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HIV-1 drug resistance testing from dried blood spots collected in rural Tanzania using the ViroSeq HIV-1 Genotyping System

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Objectives: To assess whether the commercial ViroSeq HIV-1 Genotyping System (Abbott Molecular, Des Plains, IL, USA) can be used in conjunction with dried blood spots (DBS) for clinical monitoring of drug resistance in patients who fail antiretroviral treatment (ART) in rural Tanzania.

Patients and methods: Patients at Haydom Lutheran Hospital with confirmed treatment failure (viral load >1000 copies/mL) of a first-line ART regimen were selected for resistance testing. DBS were stored with desiccant at −20°C for a median of 126 days (range 0–203) and shipped at ambient temperature for 20 days. After manual extraction of nucleic acids, the ViroSeq kit was used for amplification and sequencing. DBS-derived genotypes were compared with those of a plasma-based assay.

Results: Seventeen of 36 (47%) DBS specimens were successfully genotyped. Only 2 of 16 (13%) DBS with a viral load <10000 copies/mL could be amplified, compared with 15 of 20 (75%) DBS with a viral load >10000 copies/mL ($P=0.001$). In samples that yielded a sequence, all 23 clinically significant reverse transcriptase (RT) mutations in plasma were also detected in DBS. One RT mutation was found in DBS only. In the protease region, 77 polymorphisms were found in plasma, of which 70 (91%) were also detected in DBS. Sixteen of 17 (94%) patients had identical resistance profiles to antiretroviral drugs in plasma and DBS.

Conclusions: The ViroSeq kit performed well in patients with a high viral load, but failed to genotype most DBS with a viral load <10000 copies/mL. In DBS that yielded a genotype, there was high concordance with a plasma-based assay.

Keywords: HIV infections, antiretroviral therapy, molecular diagnostic techniques, sub-Saharan Africa

Introduction

By the end of 2008, >4 million people were receiving antiretroviral treatment (ART) of HIV/AIDS in low- and middle-income countries, which is a 10-fold expansion over the past 5 years.[1] The probability of drug resistance increases with duration of treatment and has been estimated to reach 27% by 6 years.[2] Thus, the number of individuals in need of second-line ART in low- and middle-income countries can be expected to rise sharply in the coming years.

Drug resistance testing is standard of care in industrialized countries in order to tailor new fully active regimens in patients with treatment failure, but is rarely available in resource-limited settings due to high costs and stringent requirements for storage and transport of plasma. Hence, patients with treatment failure in such settings usually switch blindly from a non-nucleoside reverse transcriptase inhibitor (NNRTI)-based regimen to a protease inhibitor-based regimen. However, since most patients with treatment failure are detected late, at which stage widespread resistance is common, patients risk undergoing switches to regimens with limited efficacy. Further down the line, switching blindly to a third- or fourth-line regimen will be virtually impossible without access to resistance testing.

Dried blood spots (DBS) are easy to collect and store, and can be a convenient alternative to plasma. Recently, several studies have demonstrated the feasibility and reliability of using DBS to monitor HIV drug resistance.[3–6] However, most studies utilized in-house genotyping assays, and few used specimens collected under 'real-life' field conditions. Commercial genotyping kits have several advantages over in-house assays, including quality-controlled reagents, standardized protocols and validated interpretation tools, and would facilitate routine use in

high-throughput settings; however, these kits have only been validated with plasma. We therefore aimed to assess the performance of the ViroSeq assay in conjunction with DBS obtained under field conditions from patients failing ART in rural Tanzania.

Patients and methods

We utilized 36 remnant DBS cards collected from patients with known treatment failure of an NNRTI-based first-line regimen at Haydom Lutheran Hospital. Median plasma viral load was 15 180 copies/mL (range 1350–3683 000), and a plasma genotype was available from all patients. HIV-1 subtypes were A ($n=11$), C ($n=10$), D ($n=12$) and CRF01_AE ($n=3$). A more detailed description of the study population has been published previously.[7] All patients gave written consent to participate in the study, and the study was approved by the National Institute for Medical Research (Tanzania) and Regional Committee for Medical Research Ethics (Norway).

