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Subtype variability, virological response and drug resistance assessed on dried blood spots collected from HIV patients on antiretroviral therapy in Angola

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***Background*: Subtype variability may influence treatment response and selection of drug resistance mutations in HIV-positive patients on antiretroviral therapy.**

***Patients and methods*: A retrospective study was performed on specimens collected on dried blood spots (DBS) from HIV-positive individuals receiving antiretroviral therapy in Luanda, Angola. HIV-RNA, drug resistance mutations and subtypes were examined in 294 HIV-positive patients treated with two nucleoside analogues (NA) plus one non-nucleoside reverse transcriptase inhibitor (NNRTI).**

***Results*: Overall, 217 (74%) had <1000 HIV-RNA copies/mL after a median of 12 months (range 7–24) of therapy. CD4 count was significantly higher in subjects with undetectable viraemia compared with viraemic patients (294 versus 220 cells/mm^3; *P* = 0.003). Reverse transcriptase and/or gp41 genes could be genotyped in only 45 (58%) of viraemic patients, probably due to poor storage conditions of DBS. The most frequent resistance mutations were M184V (70%) and K103N (39%); 65% had mutations conferring resistance to both NA and NNRTI. Only five patients did not show resistance mutations. A wide HIV-1 subtype heterogeneity was found: 6 C (18.2%), 2 F (6%), 2 H (6%), 1 D (3%), 1 G (3%), 8 CRF02_AG (24.2%), 2 CRF06 (6%), 1 CRF01_AE (3%), 1 CRF14_BG (3%), 1 CRF25 (3%) and 1 CRF19 (3%). HIV clade could not be assigned in 7 (21%).**

***Conclusions*: Nearly three-quarters of HIV-positive individuals who began an NNRTI-based triple regimen in Angola showed undetectable viraemia after a median of 12 months of therapy, a rate similar to that reported in Western countries. Specimens collected on DBS may allow monitoring of treatment response in resource-limited regions, although adequate temperature and humidity storage conditions are important to ensure RNA stability and further successful testing.**

Keywords: DBS, viral load, HIV subtypes, non-B subtypes

Introduction

HIV-1 strains can be assigned to M, O and N groups. Group M viruses are so far classified into 9 subtypes and at least 34 inter-subtype circulating recombinant forms (CRFs). Although subtype B is the most frequent HIV-1 variant in Western countries, non-B clades are predominant in sub-Saharan Africa, where around 25 million of the 40 million people living with HIV are found.[1]

Diagnostic tools and antiviral drugs for HIV-1 infection have been mainly designed based on subtype B. With a few exceptions, preliminary evidence suggests that most viral load assays and drug resistance methods are useful for testing specimens from distinct HIV-1 variants.[2] Antiretroviral therapy seems to be similarly effective in populations infected with distinct HIV-1 subtypes.[3] However, several *in vitro* studies have revealed that natural polymorphisms in non-B subtypes might compromise the

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effectiveness of individual drugs. Therefore, resistance interpretation algorithms have recently incorporated particular considerations for some non-B clades.[4]

The current raised interest in the clinical and therapeutic implications of HIV-1 diversity is mainly derived from the major efforts that are being made to provide access to antiretroviral treatment in developing countries, as highlighted by the recently finished WHO '3 by 5' programme and the current 'Universal Access by 2010' programme. Moreover, there is increased awareness that expansion of HIV-1 non-B subtypes within Western regions is rapidly growing due to immigration and other population movements.[5] Thus, it is warranted to know to what extent current diagnostic tools and antiretroviral regimens perform in patients infected with non-B variants.

Patients and methods

Study population

All HIV-1-infected individuals on regular follow-up at one clinic located in Luanda, Angola, who had initiated triple antiretroviral therapy, were identified. For each of them, blood samples were collected on dried blood spots (DBS) after at least 6 months of therapy. DBS specimens were prepared applying a few drops of blood, freshly drawn by finger stick with a lancet, onto an absorbent specimen collection S&S903 filter paper (Schleicher & Schull, BioScience GmbH, Barcelona, Spain). The filters were left at room temperature overnight and stored at 4°C in the absence of desiccant bags until shipment to Hospital Carlos III, Madrid, Spain. The study was approved by the Ethics Committee of the hospital and all participants signed a written informed consent.

