AIDS Rev. 2010;12:195-208

Dried blood spots for HIV-1 Drug Resistance and Viral Load Testing: A Review of Current Knowledge and WHO Efforts for Global HIV Drug Resistance Surveillance

Silvia Bertagnolio[1], Neil T. Parkin[2], Michael Jordan[1,3], James Brooks[4] and J. Gerardo García-Lerma[5]

[1]World Health Organization, Geneva, Switzerland; [2]Data First Consulting, Inc., Menlo Park, CA, USA; [3]Tufts University School of Medicine, Boston, USA; [4]National HIV and Retrovirology Laboratories, Public Health Agency of Canada, Ottawa, Ontario, Canada; [5]Laboratory Branch, Division of HIV/AIDS Prevention, Centers for Disease Control, Atlanta, GA, USA

Abstract

HIV-1 drug resistance genotyping is an essential component of the World Health Organization global HIV Drug Resistance (HIVDR) prevention and assessment strategy. Plasma is considered to be the most appropriate specimen type for HIV-1 drug resistance genotyping. However, use of plasma may not be feasible in rural, remote areas in resource-limited settings since its preparation and storage requires personnel and laboratory infrastructure that is often lacking. An alternative specimen type for HIVDR genotyping is dried blood spots (DBS). DBS can be made from blood drawn for routine clinical or surveillance purposes without special laboratory processing. The filter paper used is relatively inexpensive, easily obtained and stored, and although procedures for making DBS must be followed precisely, the training required is less intensive than that required for plasma separation. HIV nucleic acids are generally stable over long periods of time and freezing is not required unless storage over two weeks is planned. In addition, DBS are more easily transported than plasma because they can be shipped as non-hazardous materials using regular mail or courier services. Many studies have reported the successful genotyping of HIV-1 from DBS and some have shown a high genotypic concordance with plasma genotypes despite potential DNA interferences. During the past few years DBS have started to be widely used for HIV-1 drug resistance testing, and an increased number of reports from resource-limited areas have indicated DBS as the preferred specimen type for transmitted HIV-1 drug resistance surveillance where plasma collection is not feasible. The World Health Organization has brought together a group of experts (WHO HIVResNet DBS working group) to review current data on DBS preparation, storage, and transport conditions, and provide a reference protocol, which is also summarized in this article. **(AIDS Rev. 2010;12:195-208)**

Corresponding author: Silvia Bertagnolio, bertagnolios@who.int

Key words

HIV-1. Drug resistance. DBS. Filter paper. Genotyping. Viral load. Surveillance.

Introduction

As of December 2002, UNAIDS reported that only 300,000 people infected with HIV in resource-limited countries of the five to seven million in need had access to antiretroviral therapy (ART), with an overall 5% coverage[1]. In 2003, the World Health Organization (WHO), UNAIDS, and partners launched the "3 by 5" initiative which galvanized the unprecedented expansion of ART in low- and middle-income countries. Currently, the HIV epidemic disproportionately affects resource-limited settings[2]. Over the past five years, through funding sources that include government programs, the Global Fund to fight AIDS, Tuberculosis, and Malaria, and the U.S. President's Emergency Plan for AIDS Relief, over four million individuals have been started on ART. At the end of 2007, three million people in low- and middle-income countries had access to HIV treatment, increasing to more than four million at the end of 2008. However, despite considerable

Correspondence to:
Silvia Bertagnolio
20 Avenue Appia
CH-1211 Geneva 27, Switzerland
E-mail: bertagnolios@who.int

progress and the achievement of near universal access to therapy for those in need in several low- and middle-income countries, 2008 estimates suggest that 58% of patients in immediate need still do not receive ART[2].

WHO strategy for HIV drug resistance prevention and assessment in resource-limited settings

WHO advocates a public health approach for the management of HIV in resource-limited settings[3]. In this context, while routine HIV drug resistance (HIVDR) testing for individual clinical monitoring is not recommended until capacity is expanded to provide more basic diagnostic and clinical tests to support HIV diagnosis and treatment, WHO strongly supports the use of HIVDR testing to assess the burden of transmitted and acquired resistance at the population level in countries scaling up ART[3,4]. The consequences of HIVDR include suboptimal response and more rapid virologic failure of patients on first-line regimens, increased direct and indirect health costs associated with the need to start more costly second-line treatments, the spread of resistant strains of HIV, and the potential impact on pre- and postexposure antiretroviral prophylaxis, topical microbicides, or regimens to prevent mother-to-child transmission (PMTCT).

As access to ART expands, the emergence of some degree of HIV drug resistance is inevitable even if appropriate regimens are provided and optimal adherence to therapy is supported. The error-prone nature of HIV replication, its high mutation rate in the presence of drug selective pressure, viral recombination, and the need for lifelong treatment can all contribute to drug resistance emergence. However, the emergence and transmission of HIVDR can be minimized through public health action at the clinical, country, regional, and global level. Improving quality of care by ensuring appropriate prescribing practices, minimizing the number of patients lost to follow up, ensuring drug supply continuity, and supporting patient adherence to ART can minimize the risk of HIVDR emergence.

Global estimates of the burden of transmitted and acquired HIVDR are difficult to gather due to the heterogeneity of laboratory and epidemiological methodologies across studies in different countries. The lack of standardization makes interpretation of results difficult, and the application of this information towards public health action particularly challenging. As a key contribution to the global HIV response, WHO is leading the global effort to assess and minimize HIVDR, with the overall goal of promoting the long-term effectiveness of the available first- and second-line regimens, improving quality of care, and optimizing treatment program efficiency. To achieve this ambitious goal, WHO has created HIVResNet, a network of over 50 institutions, laboratories, clinicians, epidemiologists, and HIVDR experts with the function to advise and support WHO through the development of standardized tools, training, technical assistance, laboratory quality assurance, analysis of results, and recommendations for guidelines and public health action.

Together with its partners, WHO has developed and is promoting the implementation of the global HIVDR prevention and assessment strategy[4]. The strategy relies upon implementation of standardized methodologies to evaluate the emergence and transmission of HIVDR, both in adult and pediatric populations, as well as the regular evaluation of HIVDR early warning indicators, which are site and programmatic factors potentially associated with HIVDR emergence. In countries adopting and implementing the global HIVDR strategy, the evidence generated through HIVDR assessment is being used to make important, positive public health action to improve ART programs.

WHO/HIVResNet global HIVDR prevention and assessment strategy includes eight key elements, which have been described in detail elsewhere (http://www.who.int/hiv/topics/drugresistance/en/)[4]. Two of these elements require HIVDR laboratory testing.

The first element monitors HIVDR emerging during treatment and is commonly referred to as HIVDR monitoring survey. This survey utilizes a minimum resource, prospective, clinic-based approach for monitoring the emergence of HIVDR during the first 12 months of treatment, and for identifying factors associated with failure of viral suppression and HIVDR emergence[5]. HIVDR monitoring surveys include resistance testing before initiation of ART and one year thereafter (or before switching to a second-line regimen). The survey should be integrated into routine national assessments of ART programs and is designed to use remnant specimens (plasma or dried blood spots [DBS]) collected for routine testing. The data generated promote efforts to minimize the emergence of HIVDR at a population level by implementing positive site/programmatic adjustments as needed.

A second key element of WHO/HIVResNet global HIVDR prevention and assessment strategy is the surveillance of transmitted drug resistance in untreated, recently infected populations. The surveillance targets individuals infected with HIV-1 within the past 3-5 years who remain ART-naive (aged < 25 years and at first pregnancy, if female) and/or, when available, have laboratory evidence of recent infection or seroconversion, have no previous positive HIV test,

a CD4 cell count above 500 cells/µl, and the first risk-defining event within the past three years. The survey targets specific geographical areas within each country where ART has been widespread for 3-5 years and where HIVDR is most likely to emerge first[6]. The survey is called the HIVDR threshold survey because results categorize transmitted resistance to relevant antiretroviral drugs and drug classes as above or below two thresholds: 5 and 15%[7].

