

# Viral Load Detection Using Dried Blood Spots in a Cohort of HIV-1-Infected Children in Uganda: Correlations with Clinical and Immunological Criteria for Treatment Failure

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**Correlations between clinical/immunological treatment failure and viral load (VL) detected by dried blood spot (DBS) sampling were explored in HIV-1-infected children in Uganda. Of 104 children on combined antiretroviral treatment (cART), 12.5% experienced clinical and/or immunological failure, while 28.8%, 44.2%, and 26.9% had VLs of <1,000, 1,000 to 5,000, and >5,000 copies/ml, respectively. Clinical/immunological failure poorly predicted virological failure.**

High rates of virological suppression were reported among HIV-1-infected children of low- and middle-income countries (LMIC) up to 5 to 6 years after receiving combined antiretroviral treatment (cART) (1). In 2013, the World Health Organization (WHO) recommended viral load (VL) monitoring as the preferred approach to detecting treatment failure (TF) among HIV-1-infected children (2). High costs, a lack of adequate facilities, and inappropriate handling of specimens still limit the implementation of VL monitoring in LMIC, leading to delays in detecting treatment failure when immunological and/or clinical criteria are used instead (3, 4).

Dried blood spot (DBS) sampling is a promising tool for virological monitoring in LMIC (5–8). Although the accuracy of VL testing with DBS sampling may be affected by prolonged storage at high temperatures and humidity (5) and by variability in nucleic acid extraction and amplification results using commercial and in-house methods (6, 9), most studies have reported high correlations between plasma viral loads and DBS results when plasma VLs exceed 5,000 HIV-1 RNA copies (cp)/ml (6, 7, 9, 10). Whole-blood samples from DBS reflect the contribution of plasma plus cell-associated viral nucleic acids and may yield higher levels of HIV-1 RNA than those from plasma, particularly in the case of lower plasma VLs (7, 9).

The aim of this study was to evaluate correlations between clinical and immunological TF criteria and VLs of >1,000 and >5,000 cp/ml in whole blood from DBS in a cohort of HIV-1-infected children enrolled at the home-care department of St. Raphael of St. Francis Hospital (Kampala, Uganda). Cotrimoxazole prophylaxis, cART, and opportunistic infections were managed according to 2006 or 2010 WHO guidelines (11, 12), which were applicable at the time; cART was initiated after obtaining written consent from the caregivers. The children were evaluated monthly for clinical follow-up and self-reported adherence. Laboratory investigations, including CD4 cell counts, were carried out every 6 months or more frequently, if clinically required. Quantitative HIV-1 RNA detection was not part of the routine examinations. In 2010, DBS samples were collected for quantitative HIV-1 RNA detection in HIV-1-infected children enrolled in a parallel study investigating the Epstein-Barr virus (13).

Whole-blood samples were collected onto DBS (903 protein saver card; Whatman GmbH, Hahnestra) as previously described (13), left to dry overnight at room temperature, and then stored in individual resealable plastic bags containing a desiccant. Median time from blood collection in Uganda to DBS shipment to the laboratory in Italy was 49 days (interquartile range [IQR], 34 to 64 days). For each sample, RNA was extracted from two 50- $\mu$ l samples of DBS (total volume, 100  $\mu$ l whole blood); the total HIV-1 RNA (cell-associated plus plasma) levels were determined using the Amplicor HIV-1 monitor test (Roche diagnostic systems), and the limit of detection was 170 HIV-1 RNA cp/ml in whole blood, taking into account the spot volume (13).

Clinical and immunological failures were defined according to 2010 WHO criteria (Table 1) (12). The differences in demographic and clinical characteristics by VL category were assessed using Kruskal-Wallis chi-square tests. The predictive value of clinical and/or immunological failure for a VL of >1,000 or >5,000 cp/ml was assessed using sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the curve (AUC) statistics from receiver operating characteristic (ROC) curves. All analyses were carried out with Stata 12 (College Station, TX).

