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Low prevalence of transmitted HIV-1 drug resistance detected by a dried blood spot (DBS)-based next-generation sequencing (NGS) method in newly diagnosed individuals in Cameroon in the years 2015–16

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Objectives: To determine the most recent prevalence, transmission patterns and risk factors of transmitted drug-resistance mutations (TDRMs) in Cameroon, we initiated a multicentre study monitoring HIV-1 drug resistance in newly HIV-1-diagnosed individuals using a novel next-generation sequencing (NGS) assay applicable to fingerprick dried blood spot (DBS) samples.

Methods: Fingerprick DBS samples and questionnaires were collected from 360 newly HIV-1-diagnosed individuals in four hospitals in urban areas in Cameroon in the years 2015–16. We developed an HIV-1 protease and reverse transcriptase drug resistance genotyping assay applicable to DBS samples and HIV-1 genomes of groups M, N and O. The WHO 2009 list of mutations for surveillance of transmitted drug-resistant HIV strains was used to analyse TDRMs.

Results: Applying our 'DBS-NGS-genotypic resistance test', baseline HIV-1 drug resistance data were successfully obtained from 82.8% (298/360) of newly diagnosed individuals. At nucleotide frequencies >15%, TDRMs to NRTIs were observed in 3.0% (9/298), to NNRTIs in 4.0% (12/298) and to PIs in 1.3% (3/240). The NNRTI mutation K103N was most commonly detected (2.7%). Expanding the analysis to low-abundance TDRMs, i.e. 3%–15%, 12 additional individuals (4.0%) harbouring TDRMs were identified. Having unprotected sex with a known HIV-1-positive person was significantly associated with the transmission of DRMs (adjusted OR 9.6; 95% CI 1.79–51.3).

Conclusions: The prevalence of transmitted HIV-1 drug resistance is currently low in the study sites in Cameroon. Evidence of some risky sexual behaviours depicts a public health problem with possible implications for the prevention of new HIV-1 infections.

Introduction

Since its implementation, combination ART has greatly reduced the morbidity and mortality caused by HIV-1 infections worldwide.[1] However, this improvement is being hampered by the emergence of drug-resistant viruses,[2] posing a more serious problem today in resource-limited settings compared with resource-rich settings.[3,4] Acquired HIV-1 drug resistance develops when viral mutations emerge due to viral replication in the presence of insufficient levels of antiretroviral drugs; meanwhile, transmitted HIV-1 drug resistance occurs when individuals are infected with a drug-resistant virus.[5] Also, minority drug-resistant HIV-1 variants, undetected by conventional genotyping may impair the outcome of ART.[6–8] However, the impact of pre-existing minority drug-resistant HIV-1 variants on first-line ART still remains unclear.[9–12]

Cameroon, like many other developing countries, is increasing its HIV prevention and treatment efforts, which has led to the widespread use of ART through national scale-up plans. In 2015,

the Joint UN Programme on HIV and AIDS (UNAIDS) estimated the adult HIV-1 prevalence in Cameroon to be 4.5%, with about 620 000 people living with HIV-1.[13] In May 2016, ART eligibility in the country was modified to include all individuals diagnosed with HIV-1 in over 145 treatment centres (HIV Approved Treatment Centres and HIV Management Units) nationwide. In 2013, a survey conducted in 15 HIV-1 treatment centres in Cameroon evaluating HIV-1 drug resistance early warning indicators (EWIs) revealed that only 33.3% of centres reached the desirable performance for 'on-time pill pick-up' while just 14.4% reached the desirable performance of 'no pharmacy stock-outs'.[14] With limited healthcare infrastructure for care and monitoring, Cameroon may face increasing rates of HIV-1 drug resistance in the near future.

Surveillance of transmitted and acquired HIV-1 drug resistance is essential for the management of ART programmes.[5] Recent systematic reviews of published literature between 2014 and 2017 on pretreatment HIV-1 drug resistance in adults from low- and middle-income countries, including Cameroon, found that NNRTI resistance was higher in more recent studies across Eastern Africa, Southern Africa, Western and Central Africa, Latin America and Asia.[4,15] In Cameroon, very few studies on HIV-1 drug resistance in untreated patients have been conducted so far, and report low to moderate prevalence rates (< 8%) of transmitted drug resistance mutations (TDRMs).[16–18] These studies were performed with few samples (median 141), targeting special groups of people, such as pregnant women and blood donors, and/or conducted in specific geographical areas, mainly the south-eastern part of the country. Findings from these data are quite useful but a larger and more diverse sample population would better estimate the prevalence of TDRMs in Cameroon. Another unique circumstance in Cameroon is that all major groups and subtypes of HIV-1 co-circulate,[19,20] which represents a challenge for genotypic HIV-1 drug resistance testing.

Here, we have developed a next-generation sequencing (NGS) assay for genotypic HIV-1 drug resistance testing applicable to dried blood spot (DBS) samples. Using DBS samples overcomes the challenges with handling plasma samples in resource-limited settings. Using this test, we determined the recent prevalence of transmitted HIV-1 drug resistance and its associated risk factors in newly diagnosed patients from the western and northern areas of Cameroon.

Materials and methods

Establishment of the genotypic HIV-1 drug resistance assay

The newly designed genotypic HIV-1 drug-resistance assay, DBS-NGS-genotypic resistance test (DBS-NGS-GRT), encompasses amino acids 1–99 of the HIV-1 protease (PR) and 1–321 of the reverse transcriptase (RT). PR and RT genes are amplified in two fragments, each fragment comprising a semi-nested PCR of cDNA transcribed from RNA. The virus stock HIV-1_{NL4-3} subtype B, several primary virus isolates and HIV-1 subtypes A, C, D, G, F and CRF02_AE were used to develop and validate the assay. Details regarding the establishment and validation of our DBS-NGS-GRT assay are described in File S1 (available as Supplementary data at *JAC* Online).