DBS were prepared by spotting whole blood from a plasma preparation tube (PPT; Becton Dickinson, Franklin Lakes, NJ, USA) onto a 903 filter paper card (Whatman plc, Maidstone, UK) to completely fill the circles. The cards were dried overnight (temperature range 24–28°C) and stored in zip-lock plastic bags (purchased locally) with a silica desiccant (Elcon-Broker AS, Holmestrand, Norway). DBS were stored at the collection site at −20°C for a median of 126 days

Table 1. Patient characteristics and efficiency of HIV-1 genotyping from DBS in 36 individuals failing ART in rural Tanzania

ID no.	Viral load (copies/mL)	CD4 (cells/mm^3)	Subtype	Current ART regimen[a]	Months since starting ART	Plasma genotype	DBS genotype in-house assay[b]	DBS genotype ViroSeq assay
1	3683000	381	D	d4T, 3TC, EFV	14.6	+	+	+
2	477500	19	D	d4T, 3TC, NVP	32.3	+	+	+
3	434100	31	C	ZDV, 3TC, EVF	42.1	+	+	+
4	420000	969	C	d4T, 3TC, EFV	24.0	+	+	+
5	281500	214	A	no (d4T, 3TC, NVP)	45.9	+	+	−
6	229000	371	C	no (d4T, 3TC, EFV)	24.0	+	+	+
7	154200	62	D	no (d4T, 3TC, NVP)	16.7	+	+	+
8	132000	223	D	no (d4T, 3TC, NVP)	30.0	+	+	+
9	122900	488	A	no (d4T, 3TC, NVP)	9.7	+	+	−
10	120200	469	A	no (d4T, 3TC, NVP)	30.0	+	+	+
11	113400	432	C	ZDV, 3TC, NVP	24.5	+	+	+
12	81690	673	CRF01_AE	d4T, 3TC, NVP	35.5	+	+	+
13	76700	224	A	d4T, 3TC, NVP	9.2	+	+	+
14	73400	526	A	no (ZDV, 3TC, NVP)	27.3	+	+	+
15	44250	547	A	no (d4T, 3TC, NVP)	9.2	+	+	+
16	35400	302	A	d4T, 3TC, NVP	19.0	+	+	−
17	20500	122	A	d4T, 3TC, EFV	28.3	+	+	−
18	15600	117	C	d4T, 3TC, NVP	17.8	+	+	−
19	14750	445	A	d4T, 3TC, EFV	24.2	+	+	+
20	12390	148	D	no (d4T, 3TC, NVP)	30.9	+	+	+
21	8690	162	D	d4T, 3TC, NVP	49.1	+	+	−
22	5990	768	D	d4T, 3TC, NVP	36.9	+	+	+
23	5560	388	CRF01_AE	no (ZDV, 3TC, NVP)	9.8	+	+	−
24	3965	144	D	d4T, 3TC, NVP	27.3	+	+	−
25	3198	190	D	no (ZDV, 3TC, EFV)	16.2	+	+	+[c]
26	3101	299	A	d4T, 3TC, NVP	14.5	+	+	−
27	3000	223	C	d4T, 3TC, NVP	28.3	+	+	−
28	2621	244	A	d4T, 3TC, NVP	32.4	+	−	−
29	2400	271	D	d4T, 3TC, NVP	31.3	+	+	−
30	2290	454	C	d4T, 3TC, NVP	28.6	+	+	−
31	2260	495	C	ZDV, 3TC, NVP	25.9	+	+	−
32	1980	635	D	d4T, 3TC, NVP	26.5	+	+	−
33	1886	432	C	d4T, 3TC, NVP	24.0	+	+	−
34	1504	354	D	d4T, 3TC, NVP	30.4	+	+	−
35	1432	810	CRF01_AE	d4T, 3TC, NVP	21.1	+	−	−
36	1349	419	C	d4T, 3TC, NVP	34.7	+	+	−

d4T, stavudine; ZDV, zidovudine; 3TC, lamivudine; NVP, nevirapine; EFV, efavirenz.
[a]In patients who discontinued ART, past treatment is shown in parentheses.
[b]Results by the use of an in-house assay have been published previously.[7]
[c]Only the protease region was amplified.

(range 0–203), and exposed to ambient temperature for 20 days during transport. Before shipment, desiccants were replaced with new ones, after allowing the DBS to equilibrate at room temperature for a minimum of 1 h. After arrival at the reference laboratory (Hospital Carlos III, Madrid, Spain), samples were kept at −20°C until processing 1 month later.

