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HIV-1 non-B subtypes: High transmitted NNRTI-resistance in Spain and impaired genotypic resistance interpretation due to variability

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ABSTRACT

Genotypic resistance algorithms interpret drug-resistance mutations, but are mainly developed for HIV-1 subtype B, meanwhile non-B subtypes cause 90% of worldwide infections. They include clade-specific amino acid at drug-resistance positions different than subtype B.

This study explores: (i) the variability at resistance-related positions in 128 non-B and 226 B sequences from 354 treatment-naïve patients diagnosed in Spain (1999–2007); (ii) the discordances between five resistance interpretation algorithms (ANRS, Stanford, Rega, Geno2pheno, RIS); and (iii) the reliability of five subtyping tools (Stanford, Geno2pheno, Rega, NCBI, EuResist) for each HIV-1 variant.

Primary drug-resistance prevalence was 13.6%, although higher in non-B *vs.* B subtypes (18.7% *vs.* 10.6%), due to a twofold higher NNRTI-resistance prevalence (15.7% *vs.* 7.6%). Most secondary PI-resistances, more frequent in non-B, were in fact clade-specific residues. Most sequences were interpreted as susceptible to all antiretrovirals by the five resistance algorithms, except for tipranavir by ANRS in non-B clades. Interalgorithm discordances were significantly higher in non-B variants for specific drugs. The agreement with phylogenetic analysis differed among subtyping tools testing non-B variants.

We found a higher prevalence of NNRTI-resistance mutations in non-B subtypes. Certain algorithms overestimate the resistance in non-B subtypes due to natural patterns of mutations. Subtyping tools should be optimised for non-B variants.

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1. Introduction

The expanding use of antiretroviral drugs for the treatment against human immunodeficiency virus type-1 (HIV-1) favours the emergence of virus harbouring resistance mutations. This can generate an increasing prevalence of primary resistance mutations in viruses from treatment-naïve patients who have been infected by pre-treated subjects, compromising the effectiveness of the first antiretroviral therapy. Transmission of drug resistant viruses has been widely reported in Europe and the USA, with a prevalence ranging from 5% to 15% (Booth and Geretti, 2007; Sagir et al., 2007; Wensing et al., 2005; Wheeler et al., 2007). In Spain, the rate of primary resistance mutations differs among regions and time periods, but these mutations are present in around 10% of treatment-naïve patients (de Mendoza et al., 2005; Martínez-Picado et al., 2005; Palacios et al., 2008; Sanchez-Oñoro et al., 2007). The rate rarely reaches 10% in treatment-naïve patients from developing countries (Geretti, 2007; Nyombi et al., 2008; Ojesina et al., 2006), and is mainly limited to a few reverse transcriptase inhibitors (RTI), which are the most available drugs in these countries.

International guidelines recommend routine HIV resistance testing for the selection of an optimal antiretroviral therapy selection. Genotypic resistance tests are used more than phenotypic tests, due to their lower costs and easier implementation. Several online algorithms have been developed by correlating genotypic patterns with clinical or phenotypic data. Recent reports have demonstrated their utility to predict virological response in the clinical settings (Rhee et al., 2009). Furthermore, they are inexpensive and widely used for detection and interpretation of resistance mutations using *pol* (protease, PR and reverse transcriptase, RT) sequences.

Both genotypic drug-resistance interpretation algorithms and resistance prevalence studies have been mainly based on results derived from patients infected by subtype B. This is the most prevalent HIV-1 variant in industrialized countries where all antiretroviral drugs are available. However, the remaining HIV-1 variants (non-B subtypes and recombinants), traditionally ignored in the studies, are responsible for 90% of the 33 million infections worldwide (Hemelaar et al., 2006; UNAIDS, 2009). They are prevalent in developing regions and are continuously increasing among new infections in Western countries, including Spain (Holguín et

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al., 2008a). HIV-1 non-B subtypes and recombinant variants have a high genetic variability when the consensus of subtype B is used as reference for the definition of resistance mutations. HIV-1 variants show clade-specific polymorphisms (substitutions present in more than 10% of samples from treatment-naïve patients). Non-B subtypes can also present different wild-type amino acids than subtype B in positions related to drug-resistance to RTI and protease inhibitors (PIs) (Kantor and Katzenstein, 2003). This can be misinterpreted by algorithms. Moreover, scored drug-resistance mutations still vary among different algorithms (Champenois et al., 2008), which complicates the comparison of genotypic resistance among them, mostly when HIV-1 non-B variants are studied and secondary or minor resistance mutations are analyzed. The influence of this clade-specific pattern in the antiretroviral long-term response for each drug in each HIV-1 subtype and recombinant is still unknown. Thus, it is important to clarify the nature and frequency of clade-specific residues in viral positions related to drug-resistance in treatment-naïve patients infected by different HIV-1 subtypes and recombinants. Also it is important to study their influence in interalgorithm discordances.

Hence it is essential to identify HIV-1 subtypes and circulating recombinant forms (CRF). Phylogenetic analysis (phy) is the gold standard method for subtyping, although it is not widely implemented in clinical settings because of its complexity. Most clinicians use online subtyping tools, despite their limitations for the correct classification of some non-B subtypes and most recombinants different to CRF02_AG (Holguín et al., 2008b). This study reports: (i) the prevalence of primary and secondary drug-resistance mutations in 128 HIV-1 non-B and recombinant variants *vs*. 226 clade B sequences from 354 treatment-naïve patients diagnosed in Spain from 1999 through 2007; (ii) the reliability of interpretation of drug-resistance by five algorithms free of charge and available online used in clinical practice in all analyzed sequences belonging to different HIV-1 subtypes and recombinants; and (iii) the reliability of five rapid subtyping tools in detecting HIV-1 subtypes and circulating recombinant forms (CRF) compared to phylogenetic analysis.