The threshold survey is, whenever possible, embedded into regularly performed serosurveys to estimate HIV prevalence, which are already in place in most resource-limited settings. Generally, consecutive HIV-seropositive diagnostic specimens (plasma, sera, or DBS) from HIV serosurveillance performed in antenatal clinics, voluntary counseling and testing centers or other HIV diagnostic settings are used, along with information collected routinely at these sites. The results of threshold surveys contribute to ART policy decisions, including ART regimens and HIV prophylaxis guidelines.

Dried blood spots for genotypic drug resistance testing

Resistance to antiretroviral drugs can be determined phenotypically in cell culture-based assays, and genotypically by examining the HIV coding regions corresponding to the target(s) of the drug(s) of interest (for this review, limited to reverse transcriptase and protease)[8,9]. Genotypic drug resistance testing by sequence analysis is widely used for clinical and surveillance purposes by laboratories in developed countries, and is the technique recommended by WHO for HIVDR surveillance and monitoring surveys. Genotyping is performed using either commercial kits (primarily ViroSeq™ or TRUGENE®) or in-house developed assays.

Because the majority of HIV-1 infections occur in low- and middle-income countries, and the availability of ART has expanded greatly in recent years, the need to perform population-based surveys to assess HIVDR calls for simplified, field-friendly methods for specimen collection, storage, and transport. Plasma is considered to be the most appropriate specimen type for HIVDR genotyping; however, its use may not be feasible in rural, remote areas in resource-limited settings, since its preparation and storage requires personnel and laboratory infrastructure that is often lacking. Alternative specimen types for HIVDR genotyping include DBS, dried serum spots (DSS), and dried plasma spots (DPS). DBS can be made from blood drawn for routine clinical or surveillance purposes without special laboratory processing. Given the comparative advantage of collecting DBS compared to DPS and DSS, DBS will be the focus of this review (Fig. 1).

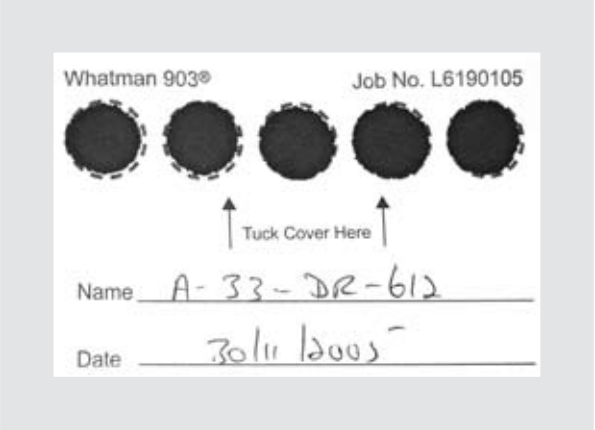

Figure 1. *Whatman 903 Filter Paper with dried blood spots.*

The filter paper used for DBS collection is easily obtained and stored, and although procedures for making DBS must be followed precisely, the training required is less intensive than that required for plasma separation. HIV nucleic acids on DBS are stable over long periods at ambient temperature and freezing is not required unless longer storage is planned. Finally, in resource-limited settings, DBS are more easily transported than plasma because they can be shipped as non-hazardous material using regular mail or courier services.

Despite the potential advantages of DBS as a method of specimen collection, there are some disadvantages, the foremost being reduced sensitivity of viral RNA amplification because of small input volumes and impaired nucleic acid extraction as well as nucleic acid degradation under extreme environmental storage conditions. Additionally, in specimens with low viral loads, proviral DNA from peripheral blood mononuclear cells (PBMC) may contribute a significant proportion of information to genotyping results. Thus, in some patients with low viral loads, genotyping results from DBS specimens may not reflect the current status of replicating viruses circulating in the patient's plasma accurately. Finally, the variability around methods for DBS collection, transportation, and manipulation may make interpretation of results challenging.

Recommendations for dried blood spot-based HIV drug resistance genotyping from WHO HIVResNet Dried blood spots Working Group

Because of the need to develop standard methods for DBS collection, transport, and manipulation as part of the wider global effort to scale up HIVDR assessment and prevention strategies in resource-limited settings, WHO has brought together a sub-group of WHO/HIV

Table 1. Summary of studies investigating impact of DBS storage conditions on HIV-1 drug resistance genotyping

	Storage conditions tested			
Study	**Time**	**Temperature/Humidity**	**Desiccant**	**Outcomes**
Garcia-Lerma[15]	1 to 16 weeks	37 °C/high humidity, –20 °C	Yes	DBS stable at 37 °C only for 1-2 weeks. –20 °C recommended for long-term storage; –20 °C superior for short and long term.
Buckton[13]	3 months	–20 °C, 4 °C, 20 °C	Yes	HIV DNA PCR only. No observed degradation in HIV DNA during 3-month study period.
Bertagnolio[12]	3 months	37 °C/85% humidity	Yes	Good amplification rate (90%)
McNulty[19]	6 years 5 years 2-3 years	–30 °C Ambient temperature and –70 °C, –20 °C	Yes	Complete degradation at ambient temperature; stable at –30 °C and –70 °C; –20 °C recommended for long-term storage
Nelson[21]	3 to 6 years	Ambient temperature	Yes	Moderately successful amplification rate (69%); 1 log drop in viral load
Wallis[30]	3 months	Ambient temperature, 4 °C, –20 °C	Yes	Some reduction in amplification rate at ambient temperature vs. 4 °C or –20 °C

DBS: dried blood spots; PCR: polymerase chain reaction.

ResNet (WHO HIVResNet DBS Working Group), a panel of international laboratory experts on DBS, with the objective to review available data and develop, validate, and standardize methods for HIVDR genotyping from DBS. WHO and the HIVResNet DBS Working Group has recently developed a protocol for DBS preparation, handling, and testing, with the ultimate goal to improve the efficiency and standardization of DBS testing and finally the overall quality of HIVDR survey data. A full description of WHO protocol for DBS collection, storage, and transport is available on WHO website at (http://www.who.int/hiv/topics/drugresistance/laboratory/en/index.html).This guidance document is regularly updated as needed, based on new data and other developments in the field.

Methods for HIV drug resistance genotyping from dried blood spots

Successful amplification of HIV from DBS is directly linked to the use of proper and adequate specimen collection, drying, packaging, storage, transport, and nucleic acid extraction techniques.

Collection and drying

Most published studies have employed similar collection methods and used Whatman 903 filter paper (Whatman, Maidstone, UK)[10]. DBS are prepared using whole blood, either obtained by venipuncture or finger prick with a range of 50-100 µl of whole blood placed on a minimum of 3-5 circles on the filter paper. Drying times generally range from one hour to overnight, with the majority of studies drying overnight at 25 °C. One study reported a drying time of 18-24 hours[11]. Generally, best results were obtained when DBS were left to dry for at least four hours (though preferably overnight) in a suspended horizontal position (on a drying rack, if available), or laid flat on a clean paper towel in a biohazard safety cabinet. In general, available data support the need for complete drying of DBS prior to packaging for short or long-term storage.

Packaging and short- and long-term storage

Correct packaging and storage of DBS are critical elements ensuring successful amplification. A number of different methodologies for performing HIVDR genotyping using DBS (or DSS), including some comparisons of various storage conditions, have been developed and reported in the literature[12-25] (Table 1). Studies have used various packaging techniques, primarily placing individual DBS specimens into low gas-permeable sealed bags with or without silica desiccant. Few studies have reported on or have assessed the effect of short-term storage conditions (time between DBS collection and testing or freezing) on the success of HIV amplification and even fewer have documented humidity conditions. For those reporting short-term storage conditions, the conditions tested varied widely: 22-28 °C for up to six weeks[26], ambient temperature for 48 hours[22], 35.8 °C with 73% humidity for 1-6 days[27], 100% humidity for eight and 15 days[28], or 100% humidity at –20 °C or at 37 °C for one, two, eight, and 16 weeks[15] (Table 1). With some exceptions[12], DBS stored under conditions of high temperature and high humidity demonstrate impaired amplification efficiency. In one study[29], DBS stored at 37 °C and > 90% humidity for two weeks showed a reduced amplification success

(n = 8; 38%) compared to DBS stored at either –20°C or 4 °C (p-value = 0.077 and 0.026 for 37 °C and 37 °C and humidity > 90%, respectively, compared to plasma; Fisher's exact test). Storage at 37 °C with low humidity (< 20%) was determined to be less detrimental than the same temperature with > 90% humidity at all time points. The results of one study suggest that short-term storage of samples at either 4 °C or –20 °C is preferable[30].

