JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2007, p. 517–521 Vol. 45, No. 2
0095-1137/07/$08.00+0 doi:10.1128/JCM.02016-06

Evaluation of Dried Blood Spots for Human Immunodeficiency Virus Type 1 Drug Resistance Testing▽

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Received 29 September 2006/Returned for modification 23 November 2006/Accepted 3 December 2006

Dried blood spots (DBS) are simpler to prepare, store, and transport than plasma or serum and may represent a good alternative for drug resistance genotyping, particularly in resource-limited settings. However, the utility of DBS for drug resistance testing is unknown. We investigated the efficiency of amplification of large human immunodeficiency virus type 1 (HIV-1) *pol* fragments (1,023 bp) from DBS stored at different temperatures, the type of amplified product(s) (RNA and/or DNA), and the similarity between plasma and DBS sequences. We evaluated two matched plasma/DBS panels stored for 5 to 6 years at several temperatures and 40 plasma/DBS specimens collected from untreated persons in Cameroon and stored for 2 to 3 years at −20°C. The amplification of HIV-1 *pol* was done using an in-house reverse transcriptase-nested PCR assay. Reactions were done with and without reverse transcription to evaluate the contribution of HIV DNA to *pol* sequences from DBS. Amplification was successful for the DBS samples stored for 5 to 6 years at −20°C or at −70°C but not for those stored at room temperature. Thirty-seven of the 40 (92.5%) DBS from Cameroon were amplifiable, including 8/11 (72.7%) with plasma virus loads of <10,000 RNA copies/ml and all 29 with plasma virus loads of >10,000. Proviral DNA contributed significantly to DBS sequences in 24 of the 37 (65%) specimens from Cameroon. The overall similarity between plasma and DBS sequences was 98.1%. Our results demonstrate the feasibility of DBS for drug resistance testing and indicate that −20°C is a suitable temperature for long-term storage of DBS. The amplification of proviral DNA from DBS highlights the need for a wider evaluation of the concordance of resistance genotypes between plasma and DBS.

The introduction of highly active antiretroviral therapy and the demonstration of dramatic improvements in human immunodeficiency virus (HIV)- and AIDS-related mortality and morbidity in North America and Europe have fueled international efforts to expand access to care and treatment in less-developed countries. Several major initiatives to provide treatment in resource-limited settings, including the U.S. President's Emergency Plan for AIDS Relief and the Global Fund against AIDS, TB and Malaria, are now in progress (16). The implementation of these programs requires the development of appropriate and effective patient-monitoring systems, including surveillance for antiretroviral drug resistance. Sentinel drug resistance surveillance systems are important public health tools that can provide information on trends in the prevalence of resistance at the population level and can be used to modify treatment guidelines.

Plasma and serum are considered the preferred specimen types for HIV type 1 (HIV-1) drug resistance testing. However, these types of specimens are not optimal in resource-limited settings where the equipment necessary for PCR amplification and sequencing may not be available at collection sites and resistance testing requires transportation of the samples to a reference center. In these settings, alternative specimen types with simplified processing and less-stringent storage and transport requirements are preferable. Dried blood spots (DBS) and dried plasma spots (DPS) are inexpensive to prepare and easy to transport and potentially represent a good alternative to plasma for drug resistance testing. DBS and DPS have been extensively used for HIV-1 antibody testing (20, 21), molecular diagnostics (2, 18), and virus load quantification (5, 10, 15, 17). However, little is known regarding the utility of DBS/DPS for drug resistance testing. Important concerns related to the use of DBS/DPS for resistance testing include the need to amplify large *pol* fragments that may easily degrade under suboptimal storage conditions and sensitivity limitations due to the low (50- to 100-μl) specimen volumes used to prepare DBS. Furthermore, the correlation between genotypic test results generated from plasma and DBS is unknown.

Here, we assessed the feasibility of using DBS for drug resistance testing. We investigated the efficiency of amplification of large HIV-1 *pol* fragments from DBS collected and stored under defined conditions and from DBS collected in the field. We also evaluated the amplification of proviral DNA and assessed the similarity between *pol* sequences from paired plasma and DBS specimens. We show that despite the small volumes of blood used, DBS represent a feasible alternative to plasma for drug resistance surveillance and monitoring.