2. Patients and methods

2.1. Study population

Pol sequences from plasma samples from 354 HIV-1-infected patients unexposed to antiretroviral therapy, according to the clinical reports, were collected. Patients were diagnosed from 1999 through 2007 in four Spanish HIV/AIDS clinics: 210 in Centro Sanitario Sandoval (Madrid), 130 in Hospital Carlos III (Madrid), 5 in Hospital de Móstoles (Madrid), 5 in Hospital Doctor Negrín (Las Palmas, Canary Islands) and 4 in NGO Medicus Mundi facilities (Madrid). The patients were native Spaniards (45.8%), South-Americans (20.6%), Africans (18.6%), other Europeans (5.9%), Asians (0.3%), and of unknown origin (8.8%). Epidemiological data suggested that most of the migrants had probably been infected overseas.

PR and RT sequences were available from 210 of 226 subtype B sequences and 108 of 128 non-B subtypes. In the remaining cases only PR was available. Of the 354 sequences, 64 non-B and 67 subtype B *pol* sequences were previously published (Gutiérrez et al., 2004; Holguín et al., 2007, 2008a). The new HIV-1 sequences were from patients under follow up in Centro Sanitario Sandoval ($n = 191$), Hospital Carlos III ($n = 29$), Hospital de Móstoles ($n = 3$), and NGO Medicus Mundi ($n = 4$), all located in Madrid, Spain.

This study was part of a project approved by a review board and Ethical Committee of our institution. It was designed to protect the rights of all subjects involved under the appropriate local regulations. To maintain subject confidentiality, a unique ID number was assigned to each specimen.

2.2. HIV-1 subtyping

Direct sequencing of nested PCR purified products from viral RNA was performed in the HIV-1 *pol* coding region. *Pol* sequences included the complete protease (codons 1–99) and part of the reverse transcriptase (codons 1–247 or 1–335) using Trugene (Siemens, Barcelona, Spain) or Viroseq (Celera Diagnostics, Alameda, CA, USA) assays, respectively. In some specimens with amplification difficulties, *PR* and/or *RT* amplification was made using primers and conditions reported elsewhere, utilizing an automatic sequencer for sequencing (ABI Prism, Applied Biosystems, Foster City, CA, USA) (Holguín et al., 2005, 2006a).

All 354 *pol* sequences were subtyped by phylogenetic analysis. At least two representative sequences of each subtype/CRF within HIV-1 group M available at the moment of the analysis were taken as references. DNA sequences were aligned using the ClustalW program. The tree topology was obtained using the Neighbour-Joining method. The pairwise distance matrix was estimated using the Kimura two-parameter model within the DNADIST program, as implemented in the PHYLIP software package. Bootstrap re-sampling (1000 data sets) of the multiple alignments was performed to test the statistical robustness of the tree. Bootstrap cut-off was set at 700.

Besides using phylogenetic analysis, HIV-1 subtyping was also assessed in all *pol* sequences by five online rapid subtyping tools: Stanford 4.3.7, Geno2pheno 3.0, Rega 2.0, NCBI 2008 and EuResist 2008. They were available at: http://hivdb.stanford.edu (HIV-1 Drug Resistance Database; Stanford University, Palo Alto, CA); http://www.geno2pheno.org (Max Planck Institute for Informatics, Saarbrücken, Germany); http://www.bioafrica.net/subtypetool/html (Rega University, Leuven, Belgium); http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi (National Center for Biotechnology Information, Bethesda, MD, USA) and http://engine.euresist.org/data_analysis/viral_sequence/new (EuResist Project, Roma, Italy). A discrepancy was considered when the subtyping tool assigned a different HIV-1 subtype or CRF than that provided by phy.

2.3. Genotypic drug-resistance interpretation algorithms

Drug-resistance mutations defined by the International AIDS Society-USA (IAS) (Johnson et al., 2008) were manually located in the *pol* sequences. Resistance mutations at PR gene were classified as primary (major) or secondary (minor) following the IAS-USA nomenclature. We considered primary or major mutations at PR: D30N, V32I, L33F, M46I/L, I47V/A, G48V, I50L/V, I54M/L, Q58E, T74P, L76V, V82A/T/F/S/L, I84V, N88S, L90M; as secondary mutations at PR: L10V/I/R/F/C, V11I, I13V, G16E, K20R/M/I/T/V, L24I, L33I/V/F, E34Q, E35G, M36I/L/V, K43T, F53L/Y, I54V/T/A/S, D60E, I62V, L63P, I64L/M/V, H69K, A71V/I/T/L, G73C/S/T/A, V77I, V82I, N83D, I85V, N88D, L89V, I93L/M. Resistance mutations at RT: M41L, A62V, K65R, D67N, T69 insertion, K70R/E, L74V, V75I, F77L, V90I, A98G, L100I, K101E/H/P, K103N, V106M/A/I, V108I, Y115F, F116Y, E138A, Q151M, V179D/F/T, Y181C/I/V, M184V/I, Y188C/L/H, G190S/A, L210W, T215Y/F, T215rev, K219Q/E, P225H and M230L. The objective was to study their prevalence among each HIV-1 subtype and CRF from naïve subjects, as well as their influence in the prediction of resistance to each PR or RT inhibitor in current therapeutic use.

The concordance between five different genotypic resistance algorithms (ANRS 2008.07, Stanford 4.3.7, Rega 7.1.1, Geno2pheno 3.0, and RIS-2008) was also studied. For that purpose, all 354 *pol* sequences were introduced in the corresponding web-

Table 1
Substitutions in PR (a) and RT (b) associated with drug-resistance in sequences from 354 treatment-naïve patients carrying different HIV-1 variants.

