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Sensitivity of seven HIV subtyping tools differs among subtypes/recombinants in the Spanish cohort of naïve HIV-infected patients (CoRIS)

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ABSTRACT

Background: HIV-1 group M is classified into 9 subtypes and recombinants (CRFs/URFs). Variants other than subtype B (non-B) cause 90% of infections worldwide. HIV is often subtyped using automated tools instead of the gold-standard phylogenetic analysis. We evaluated the reliability of subtyping tools *vs.* phylogeny in a panel of HIV-1 *pol* sequences from the cohort of naïve patients of the HIV/AIDS Spanish Research Network (CoRIS).
Methods: HIV-1 subtyping was performed using seven automated subtyping tools (Stanford, Geno2pheno, Rega, NCBI, EuResist, STAR, TherapyEdge) in HIV-1 *pol* sequences from 670 CoRIS patients previously subtyped by phylogeny (587 subtype B/83 non-B). Sensitivity with respect to phylogeny was assessed.
Results: Most tools correctly classified subtype B, although up to 15% of non-B sequences were wrongly identified as B depending on the tool. For subtype B and CRF02_AG identification, Stanford/NCBI and Geno2pheno/Rega presented the highest/lowest sensitivities, respectively. EuResist and Geno2pheno correctly classified all 13 non-B "pure"subtypes at *pol*. The efficacy of all subtyping tools dropped clearly when identifying recombinants different from CRF02_AG. Only NCBI05, Rega and STAR identified URF, but with very low sensitivities. NCBI classified the highest number of subtypes B as non-B, and overestimated recombinants, especially when including references of 2009.
Conclusions: Automated tools are useful for subtype B identification, although they present serious limitations in classifying variants uncommon in developed regions, especially recombinants. Their sensitivity depends on the prevalence of non-B variants in the population, and decreases drastically when the frequency of recombinants increases. Furthermore, HIV-1 variant distribution differs according to the tool used.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) has been divided in four groups: M (main), O (outlier), N (non-M, non-O) and the recently identified P (Plantier et al., 2009). Most variants are included in group M, which is subdivided into 9 subtypes (A–D, F–H, J, K), at least 45 circulating recombinant forms (CRFs) (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html) and multiple unique recombinant forms (URFs). URFs are widely spread throughout the world, with different recombination breakpoints from those found in CRFs. The global distribution of HIV-1 clades is unequal. Subtype B only accounts for around 10% of the total infections worldwide (Hemelaar et al., 2006), but is prevalent in developed countries, including Western Europe and North America. Thus, most clinical and biological studies are based on this clade. The remaining subtypes and the recombinant forms (grouped as "non-B" variants) have been studied less, even though they cause about 90% of the estimated 33 million HIV

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infections. Among them, subtypes C and A and recombinants CRF01_AE and CRF02_AG are responsible for nearly 70% of all HIV infections. HIV-1 molecular epidemiology studies have revealed an increasing prevalence of non-B subtypes and recombinants in developed countries in the last decade (Wensing et al., 2005; Sagir et al., 2007; Yerly et al., 2007; Frange et al., 2008; Holguín et al., 2008a). In Spain, non-B variants have been increasing among newly diagnosed HIV-1 natives and immigrants in the last years, and their current prevalence is about 15% (Holguín et al., 2008a; Cuevas et al., 2009; De Mendoza et al., 2009), although it is higher when the surveillance studies include a larger immigrant population.

In addition to the epidemiological impact, the presence of non-B subtypes and recombinants has implications for the diagnosis (Candotti et al., 2000), the viral load quantification (Gottesman et al., 2004; Kim et al., 2007; Rouet et al., 2007, 2010; Steegen et al., 2007; Holguín et al., 2008b; Korn et al., 2009; Wirden et al., 2009), the vaccine design (Zhang et al., 2010), the progression to AIDS (Vasan et al., 2006; Baeten et al., 2007) and the cognitive impairment (Sacktor et al., 2009). The genetic peculiarities of non-B variants (Kantor and Katzenstein, 2003; Yebra et al., 2010) could affect the emergence of resistance (Grossman et al., 2004; Gonzalez et al., 2008), the viral replicative capacity (Holguín et al., 2006), the genetic barrier of certain drugs (Van de Vijver et al., 2005), the drug-binding affinity (Kinomoto et al., 2005) and the reliability of algorithms of genetic resistance interpretation (Snoeck et al., 2006; Champenois et al., 2008; Yebra et al., 2010). Thus, a proper detection and description of HIV-1 variants in representative cohorts are of clinical importance. Phylogenetic analysis (phy) is the gold standard method for subtyping and discrimination between subtypes and/or CRFs. However, it is not widely implemented in clinical settings because of its complexity. Several automated subtyping tools have been developed for HIV-1 classification in the clinical routine. They are fast and easy to use, and most are free-of-charge. However, they have considerable limitations *vs*. phy that confound their results especially in the analysis of non-B variants (Smith et al., 2005; Holguín et al., 2008c,d; Ntemgwa et al., 2008; Galán et al., 2009; Wilkinson and Engelbrecht, 2009; Yebra et al., 2010). Furthermore, the results of different tools are usually in disagreement (Gifford et al., 2006; Loveday and MacRae, 2006), especially in the analysis of recombinants.