The baseline characteristics of the 104 children (51 male, 53 female) are listed in Table 2. The median age of the children at specimen collection was 6.55 years (IQR, 3.67 to 9.21 years). At birth, 2 of the children were exposed to nevirapine (NVP) for the prevention of mother-to-child transmission (PMTCT), 35 children were not exposed to NVP, and 67 children had an unknown PMTCT history. DBS samples were collected at a median of 34.19

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**TABLE 1** Immunological and clinical failure to predict virological failure for VLs of >1,000 and >5,000 cp/ml in whole blood in cART-experienced children<sup>c</sup>

2010 WHO criteria for immunological and/or clinical failure <sup>a</sup>	Virological failure at VL of:	
	>1,000 cp/ml <sup>b</sup>	>5,000 cp/ml <sup>c</sup>
AUC	0.5176	0.5122
Sensitivity (%)	13.51	14.29
Specificity (%)	90.00	88.16
PPV (%)	76.92	30.77
NPV (%)	29.67	73.63

<sup>a</sup> 2010 WHO criteria for immunological failure, CD4 count of  $\leq 200$  cells/mm<sup>3</sup> or CD4% of  $\leq 10\%$  for children 2 to 5 years old and CD4 count of  $\leq 100$  cells/mm<sup>3</sup> for children >5 years old; 2010 WHO criterion for clinical failure, a new WHO stage III to IV event occurring at >24 weeks after cART initiation.

<sup>b</sup>  $n = 30$  for VLs of <1,000 cp/ml, and  $n = 74$  for VLs of >1,000 cp/ml in whole blood.

<sup>c</sup>  $n = 76$  for VLs of <5,000 cp/ml, and  $n = 28$  for VLs of >5,000 cp/ml in whole blood.

months (IQR, 19.57 to 52.44 months) after cART initiation. At the time of specimen collection, 68 children were still on their first cART regimen, and 36 had substituted at least one drug (cART was switched for one child due to clinical failure). Poor adherence between the first cART and DBS collection (defined as self-reported consumption of <95% of doses received) was reported for one child.

Thirty children (28.8%) had a VL of <1,000 cp/ml after a median time of 41.63 months (IQR, 28.52 to 54.28 months) from cART initiation; VLs of 1,000 to 5,000 and >5,000 cp/ml, respectively, were detected in 46 (44.2%) and 28 (26.9%) of the children at median times of 31.31 months (IQR, 15.47 to 45.60 months) and 37.72 months (IQR, 21.16 to 55.62 months) after cART initiation (Table 2). At the time of sample collection, 13 children (12.5%) had experienced TF according to immunological (1) or clinical (12) criteria; 3 children with clinical failures had a VL of <1,000 cp/ml, 6 had a VL of 1,000 to 5,000 cp/ml, and 3 with clinical failure and 1 with immunological failure had a VL of

**TABLE 2** Univariate analysis of 104 cART-experienced HIV-positive children with VLs of <1,000, 1,000 to 5,000, or >5,000 cp/ml in whole blood

Characteristic <sup>a</sup>	Overall	Children with VL of:			P value for trends
		<1,000	1,000–5,000	>5,000	
No. (%) of children	104	30 (28.8)	46 (44.2)	28 (26.9)	
Male (no. [%])	51 (49.04)	14 (46.67)	19 (41.30)	18 (64.29)	0.152
At cART initiation					
Median age (IQR) (yr)	6.55 (3.67–9.21)	6.44 (4.05–8.72)	6.81 (4.22–9.77)	5.75 (2.70–8.92)	0.534
CD4 cell count (median [IQR]) (cells per mm <sup>3</sup> )	412 (235–674)	527 (245–732)	341 (254–628)	567 (198–574)	0.849
CD4% (median [IQR])	11.8 (7.9–18)	12.1 (9.07–18.59)	9.82 (7.38–17.40)	10.64 (7.62–16.01)	0.685
WHO HIV stage (no. [%])					
I	10 (9.62)	3 (10.00)	3 (6.52)	4 (14.29)	0.542
II	66 (63.46)	21 (70.00)	28 (60.87)	17 (60.71)	
III	26 (25.00)	5 (16.67)	15 (32.61)	6 (21.43)	
IV	2 (1.92)	1 (3.33)	0 (0.00)	1 (3.57)	
First cART regimen (no. [%])					
3TC-D4T-EFV	11 (10.58)	4 (3.85)	6 (13.04)	1 (3.57)	0.117
3TC-D4T-LPV/r	2 (1.92)	1 (3.33)	0 (0)	1 (3.57)	
3TC-D4T-NVP	20 (19.23)	6 (20)	9 (19.57)	5 (17.86)	
AZT-3TC-EFV	25 (24.04)	8 (26.67)	12 (26.09)	5 (17.86)	
AZT-3TC-LPV/r	5 (4.81)	1 (3.33)	3 (6.52)	1 (3.57)	
AZT-3TC-NVP	41 (39.42)	10 (33.33)	16 (34.78)	15 (53.57)	
NNRTI-based first regimen (no. [%])	97 (93.27)	28 (93.33)	43 (93.48)	26 (92.86)	0.117
PI-based first regimen (no. [%])	7 (6.73)	2 (6.67)	3 (6.52)	2 (7.14)	0.117
cART regimen at VL testing <sup>b</sup> (no. [%])					
3TC-D4T-EFV	8 (7.69)	3 (10)	5 (10.87)	0 (0)	0.687
3TC-D4T-LPV/r	1 (0.96)	0 (0)	1 (2.17)	1 (3.57)	
3TC-D4T-NVP	26 (25.00)	2 (6.66)	11 (23.91)	10 (35.71)	
AZT-3TC-EFV	23 (22.12)	9 (30)	9 (1.96)	6 (21.43)	
AZT-3TC-LPV/r	7 (6.73)	3 (10)	2 (4.35)	1 (3.57)	
AZT-3TC-NVP	39 (37.50)	13 (43.33)	18 (39.13)	10 (35.71)	
NNRTI-based regimen at VL testing (no. [%])	96 (92.31)	27 (90.00)	43 (93.48)	26 (92.86)	
PI-based regimen at VL testing (no. [%])	8 (7.69)	3 (10.00)	3 (6.52)	2 (7.14)	0.850
Median (IQR) time from cART initiation to DBS collection (mo)	34.19 (19.57–52.44)	41.63 (28.52–54.28)	31.31 (15.47–45.60)	37.72 (21.16–55.62)	0.255

