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Publication date
2013

Link to publication

Citation for published version (APA):
Hamers, R. L. (2013). *Drug-resistant HIV-1 in sub-Saharan Africa: clinical and public health studies*. [Thesis, fully internal, Universiteit van Amsterdam].

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Chapter 15

Dried fluid spots for HIV type-1 viral load and resistance genotyping: a systematic review

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Antiviral Therapy 2009;14(5):619–629

ABSTRACT

Background

Dried spots on filter paper made of whole blood (dried blood spots; DBS), plasma (dried plasma spots; DPS) or serum (dried serum spots) hold promise as an affordable and practical alternative specimen source to liquid plasma for HIV type-1 (HIV-1) viral load determination and drug resistance genotyping in the context of the rapidly expanding access to antiretroviral therapy (ART) for HIV-1-infected individuals in low- and middle-income countries. This report reviews the current evidence for their utility.

Methods

We systematically searched the English language literature published before 2009 on Medline, the websites of the World Health Organization and US Centers for Disease Control and Prevention, abstracts presented at relevant international conferences and references from relevant articles.

Results

Data indicate that HIV-1 viral load determination and resistance genotyping from DBS and DPS is feasible, yielding comparable test performances, even after storage. Limitations include reduced analytical sensitivity resulting from small analyte volumes (approximately 3.5 $\log_{10}$ copies/ ml at 50 µl sample volume), nucleic acid degradation under extreme environmental conditions, impaired efficiency of nucleic acid extraction, potential interference of archived proviral DNA in genotypes obtained from DBS and the excision of spots from the filters in high-volume testing.

Conclusions

This technology offers the advantages of a stable specimen matrix, ease of sample collection and shipment. The current sensitivity in drug resistance testing is appropriate for public health surveillance among pretreatment populations. However, consistently improved analytical sensitivity is needed for their routine application in the therapeutic monitoring of individuals receiving ART, particularly at the onset of treatment failure.

INTRODUCTION

The use of combination antiretroviral therapy (ART) in individuals infected with HIV type-1 (HIV-1) has been shown to effectively reduce morbidity and mortality worldwide [1]. The effectiveness of ART is typically assessed by regular enumeration of CD4+ T-cells and determination of HIV-1 viral load, and, in case of suspected treatment failure, drug resistance genotyping [2]. However, standard methods of viral load determination and genotyping require the appropriate collection, processing and storage of plasma specimens, trained personnel and a molecular laboratory infrastructure, including a centrifuge and freezers. High cost and complexity render these methods unsuitable for resource-limited settings [3]. Given that more than 90% of new HIV-1 infections occur in low- and middle-income countries, and that the availability of ART in these countries has greatly expanded in recent years [4], there is a need to develop simplified methods of specimen collection, storage and transport, which are adapted to field conditions.

The most promising approach in this respect is the spotting and drying of blood specimens on an absorbent filter paper matrix. This has several technical, practical and economic advantages over using liquid plasma. Dried blood spots (DBS) can be prepared by healthcare practitioners with relatively little training, require no manipulation at the clinic level, are non-hazardous and can be dispatched to reference testing facilities by regular mail at ambient temperature without the need for expensive dry ice shipments. Moreover, DBS are particularly attractive for paediatric applications, given the challenges of phlebotomy in young children. DBS are already being used for a number of serological and molecular (qualitative) assays, such as for screening of metabolic disorders in neonates [5, 6], detection of HIV-1 antibodies [7–9] and DNA PCR for infant diagnosis of HIV-1 [10–12]. Additionally, DBS as well as dried serum spots (DSS) and/or dried plasma spots (DPS) have been evaluated for HIV-1 viral load quantification [13–26], resistance genotyping [27–38], p24 antigen quantitation [39, 40] and CD4+ T-cell enumeration [41].

Compared with liquid plasma-based methods, however, the use of dried fluid spots has some potential disadvantages, which include reduced test sensitivity in HIV-1 viral load quantification [15, 23] and genotyping assays [30, 32, 33, 35] because of small analyte input volumes and impaired efficiency of nucleic acid extraction [29], as well as nucleic acid degradation under environmental storage conditions [16, 27, 32]. Moreover, archived proviral DNA *pol* sequences might interfere with the genotypic profiles generated from DBS [27, 33, 34].

This report reviews the current evidence for the utility of dried fluid spots as a specimen matrix for HIV-1 viral load and resistance genotyping assays. Additionally, remaining challenges and recommendations for further research are discussed.

METHODS

Search strategy and selection criteria

References for this review were identified by a systematic search of the English language literature published before 1 January 2009 on Medline, the websites of the World Health Organization (WHO) and US Centers for Disease Control and Prevention, abstracts presented at relevant international conferences and references from relevant articles. Search terms used in combination were 'HIV-1', 'diagnostic test', 'dried fluid spot', 'dried blood spot', 'dried plasma spot', 'dried serum spot', 'filter paper', 'viral load', 'resistance genotyping' and 'resource-limited settings'. Two observers independently reviewed and extracted data from the studies. Disagreements about data extraction were settled by conversation. Studies that evaluated the test performance of DBS, DPS and/or DSS for HIV-1 viral load quantification and/or genotyping were selected. Studies that evaluated the performance of dried fluid spots for diagnosis of HIV-1 infection using qualitative molecular methods were excluded. Extracted data were the laboratory methods used, test performance of dried fluid spots compared with the reference standard liquid plasma, and nucleic acid stability during storage. All RNA values are expressed as $\log_{10}$ transformed copy numbers of RNA per ml of liquid plasma, DBS, DPS or DSS equivalent.