(a) Substitutions at PR	HIV-1 variants (no.)																	% of HIV-1 variants		p
	Subtypes								CRF											
	A (9)	B (226)	C (8)	D (1)	F (8)	G (15)	02 (41)	06 (5)	10 (3)	11 (2)	12 (16)	13 (1)	14 (9)	19 (1)	22 (1)	23 (6)	31 (2)	Non-B (128)	B (226)	
Primary																				
V32I	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	0.8	0	NS
L33F	–	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	0.8	0.4	NS
M46I/L	1	3	–	–	–	–	1	–	1	–	–	–	1	–	–	–	–	3.1	1.3	NS
Q58E	–	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	2.3	0	NS
I50L/V	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0.4	NS
V82A/T/F/S/L	–	3	–	–	–	–	–	–	1	–	3	–	–	–	–	–	–	3.1	1.3	NS
L90M	–	4	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	0.8	1.8	NS
Secondary																				
L10V/I/R/F/C	3	25	–	–	3	2	8	–	–	1	5	–	1	–	–	–	1	18.7	11.1	<0.05
V11I	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.8	0	NS
I13V	**7**	42	2	–	1	**13**	**33**	**5**	**2**	1	3	**1**	**9**	–	**1**	6	**1**	66.4	18.6	<0.05
G16E	2	1	–	–	2	1	6	1	–	1	3	–	–	–	–	–	2	14.1	0.4	<0.05
K20R/M/I/T/V	3	8	1	–	3	**15**	**38**	**5**	–	–	5	1	**9**	–	–	**6**	2	68.7	3.5	<0.05
L24I	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0.4	NS
L33I/V	1	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.8	4.9	<0.05
E34Q	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	0.8	0	NS
E35G	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	0.8	0	NS
M36I/L/V	**8**	38	**7**	**1**	**6**	**15**	**38**	**5**	**3**	**1**	**16**	**1**	**9**	**1**	**1**	**6**	**1**	93	16.8	<0.05
K43T	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0.4	NS
F53L/Y	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	1.8	NS
I54V/T/A/S	–	3	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	2.3	1.3	NS
D60E	–	5	–	–	–	–	–	–	1	2	–	–	–	–	–	–	–	2.3	2.2	NS
I62V	–	49	–	–	1	1	1	1	1	–	1	–	1	–	–	–	–	5.5	21.7	<0.05
L63P	–	119	3	1	2	4	4	–	1	–	–	–	–	–	1	–	1	13.3	52.6	<0.05
I64L/M/V	2	59	–	–	–	1	5	3	1	–	1	–	–	–	–	3	–	12.5	26.1	<0.05
H69K	**9**	1	**8**	–	–	**15**	**40**	**4**	**2**	**2**	**2**	**1**	**9**	–	**1**	**6**	**2**	78.9	0.4	<0.05
A71V/I/T/L	–	36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	15.9	<0.05
V77I	–	74	1	–	1	2	1	–	–	1	–	1	–	–	–	–	–	5.5	32.7	<0.05
V82I	–	1	–	–	–	**12**	1	–	–	–	–	–	**9**	–	–	**6**	–	21.9	0.4	<0.05
I93L/M	1	80	**6**	–	–	1	1	–	–	–	4	–	–	–	–	1	**1**	11.7	35.4	<0.05

(b) Substitutions at RT	HIV-1 variants (*n*)														% of HIV-1 variants		p
	Subtypes						CRF										
	A (8)	B (210)	C (8)	D (1)	F (7)	G (6)	02 (38)	06 (3)	10 (3)	11 (2)	12 (16)	14 (8)	23 (6)	31 (2)	Non-B (108)	B (210)	
NRTI-resistance																	
M41L	–	4	–	–	–	–	–	–	1	–	–	–	–	–	0.9	1.9	NS
A62V	–	3	–	–	–	–	–	–	–	–	–	–	–	–	0	1.4	NS
K65R	–	1	–	–	–	–	–	–	–	–	–	–	–	–	0	0.5	NS
D67N	–	3	–	–	–	–	–	–	1	–	–	–	–	–	0.9	1.4	NS
K70R/E	–	2	–	–	–	–	–	–	–	–	–	–	–	–	0	0.9	NS
L74V	–	1	–	–	–	–	1	–	–	–	–	–	–	–	0.9	0.5	NS
F77L	–	–	–	–	1	–	–	–	–	–	–	–	–	–	0.9	–	NS
M184V/I	–	1	–	–	–	–	2	–	–	–	–	–	–	–	1.8	0.5	NS
L210W	–	3	–	–	–	–	–	–	1	–	–	–	–	–	0.9	1.4	NS
T215Y/F	–	1	–	–	–	–	–	–	–	–	–	–	–	–	0	0.5	NS
T215rev	1	2	–	–	–	–	–	–	1	–	–	–	–	–	1.8	2.9	NS
K219Q/E	–	2	–	–	–	–	–	–	–	–	–	–	1	–	0.9	0.9	NS

Table 1 (*Continued*)

(b) Substitutions at RT	HIV-1 variants (n)														% of HIV-1 variants		p
	Subtypes						CRF										
	A (8)	B (210)	C (8)	D (1)	F (7)	G (6)	02 (38)	06 (3)	10 (3)	11 (2)	12 (16)	14 (8)	23 (6)	31 (2)	Non-B (108)	B (210)	
NNRTI-resistance																	
V90I	–	1	–	–	–	–	6	–	–	–	–	–	–	–	5.6	0.5	<0.05
K101E/P	–	1	–	–	–	–	1	–	–	–	–	–	–	–	0.9	0.5	NS
K103N	1	9	–	–	–	–	1	–	1	–	2	–	–	–	5.6	4.3	NS
V106M/A/I	–	–	–	–	2	–	1	–	–	–	–	–	1	–	3.7	–	NS
V179D/F/T	–	4	–	–	–	–	–	–	–	–	–	–	–	–	0	1.9	NS
Y181C/I/V	–	1	–	–	–	–	1	–	–	–	–	–	–	–	0.9	0.5	NS
G190S/A	1	–	–	–	–	–	1	–	–	–	–	–	–	–	1.8	–	NS
P225H	–	–	–	–	–	–	1	–	–	–	–	–	–	–	0.9	–	NS