The objective of the present study was to assess the sensitivity and specificity of seven subtyping tools (Stanford, Rega, Geno2pheno, NCBI, STAR, EuResist and TherapyEdge) in classifying a panel of HIV-1 *pol* sequences (587 subtype B/83 non-B) subtyped by phy from 670 different patients included in CoRIS, a large cohort of HIV-infected treatment-naïve patients in Spain.

2. Materials and methods

2.1. Study population

The Spanish cohort of ARV-naïve HIV-infected patients included in the Research Network on HIV/AIDS (CoRIS) is a multicenter, hospital-based prospective cohort of subjects over 13-years-old seen at 31 hospitals of the 19 Autonomous Regions in Spain from January 2004. This study was approved by a review board and Ethical Committee of the CoRIS Cohort. It was designed to protect the rights of all subjects involved under the appropriate local regulations. About 60% of these patients have started ARV therapy and more than 30% of subjects are immigrants (Caro-Murillo et al., 2007). Out of the 670 patients included in this study, 375 (56%) were Spanish, 181 (27%) were immigrants (118 Central and South Americans, 17 Sub-Saharan Africans, 16 West Europeans, 11 East Europeans, 10 North Africans, 6 North Americans and 3 Asians) and 114 were of unknown origin. Basal *pol* sequences in fasta format were collected including the complete protease (PR) (codons 1–99) and part of the reverse transcriptase (RT) (codons 38–260 or 1–335) from the 670 naïve patients with available sequence.

2.2. HIV-1 variants identified by phy

HIV-1 subtypes and CRF were identified by phylogenetic analysis (phy) of the 670 *pol* sequences. The 2008 version of the subtype reference dataset provided by Los Alamos National Laboratory (available at: http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html) was used. It was updated including more sequences of CRF absent or scarcely represented (26_AU, 30_0206, 32_06A1, 34_01B, 38_BF, 41_CD and 42_BF). Therefore, at least two representative sequences of each 9 subtypes and the 43 CRF of HIV-1 group M available at the moment of the analysis were taken as references. The tree topology was obtained using the Neighbor-Joining method. DNA sequences were aligned using the ClustalX 2.0.11 program. 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, with the bootstrap cut-off set at 700. Out of the 670 sequences, 587 (87.6%) were subtype B, and 83 (12.4%) non-B variants. Only 13 (15.7%) were "pure" non-B subtypes: 1 A1, 3C, 4 F1, 4 F2, and 1 G. The remaining 70 (84.3%) non-B were recombinant. Forty-seven (67.1%) clustered with 12 different CRFs (1 CRF01_AE, 31 CRF02_AG, 2 CRF03_AB, 1 CRF06_cpx, 1 CRF11_cpx, 1 CRF12_BF, 4 CRF14_BG, 1 CRF15_01B, 2 CRF19_cpx, 1 CRF20_BG, 1 CRF28_BF, 1 CRF42_BF) and 23 were URF. They did not cluster to any known subtype/CRF, and presented complex mosaic patterns, including fragments from 8 different subtypes (A, B, C, D, F, G, J and K) and 3 different CRF (01_AE, 02_AG and 03_AB). In more detail, they carried B/CRF02_AG sequences (17.4%), A1/CRF03_AB (13%), B/A1/CRF03_AB (13%), B/F1 (8.7%), B/A1 (8.7%) or others (39.1%).

