The use of dried blood spot specimens for HIV-1 drug resistance genotyping in young children initiating antiretroviral therapy

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Running title: DBS specimens for HIVDR genotyping in children

52 **Abstract**

53 Paired plasma and dried blood spots (DBS) from 232 South African HIV-infected children
54 initiating antiretroviral therapy (ART) were genotyped for drug resistance mutations, most of
55 who had prior exposure to ART for prevention-of-mother-to-child-transmission. Non-
56 nucleoside reverse transcriptase inhibitor mutations were most commonly detected in both
57 specimen types, particularly Y181C/I and K103N/S. Resistance interpretation concordance
58 was achieved in 97% of pairs with 7 children having mutations detected in DBS only. These
59 results validate the preferential use of DBS specimens for HIVDR genotyping in this patient
60 group.

Since the implementation of the antiretroviral therapy (ART) programme in 2004, more than 140,000 children have received treatment for HIV-1 infection in South Africa (WHO, 2013). Importantly, infection rates in infants have declined to <4% at 4-8 weeks after birth (Goga, 2012), primarily due to the provision of drug interventions for prevention of mother to child transmission (PMTCT). Despite these successes, ~5,000 infants are still infected with HIV-1 each year (Sherman et al., 2014). Those infected less than 3 years of age are initiated on a ritonavir-boosted lopinavir (LPV/r)-containing regimen, and age of initiation has been revised to include all HIV-1 positive children immediately after diagnosis (DOH, 2013). HIV-1 drug resistance (HIVDR) prevalence prior to starting ART is high in young children (~60%) but is less common in older children (Hunt et al., 2011; Kuhn et al., 2014). As the scale-up of PMTCT and treatment continues, understanding the patterns of HIVDR in newly-diagnosed children is critical (Fitzgerald et al., 2013).

Plasma is currently the preferred specimen for HIVDR genotyping, but requires maintenance of a cold-chain and short transit times to preserve the integrity of the viral RNA for PCR amplification. Dried blood spots (DBS) are considered an alternative specimen type for HIVDR genotyping, as these are easily collected on filter paper cards using heel-prick or finger-prick technique and can be stored at ambient temperature. Studies on adults have shown a high rate of concordance in identifying HIVDR mutations when using plasma and whole blood specimens spotted onto DBS cards in treatment-naïve and treatment-exposed patients (Bertagnolio et al., 2010; Johannessen et al., 2010; Monleau et al., 2014; Rottinghaus et al., 2012). Limited data exists on HIVDR genotyping comparison between DBS and plasma specimens for paediatric populations, with one study showing 95% success rate in HIVDR genotyping using DBS (Ziemniak et al., 2011). The objective of this study was to assess the use of DBS specimens for detection of HIVDR mutations in a large cohort of HIV-infected infants and young children initiating ART in Johannesburg, South Africa.

Specimens from HIV-infected children under 2 years of age from 5 different sites in Johannesburg that were part of the Finding Infants with HIV disease: Evaluation of Resistance (FInHDER) Study were selected for analysis (Kuhn et al., 2014). Two-thirds had exposure to maternal and/or infant antiretrovirals for PMTCT. DBS specimens were collected by heel-prick on to Whatman 903 filter cards, approximately 50µl per spot. Whole blood specimens were collected by veni-puncture in EDTA tubes and separated according to standard protocols. Viral load assessment was performed on plasma specimens using the COBAS® Taqman® HIV-1 Test v2.0. Plasma and DBS specimens were stored at -80°C until processed for HIVDR genotyping. The study was approved by the Institutional Review Boards of the University of the Witwatersrand and Columbia University.

Two 50µl spots were excised using a DBS puncher, immersed in 2ml of NucliSENS lysis buffer (Biomerieux, Germany) and lysed for 2 hours on a roller mixer at room temperature. Total nucleic acid (TNA) was extracted using the NucliSENS EasyMAG® automated system according to the manufacturer's instructions. Amplification of a 1,084bp fragment consisting of codons 1-99 of *protease* and codons 1-250 of *reverse transcriptase* was performed as previously described (Zhou et al., 2011) except for 400µM of each primer was used for RT-PCR. Plasma samples were processed as previously described using a 1,770bp amplicon PCR spanning codons 1-99 of *protease* and 1-430 of *reverse transcriptase* (Pillay et al., 2008). Editing of sequences was performed using Recall software v2.10 (Woods et al., 2012), and drug resistant mutations were identified using the Stanford HIV database algorithm V7.0 (<http://hivdb.stanford.edu>). Inter-rater agreements were calculated using Cohen's kappa coefficient (STATA v11, StataCorp, College Station, USA).