No., number; PR, protease; RT, reverse transcriptase; NRTI, nucleoside RT inhibitors; NNRTI, non-nucleoside RT inhibitors; CRF, circulating recombinant form (the numbers indicate the specific CRF: CRF02_AG, CRF06_cpx, CRF10_CD, CRF11_cpx, CRF12_BF, CRF13_cpx, CRF14_BG, CRF19_cpx, CRF22_01A1, CRF23_BG, and CRF31_BC); NS, not significant (p-value >0.05); dash means no changes. In bold, those amino acids that are, in fact, the wild-type amino acid in the corresponding HIV-1 subtype/CRF according to Los Alamos HIV Database (www.hiv.lanl.gov). Drug-resistance mutations are listed according to IAS-USA (Johnson et al., 2008). At the PR no primary mutations D30N, I47V/A, G48V, I54M/L, T74P, L76V, I84V and N88S were found. At the RT, neither T69insertion nor changes V75I, A98G, L100I, V108I, Y115F, F116Y, E138A, Q151M, Y188C/L/H and M230L were observed. T215rev in RT means T215 revertants including changes T215A/C/D/E/G/H/I/L/N/S/V. Some of the sequences presented more than one change.

sites: ANRS (http://www.hivfrenchresistance.org), Stanford HIVDB (http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceForm), Rega (http://www.rega.kuleuven.be/cev), Geno2pheno (http://www.geno2pheno.org) and RIS (Spanish AIDS Research Network, http://www.retic-ris.net/default_principal.asp?idx=&cidioma=2). All algorithms provided different resistance levels. Thus, they were normalized for a better comparison in three resistance levels: susceptible (S), intermediate (I), and resistant (R), as in the HIValg Program in the Stanford website (http://hivdb.stanford.edu/pages/algs/HIValg.html).

Interpretations were considered concordant when all algorithms assigned the same level of resistance (S, I or R) to a specific sequence for a particular drug. We considered full discordances when one of the algorithms scored a sequence as S for a particular drug and another one as R. Partial discordances were considered when: (1) one algorithm scored a specific sequence as S for a particular drug and another one as I, or (2) when one algorithm scored a sequence as I for a particular drug and another one as R.

2.4. Statistical analysis

Chi-square test was performed with Epi Info 3.5 program (Center for Disease Control and Prevention, Atlanta, GA, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Phylogenetic characterization of HIV-1 variants

Among the 354 HIV-1 *pol* sequences, 226 (63.8%) were ascribed to clade B by phylogenetic analysis and 128 (36.2%) to non-B subtypes and recombinants: 9 A, 8 C, 1 D, 8 F, 15 G, 41 CRF02_AG, 5 CRF06_cpx, 3 CRF10_CD, 2 CRF11_cpx, 16 CRF12_BF, 1 CRF13_cpx, 9 CRF14_BG, 1 CRF19_cpx, 1 CRF22_01A1, 6 CRF23_BG and 2 CRF31_BC. Patients infected by non-B variants came from Spain (16.4%), sub-Saharan Africa (50%), South-America (16.4%), other European countries (6.2%), or were of unknown origin (11%). Subjects carrying subtype B came from Western Europe (68.1%), South-America (23%), North-Africa (0.9%), Asia (0.4%), or were of unknown origin (7.6%). New sequences were submitted to GenBank: from FJ481650 to FJ481713 (64 non-B variants) and from FJ481714 to FJ481872 (159 subtype B).

3.2. Twofold higher prevalence of global primary and NNRTI-resistance mutations in non-B vs. B subtypes

Global primary drug-resistance prevalence (i.e., to any antiretroviral drug class) in viruses from 354 treatment-naïve patients from Spain collected during 1999–2007 was 13.6% (Fig. 1). All carried primary or major resistance mutations in PR or/and RT. Considering the HIV-1 variant, prevalence was almost twofold higher in non-B subtypes than in clade B viruses (18.7% *vs.* 10.6%, $p < 0.05$) due to a twofold higher prevalence of NNRTI-resistances (13.1% *vs.* 7.1%, $p < 0.05$). Prevalence of drug-resistance mutations was similar in non-B *vs.* B subtype for PI (6.2% *vs.* 4.4%) and NRTI (4.6% *vs.* 5.2%) (Fig. 1). We observed a similar rate of non-B *vs.* B viruses showing resistance to 2 and 3 drug classes, ranging from 2 to 4%.

The nature and frequency of primary and secondary (or minor) drug-resistances found in each HIV-1 variant is recorded in Table 1. Most frequent primary PI-resistance mutations were M46L (3.1%) and V82A (3.1%) in non-B and M46L/I (3.1%) and L90M (1.8%) in subtype B. For NRTI-resistance substitutions, M184V (1.8%) and M41L (1.9%) were the most common changes in non-B and B, respectively. Surprisingly, the frequency of T215 revertants, normally the most