<sup>a</sup> 3TC, lamivudine; D4T, stavudine; EFV, efavirenz; LPV/r, lopinavir-ritonavir; AZT, zidovudine; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

<sup>b</sup> cART regimen at time of VL testing is first cART regimen for patients with no drug substitutions and most recent cART regimen prior to VL testing for those with substitutions.

>5,000 cp/ml. All of them were receiving a nonnucleoside reverse transcriptase inhibitor (NNRTI)-based regimen, and 8/12 substituted cART before DBS collection. Chi-square tests indicated that neither the initial regimen ( $P = 0.117$ ) nor the cART regimen at the time of specimen collection ( $P = 0.850$ ) was associated with the VL (Table 2). The sensitivities and specificities of clinical and/or immunological criteria in predicting virological failure were very low at the 1,000- and 5,000-cp/ml whole-blood thresholds (Table 1).

These findings indicate that a high proportion of children may have a detectable viral load after at least 6 months of cART in the absence of clinical and/or immunological failure. In our cohort, a single determination of the VL makes it hard to discriminate between treatment failure and a slow but progressive decrease in viral load from a very high initial viral load. However, DBS samples were collected at a median time of 34 months after cART initiation, suggesting that a slow virological response is unlikely. Self-reported adherence to cART may have been overestimated, particularly for older children/adolescents at major risk of poor adherence. Furthermore, the lack of data on cART resistances makes it difficult to give an exhaustive explanation of the poor virological outcome observed in our cohort.

As previously mentioned, HIV-1 RNA extracted from DBS comes from both plasma and cells. Cell-associated residual viral replication may persist even in cART-treated patients with undetectable plasma VLs (14). In our cohort, the VLs determined from DBS may have been slightly overestimated compared to the plasma VLs, particularly in the <1,000- and 1,000- to 5,000-cp/ml groups (7). The possibility of contamination of HIV-1 DNA cannot be excluded. However, considering the numbers of cells in the DBS samples and the low percentages of HIV-1-infected CD4<sup>+</sup> cells, the amount of viral DNA should have been consistently low (15).

Overall, our findings demonstrate the low sensitivity and positive predictive value of immunological and/or clinical criteria in predicting virological failure. The use of whole blood from DBS may be a useful tool for the virological monitoring of antiretroviral therapy, as recommended in the 2013 WHO guidelines (2), and may help to identify treatment failure as early as possible.