Total nucleic acids were extracted from two circles of dried blood, each of which holds 75–80 μL of whole blood,[8] using the NucliSENS silica-based extraction method (bioMérieux, Inc., Durham, NC, USA) as described previously.[7] HIV-1 genotypic analysis was done using the ViroSeq HIV-1 Genotyping System v2.0 (Abbott Molecular, Des Plains, IL, USA) following the manufacturer's instructions. This method amplifies the entire protease gene and approximately two-thirds of the reverse transcriptase (RT) gene, representing 1.8 kb of HIV-1 *pol*. The assay is performed by a single RT–PCR and has a sensitivity of detection of 2000 HIV-1-RNA copies/mL in plasma.

Genotypes obtained with DBS were compared with those obtained with use of a plasma-based assay. Plasma was genotyped using the ViroSeq HIV-1 Genotyping System v2.0 with inclusion of an in-house RT-nested PCR step as previously described.[7] Resistance profiles to antiretroviral drugs were interpreted according to the Stanford University HIV Drug Resistance Database (HIVdb Program, http://hivdb.stanford.edu). Only drug resistance mutations listed in the December 2009 update from the International AIDS Society were considered in the present study.[9] Logistic regression was used to study factors associated with amplification failure. Data were analysed with SPSS v16.0 for Windows (SPSS Inc., Chicago, IL, USA). All tests were two-sided and the level of significance was set at $P<0.05$.

Results

HIV-1 *pol* was successfully amplified in 17 of 36 (47%) DBS specimens using the ViroSeq assay, but one of these yielded a genotype in the protease region only (Table 1). Most of the amplification failures occurred in specimens with low-level viraemia. Only 2 of 16 (13%) DBS with a viral load of <10000 copies/mL could be amplified, compared with 15 of 20 (75%) DBS with a viral load of >10000 copies/mL ($P=0.001$). Amplification failures were observed with all HIV-1 subtypes in the study population: A ($n=6$); C ($n=6$); D ($n=5$); and CRF01_AE ($n=2$). In specimens with viral loads of >10000 copies/mL, four of five amplification failures were subtype A; however, numbers were small and not statistically significant. Furthermore, there was no significant association between the length of DBS storage at −20°C and amplification failure ($P=0.70$).

Seventeen plasma/DBS pairs were available for comparison (Table 2). Of 77 protease mutations found in plasma, 70 (91%) were also detected in DBS, all of which were minor mutations or polymorphisms. The seven protease mutations missed in DBS were present in plasma as mixtures with the wild-type strain. No additional protease mutations were observed in DBS. In the RT region, all 23 clinically significant mutations in plasma were also detected in DBS. In addition, one RT mutation was found in DBS only, as a mixture in the 101 position (K101EK in patient 13) with some impact on etravirine resistance

Table 2. Mutations in the protease and RT genes in 17 matched plasma/DBS specimens from patients failing ART in rural Tanzania

ID no.	Protease		RT	
	plasma	DBS	plasma	DBS
1	I13V, M36I, D60E, I64V	I13V, M36I, D60E, I64V	none	none
2	I13V, K20R, M36MV, L63P, I64V, I93IL	I13V, K20R, L63P, I64V	G190A	G190A
3	M36I, L63LP, H69K, I93L	M36I, L63LP, H69K, I93L	K103N, E138A	K103N, E138A
4	M36I, L63P, H69K, I93L	M36I, L63P, H69K, I93L	none	none
6	G16E, H69K, V77I, I93L	G16E, H69K, V77I, I93L	K103N, V108IV, M184V, P225H	K103N, V108IV, M184V, P225H
7	I13V, K20R, M36MI, I62IV, I64V	I13V, K20R, I62IV, I64V	none	none
8	L10IV, I13IV, G16GE, I62V, I64V, V77I, I85IV	L10IV, G16E, I62V, I64V, V77I	M184V, G190A	M184V, G190A
10	I13V, M36I, L63P, I64V, H69K, V77I	I13V, M36I, L63P, I64V, H69K, V77I	K103KN	K103KN
11	M36I, H69K, I93L	M36I, H69K, I93L	none	none
12	I13V, M36I, L63P, H69K	I13V, M36I, L63P, H69K	D67N, K70R, K103N, V179TV, M184V, K219Q	D67N, K70R, K103N, V179T, M184V, K219Q
13	I13V, M36I, L63LP, H69K, V82VI	I13V, M36I, H69K, V82IV	V179T, M184V, G190A	**K101EK**, V179T, M184V, G190A
14	I13V, M36I, H69K	I13V, M36I, H69K	none	none
15	L10V, I13V, G16E, M36I, I62IV, H69K	L10V, I13V, G16E, M36I, H69K	E138A	E138A
19	I13V, M36I, D60E, L63P, H69K	I13V, M36I, D60E, L63P, H69K	none	none
20	L63P, V77VI	L63LP, V77IV	none	none
22	I13V, L63P, I64V, V77I	I13V, L63LP, I64V, V77I	K101E, M184V, G190A	K101E, M184V, G190A
25	I13V, K20R, M36I, I62IV, I64IV	I13V, K20R, M36I, I62V, I64IV	none	failed