2.3. Automated HIV-1 subtyping tools

HIV-1 subtyping by seven automated tools was also assessed using all sequences. Six were free-of-charge and available online: Stanford HIVdb 6.0.5, Geno2pheno 3.0, Rega 2.0, NCBI (including both 2005 and 2009 reference sets), EuResist 2009, STAR 2006; however TherapyEdge-HIV 2009 was a commercial tool. They were available at: http://hivdb.stanford.edu/pages/algs/sierra_sequence.html (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 Institute for Medical Research, Leuven, Belgium); http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi (National Center for Biotechnology Information, Bethesda, MD); http://engine.euresist.org/data_analysis/viral_sequence/new (EuResist Project, Rome, Italy); http://www.vgb.ucl.ac.uk/starn.shtml (UCL Division of Infection and Immunity, Royal Free & University College Medical School, London, UK) and http://www.therapyedge.com (TherapyEdge-HIV, ABL, Luxembourg). A discrepancy was considered when the tool assigned a different HIV-1 subtype/CRF than phy. For NCBI tool, we used two different reference sequences' sets (2005 and 2009) in the analysis, because NCBI09 included 22 more CRFs than NCBI05.

Table 1
Sensitivity of the automated subtyping tools *vs.* phy in the 670 HIV-1 *pol* sequences classification.

HIV-1 variants (no.)	% Sensitivity (95% CI)							
	Stanford 6.0.5	Rega 2.0	Geno2pheno 3.0	TherapyEdge 2009	STAR 2006	EuResist 2009	NCBI 2005	NCBI 2009
Subtype B (587)	98.6 (98;99)	85.9 (83;89)	95.9 (94;97)	95.6 (94;97)	95.1 (93;97)	97.6 (96;99)	81.3 (78;84)	48.7 (45;53)
Non-B variants (83)	47 (36;58)	48.2 (37;59)	59 (48;70)	55.4 (45;66)	50.6 (40;61)	57.8 (47;68)	54.2 (43;65)	16.9 (9;53)
Pure non-B subtypes (13)	76.9 (54;100)	92.3 (78;100)	100	92.3 (78;100)	84.6 (65;100)	100	76.9 (54;100)	53.8 (27;81)
CRF02_AG (31)	90.3 (80;100)	71 (55;87)	93.5 (85;100)	83.9 (71;97)	83.9 (71;97)	87.1 (75;99)	77.4 (63;92)	9.7 (−1;20)
CRF non-02_AG (16)	6.2 (−6;18)	12.5 (−4;29)	43.75 (19;68)	50 (25;74)	6.2 (−6;18)	50 (25;74)	31.2 (8;54)	25 (4;46)
URF (23)	0	17.4[a] (2;33)	0	0	17.4[a] (2;33)	0	26.1 (8;44)	0
Total (670)	77.3 (74;80)	81.2 (78;84)	91.3 (89;93)	90.6 (88;93)	89.5 (87;92)	92.7 (91;95)	77.9 (75;81)	44.8 (41;48)

No., number of sequences; CI, confidence interval; CRF, circulating recombinant form; URF, unique recombinant form; Phy, phylogenetic analysis. We considered as correct classification when the subtyping tool assigned the same HIV-1 subtype or CRF as that provided by phylogenetic analysis for each *pol* sequence. Pure non-B subtypes included: 1 A1, 3C, 4 F1, 4 F2 and 1 G. CRF non-02_AG included: 1 CRF01_AE, 2 CRF03_AB, 1 CRF06_cpx, 1 CRF11_cpx, 1 CRF12_BF, 4 CRF14_BG, 1 CRF15_01B, 2 CRF19_cpx, 1 CRF20_BG, 1 CRF28_BF and 1 CRF42_BF (see Table 2). Complex recombinants or URFs not ascribed to any HIV-1 subtype or circulating recombinant (CRF) were defined using a bootscanning method (Simplot) (Yebra et al., unpublished data).

[a] All the URFs whose mosaic patterns were correctly identified by Rega and STAR (4/23 each) were, however, unassigned by each tool.

2.4. Statistical analysis

Sensitivity and specificity were obtained using a calculator available at: http://www.hrc.es/investigacion/bioest/otras_calculadoras.html.

3. Results

3.1. Different sensitivities of automated subtyping tools for HIV-1 variant identification

3.1.1. In the complete cohort

The sensitivity of the seven automated tools for HIV-1 identification when compared to phy differed among HIV-1 variants and tools. Including our entire study cohort ($n = 670$) with predominance of clade B (87.6%), sensitivity exceeded 90% for Geno2pheno, EuResist and TherapyEdge. However, when only the 83 HIV-1 non-B subtypes and recombinants were included, Geno2pheno, EuResist, TherapyEdge and NCBI05 only identified about 55% of them (Table 1). The remaining tools identified a half at best. Of note, a high rate (9.3% and 17.2%, respectively) of the 670 *pol* sequences was not assigned to any subtype/recombinant using STAR and Rega. STAR unassigned 33 (40%) non-B variants and 83 (14.1%) subtypes B and Rega 32 (38.5%) non-B variants and 29 (4.9%) subtypes B. Stanford and TherapyEdge provided the subtype at PR and RT separately, even when the complete pol (PR and RT) in one file was used. Stanford provided very similar sensitivities for HIV-1 variant detection in PR/RT meanwhile TherapyEdge subtyped RT slightly better than PR (data not shown).