Study population and data collection

Between April 2015 and July 2016, fingerprick DBS samples were collected from 360 patients in four different hospitals in Cameroon, representing three regions of the country: the North-West (Bamenda Regional Hospital and Ndop District Hospital), South-West (Kumba District Hospital) and Adamawa (Ngaoundere Regional Hospital) regions (Figure 1a). Participants had to meet the following inclusion criteria: (i) age ≥18 years; (ii) newly diagnosed with HIV-1; and (iii) with no previous exposure to any form of ART. The procedure for fingerprick DBS collection and handling was adapted from the WHO manual for HIV drug resistance testing using DBS samples.[21] This manual was distributed to all four hospitals (File S2). A questionnaire capturing patient demographics, clinical data and sexual behaviours was also administered (File S3). The samples and completed questionnaires from all sampling sites were sent at room temperature by express mail to the Division of Infectious Diseases and Hospital Epidemiology of the University Hospital of Zurich in Switzerland, where they were stored at −80°C until further procedures. Details on the handling and shipment of fingerprick DBS samples can be found in File S1.

Ethics statement

This study was approved by the National Ethics Committee of Cameroon (Number: 2015/01/539/CE/CNERSH/SP) and written informed consent was obtained from each participant.

Laboratory methods and data analyses

HIV-1 RNA was isolated from an equivalent of 100 μL of blood (two spots) on a Whatman® 903 protein saver card (DBS sample). DNase-treated HIV-1 RNA was reverse-transcribed and amplified by two semi-nested PCRs using the primers shown in Table S1. PCR products were purified, quantified and sequenced with the Illumina MiSeq system. Sequencing reads were retrieved, processed and mutations were called with MinVar (git.io/minvar), a tool to discover and annotate mutations from NGS of HIV-1 populations.[22] Mutations were called with a cut-off of 3% and finally annotated according to the Stanford HIV Drug Resistance Database,[23] version 8.1.1 (https://hivdb.stanford.edu/). The assessment of TDRMs was done using the WHO 2009 list of mutations for surveillance of transmitted drug-resistant HIV strains[24] (https://hivdb.stanford.edu/page/who-sdrm-list/). Phylogenetic and statistical analyses were also performed on sequences and questionnaire data. The above methods are described in detail in File S1.

Results

Demographic and baseline characteristics of patients

In total, fingerprick DBS samples were collected from 360 newly diagnosed patients in four hospitals in Cameroon. Three of the hospitals are located in urban areas (sites A, B and D) and one in a semi-urban area (site C; Figure 1a). From April 2015 until July 2016, 51, 210, 35 and 64 patients were enrolled in the study by the centres A, B, C and D, respectively. DBS samples were obtained from each participant and completed questionnaires were returned by 334 (92.8%) participants (Figure 1b). In the following analysis, data from patients for whom HIV-1 RNA was successfully amplified and sequenced ($n = 298$) were included, as well as questionnaire data from patients with HIV-1 sequences ($n = 289$).

Demographic and clinical baseline data are shown in Table 1. The median age was 36 years and the majority of patients were female (63%). Patients were first diagnosed with HIV-1 infection at all WHO clinical stages[25] with 38.1% at stage 2. The median CD4+ T cell count was 303 (range 1–1112) cells/mm^3 of blood. The median HIV-1 cDNA $\log_{10}$ copy number/mL of blood in DBS samples, as estimated by quantitative PCR (qPCR) of HIV-1 RNA recovered from DBS samples, was 4.4 (range 3.7–5.9). No patients

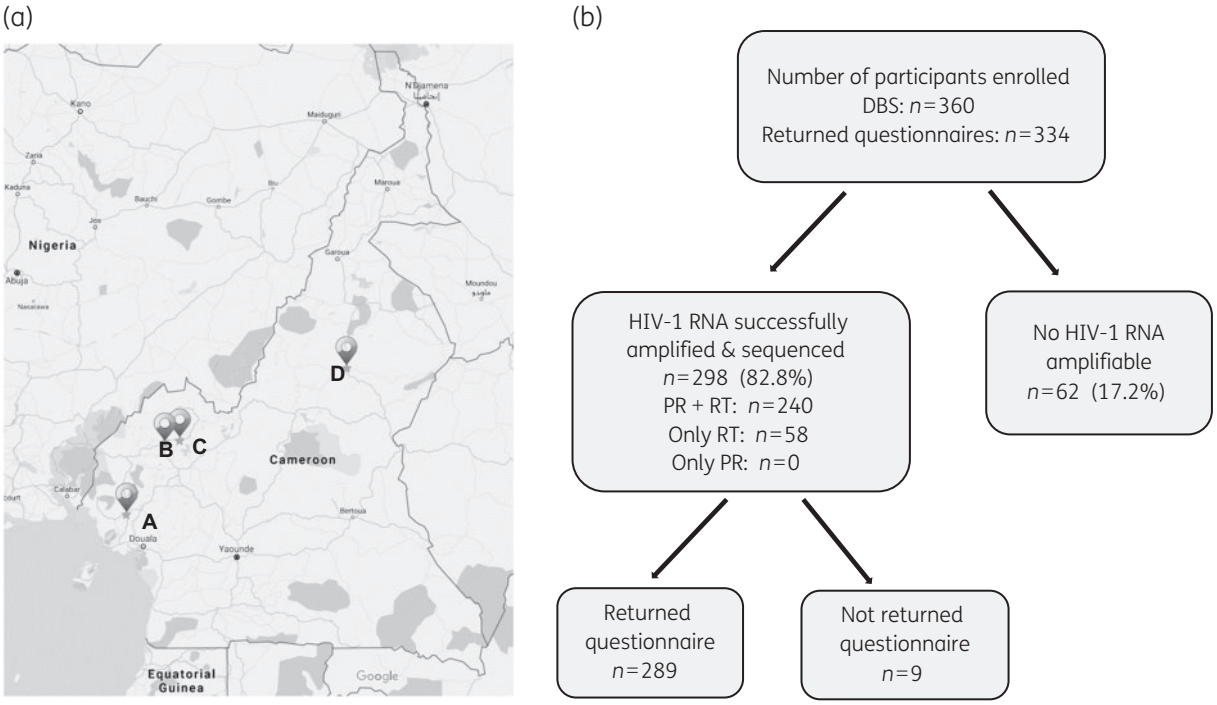

Figure 1. Geographical distribution of participating hospitals and study outcome. (a) Map of Cameroon[a] showing the participating hospitals: A, District Hospital Kumba; B, Regional Hospital Bamenda; C, District Hospital Ndop; and D, Regional Hospital Ngaoundere. (b) Enrolled participants, HIV-1 PR and RT amplification and return of completed questionnaires. [a]Map adapted from: https://www.google.ch/maps/place/Cameroon/.

had viral load records because this is hardly done for pretreatment patients due to limited resources in most hospitals in Cameroon.