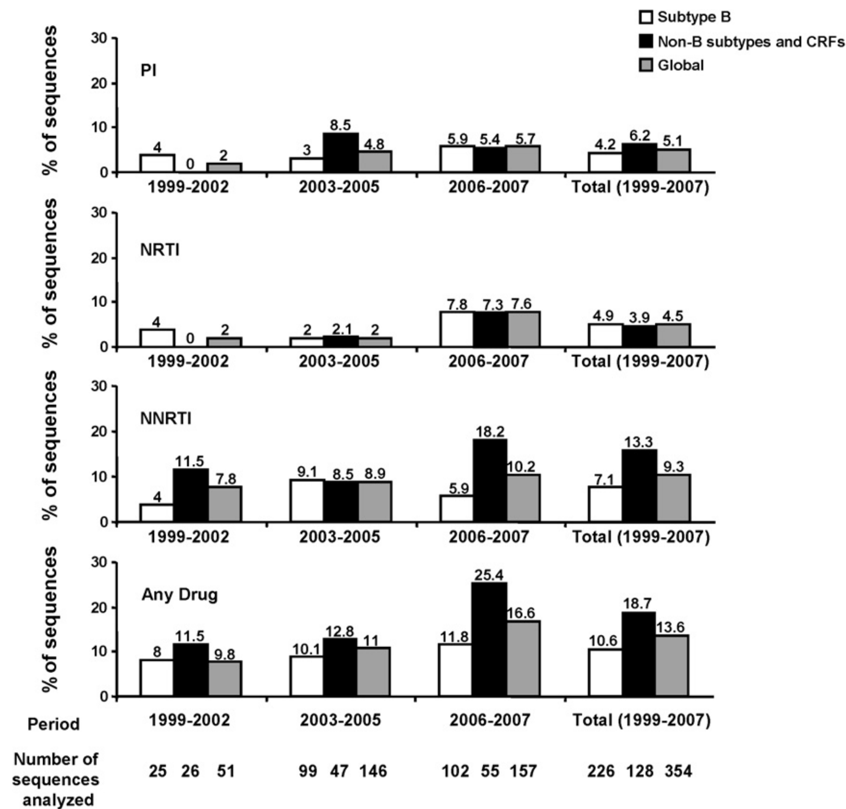

Fig. 1. Prevalence of sequences with primary drug-resistance mutations across HIV-1 subtypes from 354 treatment-naïve patients in Spain across years (1999–2007). PI, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; CRFs, circulating recombinant forms. Figures on the top of the bars indicate the percentage of sequences with primary resistance mutations.

commonly transmitted drug-resistance, was very low. Finally, the most frequent NNRTI-resistance mutations were V90I and K103N (5.6% each one) in non-B and K103N (4.3%) in clade B variants (Table 1).

3.3. Most secondary PI-resistance mutations are clade-specific amino acids

Most (100% non-B, 93% B) of 354 PR sequences presented secondary or minor PI-resistance mutations, different in frequency and nature across variants since some of them were the wild-type amino acid in some subtypes and recombinants (Table 1). This could explain the higher frequency of amino acid related to secondary resistance in residues 10, 13, 16, 20, 36, 69, and 82 in specific non-B clades and in residues 62, 63, 64, 71, 77, and 93 at clade B variants (Table 1). For instance, substitutions M36I (related to atazanavir, indinavir, nelfinavir and tipranavir-resistance) and H69K (related to tipranavir-resistance) were detected in most analyzed non-B specimens. Additionally, change V82I (related to atazanavir-resistance) appeared in all CRF14_BG, CRF23_BG, and 80% of subtype G specimens in our country.

3.4. High interalgorithm discrepancies in non-B subtypes for specific drugs

Drug susceptibility to 19 drugs (PI, NRTI and NNRTI) was predicted in all 354 non-B and B sequences using five different algorithms: French ANRS 2008.07, Stanford 4.3.7, Rega 7.1.1,

Table 2
Percentage of HIV-1 sequences from 354 treatment-naïve subjects with genotypic drug-resistance interpretation to 19 different drugs provided by five online genotypic resistance algorithms.

Drugs	HIV-1 variants (no.)	Genotypic resistance algorithms (% of sequences scored to each level of resistance)														
		ANRS 2008.07			Stanford 4.3.7			Rega 7.1.1			Geno2pheno 3.0			RIS 2008		
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
PI	Non-B (128)	9.4	14.8	75.8	94.4	5.5	0.8	94.4	3.1	3.1	94.4	3.1	3.1	94.4	3.1	3.1
	B (226)	92.5	2.6	4.9	94.7	4.9	0.4	95.6	2.6	1.8	95.6	2.2	2.2	96	3.1	0.9
NRTI	Non-B (108)	95.4	1.8	2.8	92.6	4.6	2.8	94.4	2.8	2.8	78.7	14.8	6.5	95.4	1.8	2.8
	B (210)	93.8	2.4	3.8	93.3	5.2	1.4	93.3	3.8	2.9	81	16.7	2.4	93.8	3.8	2.4
NNRTI	Non-B (108)	81.5	0	18.5	91.7	0.9	7.4	91.7	0.9	7.4	91.7	0	8.3	92.6	0	7.4
	B (210)	95.7	0	4.3	94.3	1.4	4.3	95.7	0	4.3	94.8	0	5.2	95.2	0	4.8

No., number of sequences; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; S, percentage of sequences interpreted as susceptible by the respective algorithm for a drug family; I, intermediate resistant; R, resistant. ANRS 2008.07 (http://www.hivfrenchresistance.org), Stanford HIVDB 4.3.7 (http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceForm), Rega 7.1.1 (http://www.rega.kuleuven.be), Geno2pheno 3.0 (http://www.geno2pheno.org) and RIS 2008 (http://www.retic-ris.net/default_principal.asp?idx=&cidioma=2).

Table 3
Discordances between five genotypic drug-resistance interpretation algorithms testing 128 non-B and recombinants *vs.* 226 subtype B *pol* sequences.