MATERIALS AND METHODS

Samples. We evaluated samples collected from two different sources. The first group of specimens included matched plasma, DBS, and DPS obtained from the Virology Quality Assessment program (VQA). Samples were generated from six subtype B HIV-infected donors and were part of a stability

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▽ Published ahead of print on 13 December 2006.

study of HIV-1 RNA on DBS (5). Samples were classified in two panels (A and B), with each panel consisting of three samples with a low, medium, or high virus load. Panel A was stored at −30°C for 6 years and contained samples with resistance-associated mutations. Panel B was stored at either room temperature (panel B1) or −70°C (panel B2) for 5 years and contained samples with wild-type viruses.

The second group of specimens included matched plasma and DBS samples from HIV-1-infected donors collected at Cameroonian blood banks ($n = 40$). These samples were collected as part of a surveillance study of divergent HIV variants and were prepared from units of blood that were found to be positive for HIV (13). Of these samples, 9 were classified as having subtype A, 1 as having subtype F, 1 as having circulating recombinant form 11 (CRF11), and 26 as having CRF02 based on the analysis of gp41 sequences. gp41 was not amplifiable in the remaining three samples. These three samples were classified as having subtypes CRF11 (two samples) and CRF06 (one sample) based on the analysis of p17 sequences. The DBS specimens had been stored at −20°C for a period of 2 to 3 years.

All blood and plasma spots were prepared by pipetting 50 μl of EDTA-anticoagulated whole blood or plasma onto premarked circles on 903 filter paper cards (Schleicher & Schuell, Keene, NH). Each card was dried overnight and was then individually packaged into Bitran zipper-lock bags (Fisher Scientific Company, Pittsburgh, PA) containing a silica gel pack (Mini Pax Sorbent; Multisorb Technologies, Buffalo, NY). Matched plasma samples were aliquoted and stored at −80°C or liquid nitrogen. Plasma virus loads in the Cameroonian specimens were quantified using the Amplicor HIV-1 Monitor assay (version 1.5; Roche Diagnostics, Branchburg, NJ). Plasma virus loads in the VQA panels were also quantified using the Amplicor assay (version 1.5 for panel A and version 1.0 for panel B) (5).

Nucleic acid extraction. HIV-1 RNA from plasma (100 μl) was extracted by using the Nuclisens silica-based extraction method according to the manufacturer's instructions (bioMerieux, Inc., Durham, NC). Total HIV-1 nucleic acids from DBS and DPS were also extracted using the Nuclisens method with some modifications for DBS and DPS processing. Briefly, a whole spot containing 50 μl of blood or plasma was cut with scissors and added into 9 ml of Nuclisens lysis buffer. After a 2-hour incubation at room temperature under gentle rotation, the supernatant was clarified by centrifugation at 250 × *g* for 5 min and then transferred to a clean 15-ml conical tube. Total nucleic acids were then extracted following the manufacturer's instructions, resuspended in 30 μl of elution buffer, and stored at −80°C until use.

Amplification by RT-nested PCR. A 1,023-base pair fragment of HIV-1 *pol* corresponding to amino acids 15 to 99 of the protease and 1 to 256 of the RT was amplified from plasma, DBS, and DPS using an in-house reverse transcriptase (RT)-nested PCR method. This assay has been previously validated with plasma samples from patients infected with HIV subtype B and has a sensitivity of detection of 1,000 RNA copies/ml (11). Briefly, a 5- to 10-μl volume of extracted nucleic acids was added to an RT cocktail containing murine leukemia virus (MuLV) RT enzyme (Applied Biosystems) and reverse primer RT-gen.4R (5′-ATC CCT GCA TAA ATC TGA CTT GC-3′). The RT reaction was done for 1 h at 39°C. After a first round of PCR amplification using primers RT-gen4R and Pro-Out.3F (5′-CCT CAG ATC ACT CTT TGG CAA CG-3′), 4 μl was subjected to a second round of PCR amplification using internal primers PR/RT.2F (5′-GAT CAC TCT TTG GCA ACG ACC CAT-3′) and 215/219.3R (5′-CTT CTG TAT GTC ATT GAC AGT CC-3′). Cycling conditions for the first and second amplifications were 4 min at 95°C followed by 40 cycles of 45 s at 95°C, 30 s at 50°C, and 2 min at 72°C. Parallel RT-nested PCRs were also done in the absence of MuLV RT to selectively amplify DNA. Amplifications were done with a T3 thermocycler (Biometra GmbH, Goettingen, Germany).