Several studies report the success of amplification and genotyping from DBS after longer storage times and variable conditions: three months at 37 °C and 85% humidity[12], 18-26 weeks at –20 °C[18], 2-3 years at –20 °C or six years at –30 °C[19], one year at 4 °C[24], and up to 4.9 months at ambient temperature[25]. All studies reported use of desiccant. Following one year of storage at 4 °C Youngpairoj, et al. observed a 95% amplification rate using an in-house assay, but only a 58% amplification rate using ViroSeq™[24]. As discussed below, the different amplification efficiencies seen by these two methods might reflect assay vulnerability to nucleic acid degradation that occurs under suboptimal temperatures, humidity, or both. McNulty, et al. found no amplification from DBS stored for five years at room temperature, further emphasizing the need for appropriate storage conditions[19]. Overall, these studies demonstrate failure of amplification resulting from lack of humidity control and highlight the importance of drying DBS prior to storage in zip-lock plastic bags containing 2-3 silica desiccant packs to remove residual moisture along with one humidity indicator card. The use of desiccant packs appears more appropriate as free desiccant material should not come into direct contact with the DBS. As UV light can also damage nucleic acids, DBS should be kept in the dark.

Available studies indicate that DBS should be transferred at –20 °C or lower temperatures as soon as possible; however, when this is not possible they can be kept and/or transported at ambient temperature for up to 14 days after collection. After this time, DBS must be either processed for genotyping or frozen at –20 °C or below. If genotyping cannot be performed within 14 days from the date of collection, DBS should be transported to a central facility where there is a constant electricity supply and a –70 °C freezer. In settings where –70 °C freezers are not available, non-frost free –20 °C freezers can also be used for long-term storage (at least up to two years).

Transport

The transport of specimens from the point of collection and initial processing to the laboratory performing HIV genotypic testing is challenging in most of the remote rural areas in low-resource countries. A number of recent studies report shipping at 25 °C[16,28], while others report shipping on dry ice[18,24]. Overall, published data suggest that if specimens have been stored at –20 °C or lower, it is considered best practice to ship them on dry ice to the place of genotyping to avoid unnecessary cycles of thawing and re-freezing. In settings where dry ice is not available, DBS should be removed from the freezer and be allowed to thoroughly equilibrate to room temperature for a minimum of 30 minutes prior to opening the bag. After equilibrating, the outer bag should be opened and the desiccants contained in each of the small plastic bags replaced with fresh desiccant for shipping. The equilibrated DBS should be placed in a new plastic bag containing humidity indicator and desiccant and shipped at room temperature to reduce the danger of increased humidity exposure. If the DBS have been stored at ambient temperature, they can be transported without refrigeration for up to 14 days after collection. After this time, DBS must be either processed for genotyping or frozen at –20 °C or below. However, available data suggest that the total time at ambient temperature should be minimized.

Nucleic acid extraction, polymerase chain reaction amplification, and drug resistance genotyping

The efficient extraction of HIV-1 nucleic acids from DBS is considered critical for drug resistance testing, given the limited amount of RNA and DNA that can be retrieved from spots that are usually prepared with only 50-100 µl of blood. The use of quality-controlled reagents is also essential since most genotypic assays amplify large *pol* fragments that may be particularly sensitive to degradation. Many commercial methods are now available to extract HIV-1 RNA from plasma and may potentially be used for DBS. Information regarding the performance of these methods is mostly limited to the Nuclisens® (bioMérieux, USA) assay. Modifications of the protocol that include a nucleic acid elution step under gentle rotation in different volumes of lysis buffer (2 or 9 ml) and for different periods of time (2-17 hours) and temperatures (ambient or 4 °C) have all resulted in high amplification success rates[16-19,31,32]. High amplification efficiencies have also been seen using other commercial or in-house methods that extract RNA and/or DNA[12,13,23,25,29]. It is important to note, however, that most available methods extract total HIV nucleic acids and that the presence of cell-associated DNA may potentially increase amplification efficiencies from DBS[18,19,25].

Table 2. Summary of studies related to HIV-1 drug resistance genotyping from DBS

Study	Genotyping method(s)	Amplicon size	Storage conditions	Sample characteristics	Tested (n)	Viral load of tested samples (copies/ml)	Amplification success rate*	Sequence concordance vs. plasma†
Masciotra[18]	ViroSeq™	1.8 kb	–20 °C, 18-26 weeks	Mostly treatment experienced, subtype B	60	78 to 676,694 (median: 9,135)	Overall: 83% VL > 2,000: 100% VL < 2,000: 54%	98.8%
Youngpairoj[24]	ViroSeq™ or in-house nested RT-PCR	1.8 kb or 1 kb	4 °C, 1 year	Treatment experienced, subtype B	40	518 to 676,694 (median: 13,680)	ViroSeq™: 57.5% In-house: 95%	94.5% (drug resistance mutations, DBS/in house vs. plasma/ ViroSeq®)
McNulty[19]	In-house nested RT-PCR	1 kb	–20 °C, 2-3 years	Untreated, subtypes from Cameroon, subtypes A, CRF02	40	665 to 645,256 (median: 23,715)	Overall: 92% VL > 10,000: 100% VL < 10,000: 73%	98.5%
Ziemniak[25]	In-house nested RT-PCR	RT: 663 bp	Ambient, 0-5 months	Treated and untreated patients from the USA, subtype B	9	< 50 to 94,600 (median: 17,792)	Overall: 94% VL ≥ 193: 100%	Not assessed
Bertagnolio[12]	In house nested RT-PCR	RT: 700 bp	37 °C, 85% humidity, 3 months	Untreated subjects from Mexico, subtype B	103	Not all tested	90.1% either PR or RT region; 78.2% for both regions	99.9% (in samples with resistance mutations)
Hallack[17]	TRUGENE®	1.3 kb	–20 °C	Treated and untreated patients from the USA, subtype B	33	1,178 to 414,212 (median: 11,666)	Overall: 78.8% VL > 6,000: 90.5% VL < 6,000: 58.3%	99.3%
Garrido[16]	In-house nested RT-PCR: RT and gp41 fragments	RT: 726 bp	4 °C, no desiccant	Treated patients from Angola; many subtypes	77	1,000 to 850,000	RT: 30% gp41: 43%	Not assessed
Steegen[23]	In-house nested RT-PCR	PR: 458 bp RT: 646 bp	–20 °C	Treated and untreated patients from Kenya; subtypes A, C, D, CRF16	29	55 to > 100,000	96.6% either PR or RT region; 89.7% for both regions; VL > 100: 100%	Not assessed
Buckton[13]	In-house nested RT-PCR	PR: 758 bp RT: 805 bp	–20 °C	Clinic patients from the UK; subtypes A, B, C, CRF02	12	80 to 115,300 (median 10,950)	PR: 83% RT: 100%	Not assessed

VL: viral load; DBS: dried blood spots; RT: reverse transcriptase; PCR: polymerase chain reaction; PR: protease; bp: base pair.
*It is likely that the quality of field-collected DBS is substantially inferior to that of lab-collected DBS (which are often used in comparison studies) and especially plasma, with respect to amplification success rates.
†mean nucleotide sequence identity, unless otherwise noted.