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## REFERENCES

1. Fitzgerald F, Penazzato M, Gibb D. 2013. Development of antiretroviral resistance in children with HIV in low- and middle-income countries. *J. Infect. Dis.* 207(Suppl 2):S85–S92. <http://dx.doi.org/10.1093/infdis/jit115>.
2. WHO. 30 June 2013. Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: recommendations for a public health approach. WHO, Geneva, Switzerland.
3. Westley BP, DeLong AK, Tray CS, Sophearin D, Dufort EM, Nerrienet E, Schreier L, Harwell JJ, Kantor R. 2012. Prediction of treatment failure using 2010 World Health Organization guidelines is associated with high misclassification rates and drug resistance among HIV-infected Cambodian children. *Clin. Infect. Dis.* 55:432–440. <http://dx.doi.org/10.1093/cid/cis433>.
4. Reynolds SJ, Nakigozi G, Newell K, Ndyababo A, Galiwongo R, Boaz I, Quinn TC, Gray R, Wawer M, Serwadda D. 2009. Failure of immunologic criteria to appropriately identify antiretroviral treatment failure in Uganda. *AIDS* 23:697–700. <http://dx.doi.org/10.1097/QAD.0b013e3283262a78>.
5. Bertagnolio S, Parkin NT, Jordan M, Brooks J, Garcia-Lerma JG. 2010. 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. *AIDS Rev.* 12:195–208.
6. Parkin N, de Mendoza C, Schuurman R, Jennings C, Bremer J, Jordan MR, Bertagnolio S, WHO DBS Genotyping Working Group. 2012. Evaluation of in-house genotyping assay performance using dried blood spot specimens in the Global World Health Organization laboratory network. *Clin. Infect. Dis.* 54(Suppl. 4):S273–S279. <http://dx.doi.org/10.1093/cid/cir982>.
7. Arredondo M, Garrido C, Parkin N, Zahonero N, Bertagnolio S, Soriano V, de Mendoza C. 2012. Comparison of HIV-1 RNA measurements obtained by using plasma and dried blood spots in the automated Abbott real-time viral load assay. *J. Clin. Microbiol.* 50:569–572. <http://dx.doi.org/10.1128/JCM.00418-11>.
8. Rottinghaus EK, Ugbena R, Diallo K, Bassey O, Azeez A, Devos J, Zhang G, Aberle-Grasse J, Nkengasong J, Yang C. 2012. Dried blood spot specimens are a suitable alternative sample type for HIV-1 viral load measurement and drug resistance genotyping in patients receiving first-line antiretroviral therapy. *Clin. Infect. Dis.* 54:1187–1195. <http://dx.doi.org/10.1093/cid/cis015>.
9. Monleau M, Montavon C, Laurent C, Segondy M, Montes B, Delaporte E, Boillot F, Peeters M. 2009. 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.* 47:1107–1118. <http://dx.doi.org/10.1128/JCM.02255-08>.
10. Smit PW, Sollis KA, Fiscus S, Ford N, Vitoria M, Essajee S, Barnett D, Cheng B, Crowe SM, Denny T, Landay A, Stevens W, Habiyambere V, Perriens JH, Peeling RW. 2014. Systematic review of the use of dried blood spots for monitoring HIV viral load and for early infant diagnosis. *PLoS One* 9:e86461. <http://dx.doi.org/10.1371/journal.pone.0086461>.
11. WHO. 2006. Antiretroviral therapy of HIV infection in infants and children in resource-limited settings: towards universal access: recommendations for a public health approach: 2006. WHO, Geneva, Switzerland.
12. WHO. 2010. Antiretroviral therapy for HIV infection in infants and children: towards universal access. Recommendations for a public health approach: 2010 revision. WHO, Geneva, Switzerland.
13. Petrara MR, Penazzato M, Massavon W, Nabachwa S, Nannyonga M, Mazza A, Gianesin K, Del Bianco P, Lundin R, Sumpter C, Zanchetta M, Giaquinto C, De Rossi A. 31 March 2014. Epstein-Barr virus load in children infected with human immunodeficiency virus type 1 in Uganda. *J. Infect. Dis.* <http://dx.doi.org/10.1093/infdis/jiu099>.
14. Zanchetta M, Walker S, Burighel N, Bellanova D, Rampon O, Giaquinto C, De Rossi A. 2006. Long-term decay of the HIV-1 reservoir in HIV-1-infected children treated with highly active antiretroviral therapy. *J. Infect. Dis.* 193:1718–1727. <http://dx.doi.org/10.1086/504264>.
15. Mexas AM, Graf EH, Pace MJ, Yu JJ, Pappasavvas E, Azzoni L, Busch MP, Di Mascio M, Foulkes AS, Migueles SA, Montaner LJ, O'Doherty U. 2012. Concurrent measures of total and integrated HIV DNA monitor reservoirs and ongoing replication in eradication trials. *AIDS* 26:2295–2306. <http://dx.doi.org/10.1097/QAD.0b013e32835a5c2f>.