HIV-1 VIRAL LOAD QUANTIFICATION

Dried blood spots

Laboratory methods

Twelve studies evaluated DBS for HIV-1 viral load quantification [13–21, 24–26] (table 1). All studies used 903 filter paper (Whatman, Maidstone, UK; previously Schleicher & Schuell, Keene, NH, USA), two of which additionally used Isocode paper (catalogue number 495020; Schleicher & Schuell) [16, 20]. DBS were prepared using whole blood, either obtained by venipuncture (anti-coagulated) [13, 14, 16, 17, 19, 20, 26], by finger puncture [18] or unspecified [15, 21, 24, 25]. The specimen input volumes were 50 µl [19, 20, 26], 100 µl [13, 16, 17, 25], 200 µl [14] or unspecified [18, 21, 24]. Viral load assays used were based on nucleic acid sequence based amplification (NASBA) or reverse transcriptase (RT)-PCR (table 1).

Table 1. Summary of studies that evaluated the use of DBS for HIV viral load quantification.

Reference	Year of publication	Laboratory methods							Test performance			Nucleic acid stability				Comment
		Specimen source	No.	Viral load range#	Filter paper	Spot volume (µl)	Drying time	Quantification	Elution-extraction	Correlation coefficient*	Mean RNA difference#	LDL#	Storage conditions	Correlation coefficient*	Mean RNA difference#	LDL#	
Abdo et al. (26)	2008	ARV experienced, South Africa	200	1.7 to 4.0	903	50	N/A	NucliSens EasyQ NASBA	EasyMAG	N/A	0.35	3.6	6wks at -20°C, 4°C, 28°C, 40°C	N/A	N/A	N/A	
Abravaya et al. (25)	2008	N/A	N/A	3 to 6	903	100	N/A	Abbott RealTime RT-PCR	Abbott RealTime RT-PCR	N/A	N/A	3.3	N/A	N/A	N/A	N/A	
Alvarez Munoz et al. (13)	2005	ARV naive, Mexico	127	N/A	903	100	overnight	NucliSens QT NASBA	Nuclisens	0.99	N/A	N/A	travel (3-27h) at room temperature; 7 days at 4, 22, and 37°C	0.95@	<0.5	N/A	Good correlation DBS-DPS after 7 days storage at 37°C
Ayele et al. (14)	2007	ARV naïve and experienced, The Netherlands and Ethiopia	103	<1.7 to 6.5	903	200	30 min	Rainbow NASBA	Boom et al. (50)	0.7706	N/A	N/A	N/A	N/A	N/A	N/A	Good correlation DBS-DPS
Brambilla et al. (15)	2003	US	6	3.6 to 5.7	903	50	overnight	NucliSens QT NASBA/ Amplicor RT-PCR (modified)	NucliSens/ CORD reagent	N/A	-0.44 to 0.82	3.6	52 weeks at room temperature and -70°C	N/A	-2.4%	N/A	Amplicor has a broader dynamic range and is more sensitive compared with NucliSens
Fiscus et al. (16)	1998	US	76	N/A	903†	100	overnight	NucliSens QT NASBA	NucliSens	0.88‡	0.0478‡	N/A	28 days at room temperature	N/A	-0.026 per day	N/A	Problems with stability may exist with prolonged storage

Table 1 (continued)

Reference	Year of publication	Laboratory methods							Test performance			Nucleic acid stability				Comment
		Specimen source	No.	Viral load range[#]	Filter paper	Spot volume (µl)	Drying time	Quantification	Elution-extraction	Correlation coefficient*	Mean RNA difference[#]	LDL[#]	Storage conditions	Correlation coefficient*	Mean RNA difference[#]	LDL[#]	
Idigbe et al. (24)	2003	Nigeria	94	N/A	903	N/A	N/A	Rainbow NASBA	Boom et al. (50)	0.999	N/A	N/A	N/A	N/A	N/A	N/A	
Mwaba et al. (18)	2003	ARV naïve, Zambia	51	N/A	903	N/A	3h	NucliSens QT NASBA	NucliSens	N/A	N/A	N/A	6 weeks at room temperature	N/A	N/A	3.5	Good correlation DBS-DPS
O,Shea et al. (19)	1999	UK	27	N/A	903	50	2h	NucliSens QT NASBA	NucliSens	0.84	<0.5	N/A	1 to 13 months at -70 °C	N/A	<0.5	N/A	
Kane et al. (17)	2008	Senegal	41	2.9 to 5.4	903	100	overnight	NucliSens EasyQ real-time	NucliSens-Boom et al. (50)	0.90	N/A	2.9	8 and 15 days at 37°C	0.98, 0.94, resp.	-0.07, -0.01, resp.	N/A	
Uttayamakul et al. (20)	2005	Thailand	30	N/A	903[†]	50	18-24h	NucliSens QT NASBA	NucliSens-Boom et al. (50)	0.817	0.174	N/A	N/A	N/A	N/A	N/A	
Waters et al. (21)	2007	ARV experienced, Uganda	306	2.7 to 5.7	903	N/A	N/A	COBAS TaqMan	N/A	0.72	N/A	N/A	N/A	N/A	N/A	N/A	High rate of false-positive viral loads