Drugs	Full discordances			Partial discordances			Concordances non-B/B
	Non-B/B (%)	*p*	Variants with discordances	Non-B/B (%)	*p*	Variants with discordances	
Any PI	76.6/4.4	**<0.01**	99 non-B, 17B	10.9/6.6	NS		22.3/92.5
Atazanavir	2.3/1.3	NS		2.3/2.6	NS		95.3/96.0
Darunavir	0/0	–		0.8/0.9	NS		99.2/99.1
Fosamprenavir	3.1/0.4	**0.04**	3CRF12_BF, 1CRF10_CD, 1B	0/2.2	NS		96.9/97.3
Indinavir	2.3/1.8	NS		3.1/1.3	NS		94.5/96.0
Lopinavir	2.3/1.3	NS		0.8/0.9	NS		96.9/97.8
Saquinavir	0/0	–		4.7/3.1	NS		95.3/96.9
Nelfinavir	0/0.4	NS		6.2/3.5	NS		94.5/96.0
Tipranavir	73.4/0.9	**<0.01**	94 non-B, 2B	10.9/1.8	**<0.01**	14 non-B, 4B	12.5/97.3
Any NRTI	5.6/3.8	NS		16.7/13.8	NS		83.3/85.2
Lamivudine	0.9/0	NS		0.9/1.9	NS		98.1/98.1
Abacavir	0.9/0.4	NS		2.8/2.9	NS		95.4/96.7
Zidovudine	0.9/0.9	NS		1.8/4.3	NS		97.2/94.8
Estavudine	0/0.9	NS		9.3/12.6	NS		90.7/86.2
Didanosine	6.5/1.9	**0.03**	4CRF02_AG, 1A, 1CRF10_CD, 1CRF14_BG, 4B	6.5/3.8	NS		87.0/94.3
Emtricitabine	0/0	–		0.9/1.4	NS		99.1/98.6
Tenofovir	0.9/1.4	NS		1.8/7.6	**0.03**	16B, 1A, 1CRF10_CD	97.2/90.9
Any NNRTI	11.1/1.9	**<0.01**	8CRF14_BG, 2CRF23_BG, 1C, 1CRF10_CD, 4B	0.9/0.9	NS		87.0/97.1
Delavirdine	0/0	–		0/0.5	NS		100/99.5
Efavirenz	0.9/0	NS		0/0	–		99.1/100
Etravirine	0/0.5	NS		0.9/0.5	NS		99.1/99.0
Nevirapine	11/1.4	**<0.01**	8CRF14_BG, 2C, 2CRF23_BG, 3B	0/0.5	NS		88.9/98.1
Any RTI	15.7/5.7	**<0.01**	8CRF14_BG, 4CRF02_AG, 2C, 2CRF23_BG, 1A, 12B	17.6/14.8	NS		73.2/83.3

Drug susceptibilities to protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) were predicted using five different algorithms: ANRS 2008.07, Stanford 4.3.7, Rega 7.1.1, Geno2pheno 3.0, and RIS 2008. In bold, specific drugs showing significant ($p < 0.05$) differences in full or partial discordances comparing non-B *vs.* B sequences (128/108 non-B and 226/210 subtype B PR/RT sequences from different antiretroviral-naïve patients). The 354 analyzed sequences were: 9A, 226B, 8C, 1D, 8F, 15G, 41CRF02_AG, 5CRF06_cpx, 3CRF10_CD, 2CRF11_cpx, 16CRF12_BF, 1CRF13_cpx, 9CRF14_BG, 1CRF19_cpx, 1CRF22_01A1, 6CRF23_BG and 2CRF31_BC. Full discordances when one of the algorithms scored a sequence as S for a particular drug and another one as R. Partial discordance when one of the algorithms scored the sequences as S for a particular drug and another one scored it as I, or when one of the algorithms scored the sequences as I for a particular drug and another one scored it as R. The five algorithms analyzed the susceptibility to all the drugs shown except for darunavir, tipranavir and etravirine (not provided by Geno2pheno), nelfinavir (not provided by RIS) and delavirdine (not provided by ANRS and RIS algorithms). NS, Chi-square test showed non-significant differences ($p > 0.05$). Zalcitabine resistance was not included because it could only be tested by the Geno2pheno algorithm.

Geno2pheno 3.0, and Spanish RIS 2008. Their interpretations are shown in Table 2. Most sequences were interpreted as susceptible (S) to all antiretrovirals by the five algorithms, except for tipranavir by ANRS in non-B. On the other hand about 4–8% of the 354 sequences from treatment-naïve patients were intermediate (I) or resistant (R) to specific drugs by all algorithms. ANRS ascribed as I or R to a significantly higher number of non-B *vs.* B sequences for PI (90.6% *vs.* 7.5%, $p < 0.05$) and NNRTI (18.5% *vs.* 4.3%, $p < 0.05$). The other algorithms ascribed as I or R to PI or NNRTI were 6–7% or 8% of non-B sequences, respectively. Stanford was the algorithm that provided the lowest number of HIV-1 sequences as R to PI (Table 2). Geno2pheno ascribed as not susceptible (I or R) to NRTI a higher number of non-B and B sequences than other algorithms (around 20% *vs.* 5–7%).

Considering HIV-1 variants, interalgorithm discordances (including both full and partial) were significantly higher in non-B *vs.* B variants for NNRTIs (13% *vs.* 2.9%, $p < 0.05$), and similar for NRTI (16.7% *vs.* 14.8%, p = NS) and for PIs excluding tipranavir (8.6% *vs.* 5.7%, p = NS) (Table 3). Regarding drug families, interalgorithm discordances (full and partial) were significantly more frequent for NRTI *vs.* NNRTI or PI excluding tipranavir in all variants (15.4% *vs.* 6% or 6.8%, respectively; $p < 0.01$) but were similar among drug families when only full discordances were considered (4.4%, 5%, and 4.2%, respectively). Considering specific drugs, non-B variants displayed significantly more interalgorithm full discordances than subtype B for tipranavir (73.4% *vs.* 0.9%, $p < 0.05$), fosamprenavir (3.1% *vs.* 0.4%, $p < 0.05$), nevirapine (11% *vs.* 1.4%, $p < 0.05$) and didanosine (6.5% *vs.* 1.9%, $p < 0.05$), and lower partial discrepancies than clade B for tenofovir (1.8% *vs.* 8.6%, $p < 0.05$) (Table 3).

Sequences with changes in residues M36, H69 and L89, highly common in non-B viruses (Table 1), were considered as R to tipranavir by ANRS and as I when two of these changes appeared. For ANRS, all non-B sequences were susceptible to didanosine, since this algorithm, in contrast to the remaining tools, did not include L74V as a didanosine-resistance mutation. Geno2pheno considered sequences with I135T, in combination with V60I or T200A, as I to tenofovir, although none of them were included in the IAS-USA list. ANRS interpreted a higher number of nevirapine-resistant non-B sequences, due to the inclusion of the A98S change. This is actually the wild-type residue in CRF14_BG (www.hiv.lanl.gov), first described in Spain (Delgado et al., 2002), and it is also a natural polymorphism in CRF24_BG.