Sequence analysis. Sequence analysis of HIV-1 *pol* was done in an ABI 3100 capillary sequencer using primers A35, AV36, AV44, 215/219.3R, HIV 90 (5′-AAT GCT TTT ATT TTT TCT TCT GTC AAT GGC-3′), and PR/RT.2F (19). The Vector NTI program (suite 8) was used to analyze the data and calculate amino acid and nucleotide similarities. Protease and RT genotypes were interpreted using the Stanford Genotypic Resistance Interpretation Algorithm (http://hivdb.stanford.edu/pages/algs/HIVdb.html) and the list of resistance mutations included in the International AIDS Society USA mutation figures (12).

Nucleotide sequence accession numbers. The nucleotide sequences from 48 matched plasma and DBS specimens have been deposited in the GenBank database (accession numbers DQ869190 to DQ869237).

TABLE 1. Rate of amplification of *pol* sequences from DBS and DPS stored at different temperatures

Panel sample identification no.	Plasma RNA copies/ml	PCR result[a]				
		Plasma samples[b]	DBS (+RT)	DBS (−RT)	DPS (+RT)	DPS (−RT)
Panel A (−30°C, 6 yr)						
A1	21,567	+	+	+	−	−
A2	6,452	+	+	+	−	−
A3	338,112	+	+	+	+	−
Panel B						
B1 (room temp, 5 yr)						
B1.1	19,497	+	−	−	−	ND
B1.2	3,230	+	−	−	−	ND
B1.3	57,375	+	−	−	−	ND
B2 (−70°C, 5 yr)						
B2.1	19,497	+	+	−	−	−
B2.2	3,230	+	−	−	−	−
B2.3	57,375	+	+	+	+	−

[a] RT-nested PCR were done with (+RT) or without (−RT) MuLV RT enzyme. +, positive; −, negative; ND, not done.
[b] Plasma samples were stored only at −80°C.

RESULTS

Amplification and sequencing of large HIV-1 *pol* fragments from DBS and DPS stored at different temperatures. To investigate the impact of storage temperature on the ability to amplify from DBS, we evaluated the two VQA panels generated and stored under defined conditions. Table 1 shows the efficient amplification of *pol* in plasma and the corresponding DBS samples from panel A, indicating that long-term storage at −30°C was sufficient to preserve the integrity of nucleic acids on these DBS. Interestingly, all three DBS specimens from panel A were also amplifiable in the absence of reverse transcription, demonstrating the presence of proviral DNA (Table 1). In contrast to the case for plasma and DBS, amplification from panel A DPS samples was possible only for the specimen with the highest plasma virus load (A3; 338,112 RNA copies/ml). As expected, none of the DPS specimens were amplifiable in the absence of reverse transcription, consistent with the absence of proviral DNA in DPS.

Table 1 also shows the efficiencies of amplification of *pol* sequences from panel B samples that were stored at either room temperature (B1) or −70°C (B2). When the DBS were stored at room temperature for 5 years, none of the specimens were amplified either in the absence or the presence of reverse transcription. However, when the same specimens were stored at −70°C for the same period of time, *pol* sequences were recovered in the two DBS that had the highest plasma virus load. As we noted for panel A samples, only the DPS specimen obtained from the sample with the highest plasma virus load (B2.3; 57,375 RNA copies/ml) was successfully amplified in the presence of reverse transcription. Overall, these findings suggest that storage temperature is an important determinant for the efficient amplification of *pol* sequences from DBS stored for prolonged periods of time.