For each study a brief description is provided of laboratory methods used, DBS test performance compared with the reference standard liquid plasma (unless stated otherwise), data on nucleic acid stability under environmental storage conditions, and any relevant comments. * Correlation coefficients express correlation with the reference standard liquid plasma; [#] HIV viral load expressed as $\log_{10}$ copies/ml; [†] In addition to 903 filter paper, Isocode was used; [‡] Results are for 903 filter paper; for Isocode correlation coefficient = 0.90 and mean RNA difference = 0.22 $\log_{10}$ copies/ml; [@] For 7 days at 4, 22, and 37°C Kappa = 0.98, 0.83 and 0.94, resp. ARV, antiretroviral; LDL, lower detection limit; NA, not available; NASBA, nucleic acid sequence based amplification; RT, reverse transcriptase.

Test performance

All commercial viral load assays show the ability to quantify RNA from DBS, with a lower detection limit of 2.9 [17] to 3.3 [25] $\log_{10}$ RNA copies/ml at a specimen input volume of 100 µl, and 3.6 $\log_{10}$ copies/ml at a specimen input volume of 50 µl [15, 26]. Collectively, correlation coefficients for RNA viral loads in DBS and paired liquid plasma specimens ranged from 0.72 to 0.99 [13, 14, 16, 17, 19–21, 24]. In various studies, detection of RNA from DBS was linear over a broad dynamic range: 2.7 to 5.7 [21], 2.9 to 5.4 [17], 3.0 to 6.0 [25] and 3.6 to 5.7 [15] $\log_{10}$ copies/ ml. Median RNA differences between plasma and DBS were <0.5 $\log_{10}$ copies/ml [15, 16, 19, 20, 26] or unspecified [13, 14, 17, 18, 21, 24, 25]. One study reported a high rate of false-positive detectable RNA loads from DBS compared with plasma, which the authors attributed to a possible cross-reaction between cell-associated HIV-1 DNA and the RT-PCR assay [21]. One study reported a broader dynamic range and increased sensitivity at lower RNA loads for the Amplicor RT-PCR assay compared with the NucliSens NASBA assay, probably resulting from the differences in the amount of the actual RNA eluate that was used in the amplification steps for the two assays. For Amplicor, the specimen input volume was equivalent to 25 µl, which is comparable to the volume used for the standard Amplicor assay, whereas the specimen input volume for NucliSens was equivalent to 5 µl, which is 1/4 to 1/20 of the volume used for plasma analysis by the NucliSens assay [15] (table 1).

Nucleic acid stability

Several studies have shown that RNA in DBS is sufficiently stable under variable conditions: 7 days at 37°C and 60% humidity, 12 weeks at 22°C or freeze-thawing twice [23]; 15 days at 37°C [17]; 2 weeks at 22°C or 7 days at 37°C [13]; 3–27 h travel time at room temperature in different climates [13]; 6 weeks at room temperature (22–28°C) [18,26]; and 52 weeks at room temperature [15] or at -70°C [15,19]. However, Fiscus *et al*. [16] reported a statistically significant decrease of 0.0261 $\log_{10}$ copies/ml per day over a 28-day period at room temperature, which is equivalent to a loss of approximately 5% per day; in this study it was not clear whether the RNA degraded with time or whether there was increasing difficulty in recovering the RNA from the filter paper after prolonged storage (table 1).

Dried plasma spots

Laboratory methods

Nine studies evaluated DPS [13–15, 18, 21–24, 26] (and no study evaluated DSS) for RNA viral load quantification (table 2). All studies used 903 filter paper. The specimen input volumes were 50 µl [15, 23, 26], 100 µl [13], 200 µl [14, 22] or not specified [18, 21, 24]. Viral load assays used were based on NASBA or RT-PCR (table 2).

Test performance

All commercial viral load assays show the ability to quantify RNA from DPS, with a lower detection limit of 3.5 [23] to 3.6 [26] $\log_{10}$ copies/ml at a specimen input volume of 50 µl, and 3 $\log_{10}$ copies/ml at a specimen input volume of 200 µl [22]. Collectively, correlation coefficients for RNA levels in DPS and paired liquid plasma specimens ranged from 0.86 to 0.97 [14, 22–24]. In various studies, detection of RNA from DPS was linear over a broad dynamic range: 3.2 to 8.4 [23], 2.6 to 5.7 [22] and 3.6 to 5.7 [15] $\log_{10}$ copies/ml. Median RNA differences between plasma and DPS were 0.077 $\log_{10}$ copies/ ml [23], 0.16 $\log_{10}$ copies/ml [26], 0.64 $\log_{10}$ copies/ml [22] or unspecified [13–15, 18, 21, 24] (table 2).