Establishment and validation of the amplification of HIV-1 RNA from DBS samples

We first developed an amplification protocol applicable to DBS samples for the establishment of a genotypic HIV-1 PR and RT drug resistance assay. This assay is universal, i.e. applicable to all heterogeneous HIV-1 subtypes i.e., applicable to HIV-1 genomes of groups M, N and O. HIV-1 RNA was isolated from an equivalent of 100 μL of blood on a Whatman® 903 protein saver card, reverse-transcribed and amplified by two semi-nested PCRs. To determine the applicability in terms of diverse HIV-1 subtypes and the sensitivity, limited diluted HIV-1-spiked DBS samples (HIV-1 subtypes A, B, C, D, G, F and CRF02_AE) were tested. All these HIV-1 subtypes were successfully amplified with a lower limit of detection of 1000 HIV-1 RNA copies/mL (Table S2). The error rates generated by PCR and NGS ranged from 0.04% to 0.66% and were previously investigated using the same reagents and kits that were used in this study.[26]

We proceeded to apply our established DBS-NGS-GRT to the 360 patient fingerprick DBS samples. Amplification of HIV-1 PR and/or RT was successful in 298/360 (82.8%) DBS samples (Figure 1b). From 240/298 (80.5%) DBS samples, both amplicons were generated, while from 58/298 (19.5%) DBS samples only the RT region was successfully amplified. Investigating the possible reason for amplification failure in 62 DBS samples, we first examined the correlation to the CD4+ T cell count measured at the hospitals in Cameroon, i.e. as a surrogate marker for disease progression and viral load. A significantly lower CD4+ T cell count ($P < 0.0001$) was observed for patients whose samples were successfully amplified compared with those whose samples failed amplification (Figure S1a). Secondly, we quantified and compared the HIV-1 cDNA copy numbers in DBS samples in those two groups. Successful amplification was significantly associated with higher HIV-1 cDNA copy numbers ($P < 0.0001$) (Figure S1b). The subtype diversity in this study was quite high as all group M HIV-1 subtypes and circulating recombinant forms previously known to exist in Cameroon were detected in patients' samples (Figure 2c). In summary, we developed and applied a highly efficient, universal HIV-1 PR and RT amplification procedure for DBS samples.

Prevalence of HIV-1 transmitted drug resistance

Sequence results were obtained from 298 successful amplicons (Figure 1b). Overall, TDRMs were found in 31/298 patients (10.4%; 95% CI 7.2%–14.4%). In those individuals, the minimum coverage per nucleotide was 439 (Table 2; patient 5) and the minimum HIV-1 cDNA copy number sequenced was 131 copies/5 μL (Table 2; patient 22) allowing a cut-off for low-abundance TDRMs at 3%. TDRMs were checked for APOBEC3G/F signatures: no G-to-A hypermutations were detected in the NGS reads.

First, we investigated the prevalence of TDRMs at frequencies >15%, i.e. which would also be detectable by population sequencing commonly performed by Sanger sequencing. In 19/298 patients (6.4%; 95% CI 3.9%–9.8%), TDRMs were detected at frequencies >15% (Figure 2a). Here, TDRMs to PIs were detected in 3/240 patients (1.3%; 95% CI 0.3%–3.6%), to NRTIs in 9/298 patients (3.0%; 95% CI 1.4%–5.7%) and to NNRTIs in

Table 1. Comparisons of the demographic and clinical baseline characteristics of patients with and without TDRMs (low- and high-abundance TDRMs combined)

	Total, $N = 289$	Patients with TDRMs, $N = 31$	Patients without TDRMs, $N = 258$	P
Hospital				
A	44 (15.2)	0 (0)	44 (17.1)	**0.012**$^{*\psi}$
B	193 (66.8)	23 (74.2)	170 (65.9)	
C	13 (4.5)	0 (0)	13 (5.0)	
D	39 (13.5)	8 (25.8)	31 (12.0)	
Age (years), median (range)	36 (18–68)	41 (18–60)	36 (18–68)	0.861†
Gender				
male	107 (37.0)	9 (29.0)	98 (38.0)	0.329$^{\psi}$
female	182 (63.0)	22 (71.0)	160 (62.0)	
Occupation				
skilled worker	90 (31.1)	8 (25.8)	82 (31.8)	0.594$^{\psi}$
unskilled worker	152 (52.6)	19 (61.3)	133 (51.6)	
applicant/student	47 (16.3)	4 (12.9)	43 (16.6)	
Level of education				
none	9 (3.1)	0 (0)	9 (3.5)	0.843$^{\delta}$
primary	135 (46.7)	14 (45.2)	121 (46.9)	
secondary	108 (37.4)	14 (45.2)	94 (36.4)	
university	34 (11.8)	3 (9.7)	31 (12.0)	
other	3 (1.0)	0 (0)	3 (1.2)	
Marital status				
single	104 (36.0)	7 (22.6)	97 (37.6)	0.110$^{\psi}$
married	121 (41.9)	12 (38.7)	109 (42.2)	
divorced	17 (5.9)	4 (12.9)	13 (5.1)	
widow(er)	40 (13.8)	6 (19.4)	34 (13.2)	
cohabiting	7 (2.4)	2 (6.5)	5 (1.9)	
WHO clinical staging				
primary infection	55 (19.0)	7 (22.6)	48 (18.6)	0.866$^{\psi}$
stage 1	70 (24.2)	7 (22.6)	63 (24.4)	
stage 2	110 (38.1)	13 (41.9)	97 (37.6)	
stage 3	50 (17.3)	4 (12.9)	46 (17.8)	
stage 4	4 (1.4)	0 (0)	4 (1.6)	
CD4+ T cell count (cells/mm^3 blood), median (range)	303 (1–1112)	301 (13–940)	296 (1–1112)	0.569$^{\lambda}$
HIV-1 cDNA copy number in DBS sample (copies/mL), median (range)	25 119 (5012–794 328)	63 096 (5012–501 187)	25 119 (5012–794 328)	**0.0006**$^{*\lambda}$ 0.2713$^{\pi\lambda}$
Sexual orientation				
MSM	4 (1.4)	0 (0)	4 (1.6)	0.634$^{\delta}$
heterosexual	285 (98.6)	31 (100)	254 (98.4)	
HIV-1 status of stable partner				
positive	37 (12.8)	5 (16.1)	32 (12.4)	0.709$^{\psi}$
negative	32 (11.1)	2 (6.5)	30 (11.6)	
I don't know	207 (71.6)	24 (77.4)	183 (71)	
no partner	13 (4.5)	0 (0.0)	13 (5.0)	
Condom use before diagnosis				
never	86 (29.8)	7 (22.6)	79 (30.6)	0.478$^{\delta}$
sometimes	185 (64.0)	21 (67.7)	164 (63.6)	
always	18 (6.2)	3 (9.7)	15 (5.8)	
No. of sexual encounters with an occasional partner ≤ 1 year before diagnosis				
none	77 (26.6)	7 (22.6)	70 (27.1)	0.923$^{\psi}$
one	75 (26.0)	9 (29.0)	66 (25.6)	
two	49 (17.0)	6 (19.4)	43 (16.7)	
three and above	88 (30.4)	9 (29.0)	79 (30.6)	