Of 255 available DBS specimens, 14 did not have matched plasma specimens and 3 were duplicate specimens, leaving 238 paired plasma and DBS specimens for HIVDR testing. The majority of the specimens had a plasma viral load $>\log 5$ copies/ml. Genotyping PCR amplification rates were 97.9% (233/238) for plasma and 98.7% (235/238) for DBS specimens (Table 1). All 6 specimen pairs where DBS and/or plasma failed to amplify were removed from further analysis.

Sequencing and HIVDR genotyping was successful on all 232 specimen pairs. The median age of children when these samples were collected was 27 weeks (inter-quartile range 13 to 51 weeks). Non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations were the most commonly detected in DBS and plasma, particularly Y181C/I (28% and 27%) and K103N/S (15% and 13%) respectively (Figure 1). Nucleoside reverse transcriptase inhibitor (NRTI) mutations occurred at $\leq 5\%$. Only one specimen was predicted to be resistant to protease inhibitors (PI) due to the presence of major and minor PI mutations (L10F, M46I, I54V, L76V and V82A) while 8 specimens had minor PI mutations (A71T (n=3), L10I/V (n=3) and M46L/T (n=2)).

All specimens clustered with subtype C. Genotypic profiles between plasma and DBS specimens were concordant in 214 (92%) specimen pairs. Of the 18 discordant genotypes, 11 specimen pairs showed minor discordance in that an additional mutation was detected in either the DBS or plasma specimen, with no change in drug resistance interpretation between either specimen type. Seven (3%) specimens showed major discordance in that a single mutation was detected in the DBS and not the plasma, altering the relevant drug class resistance interpretation from susceptible to resistant (Table 2). Overall, the strength of agreement between the plasma and DBS specimens in predicting NRTI resistance ($k=0.924$, 95% CI 0.819 – 1.00) and NNRTI resistance ($k=0.956$; 95% CI 0.918 – 0.994) was almost perfect. A phylogenetic analysis on sequences showed linkage between each of the paired specimens and the mean nucleotide identity per paired sample was 99.5%.

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143 These results indicate a high concordance of HIVDR genotyping between DBS and plasma
144 specimens collected from a large cohort of HIV-infected children prior to initiating ART. The
145 detection of additional mutations in DBS is likely due to the presence of viral DNA in whole
146 blood spots (Wei et al., 2011), but may reflect natural sequence variation as a result of
147 different PCR assays used for DBS and plasma specimens. Such mutations may have faded
148 in the plasma circulating virus population following PMTCT exposure up to 2 years
149 previously. It should, however, be noted that these specimens were collected under optimal
150 study conditions and the results may not reflect viral amplification success rates in the
151 absence of effective cold-chain maintenance. A further limitation of the study is that data
152 obtained from two different PCR assays is compared. It is possible that PCR amplification
153 success rates from DBS may be lower using the longer amplicon PCR method utilised to
154 sequence plasma specimens. However, this data shows that the use of DBS specimens for
155 HIVDR genotyping in children less than 2 years of age initiating ART is thus a viable option
156 for clinical management and surveillance purposes and will greatly improve the feasibility of
157 performing studies in this group.

158

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Table 1: Viral load and PCR amplification rate for 238 DBS and plasma specimens

Table 2: Specimens with major drug resistance interpretation discordance among 232 matched DBS and plasma specimens.

Figure 1. Prevalence of NRTI and NNRTI mutations detected in 232 DBS and plasma specimen pairs

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Table 1: Viral load and PCR amplification rate for 238 DBS and plasma specimens

Viral load range (copies/ml)	N	Amplified DBS (%)	Amplified Plasma (%)
2 000 - 10 000	4	4 (100.0)	4 (100.0)
10 001 - 50 000	12	11 (91.6)	11 (91.6)
50 001 - 100 000	9	9 (100.0)	9 (100.0)
>100 000	190	188 (98.9)	186 (97.8)
unknown	23	23 (100.0)	23 (100.0)
Total	238	235 (98.7)	233 (97.9)

Table 2: Specimens with major drug resistance interpretation discordance among 232 matched DBS and plasma specimens.

ID	Age when tested for HIVDR (weeks)	Plasma			DBS		
		Mutations detected	NRTI Resistance Interpretation	NNRTI Resistance Interpretation	Mutations detected	NRTI Resistance Interpretation	NNRTI Resistance Interpretation
1	30	None	Susceptible	Susceptible	Y181CY	Susceptible	Resistance
2	84	None	Susceptible	Susceptible	K103KN	Susceptible	Resistance
3	13	None	Susceptible	Susceptible	Y188CY	Susceptible	NVP Resistance only
4	27	None	Susceptible	Susceptible	Y181CY	Susceptible	Resistance
5	99	None	Susceptible	Susceptible	G190AG	Susceptible	Resistance
6	43	Y181C	Susceptible	Resistance	Y181C,K219 E	Reduced Susceptibility to AZT and d4T	Resistance
7	13	K103N, F227FL	Susceptible	Resistance	L74V,K103N, Y181CY	Resistance to ddl and ABC	Resistance

Figure(s)