Viral load

HIV-RNA was measured using the NucliSens EasyQ HIV-1 v1.1 (bioMérieux, Boxtel, The Netherlands), which has already proven to reliably measure viraemia for distinct HIV-1 variants both in plasma[6] and DBS.[7]

HIV subtyping and drug resistance

Nucleic acids present on DBS were isolated using the Boom method. In all viraemic patients, two different genomic regions of HIV-1 were amplified to determine HIV subtype and reverse transcriptase (RT) resistance mutations. The *gp41 env* region was amplified using previously described conditions. The RT region was amplified in a nested RT–PCR using as outer primers A35 (5′-TTG GTT GCA CTT TAA ATT TTC CCA TTA GTC CTA TT-3′) and NE135 (5′-CTT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT-3′), and as inner primers RTc1 (5′-ATG GAT GGY CCA AAR GTW AAA CAA TG-3′) and RTc2 (5′-TGT CCA TTT YTC AGG ATG GAG YTC-3′). Sequencing was performed using an automatic sequencer (ABI Prism 3100; Celera Diagnostics, Madrid, Spain) and electropherograms were analysed with SeqScape v2.5 (Applied Biosystems).

For HIV-1 subtyping, nucleotide sequences were aligned with reference strains of HIV-1 groups M, N and O (http://hiv-web.lanl.gov) using the CLUSTAL X method (MegAlign, Lasergene, DNASTAR Inc., Madison, WI, USA). Phylogenetic analyses were performed using the PHYLIP software package (version 3.5c; J. Felsenstein, University of Washington, Seattle, WA, USA).

Statistical analyses

Baseline characteristics of the study population are recorded as percentages, mean ± standard deviations, or median values and 25% to 75% interquartile ranges (IQR). The Student *t*-test was used to compare quantitative variables, whereas the χ^2 test was used to compare qualitative parameters. Differences were only considered significant when *P* values were below 0.05. All reported *P* values were two-sided.

Results

A total of 294 patients were examined. The main characteristics of this population are recorded in Table 1. Some antiretroviral drugs were given in fixed pill combinations, such as Duovir or Combivir (zidovudine/lamivudine) and Triomune (stavudine/lamivudine/nevirapine).

After a median of 12.6 months of therapy (IQR 7.2–24), undetectable viraemia on DBS was recognized in 217 patients (73.8%). Only on-treatment analyses could be made, since data regarding loss to follow-up, deaths or toxicities were not available. Patients with viral load suppression had a significantly higher median CD4 count than patients experiencing virological failure [294 cells/mm^3 (IQR 195–435) versus 227 cells/mm^3 (IQR 90–351); $P = 0.003$]. Moreover, the median increase in CD4 counts from baseline was more pronounced in patients who reached undetectable viraemia compared with the rest [122 (IQR 67–227) versus 87 (IQR 6–197); $P = 0.018$]. There were no

Table 1. Main characteristics of the study population

No. of individuals	294
Gender (%)	
female	72.0
Median age (years)	36 (30–42)
Race (%)	
black	98.3
Route of infection (%)	
heterosexual	84.4
transfusion	3.7
others/unknown	11.9
Median weight (kg)	
men	64 (58.25–70.75)
women	54.75 (49–62)
Median baseline CD4 count (cells/mm^3)	144 (68–217)
Baseline CD4 count <200 cells/mm^3 (%)	70.3
Prior AIDS diagnosis (%)	34.4
Treatment (%)	
3TC, AZT, NVP	60.4
3TC, d4T, NVP	21.8
3TC, AZT, EFV	4.8
3TC, d4T, EFV	2.0
other combinations	6.8
Median length of current therapy (months)	12.6 (7.2–24)
Prior exposure to any antiretroviral drug (%)	19.7

AZT, zidovudine; 3TC, lamivudine; d4T, stavudine; NVP, nevirapine; EFV, efavirenz.

Table 2. Drug resistance mutations in patients experiencing virological failure in Angola