Studies on the efficiency of drug resistance genotyping from DBS have been generally encouraging. Only three studies have evaluated the effectiveness of commercially available genotypic assays (Table 2). In one study using the TRUGENE® HIV-1 Genotyping kit, complete *pol* genotypes were obtained in 19 of 21 DBS specimens with plasma viral load higher than 6,000 copies/ml[17]. Of the 12 specimens with viral loads between 1,000 and 6,000 copies/ml, only seven were successfully genotyped. However, improved genotyping efficiencies from low viral load specimens (1,000-6,000 copies/ml) were possible by extracting nucleic acids from two spots[17]. Similar findings have been reported using the ViroSeq™ HIV-1 genotyping system. Using this method, 50 of 60 DBS specimens tested were successfully genotyped, including all the specimens collected from patients with plasma virus loads greater than 2,000 copies/ml and 12 of the 22 specimens with viral loads less

than 2,000 copies/ml. This study also found that nucleic acid extraction from two 50 µl spots improved the genotypic efficiency in specimens with low (< 2,000 copies/ml) plasma viremia[18]. Higher sensitivity of genotyping may be particularly critical for routine use of DBS for drug resistance monitoring in treated individuals. It is important to note, however, that the high amplification success rates seen by both commercial assays were obtained using DBS prepared and stored under optimal conditions. Under less ideal conditions, the performance of these assays may be reduced since they both rely on the amplification of large (1.3 or 1.8 kb) *pol* fragments that may be particularly sensitive to degradation. The impact of DBS storage conditions on the performance of the ViroSeq™ assay was evident in a recent study that noted a low (38.6%) amplification efficiency in samples stored in a non-frost freezer for long periods of time without desiccant changes[33]. A second study noted reduced amplification efficiency with ViroSeq™ assay upon storage of DBS at 4 °C for one year[24]. A close examination of the amplification signals in agarose gels was certainly suggestive of some RNA degradation in the specimens stored at 4 °C compared to parallel specimens stored at –20 °C. In both studies, the low genotyping efficiency by the ViroSeq™ assay was circumvented by using an in-house method that amplified a smaller fragment in a nested-PCR protocol[24,33]. High amplification efficiencies have been also reported using other in-house assays that amplify shorter (0.5-1 kb) or separate reverse transcriptase (RT) and protease fragments from both RNA and DNA[12,13,19,23,25,31,32]. In one of these studies, RT or protease sequences from 93 of 103 specimens (90%) were successfully generated upon storage for three months under challenging conditions (37 °C and 85% humidity)[12]. Another study showed the efficient genotyping of both RT and protease in 11/12 specimens with plasma viral loads between 50 and 115,300 copies/ml[13].

The presence of PBMC in DBS has raised questions regarding the amplification of cell-associated DNA sequences and their contribution to the high amplification success rates observed in many studies. As expected, a relatively high frequency of amplification of proviral DNA sequences from DBS has been noted using selective DNA PCR amplification[18,19,25]. Amplifications of proviral DNA sequences might explain the successful genotyping observed in DBS with low (< 400 copies/ml) plasma viremia, although DNA amplification has been more frequently observed among specimens with higher plasma viral loads[18,25].

Equivalence of viral load in plasma versus dried blood spots

The preservation of nucleic acid in a desiccated form means that DBS can serve as the substrate for a number of nucleic acid-based HIV tests. Recent reviews have summarized studies on viral load testing on DBS[10]. In addition, Stevens, et al.[34] have reviewed viral load testing in resource-limited settings and highlighted the role of DBS as a sample collection strategy. From the literature, there is little doubt that viral load testing can be performed on DBS. However, key issues remain regarding the limits of detection, the durability of results during storage, and the lack of standardization of methodology.

More than 10 publications on DBS and viral load have become available since the review by Hamers, et al. was published[10]. Regardless of the specific focus of the studies, most of the papers explored lower limits of detection, usually defined as the threshold at which the number of false negatives is reduced to an acceptable level. Most recent reports confirm that the lower limit of detection for viral load on DBS appears to be in the 3-4 $\log_{10}$ copies/ml range. A paper by Mbida, et al. reported a lower limit of detection of 3 logs, based on consistency of plasma and DBS results in the 3-4 $\log_{10}$ range[35]. However, a close examination of the data shows that only one of the 16 specimens had a viral load that fell within this range. Similarly, Andreotti, et al. reports a 96.4% sensitivity with the Roche COBAS® TaqMan® assay, with a lower limit of detection of 3 $\log_{10}$ copies/ml based on analysis of specimens that fall in the 3-4 $\log_{10}$ range, although the distribution of viral load values was not fully described[36]. A more detailed study described the actual lower limit of detection as 3.72 $\log_{10}$ copies/ml[37]. Lower limit of detection cut-offs of 3 and 3.72 logs are quite different from a monitoring perspective as they represent viral load values of 1,000 and 5,000 copies/ml. Claims of enhanced sensitivity to 748 copies/ml, using the Generic HIV Charge Virale assay (Biocentric, Bandol, France) were not supported as 25% of the specimens in the 3-4 $\log_{10}$ copies/ml range were not detected[38]. Thus, it appears that current methods for viral load testing on DBS have a practical lower limit of detection of 5,000 copies/ml and remain 2 logs less sensitive than commercial plasma viral load assays.

The specificity of viral load testing on DBS is also influenced by the overall plasma viral load. In general, most studies describe DBS viral load as being lower than plasma viral load when comparing mean values derived from each specimen type. However, a few studies using different methods have noted higher viral load values from DBS compared to plasma, particularly

at low plasma viremias. These observations are likely reflecting proviral DNA contributions that may be more apparent at low viral load values[20,39-41]. While some theoretical considerations should be given to viral load assays that are specific for RNA (nucleic acid sequence-based amplification, or NASBA) as a way to improve specificity[42,43], DNA contributions from DBS may not be an issue above 5,000 copies/ml, which is the practical lower limit of detection for viral load assays for DBS.

A study by Monleau, et al. using viral load and genotypic testing from DBS has suggested that viral load testing from DBS may be less affected by storage conditions than genotype testing[20]. One possible explanation is the short intact target sequences (150 bp) that are generally amplified in viral load assays compared to the relatively long (> 600 bp) *pol* fragments that are amplified for HIVDR testing. While there is likely a correlation between the quantity of the virus on the DBS and stability[44], this is more pronounced for DPS than DBS[35]. Further studies support the notion that viral load testing on DBS may be successfully performed on specimens with viral load > 5,000 copies/ml properly stored at room temperature for 9-12 weeks[35,39,40]. However, as the readout from viral load testing is not binary as it is for genotyping, care must be taken in evaluating a quantitative assay where the specimen is subject to ongoing degradation. Thus, if specimens are to be analyzed at some future date in the context of research, then rapid storage at –20 °C or –70 °C is recommended, again with appropriate validation.

Meaningful comparisons among studies of viral load tests on DBS are confounded not only by the different tests, but also by the nucleic acid extraction techniques employed. Andreotti, et al. reported a higher correlation between plasma and DBS viral load with the COBAS® assay when the Nuclisens® extraction method was used[36]. Significant differences in the performance of extraction methods were demonstrated for viral load testing, with the Nuclisens® and Abbott sample preparation methods proving to be more efficient than the Quiagen QIAamp® or Roche High Pure Kit[20]. To further complicate matters, Wan, et al. published a recent report[43] identifying the contribution of proviral DNA to virus load detected in plasma by the Roche Amplicor COBAS® assay when collected in PPT tubes. Since high efficiency nucleic acid extraction kits such as Nuclisens® rely on total nucleic acid extraction, proviral DNA will inevitable contribute to DBS viral load assays. Further studies are needed to more precisely define the operating characteristics of a specific viral load assay with a given RNA extraction method. Cross-platform comparisons of viral load on DBS may prove to be challenging from the perspective of absolute quantification. Instead, consideration should be given to defining viral breakthrough thresholds for each platform.

Deriving a consensus for viral load testing on DBS is extremely complex due to the lack of consistency among studies. Most studies on viral load testing on DBS use commercial assays developed for plasma viral load testing. Recent in-house assays designed for viral load testing on DBS and open source real-time assays such as the Generic HIV Charge Virale[45] have not yet been properly validated. Additionally, there are either integrated, external commercial or in-house extraction methods that can be used for each assay. Further complexity is added by inconsistencies in specimen collection (phlebotomized blood or directly spotted onto cards), number of spots used in the extraction, and corrections for hematocrit values.

