−	−	+	+	+	−	−	−
4.46	+	+	+	−	+	−	+	+	+	+	−	+	+	−	NA	NA	NA
4.54	+	+	+	+	+	−	+	+	NA	+	−	+	+	+	−	−	−
4.65	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−
4.69	+	+	+	+	+	−	+	+	+	+	−	+	+	−	NA	−	NA
5.42	+	+	+	+	+	−	+	+	+	+	+	+	+	+	NA	−	NA
Total[c]	12/12	12/12	11/12	5/8	11/12	1/8	10/11	10/12	4/7	7/12	1/8	8/10	10/12	4/12	1/8	0/11	0/7

−, No amplification; +, amplification; NA, not applicable.
Amplification products were visualized by 1% agarose gel electrophoresis with ethidium bromide staining.
[a]Measured in our previous study using the same method.[4]
[b]M0=5 months of storage at −20°C.
[c]Number of specimens amplified/total number of specimens tested.

quantification and PCR amplification are still comparable after 3 months at 37°C when plasma VL is >1000 and >5000 copies/mL, respectively.

Using the same extraction method, Garcia-Lerma et al.[5] showed that PCR amplification of a 1023 bp fragment was only feasible from DBS for up to 2 weeks of storage at 37°C, which is lower than what we observed here in our study, although this could be related to different PCR protocols or the shorter fragment used in our study. Recently, van Deursen et al.[11] showed that VL measurement was only possible for up to 9 weeks at 37°C/40% humidity and 3 weeks at 37°C/80% humidity. Interestingly, they showed good quantification results after a shipment simulation cycle of 3 weeks with variation of temperature and humidity followed by storage at 37°C until 9 weeks.

Several studies have shown that DNA contributes to a significant proportion of the nucleic acid extract and consensus sequence when using DBS.[12–14] Here we showed that the contribution of DNA in VL and PCR efficiencies accounts for a significant part of the final results after long-term storage. However, if we consider the VL in plasma samples as the reference, the DNA contribution of DBS in the final results seems to balance RNA degradation over time and reflects plasma VL, suggesting that DBS could potentially be used to identify patients with virological failure under ART. However, this should be confirmed on a larger scale on patient samples and under field conditions. Importantly, when the NASBA technology is used for VL measurements, double-stranded DNA does not participate in the amplification process and results will be comparable to those for DPS over time.[3]

For PCR amplifications, DNA is also predominantly amplified from DBS over time, and as a consequence the final drug resistance mutations will not necessarily reflect the replicating virus in the patient. This could be less important in population-based studies for surveillance of transmitted drug resistance, but could be problematic in studies on monitoring of HIV drug resistance and at the individual patient level where it is important to know the profile of the replicating virus. Some studies have reported concordance between the two specimen types[15,16] and others have reported a higher frequency of resistance mutations in the plasma, suggesting a higher sensitivity to detect early treatment failure in plasma than in DBS.[13,17,18] Moreover, no data are yet available on mutation profiles over time for DBS. Due to the limited number of samples, we could not study this parameter over time in this report.

In conclusion, virological monitoring is still feasible for DBS after 3 months of storage at 37°C when VL is >5000 copies/mL, but DNA contributes largely to the final results. Further studies are needed on a larger sample set to know to what extent VL and drug resistance testing on DBS after transport and/or storage under field conditions can be used to identify virological failure and to switch treatment at the individual patient level for which a threshold of 1000 copies/mL is usually recommended, which also corresponds to the lower detection limit of most of the genotypic drug resistance tests.

Transparency declarations

None to declare.

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