Sample ID	Antiretroviral treatment	Plasma HIV-RNA (copies/mL)	HIV-1 subtype RT[a]	NRTI resistance mutations	NNRTI resistance mutations
1	3TC, d4T, NVP	22 000	CRF14_BG	M184V	Y181C, F227V
3	3TC, d4T, NVP	310 000	CRF01_AE	D67N, T69N, T215Y, K70R, M184V, K219E	A98G, K101E, Y181C, G190A
4	3TC, d4T, NVP	82 000	B	M41L, F116F/L, V118D, M184V, T215F	A98G, K103R, V179D Y181C
5	3TC, d4T, NVP	27 000	C	M184V	K101E, V108I/V, G190A
10	3TC, AZT, NVP	370 000	C	M184V	K103N
14	3TC, AZT, NVP	570 000	F	—	K103K/N
15	3TC, d4T, NVP	8000	H	M184V	—
17	3TC, d4T, NVP	42 000	CRF02_AG	—	—
18	3TC, d4T, NVP	3100	CRF14_BG	M184V	K103N
19	3TC, AZT, EFV	57 000	C	K70R M184V	K103N
20	3TC, AZT, NVP	850 000	CRF13/CRF11	—	—
25	3TC, AZT, EFV	25 000	F	M41L	K103K/N
31	3TC, AZT, NVP	5000	A	D67DG, T69AT, V118GV, M184GV	K103N, V179FV
32	3TC, d4T, EFV	120 000	H	—	—
34	3TC, AZT, NVP	11 000	A	M184V, T215NY	A98G, K101E, G190A
35	3TC, AZT, NVP	130 000	A	D67N, T69N, K70R, L74I, M184V, T215FI, K219E	K101E, G190A
36	3TC, AZT, NVP	3300	B	M184V	K103N
38	3TC, AZT, NVP	9100	G	M184V	Y181C
39	3TC, AZT, NVP	8100	A	—	—
40	3TC, AZT, NVP	7100	CRF01_AE	A62V, K70R, M184V	A98G, G190A
41	3TC, AZT, NVP	260 000	G	M41L, M184V, T215NY	A98G, K103N
43	3TC, AZT, NVP	490 000	CRF01_AE	M184V	K103, V108I, Y181C
44	3TC, AZT, NVP	410 000	CRF01_AE	—	—

[a]HIV-1 subtype was determined by phylogenetic analysis using a fragment of 650 nucleotides from the reverse transcriptase.
NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; AZT, zidovudine; 3TC, lamivudine; NVP, nevirapine; EFV, efavirenz; d4T, stavudine.

significant differences in median CD4 counts at baseline comparing virological responders and non-responders [155 CD4 cells/mm^3 (IQR 73–220) versus 120 (IQR 49.5–195.5); $P = 0.129$].

Genetic characterization of subtypes and drug resistance mutations could only be obtained in 45 out of 77 viraemic specimens (58.5%); this was not possible in the rest even after repeated attempts using different primers and PCR conditions. RT and gp41 sequences were obtained from 23 (30%) and 33 (43%) samples, respectively.

From the 23 RT sequences, mutation M184V was recognized in 15 and K103N in 9 cases. Other mutations such as T215F/Y, Y181C and G190A were seen in five patients each, and K70R and K101E in four patients (Table 2). All viraemic patients but five harboured drug resistance mutations. HIV subtyping based on RT sequences was as follows: 4 A (17.4%), 3 C (13%), 2 B (8.7%), 2 F (8.7%), 2 G (8.7%), 2 H (8.7%), 4 CRF01_AE (17.4%), 2 CFR14_BG (8.7%), 1 CRF02_AG (4.3%) and 1 CRF13/CRF11 (4.3%).

The gp41 region could be sequenced in 33 (43%) of 77 viraemic patients. One patient harboured mutation S138A, which has been associated with enfuvirtide resistance, and 14 harboured the N42S polymorphism. Eleven different HIV-1 variants could be identified based on gp41 sequences, including 6 clade C (18.2%), 2 F (6%), 2 H (6%), 1 D (3%), 1 G (3%), 8 CRF02_AG (24.2%), 2 CRF06 (6%), 1 CRF01_AE (3%), 1 CRF14_BG (3%), 1 CRF25 (3%) and 1 CRF19 (3%). For 7 (21%) subtyping was undetermined.

Discussion

Several studies have suggested that differences may exist between distinct HIV-1 subtypes in disease progression and, at least *in vitro*, susceptibility to antiretroviral drugs.[4] These aspects have acquired particular relevance given the growing introduction of antiretroviral therapy in developing countries and because of the increased circulation of non-B subtypes in Western regions.[8] In this cross-sectional study, the rate of virological suppression inferred from testing on DBS was 74%, which may be regarded as satisfactory. The lack of baseline specimens precluded determination of the individual benefit of therapy and represents a limitation of this study. Drug resistance mutations in the RT gene emerged at the same positions and apparently at similar rates as in clade B viruses. Not surprisingly M184V and K103N were the most frequent changes, as they rapidly appear following lamivudine and nevirapine/efavirenz failure, respectively. In addition, CD4 gains were significantly greater in virological responders than in viraemic patients. Altogether, these results suggest that antiretroviral therapy may be equally effective in patients infected with non-B subtypes and living in developing regions as in persons infected with clade B viruses and treated in Western countries. This is in agreement with other recent reports from Senegal, Uganda and Kenya[3] and provides strong evidence for encouraging the use of antiretroviral therapy in resource-limited settings.