Concordance between genotype data generated using plasma and dried blood spots

While many studies have now reported the successful genotyping of HIV-1 from DBS, only a few have compared DBS genotypes with those derived from plasma[12,16-19,24,25,31] (Table 2). A comparison of RT and protease sequences generated using the ViroSeq™ assay in plasma and DBS collected from 40 highly antiretroviral-experienced persons showed a high concordance in resistance mutations. Of the 316 resistance mutations found in plasma sequences, 306 were also found in sequences from DBS. Most discrepancies were due to mixtures or unusual amino acid changes, and in only two cases were caused by major protease or RT mutations[18]. Interestingly, most of the mutations found on DBS were also detected in plasma sequences, although some mutations were exclusively found in the DBS. A similarly high (96.4%) concordance in genotypic interpretations using the ViroSeq™ assay was noted in a more recent study[33]. High concordance between plasma and DBS genotypes was also found in 26 antiretroviral-treated patients genotyped using the TRUGENE® method; all 58 resistance-associated mutations detected in plasma were also detected in the corresponding DBS[17]. Similar high correlations have also been noted in studies done in small numbers of treatment-naive, newly diagnosed persons using in-house methods, although the number of resistance-associated mutations detected in plasma or DBS was minimal in most cases[12,19,25] (Table 2). In one of these studies, replicate amplifications from DBS specimens with detectable resistance-associated mutations consistently confirmed the genotypes observed in plasma[12].

The amplification of proviral DNA from DBS has raised questions regarding the potential interference of archived *pol* sequences in the genotypic profiles generated from DBS. However, it is important to note that none of the studies mentioned above have quantified the exact contribution of proviral DNA to the overall recovery rate and population-based sequences generated from DBS[18,19,25]. Potential DNA interferences might also differ according to disease stage, level of CD4 cell counts, and treatment characteristics of the population due to the different dynamics of emergence and persistence of resistance mutations in plasma and PBMC[46-48]. For instance, patients who fail treatment tend to have more detectable mutations in plasma sequences than in PBMC, particularly at lower virus loads. In these patients, mutations are generally detected first in plasma then in PBMC, with delays of up to one year[49]. The opposite usually occurs in patients undergoing treatment interruptions who typically have more detectable mutations in PBMC[49-53]. In contrast, drug resistance genotypes from plasma and PBMC are generally comparable in treatment-naive persons with unknown duration of infection[54]. While available studies have shown a high genotypic concordance with plasma genotypes despite potential DNA interferences, larger studies with more patients with diverse treatment characteristics are necessary to fully understand the correlation between resistance genotypes generated from plasma and DBS. It will also be important to compare genotypic concordance using more sensitive tools that detect minority resistant variants, since DNA amplifications from DBS might better detect archived, fossil records of past antiretroviral treatment.

Application in the field: HIV-1 drug resistance surveys in resource-limited countries using dried blood spot-based HIV drug resistance genotyping

Dried blood spots have now been widely used for HIVDR testing and an increasing number of reports from resource-limited settings have indicated DBS as the preferred specimen type for transmitted HIVDR surveillance where plasma collection is not feasible. In Tanzania for the 2005 HIVDR surveillance, 60 DBS were collected from antenatal care sites. DBS specimens were dried overnight at each survey site and shipped to a central laboratory the day following specimen collection for storage. Fifty of the 60 specimens (83%) were successfully amplified using RT PCR[31]. In Malawi, a similar HIVDR surveillance was conducted in 2006. DBS were prepared from residual blood samples from antenatal clinic attendees receiving PMTCT services in Lilongwe city. Of the 59 samples collected, 54 (92%) were successfully amplified, indicating good specimen quality and processing[32]. In China, DBS were collected from HIV-1 newly diagnosed patients in the Shandong Province in 2009. Of the 53 DBS collected, 88.7% were successfully amplified[55]. Iran performed a pilot surveillance of transmitted HIVDR covering 19 of the 60 Risk Behavior Consultation Centers (RBCC) in the country. In particular, all ten centers in Tehran and nine RBCC from nine provinces were selected for the project. Eligible participants included women and men who were less than 25 years old and testing HIV positive for the first time, with no history of ART. Each DBS card was packed in gas impermeable zip-lock bags with two desiccant packs and one humidity indicator card and stored in a refrigerator at 4-8 °C before shipping to the CDC lab in Tehran. The DBS were transported to the CDC lab in Tehran by a rapid post service or by hand. All specimens received at the national CDC lab were immediately placed in a freezer at –20 °C and then shipped overseas on dry ice to the genotyping laboratory. Seventy-three DBS were collected, with 39 (53%) specimens yielding sequence from both protease and at least part of RT[56]. In Mexico City, DBS were prospectively collected from newly diagnosed, treatment-naive, HIV-positive subjects to conduct a pilot HIVDR surveillance. Whole blood was spotted onto filter cards, air dried at ambient temperature, and stored with desiccant at 37 °C and 85% humidity for three months. Of the 103 DBS specimens collected, 90.1% could be amplified in either the region of HIV protease or the region of RT[12].

Many more countries have just completed or are in the process to complete their HIVDR surveys using DBS. The HIVDR survey results reported above show how amplification success rates varies widely (from 53 to 92%) from one survey to another, depending on DBS preparation, storage, and manipulation conditions.

Recommendations for the use of dried blood spots in the context of WHO HIV drug resistance surveys

The design of WHO HIVDR surveys in drug-naive (transmission) and treated (monitoring) surveys is described in detail elsewhere[5,6]. In situations where plasma cannot be processed and stored under appropriate conditions, DBS may be collected for transmission surveys and before ART initiation at the sentinel site for

HIVDR monitoring surveys, since most patients will have relatively high viral loads. The main outcome of WHO-recommended HIVDR monitoring surveys in treated populations is "HIVDR prevention" at 12 months after ART initiation. Because the definition for "failure of HIVDR prevention" is a viral load > 1,000 copies/ml in plasma and because if incompletely suppressed, treatment-experienced patients are more likely to have circulating viral loads of < 10,000 copies/ml compared to treatment-naive patients, DBS should not be used as a specimen type at the 12-month endpoint in monitoring surveys, as a proportion of viral loads are expected to be below the limit of amplification sensitivity of most DBS-based genotyping assays. In these settings, plasma is the preferable specimen type for resistance testing.

WHO HIV drug resistance laboratory network: Accreditation process and quality assurance

Although many laboratories are experienced in genotyping, and several of them also have considerable experience with DBS, the variety of methods employed and the lack of comparable performance standards limit the production of comparable and reliable results. Existing networks have made attempts to standardize practices and procedures, but there is still a need to develop a common approach for quality control and quality assurance. In resource-limited settings, the lack of infrastructure and the costs associated with genotypic testing limit the resources that can be directed towards these important areas. Nevertheless, a number of laboratories performing genotyping have been set up in resource-limited settings and have well-established collaborations with several centers of excellence in Europe or North America.

WHO has developed a global network of national, regional, and specialized laboratories accredited to perform HIVDR testing using a common set of WHO standard and performance indicators. Moreover, WHO has developed recommendations of acceptable methods for collection, handling, shipment, and storage of specimens in field conditions and has established a quality control and external quality assurance (EQA) system for network laboratories[57]. Accreditation (using plasma or DBS specimens) provides documentation that the laboratory has the capacity to detect, identify, and promptly submit quality HIVDR genotyping sequence data. The accreditation process further provides a learning opportunity, a mechanism for identifying resource and training needs, a measure of progress, and a link to WHO HIVResNet Lab Network. Additional details regarding WHO laboratory accreditation can be found on WHO website at http://www.who.int/hiv/topics/drugresistance/laboratory/en/index.html.

In order to obtain WHO accreditation as a national drug resistance laboratory for DBS testing, a laboratory must fulfill minimal criteria, including being already accredited by WHO for performance of HIVDR genotyping from plasma[57], at least one year of experience in DBS-based genotyping and at least 100 DBS specimens successfully amplified, successful testing of a WHO-recognized proficiency panel consisting of DBS specimens, and successful validation of a DBS-based, in-house assay for genotyping using WHO standardized criteria.

Given the diversity of methods across countries and labs, it is also essential to establish uniform standards for DBS testing as more and more countries are using DBS for their HIVDR surveys. WHO and HIVResNet is taking a multifaceted approach to address this need, including the production and regular updating of WHO guidance for DBS testing by WHO HIVResNet DBS Working Group (see above), the implementation of EQA panels consisting of DBS specimens, and the standardization of DBS-based assay validation.