Continued

Table 1. *Continued*

	Total, $N = 289$	Patients with TDRMs, $N = 31$	Patients without TDRMs, $N = 258$	P
Blood transfusion				
no	265 (91.7)	28 (90.3)	237 (91.9)	0.731$^{\delta}$
yes	24 (8.3)	3 (9.7)	21 (8.1)	
Ever paid for sex				
no	269 (93.1)	30 (96.8)	239 (92.6)	0.495$^{\delta}$
yes	20 (6.9)	1 (3.2)	19 (7.4)	
Ever received money for sex				
no	272 (94.1)	31 (100.0)	241 (93.4)	0.232$^{\delta}$
yes	17 (5.9)	0 (0.0)	17 (6.6)	
Unprotected sex with someone known to be HIV-1-positive				
no	283 (97.9)	28 (90.3)	255 (98.8)	**0.018**$^{*\delta}$
yes	6 (2.1)	3 (9.7)	3 (1.2)	

Data are presented as *n* (%), unless otherwise stated.
*Statistically significant *P* values are shown in bold.
$^{\pi}$Excluding data from patients with low-abundance TDRMs.
$^{\delta}$*P* value obtained with the Fisher's exact test.
$^{\psi}$*P* value obtained with the χ^2 test.
$^{\lambda}$*P* value obtained with the Mann–Whitney *U*-test.
†*P* value obtained with the *t*-test.

12/298 patients (4.0%; 95% CI 2.1%–6.9%). Five patients (1.7%; 5/298; 95% CI 0.5%–3.9%) had TDRMs to both NRTIs and NNRTIs, while no individual harboured mutations to both PR and RT drug classes. Expanding the analysis to low-abundance TDRMs, i.e. 3%–15%, 12/298 additional individuals (4.0%; 95% CI 2.1%–6.9%) were identified. As observed for high-abundance TDRMs, the majority of low-abundance TDRMs were associated with resistance to NNRTIs (Figure 2b).

Details of the different characteristics, mutation types and their percentage abundance for the 31 individuals with low- and high-abundance TDRMs are shown in Table 2. The most commonly detected TDRM was the K103N mutation: 8/298 patients (2.7%; 95% CI 1.2%–5.2%), in seven of those patients occurring as a high-abundance TDRM (Table 2). The M184V mutation was detected in 4/298 patients (1.3%; 95% CI 0.4%–3.4%) at high frequencies and the M184I mutation also in 4/298 patients (1.3%; 95% CI 0.4%–3.4%) at high ($n = 3$) and low ($n = 1$) frequencies. One patient harboured both the M184V and M184I mutations (Table 2).

The impact of TDRMs on future therapy response in patients was assessed with the scoring system of the Stanford HIV Drug Resistance Database. In 51.6% (16/31; 95% CI 33.1%–69.8%) cases, only low-level or potential low-level resistance was observed. Some patients had a combination of two or three resistance profiles (low, intermediate or high) for different drugs (Table 2).

Transmission clusters of DRMs were not found within and between study sites

To investigate any transmission clusters of drug resistance in the study population, phylogenetic analysis was performed. From the phylogenetic tree generated with our sequences, together with the background sequences (Figure 3a and File S4), cluster analysis revealed 200 clusters in total (bootstrap values >90%). Twenty-five of these clusters included individuals from our study population. Seventeen of those clusters included more than one individual of our study population. Of these, two clusters had patients with a DRM. One cluster with two study patients was found with both of them having a drug resistance mutation (E138G) (Figure 3b). Another cluster had two study patients with one of them having a DRM (M46I) (Figure 3c).

Comparison of demographic, clinical and risky sexual parameters between individuals with and without TDRMs

Besides acquiring patients' clinical and demographic data, the questionnaire also sought to examine the sexual attitudes of patients for any risky behaviours before HIV-1 diagnosis. This analysis could only be performed for the 289 patients with returned questionnaires and NGS data, out of the 298 patients with successfully amplified and sequenced samples, leaving out 9 patients with NGS data but no questionnaires (Table 1). There was a significant difference in the number of patients with and without TDRMs between the study sites, with site B having the highest frequency of patients with TDRMs. We found a statistically significant difference (Mann–Whitney *U*-test $P = 0.0006$) between the cDNA copy numbers for both groups (with and without TDRMs) but this difference became not significant once we excluded data from patients with low-abundance TDRMs. In a univariable logistic regression, it was found that having unprotected sex with a known HIV-1-positive person was significantly associated with the transmission of DRMs [OR 9.1 (95% CI 1.75–47.2, $P = 0.009$)]. In a multivariable logistic