We next sequenced all five positive DBS samples from panels A and B and compared the sequences with those seen in viruses from plasma. The mean (± standard deviation) similarity between nucleotide *pol* sequences from plasma and DBS was 98.6% ± 1.14% and ranged between 97% and 100%. A high similarity between amino acid sequences was also noted

TABLE 2. Resistance-associated mutations in protease and RT from matched plasma/DBS samples from VQA panel A

Panel sample identification no.	Mutation(s) for resistance to indicated enzyme[a]					
	Protease			Reverse transcriptase		
	Plasma	DBS (+RT)	DBS (−RT)	Plasma	DBS (+RT)	DBS (−RT)
A1	M36M**I**, A71AT	A71AT	A71AT	D67N, T69TN, K70R, V118I, M184V, T215TYSN, K219Q	D67DN, T69TN, K70KR, V118I, M184V, T215TYSN, K219Q	D67DN, K70KR, V118I, M184MV, T215TYSN, K219Q
A2	M36M**L**			M184V, Y181Y**C**	M184V	M184V
A3	L63P	L63P	L63P	T69N, Y181C	T69N, Y181C	T69N, Y181C

[a] RT-nested PCR from DBS were done with (+RT) or without (−RT) MuLV RT enzyme. Mutations identified only in DBS or plasma are in bold and underlined.

(mean ± standard deviation, 98.8% ± 1.6%; range, 96% to 100%), further indicating a high concordance between plasma and DBS sequences. Samples from panel A had drug resistance mutations which allowed us to compare the genotypic profiles between plasma and DBS. When this analysis was done, we noticed an overall agreement between the resistance profiles from plasma and DBS (Table 2). Of the 17 protease and RT mutations seen in plasma viruses, 14 were also found in viruses from DBS. Of the three mutations that were absent in DBS sequences, one (Y181C) was a major nonnucleoside RT mutation and two were minor protease mutations (M36L and M36I) that are weakly associated with protease resistance only when present with other mutations. Conversely, all 14 mutations seen in DBS sequences were also found in plasma viruses. Taken together, these findings indicate an overall concordance between protease and RT sequences from plasma and DBS. We also analyzed the patterns of mutations seen in HIV DNA from DBS. Table 2 shows that the T69N mutation from plasma A.1 and the Y181C and M36L mutations from plasma A.2 were undetectable in DNA sequences from DBS.

Amplification of HIV-1 *pol* from DBS collected in Cameroon. We next evaluated the efficiency of amplification of protease and RT sequences from DBS prepared under field conditions and stored at −20°C. We selected 40 matched plasma/DBS samples from Cameroon representing a wide range of plasma virus load. The median plasma virus load in these samples was 23,715 RNA copies/ml and ranged from 665 to 645,256 RNA copies/ml. Of these samples, 11 had virus loads between 100 and 10,000 RNA copies/ml, 13 had virus loads between 10,000 and 50,000 RNA copies/ml, and 16 had virus loads above 50,000 RNA copies/ml. Of the 40 plasma specimens, HIV-1 *pol* sequences were successfully amplified in 37 samples (92.5%) and 3 were consistently negative after repeated testing (Table 3). Of the negative plasma samples, one (sample 19) had a virus load of 16,515 RNA copies/ml and the remaining two (samples 1 and 2) had virus loads of 665 and 2,271 RNA copies/ml.

We also investigated the amplification of HIV *pol* sequences from these DBS in the absence of reverse transcription. Similar to that seen in the VQA panels, a significant proportion of the RT-PCR positive samples (24 of 37; 64.9%) were amplifiable in the absence of reverse transcription, demonstrating the presence of proviral DNA. Interestingly, amplification of HIV DNA was more frequent among samples with high plasma virus loads. Table 3 shows that only 11 of the 21 (52.4%) DBS samples with plasma virus loads between 1,000 and 50,000 RNA copies/ml were DNA

TABLE 3. Rate of amplification of HIV-1 *pol* in Cameroonian DBS stratified according to plasma virus loads