Nucleic acid stability

Several studies have shown that for viral load determination RNA in DPS is sufficiently stable under variable conditions: 1 week at 4°C, 22°C [22] or 37°C [13]; 2 weeks at 4°C or 20°C [23]; 3 days at 37°C with high humidity [23]; 6 weeks at room temperature (22–28°C) [18, 26]; 1 year at room temperature or -70°C [15]. However, Amellal *et al*. [22] reported a significant loss of RNA (0.92 $\log_{10}$ copies/ml) in DPS stored at 37°C for 1 week compared with plasma at -80°C. As the greatest RNA depletion occurred in the experiment with the longest drying time, the authors speculated that the loss might be attributable to the absence of desiccant during storage (table 2).

Correlation of dried blood spots and dried plasma spots

Five studies evaluated the correlation of DBS and DPS for RNA quantification [13–15, 18, 21]. Significant correlations were found by most studies [13, 14, 18, 21], including evaluations done after storage for 6 weeks at 22–28°C [18] and for 7 days at 37°C [13]. Waters *et al*. [21] found that agreement between 122 DBS/ DPS pairs was fair (Cohen's Kappa=73%). One study reported that RNA loads from DBS were, on average, 0.11 $\log_{10}$ copies/ml (29%) higher than those from DPS [15], whereas Ayele *et al*. [14] found that RNA loads generated from DBS tended to be slightly lower than those from DPS.

RESISTANCE GENOTYPING

Dried blood spots

Laboratory methods

Nine studies evaluated DBS for HIV-1 genotyping [27, 28, 31–34, 36–38], one of which sequenced the cellular proviral DNA instead of viral RNA [36] (table 3). All studies used 903 filter paper, one of which additionally used the FTA matrix (Whatman) [28]. DBS were prepared using whole blood, either obtained by venipuncture (anti-coagulated)

Table 2. Summary of studies that evaluated the use of DPS/DSS for HIV viral load quantification

Reference	Year of publication	Laboratory methods							Test performance			Nucleic acid stability				Comment
		Specimens	No.	Viral load range#	Filter paper	Spot volume (µl)	Drying time	Quantification	Elution-extraction	Correlation coefficient*	Mean RNA difference#	LDL#	Storage conditions	Correlation coefficient*	Mean RNA difference#	LDL#	
Abdo et al. (26)	2008	ARV experienced, South Africa	200	1.7 to 4.0	903	50	N/A	NucliSens EasyQ NASBA	EasyMAG	N/A	0.16	3.6	N/A	N/A	N/A	N/A	
Alvarez Munoz et al. (13)	2005	ARV naive, Mexico	127	N/A	903	100	overnight	NucliSens QT NASBA	NucliSens	N/A	N/A	N/A	travel (3-27h) at room temperature; 7 days at 37°C	N/A	-0.19	N/A	Good correlation DBS-DPS after 7 days storage at 37°C
Amellal et al. (22)	2007	ARV naïve and experienced, France	45	2.6 to 5.7	903	200	4h	Amplicor RT-PCR	PBS buffer	0.92	-0.64	3	7 days at 4, 22 and 37°C, resp.	0.98, 0.97, 0.84, resp.	-0.20, 0.03, -0.92, resp.	N/A	Significant RNA loss after 7 days at 37°C, but not at room temperature. RNA loss associated with lack of desiccation.
Ayele et al. (14)	2007	ARV naïve and experienced, The Netherlands and Ethiopia	103	<1.7 to 6.5	903	200	30 min	Rainbow NASBA	Boom et al. (50)	0.92 /0.96†	N/A	N/A	N/A	N/A	N/A	N/A	Good correlation DBS-DPS
Brambilla et al. (15)	2003	US	6	3.6 to 5.7	903	50	overnight	NucliSens QT NASBA/ Amplicor RT-PCR (modified)	NucliSens/ CORD reagent	N/A	N/A	3.6	52 weeks at room temperature and -70°C	N/A	-2.4%	N/A	Amplicor has a broader dynamic range and is more sensitive compared with NucliSens

Table 2 (continued)

Reference	Year of publication	Laboratory methods								Test performance			Nucleic acid stability				Comment
		Specimens	No.	Viral load range#	Filter paper	Spot volume (µl)	Drying time	Quantification	Elution-extraction	Correlation coefficient*	Mean RNA difference#	LDL#	Storage conditions	Correlation coefficient*	Mean RNA difference#	LDL#	
Cassol et al. (23)	1997	ARV naïve, Canada	73	3.2 to 8.4	903	50	3h	NucliSens QT NASBA/ Amplicor RT-PCR (modified)	NucliSens/ Amplicor	0.97	-0.077	3	16 days at 4 and 22C, low-humidity; 3 days at 37°C high-humidity, resp.	0.98, 0.97, 0.97, resp.	-0.063, -0.080, -0.055, resp	N/A	Good correlation NucliSens-Amplicor over a 5 log_{10} dynamic range
Idigbe et al. (24)	2003	Nigeria	94	N/A	903	N/A	N/A	Rainbow NASBA	Boom et al. (50)	0.858	N/A	N/A	N/A	N/A	N/A	N/A	
Mwaba et al. (18)	2003	ARV naïve, Zambia	51	N/A	903	N/A	3h	NucliSens QT NASBA	N/A	N/A	N/A	N/A	6 weeks at room temperature	N/A	N/A	3.2	Good correlation DBS-DPS
Waters et al. (21)	2007	ARV experienced, Uganda	218	2.7 to 5.7	903	N/A	N/A	COBAS TaqMan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Excellent agreement with reference standard