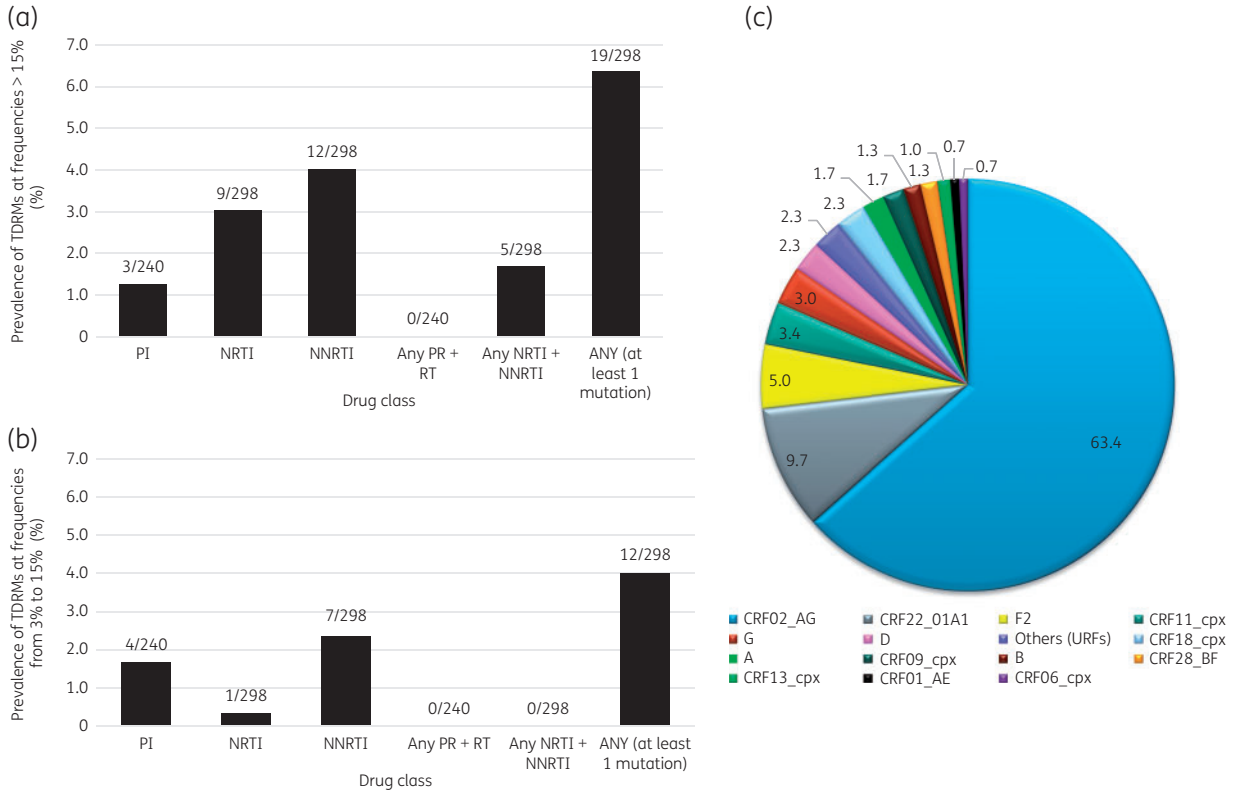

Figure 2. TDRMs detected at high (>15%) (a) and low (3%–15%) (b) frequencies in 298 newly HIV-1-diagnosed patients in Cameroon from April 2015 to July 2016 and HIV-1 subtype distribution (c). URFs, unique recombinant forms. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

regression adjusted for age and sex, this was still significant [OR 9.6 (95% CI 1.79–51.3, $P = 0.008$)].

Discussion

We have developed a sensitive, specific and universal assay for the determination of HIV-1 drug resistance using DBS samples and applying NGS, suitable for up to 96 samples per MiSeq flow cell. DBS samples serve as an attractive sampling alternative to plasma especially in developing countries (resource-limited), overcoming the challenges that come with handling plasma, such as successfully maintaining the cold chain at −80°C.

Our DBS-NGS-GRT was designed taking into account the very high HIV-1 subtype distribution in Central Africa. The primers were carefully designed to cover all major HIV-1 group M subtypes and circulating recombinant forms (CRFs) as well. The overlapping amplicon strategy used in the PCRs improved the sensitivity limit for the DBS samples as it has been previously suggested that RNA extracted from DBS samples can sometimes be fragmented or broken[27,28] (data not shown). These fingerprick DBS samples were stored at various temperatures during collection, temporary storage until shipment, transportation and storage until processing. Some loss of HIV-1 RNA was even observed in all spiked DBS samples (Table S2), which were prepared and immediately stored at −80°C after desiccation. This is a common phenomenon with DBS samples in general; furthermore, the degree of HIV-1 RNA loss can also vary depending on the viral RNA extraction technique used, some of which are not suitable for DBS samples.[21,29]

When patients' fingerprick DBS samples were used, an 82.8% amplification success rate was obtained. We found that the remaining 17.2% of patients whose samples failed to amplify probably had a low viral load at the time of sampling. Previously, it has been suggested that DBS samples may be limited for the analysis of viral RNA if the viral load is too low, i.e. less than 1000 HIV-1 RNA copies/mL of plasma.[29–31] Using spiked DBS samples, we also reached a lower limit of detection at 1000 HIV-1 RNA copies/mL. The lower limit of detection for our patients' DBS samples seemed higher, i.e. ∼5000 HIV-1 cDNA copies/mL of blood on DBSs. However, these data might be difficult to compare since different assays for the quantification were used and we cannot fully exclude some primer mismatches in some of the failing-to-be-amplified samples. Another possible reason for the drop in assay sensitivity seen with patients' fingerprick DBS samples compared with spiked DBS samples is that the spiked DBS samples used for assay development went through more ideal conditions of preparation and storage.