Sample identification no. (grouped by viral load in RNA/ml)	RNA copies/ml	RT-PCR result[a]		
		Plasma (+RT)	DBS	
			+RT	−RT
100–10,000				
1	2,271	−	+	+
2	665	−	−	−
3	1,277	+	−	−
4	8,141	+	+	−
5	3,711	+	+	−
6	5,429	+	−	−
7	3,219	+	+	+
8	2,224	+	+	−
9	4,836	+	+	+
10	4,558	+	+	+
11	5,710	+	+	−
10,000–50,000				
12	17,451	+	+	+
13	16,077	+	+	+
14	11,738	+	+	−
15	17,771	+	+	−
16	17,507	+	+	−
17	13,316	+	+	+
18	11,433	+	+	−
19	16,515	−	+	−
20	28,240	+	+	−
21	23,715	+	+	+
22	45,510	+	+	+
23	34,067	+	+	+
24	31,292	+	+	+
>50,000				
25	54,214	+	+	+
26	56,269	+	+	+
27	71,492	+	+	+
28	82,693	+	+	+
29	81,892	+	+	−
30	89,689	+	+	+
31	89,889	+	+	−
32	158,600	+	+	+
33	413,070	+	+	+
34	241,077	+	+	+
35	413,070	+	+	+
36	129,471	+	+	−
37	140,673	+	+	+
38	645,256	+	+	+
39	254,810	+	+	+
40	198,400	+	+	+

[a] RT-PCR in DBS were done with (+RT) or without (−RT) MuLV RT enzyme.+, positive; −, negative.

TABLE 4. Protease (major and minor) and RT resistance mutations found in 24 matched plasma/DBS from Cameroonian blood banks

Sample identification no.	Mutation(s) for resistance to indicated enzyme[a]			
	Protease		Reverse transcriptase	
	Plasma	DBS	Plasma	DBS
4	K20I, M36I	K20I, M36I		**L210C**
5	M36I, D60E, L63V	M36I, D60E, L63V		
8	K20R, M36I	K20R, M36I		
10	L33F, M36I, V77I	L33F, **K20I,** M36I, V77I		
11	M36I, D60E, L63T, I93IFLV	M36I, D60E, L63T, I93IFLV		
20	M36I, L63P	M36I, L639		**M184ML,**
24	K20I, M36I, L63Q	K20I, M36I, L63Q		**Y188YD**
25	K20I, M36I, L63I	K20I, M36I, L63I		
29	M36I, D60E, L63V, V77I	M36I, D60E, L63V, V77I		
32	K20I, M36I, L63GISV	K20I, M36I, L63GISV	V118I	V118I
12, 16, 26, 27, 28, 30, 33, 35, 36, 37, 40	K20I, M36I	K20I, M36I		
23, 38, 39	K20I, M36I, L63P	K20I, M36I, L63P		

[a] Mutations in bold were identified in DBS sequences but not in plasma.

positive, compared to 13 of 16 (81%) samples with virus loads greater than 50,000 RNA copies/ml. These findings suggest that the DNA contribution to DBS sequences may vary with plasma virus load levels.

We next compared *pol* sequences from plasma and DBS in a subset of 24 matched samples that had complete RT and protease sequences (data not shown). Similar to that seen in the VQA specimens, the similarity between nucleotide sequences from plasma and DBS was high (mean ± standard deviation, 98.5% ± 1.4%; range, 95% to 100%). A high amino acid similarity was also noted (mean ± standard deviation, 98.0% ± 1.8%) with only one plasma/DBS pair showing a similarity of 94% (patient 8). This particular pair had 10-amino-acid differences between positions 96 to 109 of the RT.

The analysis of protease and RT genotypes in all the 24 plasma and DBS samples showed absence of any major resistance mutations (Table 4). Table 4 also shows a high concordance between minor protease mutations, and only one sample (sample 10) had a K20I mutation in plasma but not in DBS. Two additional samples had unusual amino acids changes at RT positions 210 (sample 4) or 184 and 118 (sample 20) in DBS but not in plasma sequences (Table 4).