Discordances between plasma and DBS are underlined. Discordances that represent drug-resistant mutations are bold and underlined.

interpretation. Thus, 16 of 17 (94%) patients had identical resistance profiles to antiretroviral drugs in plasma and DBS.

The whole sequence of 1302 bases was compared between plasma and DBS, with mixed bases being classified as different. Mean nucleotide similarity was 98.7% (SD 0.62), ranging from 97.5% to 99.7%.

DBS genotypes obtained with the ViroSeq assay were highly concordant with those obtained with use of a previously published in-house DBS method.[7] Of 94 mutations found with the ViroSeq assay, 88 (93.6%) were also detected with the in-house assay, and vice versa.

Discussion

The ViroSeq kit successfully genotyped approximately half of DBS specimens from patients with ART failure in rural Tanzania. Although efficient in patients with high viral loads, the ViroSeq kit failed to genotype most DBS specimens with viral loads of <10000 copies/mL. In the specimens that yielded a genotype, however, there was high concordance with results from a plasma-based assay.

Three previous studies have assessed the ViroSeq kit in conjunction with DBS. Masciotra *et al.*[4] successfully genotyped all DBS with viral loads of >2000 copies/mL, compared with 55% of those with viral loads of <2000 copies/mL. Youngpairoj *et al.*[5] reported that only 8% of DBS could be genotyped when viral load was <10000 copies/mL, compared with 81% when viral load was >10000 copies/mL, similar to our results. Finally, in a recent study from Mexico, Lira *et al.*[10] successfully genotyped 60% of DBS with viral loads of >14000 copies/mL, but none of the DBS with viral loads below this level. All these studies found high concordance between nucleotide sequences derived from DBS and plasma, in line with our findings. Our study, however, is the first to assess the ViroSeq kit with DBS from patients infected with non-B subtypes. The ViroSeq kit was originally optimized for HIV-1 subtype B;[11] hence, future DBS studies should aim to include various subtypes, including subtype A, which frequently failed amplification in our study, even in specimens with high viral loads.

Previously, we reported 94% amplification success from DBS collected in duplicate with DBS utilized in the present study and stored under less favourable conditions, using an in-house RT-nested PCR method.[7] Youngpairoj *et al.*[5] reported similar results; overall amplification success was 57.5% with the ViroSeq kit and 95% with an in-house nested assay. In-house assays typically reduce the costs by >50% compared with commercial kits.[12] Furthermore, the use of a nested PCR protocol appears to increase sensitivity, particularly in samples with low-level viraemia, such as patients with early treatment failure. However, in resource-limited settings, where the selection of second-line antiretroviral drugs is scarce, the WHO recommends that first-line treatment be conserved as long as viral load does not exceed 10000 copies/mL.[13] Thus, the ViroSeq kit in conjunction with DBS could be an acceptable option for resistance testing under the WHO guidelines, given that genotyping is restricted to patients who reach the viral load threshold for regimen switch. Nonetheless, further refinement of the ViroSeq kit with DBS is warranted in order to increase sensitivity.

Our study was limited by a relatively small sample size. Moreover, although DBS and plasma were both genotyped using the ViroSeq kit, the use of different amplification methods might have contributed to discordances between the two specimen types. The main strength of our study was that we used samples obtained in rural Africa, and we believe our results reflect 'real-life' performance of DBS-based resistance monitoring in the field. However, temperature and humidity conditions differ from place to place, and the effect of various storage conditions should be studied in more detail.

In conclusion, we found that the ViroSeq kit performed well using DBS from patients with major virological failure, but failed to genotype most DBS with viral loads of <10000 copies/mL. In DBS samples that yielded a genotype, there was high concordance between mutations found in DBS and plasma. Using the WHO guidelines for ART in resource-limited settings, recommending regimen switch only in patients with viral loads of >10000 copies/mL, the ViroSeq kit in conjunction with DBS could be an acceptable option for drug resistance testing.

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Transparency declarations

None to declare.

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