So far, studies on transmitted HIV-1 drug resistance in Cameroon are scarce and have focused on certain parts of the country.[16–18] For our study sites, no or only very few surveillance data on HIV-1 TDRMs are available and they were obtained more than a decade ago.[32] Our study provides a comprehensive and contemporary description of the prevalence of transmitted HIV-1 drug resistance among newly diagnosed HIV-1 patients in the western and northern parts of Cameroon. By WHO classification, the prevalence is low (<5%) for all three drug classes separately.[33]

Table 2. Clinical and sequencing characteristics of patients with high- and low-abundance TDRMs

Participant no.	Gender	HIV-1 subtype	CD4+ T cell count (cells/mm^3 of blood)	HIV-1 cDNA copy number in DBS sample (copies/mL of blood)	HIV-1 cDNA copy number sequenced (copies/5 μL of blood)[a]	NGS coverage/ nucleotide		TDRMs (frequency in %) [NGS coverage at position]			Stanford HIV Drug Resistance Database mutation scores[b]
						min	max	PI	NRTI	NNRTI	
Patients with TDRMs above 15%											
1	female	CRF28_BF	682	62 017	310	468	30 159	D30N (71) [1015], M46I (74) [1221]			ATV (10), FPV (10), IDV (10), LPV (10), SQV (10), TPV (5)
2	female	CRF02_AG	397	27 920	140	755	17 460		M184V (≥97)[c] [>10 000]	K103N (≥97) [>10 000]	ABC (15), ddI (10), FTC (60), 3TC (60), EFV (60), NVP (60)
3	female	CRF22_01A1	475	34 750	174	1761	28 717			Y181C (≥97) [>10 000]	EFV (30), ETR (30), NVP (60), RPV (45)
4	female	CRF02_AG	523	36 503	183	1356	51 974		D67N (90) [>10 000], K70R (≥97) [>10 000], M184V (≥97) [>10 000], T215I (≥97) [>10 000], K219E (≥97) [>10 000]	A98G (≥97) [>10 000], K103N (≥97) [>10 000]	ABC (65), AZT (75), d4T (60), ddI (50), FTC (70), 3TC (70), TDF (20), EFV (75), ETR (10), NVP (90) RPV (15)
5	female	CRF02_AG	312	39 458	197	439	10 036		M41L (≥97) [8845], D67N (94) [9023], K70R (≥97) [9213], M184V (90) [9754], T215Y (88) [7982], K219E (≥97) [7975]	Y181C (91) [9231], G190A (≥97) [9815]	ABC (105), AZT (135), d4T (120), ddI (90), FTC (80), 3TC (80), TDF (55), EFV (75), ETR (50), NVP (120), RPV (70)
6	female	CRF02_AG	47	203 149	1016	697	50 286			G190A (64) [>10 000]	EFV (45), ETR (10), NVP (60), RPV (15)
7	female	F2	40	159 376	797	453	7467		L210W (≥97) [6952], T215S (88) [7012]		ABC (15), AZT (40), d4T (40), ddI (25), TDF (15)
8	male	CRF02_AG	102	233 307	1167	637	5857			K103N (92) [3873]	EFV (60), NVP (60)
9	female	CRF02_AG	74	35 502	178	444	9693		M184I (58) [6489], M184V (36) [6481]	V106A (54) [5107], Y188H (63) [6392], G190A (≥97) [6480], K101E (57) [5102]	ABC (15), ddI (10), FTC (60), 3TC (60), EFV (120), ETR (10), NVP (180), RPV (15)

Continued

Table 2. *Continued*

Participant no.	Gender	HIV-1 subtype	CD4+ T cell count (cells/mm^3 of blood)	HIV-1 cDNA copy number in DBS sample (copies/mL of blood)	HIV-1 cDNA copy number sequenced (copies/5 μL of blood)[a]	NGS coverage/ nucleotide		TDRMs (frequency in %) [NGS coverage at position]			Stanford HIV Drug Resistance Database mutation scores[b]
						min	max	PI	NRTI	NNRTI	
10	male	CRF22_01A1	274	29 250	146	1438	61 025	M46I (≥97) [>10 000]			ATV (10), FPV (10), IDV (10), LPV (10), NFV (30), SQV (10), TPV (5)
11	female	CRF02_AG	129	60 368	302	1546	47 840			K103N (≥97) [>10 000], P225H (79) [>10 000]	EFV (105), NVP (105)
12	male	CRF02_AG	13	473 734	2369	1327	56 169		K219Q (69) [>10 000]		ABC (5), AZT (10), d4T (10), ddI (5), TDF (5)
13	male	CRF18_cpx	437	40 977	205	843	26 082		Q151M (94) [>10 000]	L100I (≥97) [>10 000], K103N (≥97) [>10 000]	ABC (60), AZT (60), d4T (60), ddI (60), FTC (15), 3TC (15), TDF (15), EFV (120), ETR (30), NVP (120), RPV (60)
14	female	CRF02_AG	239	31 020	155	546	63 841			K103N (≥97) [>10 000]	EFV (60), NVP (60)
15	female	CRF22_01A1	132	52 215	261	535	69 471		M184I (28) [>10 000]		ABC (15), ddI (10), FTC (60), 3TC (60)
16	female	CRF02_AG	743	61 584	308	1975	25 196			K103N (37) [>10 000]	EFV (60), NVP (60)
17	female	CRF02_AG	330	88 414	442	584	13 449			G190E (37) [>10 000]	EFV (60), ETR (45), NVP (60), RPV (60)
18	male	CRF02_AG	289	94 511	473	659	10 398		D67N (16) [6843], M184I (18) [9947]		ABC (20), AZT (5), d4T (5), ddI (15), FTC (60), 3TC (60)
19	male	G	155	102 333	512	773	30 526	L90M (17) [>10 000]			ATV (25), FPV (20), IDV (30), LPV (15), NFV (60), SQV (45)
Patients with TDRMs below 15%											
20	female	CRF22_01A1	290	182 295	911	495	6144			Y181C (3) [3219]	EFV (30), ETR (30), NVP (60), RPV (45)
21	female	F2	406	56 697	283	941	8995			V106A (3) [6054]	EFV (45), NVP (60)