Patients experiencing virological failure of antiretroviral therapy in our study underwent further virological characterization. The high HIV-1 genetic variability we recognized in Angola has already been noted by others.[9] This is consistent with the close geographical proximity to Cameroon, where the postulated origin of HIV-1 took place around 70 years ago. However, adequate genetic amplicons could only be obtained from half of viraemic specimens. It is important to determine the reasons for this poor success rate. First, the high HIV-1 genetic diversity might compromise primer binding, even when they were designed based on highly conserved regions of the *pol* gene. Second, and most important, we learned later that no special control of humidity using dessiccant bags was taken during storage, although the protocol dictated preservation at 4°C. While short DNA/RNA fragments are less likely to be affected by degradation, and therefore may impact less on viral load measurements, the longer nucleic acid fragments required for sequencing may be badly affected. Conditions for DBS preservation, especially humidity, may have a large impact on the degradation of nucleic acids.[10]

We could not find any relationship between any specific HIV-1 subtype and emergence of signature drug resistance mutations, although the limited size of the study population failing antiretroviral therapy precluded examination of this aspect in more detail. Information on prior use of nevirapine in pregnant women or baseline resistance was not available. This information might be relevant since 70% of the study population were women and information derived from mother-to-child prevention trials have already underlined that either Y181C or K103N are the main changes emerging following single-dose nevirapine exposure in non-B subtypes.[11]

In summary, the virological response to NNRTI-based triple regimens assessed using DBS specimens seems to be similar in Angola to that in Western countries, despite the large HIV-1 subtype heterogeneity. DBS may be a useful tool for collecting and shipping blood for testing in adequate facilities, as it does not require rigorous storage conditions nor does it represent a potential source of infectious material. However, adequate storage conditions, including control of humidity, are warranted to ensure success of nucleic acid amplification procedures.

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Transparency declarations

None to declare.

References

1. McCutchan F. Global epidemiology of HIV. *J Med Virol* 2006; **78** Suppl 1: 7–12.

2. Holguin A, Hertogs K, Soriano V. Performance of drug resistance assays in testing HIV-1 non-B subtypes. *Clin Microbiol Infect* 2003; **9**: 323–6.

3. Weidle S, Malamba R, Mwebaze C *et al*. Assessment of a pilot antiretroviral drug therapy programme in Uganda: patients' response, survival, and drug resistance. *Lancet* 2002; **360**: 34–40.

4. Geretti AM. HIV-1 subtypes: epidemiology and significance for HIV management. *Curr Opin Infect Dis* 2006; **19**: 1–7.

5. Perrin L, Kaise L, Yerly S. Travel and the spread of HIV-1 genetic variants. *Lancet Infect Dis* 2003; **3**: 22–7.

6. De Mendoza C, Koppelman M, Montes B *et al*. Multicenter evaluation of the Nuclisens EasyQ HIV-1 v1.1 assay for the quantitative detection of HIV-1 RNA in plasma. *J Virol Methods* 2005; **127**: 54–9.

7. Garrido C, Zahonero N, Soriano V *et al*. Correlation between HIV-1 viral load quantification measured in dried blood spots (DBS) and plasma using two commercial assays. *Conference on Retroviruses & Opportunistic Infections, Boston, 3–6 February 2008* [abstract U-161] (in press).

8. Steinbrook R. Message from Toronto—Deliver AIDS treatment and prevention. *N Engl J Med* 2006; **355**: 1081–4.

9. Abecasis A, Paraskevis D, Epalanga M *et al*. HIV-1 genetic variants circulation in the North of Angola. *Infect Genet Evol* 2005; **5**: 231–7.

10. Masciotra S, Garrido C, Youngpairoj A *et al*. High concordance between HIV-1 drug resistance genotypes generated from plasma and dried blood spots in treated persons. *AIDS* 2007; **21**: 2503–12.

11. Eshleman S, Becker-Pergola G, Deseyve M *et al*. Impact of HIV-1 subtype on women receiving single-dose nevirapine prophylaxis to prevent HIV-1 vertical transmission (HIV Network for prevention trials 012 study). *J Infect Dis* 2001; **184**: 914–7.