External quality assurance programs are a key component that helps to ensure the quality of laboratory results. Studies done by experienced genotyping laboratories have shown that the quality of data can vary significantly between laboratories[58-64]. Factors that contribute to the quality of the results include the type of assay/kit used, the level of experience of the technician performing the analysis, the extent of heterogeneity (mixtures) in the sequences, and the viral subtype present in the clinical sample. Results from sequential rounds of proficiency testing indicate that over time, the quality of genotyping data can increase[65]. The use of EQA and proficiency testing helps to monitor and control this variability, and therefore DBS-based EQA panels are needed, and will be based on the successful plasma-based EQA program already in place, which is described below.

Since 2007, labs applying for WHO accreditation have been required to test a blinded proficiency panel of five plasma specimens prepared by the Virology Quality Assurance program (VQA)[59] according to WHO/HIVResNet specifications. Sequence results are analyzed for overall concordance with a consensus derived from all participating labs, as well as specifically at positions associated with drug resistance[66]. Positions

Box 1. The minimum required components of a validation of an in-house genotyping assay

Precision
Assessment of sequence similarity, including mixtures, by repeated testing of the same sample in the same test run. Recommended design: ≥ 5 replicates of ≥ 3 different samples representing multiple subtypes and resistance patterns. Sequences from each replicate are compared to others from the same specimen and the number of discrepancies quantified.

Reproducibility
Assessment of sequence similarity, including mixtures, by repeated testing of the same sample across multiple test runs, and including potential sources of variability such as operator, critical reagent lot number, key pieces of equipment, and time (e.g. over 2 weeks or more). Recommended design: ≥ 5 replicates of ≥ 3 different samples representing multiple subtypes and resistance patterns. May be supplemented by duplicate testing of a larger number of specimens (e.g. 10-20). Sequences from each replicate are compared to others from the same specimen and the number of discrepancies quantified.

Amplification sensitivity
Assessment of minimum required copy number (usually reported as equivalent number of RNA copies per ml in plasma) for reproducible amplification and sequencing. Include HIV-negative controls interspersed with the positive specimens. Two general design approaches, which are not mutually exclusive, are as follows:
- Serial dilution of a specimen with high viral load in an appropriate diluent (for DBS, whole blood from an HIV-negative donor) to achieve a range of viral copy number followed by replicate testing of each dilution. Amplification sensitivity may be defined as the viral load at which a majority of amplification reactions are successful.
- Testing of a large number (> 50) of samples over a wide range of copy number, concentrated in the range of the anticipated sensitivity limit; amplification sensitivity may be defined as the percentage of samples that can be amplified within a defined range (e.g. 95% positive for samples with viral load between 1,000 and 4,000 copies/ml).

Linearity
Assessment of sequence similarity, including mixtures, by testing a known sample over a range of input copy number including the amplification sensitivity limit.

where at least 80% of labs did not report the same base are excluded from the analysis. Three plasma-based proficiency panels were developed in collaboration with the VQA and NIH and sent to 58 network members or candidate laboratories in Europe, North America, Asia, Africa, and the Caribbean during 2007-2009. In general, the performance of the participating labs was good, with mean sequence identity vs. consensus in the range of 98.5-100%[64]. The majority of discrepancies were due to only one nucleotide being reported at a position containing a mixture of two or more nucleotides in the consensus; however, many of these discrepancies did not result in a change in the encoded amino acid. Specimens with the most mixtures at resistance-associated positions had the lowest mean sequence identity scores. Based on comparison of chromatogram data from several labs, it was concluded that subjectivity in base-calling and PCR amplification bias ("founder effect") can contribute to lower reproducibility when mixtures are present[64].

For DBS-based genotyping, well-characterized specimens will be used to prepare a large number of DBS cards under optimal conditions. Initially, specimens will be shipped on dry ice or at ambient temperature in parallel in order to assess whether shipping conditions impact genotyping assay performance. Otherwise, the DBS EQA program will resemble that described above for plasma. The first results of this study are expected in early 2011.

Laboratories performing HIVDR genotyping using DBS in the context of WHO HIVDR surveys should use a standardized methodology that has been validated according to WHO/HIVResNet guidance. The minimum required components of a validation of an in-house genotyping assay are outlined in Box 1. This list of requirements is predicated on the assumption that the DBS-based assay shares the same post-RNA extraction procedures as an existing and validated plasma-based assay. The primary concerns to be addressed during the DBS validation are amplification sensitivity, reproducibility of the sequence produced, representation of mixed species (especially at viral loads that are close to the amplification sensitivity limit), and contamination.

Careful selection of the specimens used for assay validation is crucial and will also partly determine the validation acceptance criteria, which should be established in advance of the laboratory testing. The specimens should be well characterized in advance and be representative of those expected to be encountered during routine lab operations and in the performance of WHO surveys with respect to resistance and subtypes. The results should be compared

at the nucleotide and amino acid level. Specimens with an unusually high number of mixed bases (e.g. > 2% mixtures) should be avoided, or acceptance criteria made less stringent to accommodate the expectation of additional variability between replicates due to subjectivity of base-calling and differences in representation of multiple variants through RT-PCR.

To obtain a thorough understanding of the performance characteristics of DBS-based genotyping assays, WHO HIVResNet DBS Working Group has coordinated the production of a standardized panel of DBS specimens that has been used for validation and comparison of several different in-house genotyping assays in experienced laboratories. Results from this validation panel will be used to revise the recommended procedures if required, and will be presented elsewhere.

Areas for future research

There are many areas that require additional operational and field-based research to further inform optimal procedures related to the use of DBS for HIVDR genotyping and viral load testing. Important areas include a comprehensive analysis of different storage and shipping conditions using specimens collected under field conditions in resource-limited settings. It is also essential to explore potential differences between results obtained from DBS prepared from anti-coagulated blood or directly from skin puncture. Such studies should evaluate the impact of the variability in volume associated with direct spotting from finger prick, potential RNA degradation due to RNases present in skin, and the effect of anticoagulant agents[67,68]. The known contribution of proviral DNA to the genotypes obtained from DBS also reiterates the need to better understand potential differences in resistance genotypes between plasma and DBS in populations with diverse treatment characteristics. Lastly, special emphasis should be given to developing methods that improve amplification efficiencies from DBS as well as to identify reagents that may help to stabilize HIV nucleic acids on filter paper. These two areas may help to minimize the impact of suboptimal storage conditions on the efficiency of genotyping and viral load testing from DBS.

As viral load testing is intrinsically a quantitative assay, one of the key differences between this and genotyping is that the production of a result is not always a measure of success. It is the value of the result in viral load testing that is so important. Thus, there is a critical need for a DBS viral load quality assurance program. Given the variables of number of spots, storage conditions, extraction methods, viral load assays, and data interpretation, a global performance measure is required to begin to validate and compare assay results. Generation of additional data from these types of studies will allow recommendations such as those described in this article to be strengthened and expanded.

Conclusions

Over the last several years considerable effort has been directed towards understanding the strengths and limitations of using DBS for HIVDR genotyping and viral load determination. Available evidence supports the use of DBS in most cases, as long as viral loads are high and standardized procedures for collection, preparation, storage, shipping, and processing are followed. With additional research, training, quality assurance programs, and capacity building, DBS may eventually become the specimen type of choice for resource-limited settings.

Acknowledgments

The authors are grateful to WHO HIVResNet Laboratory Working group and the following participants at meetings held in December 2007 and 2008:

Paul Sandstrom, Richard Pilon, (National HIV and Retrovirology Laboratories, Public Health Agency of Canada, Ottawa, Ontrario, Canada); Andrew Buckton, Pat Cane (Health Protection Agency, London, UK); Carmen de Mendoza, Carolina Garrido (Department of Infectious Diseases, Hospital Carlos III, Madrid, Spain); Joe Fitzgibbon (National Institutes of Health/NIAID, Bethesda, Maryland, USA); Lisa Frenkel, Caroline Mitchell (University of Washington, Seattle, Washington, USA); Chunfu Yang (Centers for Disease Control and Prevention, Atlanta, Georgia, USA); Renee Hallack, Lauren Forbes, Monica Parker (Bloodborne Viruses Laboratory, Wadsworth Center, New York State Dept. of Health, New York, USA); Anna Hearps, Claire Ryan (MacFarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria, Australia); Sarah Palmer (Swedish Institute for Infectious Disease Control, Karolinska Institute, Solna, Sweden); Martine Peeters (UMR 145, Institut de Recherche pour le Développement, University of Montpellier, Montpellier, France); Deborah Persaud (Johns Hopkins University School of Medicine, Bethesda, Maryland, USA); Sandrine Reigadas (Laboratoire EA 2968, Université de Bordeaux, Bordeaux, France); Rob Schuurman (Utrecht Medical Center, Utrecht, Netherlands); Carole Wallis (University of the Witwatersrand, Johannesburg, South Africa).