22	female	CRF02_AG	294	26 250	131	505	12 624			G190E (4) [>10 000]	EFV (60), ETR (45), NVP (60), RPV (60)
23	male	CRF02_AG	326	54 192	271	809	16 923	I50V (4) [>10 000]			DRV (20), FPV (60), LPV (30), NFV (15), SQV (15)
24	female	CRF02_AG	424	52 077	260	494	14 277			G190E (4) [>10 000]	EFV (60), ETR (45), NVP (60), RPV (60)
25	male	CRF22_01A1	89	124 511	623	688	29 198	M46I (4) [>10 000]			ATV (10), FPV (10), IDV (10), LPV (10), NFV (30), SQV (10), TPV (5)
26	female	CRF02_AG	62	113 504	568	911	66 384	M46I (5) [>10 000]			ATV (10), FPV (10), IDV (10), LPV (10), NFV (30), SQV (10), TPV (5)
27	female	F2	209	38 828	194	686	23 826	D30N (5) [>10 000]			NFV (60)
28	female	F2	147	57 563	288	597	6959			G190E (6) [4793]	EFV (60), ETR (45), NVP (60), RPV (60)
29	male	CRF22_01A1	84	62 193	311	1096	53 428			K103N (9) [>10 000]	EFV (60), NVP (60)
30	male	CRF02_AG	452	58 301	292	926	43 552		M184I (11) [>10 000]		ABC (15), ddI (10), FTC (60), 3TC (60)
31	female	D	472	67 238	336	882	17 531			Y188H (11) [9846]	EFV (30), NVP (60)

3TC, lamivudine; ABC, abacavir; ATV, atazanavir; AZT, zidovudine; d4T, stavudine; ddI, didanosine; EFV, efavirenz; ETR, etravirine; FPV, fosamprenavir; FTC, emtricitabine; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; NVP, nevirapine; RPV, rilpivirine; RTV, ritonavir; SQV, saquinavir; TDF, tenofovir disoproxil fumarate; TPV, tipranavir.

[a]Numbers in the previous column were divided by 200. 100 μL of blood (10%) was used for HIV-1 RNA isolation, 25% of RNA was used for cDNA synthesis and 20% of cDNA was used for PCR, i.e. HIV-1 cDNA from 5 μL of blood was finally sequenced per sample.

[b]TDRMs were scored in the Stanford HIV Drug Resistance Database and the total scores or combination scores associated with a drug are listed, representing the impact of all mutations put together on that particular drug. The estimated level of resistance can be calculated as follows: susceptible, 0 to 9; potential low-level resistance, 10 to 14; low-level resistance, 15 to 29; intermediate resistance, 30 to 59; and high-level resistance, ≥60 (https://hivdb.stanford.edu/page/release-notes/#drm.penalty.scores.and.resistance.interpretation). All PI and RT drugs not shown are susceptible.

[c]≥97 is given for all sites where only the TDRM was detected, since our cut-off for minority HIV-1 drug-resistant variants was set at 3%. Similar results for TDRM frequencies and nucleotide coverage were seen with the PAseq tool (https://www.paseq.org/).