For each study a brief description is provided of laboratory methods used, DPS/DSS test performance compared with the reference standard liquid plasma (unless stated otherwise), data on nucleic acid stability under environmental storage conditions, and any relevant comments. * Correlation coefficients express correlation with the reference standard liquid plasma; # HIV viral load expressed as log_{10} copies/ml; † Refers to non-B/B subtypes, respectively. ARV, antiretroviral; LDL, lower detection limit; NA, not available; NASBA, nucleic acid sequence-based amplification; PBS, phosphate-buffered saline; RT, reverse transcriptase.

Table 3. Summary of studies that evaluated the use of DBS for resistance genotyping

Reference	Year of publication	Laboratory methods								Test performance			Nucleic acid stability	Comment
		Specimen source	No.	Viral load range[#]	Subtypes	Filter paper	Spot volume (µl)	Drying time	Elution-extraction	Genotyping	Amplification success	Nucleotides*	Mutations*	Storage conditions	
Bertagnolio et al. (27)	2007	ARV naïve, Mexico	103	3.7 to 5	B	903	100	3h	NucliSens	In-house nested RT-PCR; PR-RT >1008 bp (2 amplicons),	78% overall; 94% >4log10 cps/ml	99.9%	92% (12/13)	3 months at 37°C and 85% humidity	
Buckton et al. (28)	2008	UK	33	1.9 to 5.1	multiple	903[†]	100	1h	Boom et al.(50)	PR-RT 1245 bp (2 amplicons)	83%boverall; 62% <1.7 log10cps/ml	N/A	83% (10/12)	3 months at 4°C and room temperature, with desiccant	Best DNA recovery with 903 and silica/ guanidine extraction
Garrido et al. (31)	2008	ARV experienced, Angola	77	<3 to 5.9	multiple	903	N/A	overnight	Boom et al. (50)	In-house nested RT-PCR; RT-gp41	58% overall	N/A	N/A	4°C, no desiccant	Poor amplification rate because of high HIV genetic diversity and lack of humidity control
Hallack et al. (32)	2008	ARV naïve and experienced, USA	33	3.1 to 5.6	B	903	75-80	4-24h	NucliSens	Trugene (1.3kb)	79% overall; 91% >3.8 log10 cps/ml; 58% 3-3.8log10 cps/ml	99.3%	100%	N/A	Sensitivity improved using 2 spots instead of 1
Masciotra et al. (33)	2007	ARV naïve and experienced, Spain	60	1.9 to 5.8	B	903	50	overnight	NucliSens/ Boom et al. (50)	ViroSeq/ Trugene (DNA: in-house nested RT-PCR; PR-RT 1023 bp)	83% overall; 100%>3.3 log10 cps/ml; 55% <3.3 log10 cps/ml	98.%	97% (306/316)	18-26 weeks at -20°C, with desiccant	HIV DNA in 44% of DBS
McNulty et al. (34)	2007	ARV naive, USA	9	3.5 to 5.5	B	903	50	overnight	Nuclisens/ Boom et al. (50)	In-house nested RT-PCR; PR-RT 1023 bp	N/A	98.6%	82% (14/17)	5-6 yrs at -30°C, -70°C, room temperature	

Table 3 (continued)

Reference	Year of publication	Laboratory methods								Test performance			Nucleic acid stability	Comment
		Specimen source	No.	Viral load range#	Sub-types	Filter paper	Spot volume (µl)	Drying time	Elution-extraction	Genotyping	Amplification success	Nucleo-tides*	Muta-tions*	Storage condi-tions	
Idem		ARV naive, Cameroon	40	2.8 to 5.3	multiple	idem	idem	idem	idem	idem	93% overall; 73% <4 log10 cps/ml; 100% >4 log10 cps/ml	98.5%	N/A	2-3 year at -20°C	
Steegen et al. (36)	2007	ARV naïve and experienced, Kenya	60	N/A	A, D, AD, G	903	50	overnight	QIAamp DNA blood mini kit (Qiagen)	In-house nested RT-PCR; PR 458 bp, RT 646 bp	93% overall; 55% <1.7 log10 cps/ml	97.9%	44% (15/34)	N/A	RNA sequencing superior to DNA sequencing for detection of resistance mutations
Youngpai-roj et al. (37)	2008	ARV-experienced, Spain	40	2.7 to 5.8	B	903	50	overnight	NucliSens	ViroSeq (1.8kb)	58% overall; 96% >4 log10 cps/ml	N/A	97% (158/163)	1year at 4°C, with desiccant	ViroSeq shows poor amplification
idem		idem	idem	idem	idem	idem	idem	idem	idem	In-house nested RT-PCR; PR-RT 1023 bp	95% overall	N/A	95% (275/291)	idem	
Ziemniak et al. (38)	2006	ARN naïve and experienced, US and Zambia	12	2.3 to 6.5	B, C	903	250	overnight	High Pure Viral Nucleic Acid Kit (Roche)	In-house nested RT-PCR; RT 663 bp	100% overall	N/A	100% (6/6)	<4.9 months at room temperature, with desiccant	