We also thank Cheryl Jennings and Jim Bremer (VQA, Rush University, Chicago IL) and Joseph Fitzgibbon (DHHS/NIH/NIAID, Bethesda, MD) for ongoing support of the EQA proficiency testing program.

References

1. UNAIDS-WHO. AIDS epidemic update. Geneva: UNAIDS; 2002.
2. UNAIDS-WHO. Towards Universal Access: Scaling up priority HIV/AIDS interventions in the health sector. Progress Report, 2009. Geneva: UNAIDS; 2009.
3. Gilks C, Crowley S, Ekpini R, et al. The WHO public-health approach to antiretroviral treatment against HIV in resource-limited settings. Lancet. 2006;368:505-10.
4. Bennett D, Bertagnolio S, Sutherland D, Gilks C. The World Health Organization's global strategy for prevention and assessment of HIV drug resistance. Antivir Ther. 2008;13(Suppl 2):1-13.
5. Jordan M, Bennett D, Bertagnolio S, Gilks C, Sutherland D. World Health Organization surveys to monitor HIV drug resistance prevention and associated factors in sentinel antiretroviral treatment sites. Antivir Ther. 2008;13(Suppl 2):15-23.
6. Bennett D, Myatt M, Bertagnolio S, Sutherland D, Gilks C. Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment. Antivir Ther. 2008;13(Suppl 2):25-36.
7. Myatt M, Bennett D. A novel sequential sampling technique for the surveillance of transmitted HIV drug resistance by cross-sectional survey for use in low resource settings. Antivir Ther. 2008;13(Suppl 2):37-48.
8. Parkin N, Coakley E, Petropoulos C. Phenotypic Susceptibility Assays for Human Immunodeficiency Virus Type 1. In: LaFemina R, editor. Antiviral Research: Strategies in Antiviral Drug Discovery. Washington DC: ASM Press. 2009;283-99.
9. Shafer R. Genotypic testing for human immunodeficiency virus type 1 drug resistance. Clin Microbiol Rev. 2002;15:247-77.
10. Hamers R, Smit P, Stevens W, Schuurman R, Rinke de Wit T. Dried fluid spots for HIV type-1 viral load and resistance genotyping: a systematic review. Antivir Ther. 2009;14:619-29.
11. Uttayamakul S, Likanonsakul S, Sunthornkachit R, et al. Usage of dried blood spots for molecular diagnosis and monitoring HIV-1 infection. J Virol Methods. 2005;128:128-34.
12. Bertagnolio S, Soto-Ramirez L, Pilon R, et al. HIV-1 drug resistance surveillance using dried whole blood spots. Antivir Ther. 2007;12:107-13.
13. Buckton A, Bissett S, Myers R, et al. Development and optimization of an internally controlled dried blood spot assay for surveillance of human immunodeficiency virus type-1 drug resistance. J Antimicrob Chemother. 2008;62:1191-8.
14. Dachraoui R, Depatureaux A, Chakroun M, et al. Monitoring of HIV-1 resistance in Tunisia (North Africa) with a dried plasma spot strategy. J Acquir Immune Defic Syndr. 2008;47:522-5.
15. Garcia-Lerma J, McNulty A, Jennings C, Huang D, Heneine W, Bremer J. Rapid decline in the efficiency of HIV drug resistance genotyping from dried blood spots (DBS) and dried plasma spots (DPS) stored at 37 °C and high humidity. J Antimicrob Chemother. 2009:64:33-6.
16. Garrido C, Zahonero N, Fernandes D, et al. Subtype variability, virological response and drug resistance assessed on dried blood spots collected from HIV patients on antiretroviral therapy in Angola. J Antimicrob Chemother. 2008;61:694-8.
17. Hallack R, Doherty L, Wethers J, Parker M. Evaluation of dried blood spot specimens for HIV-1 drug-resistance testing using the Trugene HIV-1 genotyping assay. J Clin Virol. 2008;41:283-7.
18. Masciotra S, Garrido C, Youngpairoj A, et al. High concordance between HIV-1 drug resistance genotypes generated from plasma and dried blood spots in antiretroviral-experienced patients. AIDS. 2007;21:2503-11.
19. McNulty A, Jennings C, Bennett D, et al. Evaluation of dried blood spots for human immunodeficiency virus type 1 drug resistance testing. J Clin Microbiol. 2007;45:517-21.
20. Monleau M, Montavon C, Laurent C, et al. Evaluation of different RNA extraction methods and storage conditions of dried plasma or blood spots for human immunodeficiency virus type 1 RNA quantification and PCR amplification for drug resistance testing. J Clin Microbiol. 2009;47:1107-18.
21. Nelson J, Loftis A, Kamwendo D, et al. Nevirapine resistance in human immunodeficiency virus type 1-positive infants determined using dried blood spots stored for up to six years at room temperature. J Clin Microbiol. 2009;47:1209-11.
22. Plantier J, Dachraoui R, Lemee V, et al. HIV-1 resistance genotyping on dried serum spots. AIDS. 2005;19:391-7.
23. Steegen K, Luchters S, Demecheleer E, et al. Feasibility of detecting human immunodeficiency virus type 1 drug resistance in DNA extracted from whole blood or dried blood spots. J Clin Microbiol. 2007;45:3342-51.
24. Youngpairoj A, Masciotra S, Garrido C, Zahonero N, de Mendoza C, Garcia-Lerma J. HIV-1 drug resistance genotyping from dried blood spots stored for 1 year at 4 °C. J Antimicrob Chemother. 2008;61:1217-20.
25. Ziemniak C, George-Agwu A, Moss W, Ray S, Persaud D. A sensitive genotyping assay for detection of drug resistance mutations in reverse transcriptase of HIV-1 subtypes B and C in samples stored as dried blood spots or frozen RNA extracts. J Virol Methods. 2006;136:238-47.
26. Mwaba P, Cassol S, Nunn A, et al. Whole blood versus plasma spots for measurement of HIV-1 viral load in HIV-infected African patients. Lancet. 2003;362:2067-8.
27. Solomon SS, Solomon S, Rodriguez I, et al. Dried blood spots (DBS): a valuable tool for HIV surveillance in developing/tropical countries. Int J STD AIDS. 2002;13:25-8.
28. Kane C, Ndiaye H, Diallo S, et al. Quantitation of HIV-1 RNA in dried blood spots by the real-time NucliSENS EasyQ HIV-1 assay in Senegal. J Virolog Method. 2008;148:291-5.
29. Hearps A, Ryan C, Morris L, Plate M, Greengrass V, Crowe S. Stability of dried blood spots for HIV-1 drug resistance analysis. Curr HIV Res. 2010;8:134-40.
30. Wallis C, Bell C, Horsfield P, Rinke de Wit T, Stevens W. Affordable resistance test for Africa (ARTA): DBS storage and extraction conditions for HIV subtype C. The 5th International AIDS Society Conference on HIV Pathogenesis, Treatment and Prevention. Cape Town, South Africa; 2009.
31. Somi G, Kibuka T, Diallo K, et al. Surveillance of transmitted HIV drug resistance among women attending antenatal clinics in Dar es Salaam, Tanzania. Antivir Ther. 2008;13(Suppl 2):77-82.
32. Kamoto K, Aberle-Grasse J. Surveillance of transmitted HIV drug resistance with the World Health Organization threshold survey method in Lilongwe, Malawi. Antivir Ther. 2008;13(Suppl 2):83-7.
33. Lira R, Valdez-Salazar H, Vazquez-Rosales G, et al. Genotypic testing for HIV-1 drug resistance using dried blood samples. Arch Virol. 2010;155:1117-25.
34. Stevens W, Scott L, Crowe S. Quantifying HIV for monitoring antiretroviral therapy in resource-poor settings. J Infect Dis. 2010;201(Suppl 1):S16-26.
35. Mbida A, Sosso S, Flori P, et al. Measure of viral load by using the Abbott Real-Time HIV-1 assay on dried blood and plasma spot specimens collected in 2 rural dispensaries in Cameroon. J Acquir Immune Defic Syndr. 2009;52:9-16.
36. Andreotti M, Pirillo M, Guidotti G, et al. Correlation between HIV-1 viral load quantification in plasma, dried blood spots, and dried plasma spots using the Roche COBAS Taqman assay. J Clin Virol. 2010;47:4-7.
37. Garrido C, Zahonero N, Corral A, Arredondo M, Soriano V, de Mendoza C. Correlation between human immunodeficiency virus type 1 (HIV-1) RNA measurements obtained with dried blood spots and those obtained with plasma by use of Nuclisens EasyQ HIV-1 and Abbott RealTime HIV load tests. J Clin Microbiol. 2009;47:1031-6.
38. Reigadas S, Schrive M, Aurillac-Lavignolle V, Fleury H. Quantitation of HIV-1 RNA in dried blood and plasma spots. J Virolog Methods. 2009;161:177-80.
39. Lofgren S, Morrissey A, Chevallier C, et al. Evaluation of a dried blood spot HIV-1 RNA program for early infant diagnosis and viral load monitoring at rural and remote healthcare facilities. AIDS. 2009; 23:2459-66.
40. Monleau M, Butel C, Delaporte E, Boillot F, Peeters M. Effect of storage conditions of dried plasma and blood spots on HIV-1 RNA quantification and PCR amplification for drug resistance genotyping. J Antimicrob Chemother. 2010;65:1562-6.
41. Waters L, Kambugu A, Tibenderana H, et al. Evaluation of filter paper transfer of whole-blood and plasma samples for quantifying HIV RNA in subjects on antiretroviral therapy in Uganda. J Acquir Immune Defic Syndr. 2007;46:590-3.
42. Johannessen A, Garrido C, Zahonero N, et al. Dried blood spots perform well in viral load monitoring of patients who receive antiretroviral treatment in rural Tanzania. Clin Infect Dis. 2009;49:976-81.
43. Wan H, Seth A, Rainen L, Fernandes H. Coamplification of HIV-1 proviral DNA and viral RNA in assays used for quantification of HIV-1 RNA. J Clin Microbiol. 2010;48:2186-90.
44. Leelawiwat W, Young N, Chaowanachan T, et al. Dried blood spots for the diagnosis and quantitation of HIV-1: stability studies and evaluation of sensitivity and specificity for the diagnosis of infant HIV-1 infection in Thailand. J Virolog Methods. 2009;155:109-17.
45. Mehta N, Trzmielina S, Nonyane B, et al. Low-cost HIV-1 diagnosis and quantification in dried blood spots by real time PCR. PLoS One. 2009;4:e5819.
46. Bruisten S, Reiss P, Loeliger A, et al. Cellular proviral HIV type 1 DNA load persists after long-term RT-inhibitor therapy in HIV type 1 infected persons. AIDS Res Hum Retrovir. 1998;14:1053-8.
47. Chun T, Carruth L, Finzi D, et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature. 1997;387:183-8.
48. Escaich S, Ritter J, Rougier P, et al. Relevance of the quantitative detection of HIV proviral sequences in PBMC of infected individuals. AIDS Res Hum Retrovir. 1992;8:1833-7.