3.5. *Low reliability of five online rapid subtyping tools in the assignment of non-B subtypes and recombinants*

The 354 *pol* HIV-1 sequences were introduced in five online rapid subtyping tools (Stanford, Geno2pheno, Rega, NCBI and EuResist) to test their agreement with phy (Table 4). Most subtype B sequences, prevalent in developed countries, were correctly assigned (87.2–99.6%), the worst was NCBI and the best was Stanford. The five tools showed a low agreement with phy ascribing non-B variants and differed among tools. Only three quarters of the sequences were correctly assigned by Geno2pheno (75.8%) and EuResist (71.9%), and results were even lower for NCBI (60.9%), Rega

Table 4
Agreement of five online subtyping tools vs. phylogenetic analysis in 354 sequences from different HIV-1 variants.

HIV-1 variant[a] (no.)	HIV-1 subtyping method					
	Phy *vs.* Stanford[b]	Phy *vs.* Geno2pheno	Phy *vs.* Rega[c]	Phy *vs.* Rega[d]	Phy *vs.* NCBI	Phy *vs.* EuResist
A (9)	22.2	44.4	88.9	100	11.1	33.3
C (8)	87.5	100	87.5	100	87.5	100
D (1)	100	100	100	100	0	0
F (8)	87.5	87.5	100	100	25	100
G (15)	93.3	93.3	73.3	100	53.3	86.7
CRF02_AG (41)	82.9	95.1	63.4	84.4	92.7	92.7
CRF06_cpx (5)	0	100	60	100	60	100
CRF10_CD (3)	0	33.3	0	0	0	0
CRF11_cpx (2)	0	50	100	100	100	50
CRF12_BF (16)	0	43.7	43.7	70	43.7	37.5
CRF13_cpx (1)	0	100	0	–	100	100
CRF14_BG (9)	0	100	0	0	100	100
CRF19_cpx (1)	0	0	0	–	0	0
CRF22_01A1 (1)	0	0	0	–	0	0
CRF23_BG (6)	0	0	0	–	0	0
CRF31_BC (2)	0	0	0	0	0	0
Pure non-B subtypes (41)	75.6	82.9	85.4	100	43.9	78
Recombinants non-CRF02_AG (46)	0	52.2	26.1	50	47.8	47.8
Total Recombinants (87)	39.1	72.4	43.7	69.6	69	69
Total Non-B (128)	50.8	75.8	57	81.3	60.9	71.9
Subtype B (226)	99.6	95.1	90.7	100	87.2	97.8

No., number of sequences; Phy, phylogenetic analysis considered as the gold standard subtyping method; CRF, circulating recombinant forms; dash indicates those cases when Rega did not assign any of the sequences of the subtype/CRF. Websites: Stanford HIVDB 4.3.7, (http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceForm), Geno2pheno 3.0 (http://www.geno2pheno.org), Rega 2.0 (http://www.bioafrica.net/subtypetool/html), NCBI 2008 (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) and EuResist 2008 (http://engine.euresist.org).
[a]Subtypes in the *pol* coding region.
[b]Stanford differenced protease and reverse transcriptase in the analysis. Out of 63 failures, 6 occurred in a PR with RT correctly subtyped and 4 vice versa.
[c]Rega analysis including or [d]excluding all the sequences not assigned to a subtype by the Rega tool (6 "pure" non-B subtypes, 31 CRF and 21 clade B).

(57%) and Stanford (50.8%) (Table 4). Rega was unable to assign any subtype in 21 (9.3%) B sequences and 37 (29%) non-B variants, and 84% of them were CRF. Rega's agreement increased up to 81.3% when only assigned subtypes were considered.

Results were better for pure non-B subtypes than for CRF, except for NCBI (Table 4). The agreement with phy testing pure non-B subtypes was higher for Geno2pheno (82.9%) and Rega (85.4%), and lower for EuResist (78%), Stanford (75.6%) and NCBI (43.9%). CRF02_AG was the best assigned, reaching 90% in some tools. However, only 50% of the 46 specimens adscribed as CRF by phy other than CRF02_AG, were correctly assigned by all online tools except Stanford, which did not identify any of them. None of the online tools could correctly subtype any CRF19_cpx, CRF22_01A1, CRF23_BG or CRF31_BC (Table 4). In summary, none of the five subtyping tools showed a complete reliability in the assignment of non-B subtypes and recombinants, although Geno2pheno, EuResist and Rega showed the best global results. Rega showed the best results subtyping pure non-B subtypes and NCBI the worst. Geno2pheno had the highest agreement in recombinant assignment and Stanford the lowest.

4. Discussion

This study analyzed different aspects. Firstly, it defined the drug-resistance prevalence in a large cohort of treatment-naïve patients infected by different HIV-1 subtypes and CRF in Spain. Secondly, it reported the discordances in resistance interpretation comparing five online algorithms. Thirdly, it showed the agreement of five online tools with phylogenetic analysis (phy) for HIV-1 subtyping. The overall prevalence of drug-resistance mutations found (13.8%) was similar to other surveillance studies in Europe and Spain (Booth and Geretti, 2007; de Mendoza et al., 2005; Sanchez-Oñoro et al., 2007; Wensing et al., 2005; Wheeler et al., 2007), although it was higher in some areas (Shet et al., 2006). To our knowledge, the study cohort of treatment-naïve subjects infected by HIV-1 non-B subtypes and recombinants is one of the largest identified in Madrid and in Spain including these variants. However, the number of samples analyzed for certain subtypes and/or recombinants was very limited. It would be more appropriate to have a significant number of each HIV-1 variant instead of merging all in a single group, given that each clade has its own peculiar characteristics and specific wild-type sequence. Nevertheless, this is not always possible due to the unequal distribution of non-B subtypes in our country. In addition, the number of patients does not allow the reliable analysis of the trends of resistance over time. These trends of transmitted resistance vary depending on the time period, geography and cohort characteristics, including the country of origin. An additional source of variation is that distinct drug-resistance surveillance studies are based on different lists of drug-resistance mutations.