DISCUSSION

Dried blood spots are considered a convenient specimen type that can be used for drug resistance surveillance and monitoring, especially in areas that lack the necessary infrastructure for collection and transport of plasma or serum specimens. DBS require minimal laboratory manipulation, are less expensive to collect, store, and transport than plasma, and can be easily collected by trained nonspecialists. We sought to evaluate whether DBS could represent a feasible alternative to plasma and sera for drug resistance testing. We demonstrated that HIV *pol* can be efficiently amplified and genotyped from DBS collected from individuals with a wide range of plasma virus loads. These findings are important because genotypic testing from DBS may simplify drug resistance surveillance and monitoring in resource-limited settings (1, 16, 23). The establishment of efficient resistance surveillance networks in these settings represents an important public health tool that will help to assess the prevalence of HIV drug resistance and adjust treatment guidelines if needed.

Our high efficiency of amplification of *pol* sequences from DBS was especially significant, since our assay amplifies a large (1-kb) *pol* fragment and RNA extractions were done from spots prepared with 50 μl of blood, which represents about 25 μl of plasma. These results expand recent findings showing the efficient genotyping of smaller (663-bp) *pol* fragments from DBS (25) and are consistent with earlier reports using commercial virus load assays which showed the ability to quantify HIV-1 RNA from DBS having a nominal RNA concentration of 4,000 RNA copies/ml (5). Our results are encouraging and suggest that amplification of large fragments from DBS may achieve sensitivity levels similar to those seen in plasma samples. In this sense, the use of a nested PCR procedure may have helped to improve the sensitivity levels. It is also possible that the presence of HIV-1 DNA from peripheral blood mononuclear cells (PBMCs) has contributed to the successful amplification of HIV from DBS. However, DNA amplifications were more common for samples with high plasma virus loads and were less frequent for specimens with lower plasma viremia.

The amplification of proviral DNA from DBS raises questions regarding the potential interference of proviral/archived *pol* sequences in the genotypic profiles generated from DBS. We found that our DBS and plasma genotypes were generally concordant, and, in only one instance, a 181Y/C mixture from plasma was missed in the corresponding DBS sequence. However, the degree of interference of HIV-1 DNA sequences may differ according to disease stage, CD4 cell counts, and treatment characteristics of the population (6, 8, 9). For instance, patients who fail treatment tend to have more detectable mutations in plasma sequences than in PBMCs, particularly at lower virus loads, and the opposite usually occurs in patients undergoing treatment interruptions who typically have more detectable mutations in PBMCs (4, 7, 14, 22, 24). In contrast, drug resistance genotypes from plasma and PBMCs are generally comparable in treatment-naïve persons with an unknown duration of infection (3). Therefore, these results emphasize the need for a thorough evaluation of the concordance between plasma and DBS genotypes in populations with diverse treatment characteristics.

A major finding from our study was the efficient amplification of large HIV *pol* fragments from DBS maintained for 2 to 3 years at −20°C. While small HIV sequences have been previously amplified from DBS stored at this temperature for 1 year (5), storage conditions may be particularly critical for genotypic assays which usually rely on the amplification of large HIV RNA fragments that may be more sensitive to degradation at suboptimal humidity and temperature. Our analysis of a limited number of specimens suggests that −20°C,

but not room temperature, may be a suitable temperature for long-term storage of DBS. However, storage of DBS specimens at −20°C may not be possible at DBS collection sites in less-developed areas. In these settings, short-term storage at 4°C or room temperature may represent a more feasible alternative. However, neither the impact of 4°C or room temperature on the ability to amplify large *pol* fragments from DBS nor how different levels of humidity will affect the stability of nucleic acids on DBS have been evaluated. Such analysis is needed to define appropriate guidelines for the correct storage of DBS at collection sites.

In summary, we show that despite the use of small volumes of blood in DBS, large HIV *pol* fragments can be efficiently amplified and sequenced. Our results also suggest that −20°C may be an appropriate temperature for long-term storage of DBS. Our results show the promise of DBS as an alternative to plasma for drug resistance genotyping and support an expanded evaluation of the correlation between resistance genotypes generated from plasma and DBS. This specimen type may be particularly useful for resistance surveillance in resource-limited settings where the use of antiretroviral drugs continues to increase.

ACKNOWLEDGMENTS

We thank Jeff Johnson for his help with the RT-PCR protocol.

The Virology Quality Assessment Program is supported by a contract from NIH/NIAID/DAIDS number NO1-AI-85354.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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