For each study a brief description is provided of laboratory methods used, DBS test performance compared with the reference standard liquid plasma (unless stated otherwise), data on nucleic acid stability under environmental storage conditions, and any relevant comments. * Concordance with the reference standard liquid plasma; # HIV viral load expressed as $\log_{10}$ copies/ml; † In addition to 903 filter paper, the FTA matrix was used. ARV, antiretroviral; N/A, not available.

[27, 32, 34, 36], by finger puncture [31] or unspecified [33, 37, 38]. The specimen input volumes were 50 µl [34, 36, 37], 75–80 µl [32], 100 µl [27], 250 µl [38] or not specified [31]. The studies include a number of different nucleic acid extraction and amplification protocols, including commercial kit-based strategies [33, 37] and in-house techniques [27, 31, 32, 34, 36, 38] (table 3).

Test performance

Overall, amplification success rates ranged from 58% to 95% [27, 31–34, 36–38]. Amplification success rates tend to be high for high RNA viral loads (>4 [27, 34, 37], >3.8 [32], >3.3 [33] or >3 [28] $\log_{10}$ copies/ml), but reduced for lower viral loads [28, 32, 33]. Buckton *et al*. [28] achieved a 100% amplification success rate from DBS with >3.0 $\log_{10}$ copies/ml and 62% from DBS with undetectable (<1.7 $\log_{10}$ copies/ml) plasma viral load at a 100 µl specimen input volume, using 903 filter paper and silica/guanidine extraction (table 3). Most studies have aimed to compare the nucleotide sequences generated from paired DBS and plasma specimens. Reported concordance between nucleotide sequences generated from the two specimen types ranged from 98.5% to 99.9% [27, 34]. Although some studies also reported concordance for drug resistance-associated codons between paired DBS and plasma specimens [27, 32, 34, 38], others have reported discrepancies [28, 33, 36]. A small number of studies examined the potential interferences of archived proviral DNA in the genotypic results generated from DBS. These studies were performed by amplifying from viral extracts with and without a reverse transcription step. The rate of amplification from DBS without reverse transcription ranged between 44% and 80% [27, 33, 34], which suggests that proviral DNA might contribute to a significant proportion of DBS consensus sequences.

Nucleic acid stability

Several studies have shown high success of amplification and genotyping after DBS storage under variable conditions: 3 months at 37°C and 85% humidity [27]; 18–26 weeks at -20°C with desiccant [33]; 2–3 years at -20°C or 6 years at -30°C [34]; 1 year at 4°C with desiccant [37]; and up to 4.9 months at room temperature with desiccant [38]. Garrido *et al*. [31] found impaired success of amplification resulting from lack of humidity control. Most studies highlighted the importance of drying DBS completely prior to storage in a zip-locked plastic bag containing silica gel desiccant. Following 1 year of storage at 4°C, Youngpairoj *et al*. [37] found an excellent (95%) amplification rate using an in-house assay, but poor (58%) amplification using ViroSeq, possibly because of the fact that ViroSeq amplifies a large *pol* fragment, which might be more sensitive to nucleic acid degradation during long-term storage at suboptimal temperature, humidity or both. McNulty *et al*. [34] found that none of the DBS stored for 5 years at room temperature were amplified. Youngpairoj *et al*. [37] showed that it is possible to overcome potential

losses in RNA integrity and efficiently genotype from DBS stored at 4°C by using nested RT-PCR that amplifies a smaller fragment (table 3).

Dried plasma/serum spots

Laboratory methods

Two studies evaluated DSS [29, 35] and two studies evaluated DPS [29, 30] for HIV-1 genotyping (table 4). All studies used 903 filter paper. The specimen input volumes was 20 µl [29, 30, 35]. Amplification was done by an in-house nested RT-PCR assay. Comparison of two elution–extraction methods showed the importance of the choice of extraction buffer; in particular, extraction lysis buffer applied directly to the spots led to aggregation of the filter paper, which probably impaired the elution efficiency [29] (table 4).

Test performance

Genotyping was reliable above viral load values of 4 $\log_{10}$ copies/ml from 20 µl DSS [35] and DPS [30]. The authors attributed the reduced sensitivity to the small sample volume used for the spot (20 µl) instead of 140 µl for a standard plasma sample (table 4).

Nucleic acid stability

High success of amplification and genotyping was achieved after storage of no more than 14 days at 4°C without desiccant [35] and 7 days at room temperature [29, 30]. More extreme conditions were not examined (table 4).