Nevertheless, the knowledge of primary resistance can be cost-effective during the clinical practice and treatment proposed regimen (Smith et al., 2007). Our treatment-naïve population presented a significantly higher prevalence of drug-resistance mutations in non-B *vs.* B subtypes. This was due to the higher rate of NNRTI-resistance mutations in certain variants, mainly in CRF02_AG (19.5% of sequences with NNRTI-resistance mutations *vs.* 7.1% in subtype B, $p < 0.05$) (Table 1b). In fact, CRF02_AG variants are the most frequent HIV-1 non-B viruses in Spain (Holguín et al., 2008b). NNRTIs are frequently used in the native countries of most foreign non-B infected patients in Spain. It was also recently reported that transmission of NNRTI-resistances in Europe is rising faster than for other antiretroviral families (SPREAD programme, 2008). It would be useful if non-B variants were detected among these NNRTI-resistant viruses in future European surveillance studies.

New HIV-1 diagnoses caused by non-B variants increased in Spain in the last decade (Holguín et al., 2008a), as in other industrialized countries (Aggarwal et al., 2006; Pillonel et al., 2008). Thus, HIV-1 subtype and country of infection of treatment-naïve patients should be considered in drug-resistance studies, since resistance prevalence may differ in subjects infected in areas with fewer avail-

able drugs. Accordingly, there is a low resistance prevalence in non-B infected patients living in developing countries with scarce therapy distribution (Agwale et al., 2006; Booth and Geretti, 2007; Vessière et al., 2006).

We observed a high frequency of secondary PI-resistance mutations in all variants, which showed a different nature among HIV-1 subtypes and CRF. Despite the low number of studies correlating mutations and response to therapy in non-B subtypes (Martínez-Cajas et al., 2008), it is known that some clade-specific amino acids at resistance positions may influence drug susceptibility, selection of different resistance pathways, and/or a more rapid emergence of drug-resistance (Holguín et al., 2006b). Regardless of their therapeutic and epidemiological implications, these residues have been excluded in a specific list created in a positive attempt to homogenise transmitted drug-resistances detected in treatment-naïve patients (Bennett et al., 2009). However, this list only includes two of the 43 HIV-1 circulating recombinant forms described to date (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html), and should be improved to be applicable to all recombinants, that are increasing in global prevalence.

Some of these subtype-associated polymorphisms in positions related to drug-resistance are responsible for the discrepancies found in drug-resistance interpretation using different algorithms. Our study reports that when using five different algorithms discordances were significantly higher in non-B *vs*. B variants for didanosine, nevirapine, tipranavir, and fosamprenavir, and significantly lower for tenofovir. These discrepancies highlight that the patterns of drug-resistance mutations have not been yet completely clarified in non-B variants, especially for PI. The use of certain algorithms could lead to an overestimation of the resistance in the analysis of specific non-B subtypes because of the lack of consensus in the resistance mutations considered. Nevertheless, it does not mean that the analyzed tools are useless or that the current interpretation algorithms may be invalidated due to non-B subtypes. These algorithms are easy to use and useful in the routine practice for clinicians during the clinical follow up of their HIV-infected patients and a good concordance among them was observed for most (but not all) variants and drugs. The discrepancies between them would justify the necessity of including more samples from subtypes different from B in the databases from different algorithms to improve the excellence of the methods, reducing the potential mistakes in some non-B specimens for some drugs using specific algorithms. For instance, it would avoid interpreting as resistant a sequence which presumably is not. On the other hand, with these data we cannot affirm which of the algorithms are the most accurate. In order to clarify this aspect phenotypic assays should be performed to elucidate which algorithm is right and which is wrong.

Although discrepancies have been previously reported (Champenois et al., 2008; Kijak et al., 2003; Muñoz et al., 2005; Poonpiriya et al., 2008; Ravela et al., 2003; Snoeck et al., 2006; Vergne et al., 2006), only a few studies have compared discrepancies testing different HIV-1 subtypes and recombinants. In fact, very few studies have included different CRF ranging from 1 to 6 (Poonpiriya et al., 2008; Champenois et al., 2008; Vergne et al., 2006; Snoeck et al., 2006) whereas our work has included sequences from 11 different CRFs. Moreover, to our knowledge, no previous reports have compared as many algorithms as the present work. Some of these studies tried to correlate clade-specific substitutions and discrepancies, as previously described using ANRS algorithm for tipranavir-resistance associated with PR changes M36I, H69K and L89M (Champenois et al., 2008), which are in fact wild-type amino acid in most of the non-B variants. Our work also reports that A98S substitution, wild-type amino acid in CRF14_BG, was interpreted as a nevirapine-resistance marker by the ANRS algorithm.

Finally, HIV-1 rapid subtyping tools can be useful for clade B identification but their efficacy is lower for other variants and differs among tools, as previously reported (Holguín et al., 2008b; Ntemgwa et al., 2008). It can be due to the fact that they assign the subtype by simply applying the similarity method. Phylogenetic analysis is still the only reliable method to correctly assign HIV-1 non-B subtypes and CRF, which are growing in number and complexity. Furthermore, this analysis is complicated to perform in the routine practice. We recommend the use of several rapid subtyping tools instead of only one, in order to compare the results. Furthermore, before entering routine clinical use, rapid subtyping tools should be optimised and updated periodically, including larger numbers of different non-B subtypes and CRF sequences in reference databases. The prediction of subtyping by these tools should be improved before being used in routine clinical settings. Thus, a common global effort is needed for the databases unification in only one rapid subtyping tool to facilitate the rapid and more correct identification of HIV-1 subtypes and CRFs.

In summary, we found a higher prevalence of drug-resistance mutations in non-B *vs*. B subtypes, which reinforces the need to identify HIV-1 variants in drug-resistance surveillance studies. Databases used by online genotypic resistance algorithms and subtyping tools should be optimised and updated periodically by increasing the number of non-B and CRF sequences. This would improve their suitability for the analysis of these variants before their use in routine clinical settings.

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