49. Bi X, Gatanaga H, Ida S, et al. Emergence of protease inhibitor resistance-associated mutations in plasma HIV-1 precedes that in proviruses of peripheral blood mononuclear cells by more than a year. J Acquir Immune Defic Syndr. 2003;34:1-6.
50. Chew C, Potter S, Wang B, et al. Assessment of drug resistance mutations in plasma and peripheral blood mononuclear cells at different plasma viral loads in patients receiving HAART. J Clin Virol. 2005;33:206-16.
51. Koch N, Yahi N, Ariasi F, Fantini J, Tamalet C. Comparison of human immunodeficiency virus type 1 (HIV-1) protease mutations in HIV-1 genomes detected in plasma and in peripheral blood mononuclear cells from patients receiving combination drug therapy. J Clin Microbiol. 1999;37:1595-7.
52. Venturi G, Romano L, Carli T, et al. Divergent distribution of HIV-1 drug-resistant variants on and off antiretroviral therapy. Antivir Ther. 2002;7:245-50.
53. Verhofstede C, Wanzeele F, Van Der Gucht B, De Cabooter N, Plum J. Interruption of reverse transcriptase inhibitors or a switch from reverse transcriptase to protease inhibitors resulted in a fast reappearance of virus strains with a reverse transcriptase inhibitor-sensitive genotype. AIDS. 1999;13:2541-6.
54. Bennett D, Winters M, Shafer R, et al. Concordance of HIV drug resistance genotyping results for paired PBMC and plasma specimens from persons newly diagnosed with HIV. Antivir Ther. 2005;10:S176.
55. Zhang J, Kang D, Fu J, et al. Surveillance of transmitted HIV type 1 drug resistance in newly diagnosed hiv type 1-infected patients in Shandong Province, China. AIDS Res Hum Retrovir. 2010;26:99-103.
56. Mousavi S, Hamkar R, Gouya M, et al. Surveillance of HIV drug resistance transmission in Iran: experience gained from a pilot study. Arch Virol. 2010;155:329-34.
57. Bertagnolio S, Derdelinckx I, Parker M, et al. World Health Organization/HIVResNet Drug Resistance Laboratory Strategy. Antivir Ther. 2008; 13(Suppl 2):49-57.
58. Galli R, Sattha B, Wynhoven B, O'Shaughnessy M, Harrigan P. Sources and magnitude of intralaboratory variability in a sequence-based genotypic assay for human immunodeficiency virus type 1 drug resistance. J Clin Microbiol. 2003;41:2900-7.
59. Huang D, Bremer J, Brambilla D, et al. Model for assessment of proficiency of human immunodeficiency virus type 1 sequencing-based genotypic antiretroviral assays. J Clin Microbiol. 2005;43:3963-70.
60. Korn K, Reil H, Walter H, Schmidt B. Quality control trial for human immunodeficiency virus type 1 drug resistance testing using clinical samples reveals problems with detecting minority species and interpretation of test results. J Clin Microbiol. 2003;41:3559-65.
61. Neuwald P, Funelas M, DelCarmen J, Raybold A, Jorgensen P. Results of the 2001 AcroMetrix HIV-1 resistance proficiency program. J Clin Virol. 2002;25(Suppl 3):S55-63.
62. Sayer D, Land S, Gizzarelli L, et al. Quality assessment program for genotypic antiretroviral testing improves detection of drug resistance mutations. J Clin Microbiol. 2003;41:227-36.
63. Schuurman R, Brambilla D, de Groot T, et al. Underestimation of HIV type 1 drug resistance mutations: results from the ENVA-2 genotyping proficiency program. AIDS Res Hum Retrovir. 2002;18:243-8.
64. Parkin N, Fitzgibbon J, Bremer J, Bertagnolio S. HIV drug resistance genotyping external quality assurance (EQA) for laboratories in the World Health Organization (WHO) ResNet during 2007-2009. Antivir Ther. 2009;14(Suppl 1):A162.
65. Descamps D, Delaugerre C, Masquelier B, et al. Repeated HIV-1 resistance genotyping external quality assessments improve virology laboratory performance. J Med Virol. 2006;78:153-60.
66. Johnson V, Brun-Vezinet F, Clotet B, et al. Update of the drug resistance mutations in HIV-1: December 2009. Top HIV Med. 2009;17:138-45.
67. Probst J, Brechtel S, Scheel B, et al. Characterization of the ribonuclease activity on the skin surface. Genet Vaccines Ther. 2006;4:4.
68. Keck J, Goedken E, Marqusee S. Activation/attenuation model for RNase H. A one-metal mechanism with second-metal inhibition. J Biol Chem. 1998;273:34128-33.