DISCUSSION

Most studies published to date have indicated that HIV-1 viral load quantification [13–26] and resistance genotyping [27–38] from dried fluid spots is feasible. Overall, DBS and DPS seem to yield comparable performance, even after storage [13–15, 18, 21]. However, certain limitations and challenges to their practical use remain. In the first place, the lower limit of detection of a viral load assay with dried fluid spots might never reach that of an assay with liquid plasma as a result of the small sample volumes of the spots. The currently reached sensitivity (approximately 3.5 $\log_{10}$ copies/ ml at 50 µl input volume) might still be useful to provide clinical guidance regarding drug regimen switch in individuals receiving ART. The current sensitivity levels in drug resistance testing seem to be appropriate for public health surveillance among newly diagnosed or pretreatment populations. This has led WHO to recommend the use of DBS for this application [42]. However, consistently improved analytical sensitivity is needed for routine application of DBS for the monitoring of drug resistance in individuals receiv-

Table 4. Summary of studies that evaluated the use of DPS/DSS for resistance genotyping

Reference	Year of publication	Laboratory methods								Test performance			Nucleic acid stability	Comment
		Specimen source	No.	Viral load range#	Subtypes	Filter paper	Spot volume (µl)	Drying time	Elution-extraction	Genotyping	Amplification success	Nucleotides*	Mutations*	Storage conditions	
Plantier et al. (35)	2005	DSS, ARV naïve and experienced, France	62	2.9 to 5.9	multiple	903	20	1h	QIAamp Viral RNA minikit (Qiagen)	Nested RT-PCR; PR 507 bp, RT 798 bp	82%; 86% 4-5 log10 cps/ml; 38% <4 log10 cps/ml	N/A	N/A	≤14 days at 4°C, no desiccant	
Dachraoui et al. (30)	2008	DPS, ARV naïve and experienced	33	3.1 to 5.9	B	903	20	1h	QIAamp Viral RNA minikit (Qiagen)	Nested RT-PCR; PR 507 bp, RT 798 bp	76%; 67% 4-5 log10 cps/ml; 0% <4 log10 cps/ml	N/A	N/A	9 days at room temperature	
Dachraoui et al (29)	2008	DPS and DSS	15	4.1 to 5.7	mostly B	903	20	1h	QIAamp Viral RNA minikit (Qiagen)	Nested RT-PCR; PR 507 bp, RT 798 bp	100% overall	N/A	N/A	2, 5, 7 day at room temperature	Different efficiency of 2 extraction methods

For each study a brief description is provided of laboratory methods used, DP/DSS test performance compared with the reference standard liquid plasma (unless stated otherwise), data on nucleic acid stability under environmental storage conditions, and any relevant comments. * Concordance with the reference standard liquid plasma; # HIV viral load expressed as $\log_{10}$ copies/ml. ARV, antiretroviral; bp, base pairs; NA, not available; PR, protease; RT, reverse transcriptase.

ing ART, particularly at the onset of treatment failure. The reduced assay sensitivities are mainly caused by the smaller specimen input volumes (20–200 µl), compared with liquid plasma specimens (140–500 µl) [29, 32, 35]. This results in an equivalently reduced number of HIV-1 RNA copies as input in the PCR reaction. Thus, further research should focus on improving the sensitivity of DBS assays, for instance by extracting virus from two or more spots [32, 33, 35] and by further concentration of the nucleic acid material upon extraction. Nested PCR has been used in several studies to increase sensitivity [27, 29–31, 33–38]. The main limitation for the analytical sensitivity of amplification assays based on dried fluid spots is caused by the amount of nucleic acid that is used as input in the first round PCR reaction. All investigations to improve the analytical sensitivity of amplification strategies based on dried fluid spots should therefore focus on optimization of extraction efficiency and to maximize the nucleic acid input in first round PCR reactions. A further downside of using nested PCR is the increased risk of carryover of amplicons, which poses a major limitation to its use particularly in low-resource settings.

Many researchers have reported high stability of nucleic acids (RNA and DNA) absorbed onto filter papers (mainly 903) and stored at ambient temperatures with humidity control. However, absence of desiccant [22, 31] and exposure to ambient and higher temperatures for extended periods [34] have been associated with degradation of nucleic acids. Most studies emphasize the importance of drying DBS completely prior to storage in a zip-locked plastic bag containing silica gel desiccant. It has been suggested that (partial) nucleic acid degradation might affect the longer DNA/RNA fragments that are required for sequencing to a greater extent than the shorter fragments amplified in viral load assays [28, 29, 31, 35]. Particularly, the large *pol* amplifications generated in various in-house or kit-based genotyping procedures might be more sensitive to nucleic acid degradation after storage under environmental conditions. The use of (in-house) RT-PCR assays that amplify a smaller genome fragment might result in higher amplification success rates [34, 37]. Overall, the results of molecular assays based on dried fluid spots are encouraging and support the use of dried fluid spots in areas where these materials can be (air)mailed to strategically situated reference testing facilities within a time span of a few days. For long-term DBS storage, the currently available data indicate that -20°C is preferable to preserve optimal amplification success.