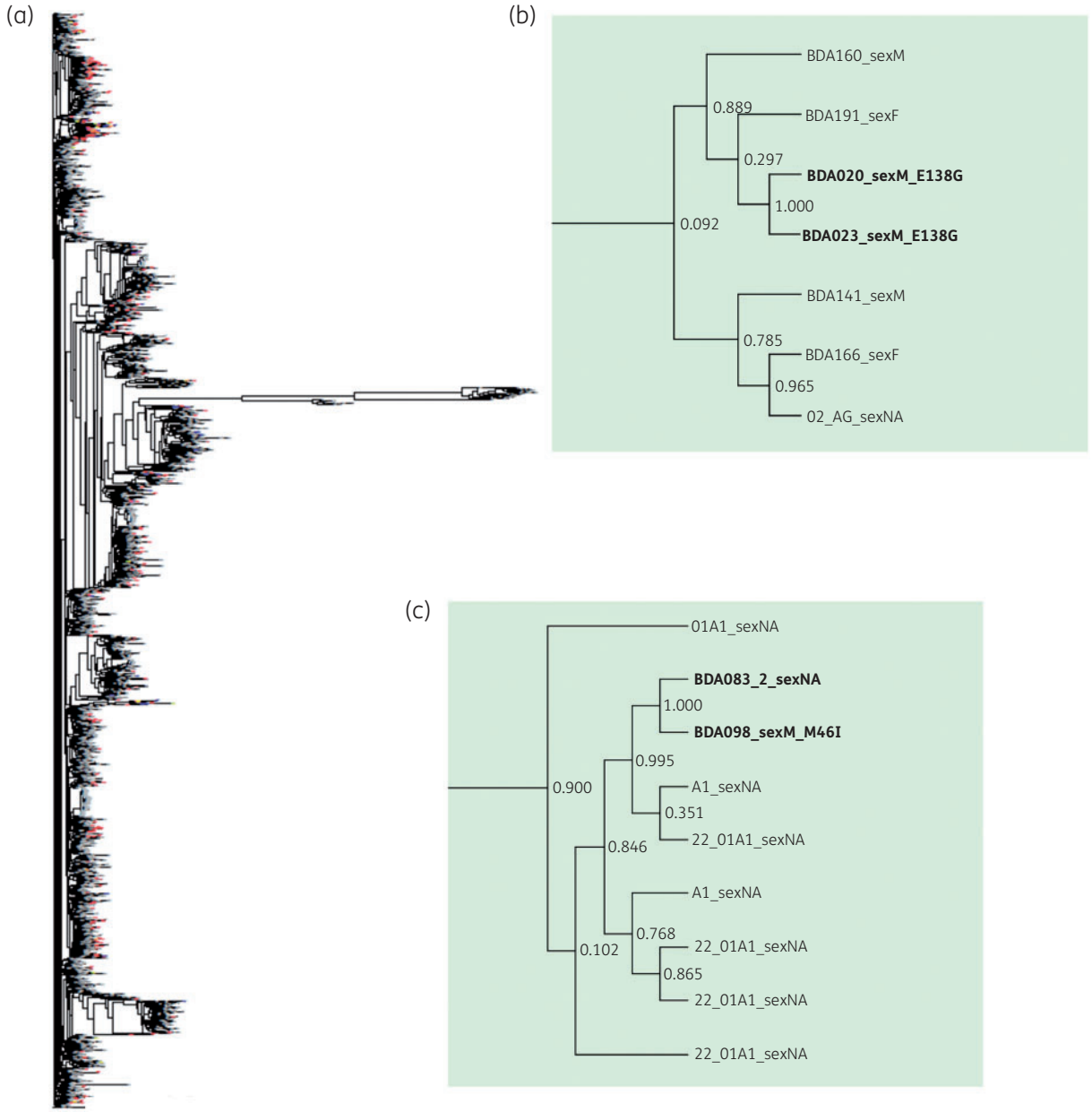

Figure 3. Phylogenetic analysis of 298 patient sequences and 2359 background sequences from Cameroon downloaded from the Los Alamos HIV database (http://www.hiv.lanl.gov). The furthest branches are HIV-1 groups O and N background sequences. (a) Phylogenetic tree with coloured tips: grey tips, background sequences; brown tips, site A; red tips, site B; yellow tips, site C; and blue tips, site D. For a high-resolution electronic file of the tree please see File S4. (b) Cluster with two study patients, both of them having a non-TDRM. (c) Cluster with two study patients, one of them having a TDRM. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

The overall prevalence we report here is slightly lower than what was reported for the eastern part of the country in 2011.[17] Those authors reported an overall prevalence of 7.8% with many more TDRMs to NRTIs (5.3%) than NNRTIs (1.9%) and PIs (1.9%). According to the WHO HIV drug resistance report for 2017,[4,15] levels of any pretreatment HIV drug resistance (PDR) was below 10% in Cameroon. This same report with 321 individuals sampled in 2015 from different hospitals in Cameroon demonstrated comparable findings to ours for their 223 ART-naive individuals, with similar prevalence rates and overlapping confidence intervals for drug resistance to PIs, NRTIs, NNRTIs or any drug class. Our study also saw unequal numbers of participants randomly enrolled from each study site. The hospital in site B is the largest compared with the others and enrolled the highest number of participants in the study. ART was also introduced earlier to this population relative to the other sites. The NNRTI mutation K103N and NRTI mutations M184V/I were most commonly detected in our study population and respectively confer high-level resistance to efavirenz/

nevirapine and lamivudine/emtricitabine,[34] commonly prescribed drugs in Cameroon. It is common to detect these DRMs among patients treated with these drugs and experiencing virological failure,[34] and they can subsequently transmit them.

We are also reporting one of the first studies on minority drug-resistant HIV-1 variants in ART-naive patients in Cameroon. The fact that the significant difference in the cDNA copy numbers between patients with and without TDRMs became not significant once data from patients with low-abundance TDRMs were excluded is an indication that this latter group of patients were possibly infected for longer periods than the other individuals. Four percent of the patients harboured TDRMs at frequencies between 3% and 15% and mostly against NNRTIs. This is lower than was previously reported in ART-naive patients in Cameroon (17%–20%)[11] at frequencies >1%. Applying our range of 3%–15% to their study population, the prevalence of low-abundance TDRMs would decrease to 1.5%. Another study with NGS data from 32 ART-naive patients with highly diverse subtypes in Cameroon found two minority variants constituting 7% (T215F/Y) and 18% (K103N) of the quasispecies.[35] Although the impact of these minority variants on the success of ART is not clearly known,[36] a substantial number of our ART-naive patients harbour them. This may give rise to the rapid selection of drug-resistant viruses, which can subsequently lead to early therapy failure in treatment-naive patients receiving ART regimens with a low genetic resistance barrier.[6]

It has been shown that phylogenetic analysis is a useful tool in identifying clusters of HIV transmission since very related viral sequences may correlate with common social or risk-behaviour patterns in individuals.[37,38] Although we found no transmitted HIV-1 drug-resistant strains spreading in our study population, a large Swiss survey on transmitted HIV-1 drug resistance conducted between 1996 and 2009 showed that DRMs are frequently transmitted among ART-naive MSM.[39] However, our sampling density is probably considerably lower compared with the Swiss settings.

We observed that having unprotected sex with a known HIV-1-positive person was significantly associated with the transmission of DRMs. These patients had 9.6-fold higher odds of harbouring genotypic drug-resistant HIV-1 strains, suggesting a more advanced disease stage and possible ART experience of the DRM transmitters. One factor we did not evaluate in this study is the patients' history of sexually transmitted diseases. This has been shown in Southern Taiwan to predispose patients to 7.8-fold increased odds of becoming infected with genotypic drug-resistant HIV-1 strains.[40]

In conclusion, we have established an assay for HIV-1 drug resistance genotyping based on NGS, applicable to DBS samples and genotyping all heterogeneous HIV-1 subtypes of at least group M with similar sensitivity and specificity to other known DBS assays.[41–43] The sample type used, the assay's sensitivity and broad subtype coverage make it ideal for HIV-1 drug resistance genotyping in areas with limited resources and a high HIV-1 subtype diversity. Although transmitted HIV-1 drug resistance is currently low in the study sites in Cameroon, continued surveillance is still necessary to detect any changes in drug resistance trends early enough. The evidence of some risky sexual behaviours found in this study depicts a public health problem with possible implications for the prevention of new HIV-1 infections in the country. This information is important for future preventive intervention strategies in the study sites and the whole country at large.

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Author contributions

H. A. M. designed the study, developed the assay, researched and analysed data, and wrote the manuscript. A. M. designed the study questionnaire and analysed data. S. S., Y. L. K. and M. S. contributed to the assay development. O. Z. analysed sequencing data. N. N. N., E. T. M., L. M. B., B. A. S. and E. O. enrolled study participants and/or collected DBS samples and questionnaires. R. D. K. co-designed the study questionnaire and edited the manuscript. H. F. G. contributed towards supervision and editing of the manuscript and leads the Zurich Primary HIV infection study. K. J. M. designed the study, supervised the work and edited the manuscript.

Supplementary data

Files S1 to S4, Tables S1 and S2 and Figure S1 are available as Supplementary data at *JAC* Online.

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