Standard genotyping methods utilize only the plasma-derived virus population for amplification and sequencing. Unlike plasma, however, DBS contain proviral DNA archived in infected peripheral blood mononuclear cells (PBMCs). Although plasma RNA represents the population of short-lived actively replicating virus, proviral DNA from PBMCs is composed of a heterogeneous mix of DNA from acutely infected cells that actively produce virus as well as quiescent cells that comprise the viral reservoir [43, 44].

Resistance-associated mutations have been reported to emerge earlier in plasma than in the proviral archive in PBMCs [45], which might result in a higher sensitivity to detect early treatment failure in the plasma than in DBS. Notably, Steegen *et al*. [36] found that sequencing of proviral DNA from DBS resulted in failure of DBS to detect all mutations present in plasma, suggesting that in (early) treatment failure RNA sequencing is possibly superior to DNA sequencing. However, the extent of interference of proviral DNA sequences in the genotypic profiles generated from DBS might differ according to the disease stage, CD4+ T-cell counts, and treatment characteristics of the population as a result of the different dynamics of emergence and persistence of resistance mutations in plasma and PBMCs [46–48]. Further studies are warranted to elucidate the relative contribution of circulating RNA and proviral DNA in genotypic profiles generated from DBS in diverse populations [27, 33, 34].

The excision of individual spots from the filter papers prior to extraction is a labour-intensive procedure and carries risks for cross-contamination, which constitutes limitations for high-volume testing [49]. Therefore, the development of automated scissors or hole-punching machines and extraction methods are required to overcome these challenges. Logistically, the use of DPS involves the added step of venipuncture, requiring blood tubes containing EDTA and electricity-dependent centrifuge equipment, which might not always be available in remote areas. DBS would be the simplest method for blood sampling in remote low-resource areas, as it only requires a simple finger puncture, spotting and drying of a drop of whole blood on the filter paper, and (air)mailing it to a central laboratory. The need for a skilled phlebotomist and laboratory technician on-site as well as centrifuges, ultra-low-temperature freezers and dry ice for shipping can thus be avoided. However, the use of DBS might require active removal of the proviral cellular DNA [35], and an appropriate extraction method to remove PCR inhibitors present in erythrocytes [50].

Future studies should be directed towards further optimization and standardization of assay protocols, sensitivity and precision, nucleic acid stability under extreme storage conditions and, additionally for DPS, eliminating the need for on-site centrifugation to separate the plasma. Elimination of this step would broaden the applicability of DPS and render it suitable for use in settings that lack reliable electricity. Comparative studies of test performance of various commercial viral load assays are warranted. There is a need to coordinate and harmonize the research and development efforts on dried fluid spots conducted by various research groups. To this end, a global working group of international experts was recently established under coordination of WHO's Global HIV Drug Resistance Surveillance Network [42]. Multi-country collaborative studies have been initiated to refine technologies and definitely prove their utility for low-resource

Box. Recommendations for research

- Assess the performance of different extraction methods and viral load and resistance genotyping assays with dried fluid spots
- Develop optimized and standardized testing algorithms compatible with dried fluid spots
- Perform additional nucleic acid stability studies under extreme storage conditions (filter paper, storage and shipping temperature, humidity, time period, and UV light)
- Study interferences of proviral DNA in genotypes obtained from DBS in populations with diverse treatment characteristics
- Develop automated spot excision and nucleic acid extraction methods to enable high-volume testing
- Coordinate research efforts under guidance of global working groups of international experts

areas. Some recommendations for future research are listed in the Box. Finally, additional applications of the dried fluid spot technology should be considered; for instance, the study of the natural history of incident HIV-1 infection, therapeutic drug level monitoring and diagnosis and monitoring of relevant conditions such as hepatitis B and C.

In conclusion, the dried fluid spot filter paper technology offers the advantages of a stable specimen matrix, ease of sample collection and shipment with minimal biohazard risks, supporting its utility for the collection, storage and transport of large numbers of field specimens in low-resource settings. Available data have suggested that HIV-1 viral load determination and resistance genotyping from dried fluid spots is feasible. Although results to date are encouraging, assay sensitivities need to be improved, to allow application in regular monitoring of individual patients on ART. In addition, further nucleic acid stability studies as well as refinement and standardization of technologies are warranted to enable dried fluid spots to become the primary specimen collection device in resource-limited settings

ACKNOWLEDGMENTS

The authors wish to thank John Dekker for useful comments on the manuscript. This work resulted from the PharmAccess African Studies to Evaluate Resistance (PASER) and the Affordable Resistance Test for Africa (ART-A) programs.

Funding

PASER is supported by a grant from the Ministry of Foreign Affairs of the Netherlands (grant 12454). ART-A is supported by a grant from the Netherlands Organisation for Scientific Research/Science for Global Development (NWO/ WOTRO), under the Netherlands African Partnership for Capacity Development and Clinical Interventions against Poverty-related Diseases (NACCAP; grant W.07.05.204.00).

Contributors

RLH, RS and TFRdW designed the study. RLH and PWS performed the literature search, reviewed and extracted the data, and wrote the first draft of the manuscript. All authors contributed to subsequent drafts and reviewed the final manuscript.

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