3.1.2. In HIV-1 pure subtypes

For subtype B ($n = 587$) identification, Stanford presented the highest sensitivity (98.6%) and only misclassified 6 subtypes B (Table 1), but only in PR and not in RT. The specificity of subtyping tools was also high, ranging from 84.3% (Geno2pheno) to 100% (Rega). However, we observed misclassifications. In fact, all rapid subtyping tools, except Rega and STAR, identified as non-B variants from 1.4% (Stanford) to 51.3% (NCBI09) of the 587 subtype B sequences defined by phy. NCBI presented the highest overestimation of non-B variants, since it only correctly classified 71.3% or 48.7% of the subtype B sequences defined by phy when the 2005 or 2009 versions were used, respectively (Table 1). In the remaining cases, NCBI included regions of other subtypes or CRF besides subtype B, especially in the case of NCBI09.

For non-B pure subtypes ($n = 13$, including A1, C, F1, F2 and G), the rate of correct identifications was high as well. EuResist and Geno2pheno correctly classified all of them. The sensitivity was 77–92% in the remaining tools (Table 1). The specificity was also high (>99%) in most cases. Of note, among the 24 sequences misclassified by Geno2pheno, 19 were assigned to subtype D. TherapyEdge provided 4.4% of false non-B variants, 11 of them subtyped as D^{PR}/B^{RT}. Thus, Geno2pheno and TherapyEdge could overestimate subtype D, which has been demonstrated to have a faster progression to AIDS and a higher pathogenicity (Vasan et al., 2006; Baeten et al., 2007; Sacktor et al., 2009).

3.1.3. In HIV-1 recombinant forms: CRFs and URFs

All subtyping tools presented a high sensitivity for CRF01_AE and CRF02_AG detection, although 7–29% of the 31 CRF02_AG were underestimated using subtyping tools excluding NCBI09, which presented important limitations in detecting both variants (Table 1). The specificity for CRF01_AE and CRF02_AG was very high, ranging from 98.4% (EuResist) to 100% (NCBI'05) and 99.7% (EuResist) to 100% (Rega and NCBI09), respectively.

However, the efficacy of all subtyping tools clearly dropped when identifying recombinants different from CRF02_AG (Table 2). Among the 15 *pol* sequences ascribed by phy to 10 different CRFs non-CRF01_AE, non-CRF02_AG, automated tools failed to detect most of them, although EuResist, TherapyEdge, and Geno2pheno provided the best results (Table 1).

Regarding the identification of the complex recombinant mosaics or URF ($n = 23$) previously characterized in our cohort by phylogenetic analysis and bootscanning, only NCBI05, Rega and

Table 2
Subtyping of HIV-1 recombinant forms by automated tools.

HIV-1 recombinants by phy (no.)	Correct identifications (no.) by each subtyping tool *vs.* phy
CRF01_AE (1)	EuR, G2p, NCBI05, Rega, Stanf, STAR, and TE (1)
CRF02_AG (31)	G2p (29); Stanf (28); EuR (27); STAR and TE (26); NCBI05 (24); Rega (22); NCBI09 (3)
CRF03_AB (2)	None
CRF06_cpx (1)	EuR, G2p and TE (1)
CRF11_cpx (1)	TE (1)
CRF12_BF (1)	G2p, NCBI05, Rega and TE (1)
CRF14_BG (4)	EuR, G2p (4); NCBI05, NCBI09 and TE (3)
CRF15_01B (1)	NCBI05 (1)
CRF19_cpx (2)	EuR (2); TE (1)
CRF20_BG (1)	NCBI09 (1)
CRF28_BF (1)	None
CRF42_BF (1)	None
URF (23)	NCBI05 (6); Rega and STAR (4)

No., number of sequences; CRF, Circulating Recombinant Form; URF, Unique Recombinant Form; EuR, EuResist 2009; G2p, Geno2pheno 3.0; Stanf, Stanford-HIVdb 6.0.5; TE, TherapyEdge-HIV 2009; NCBI05 and NCBI09, versions with reference datasets of 2005 and 2009 respectively; Rega, Rega version 2.0; STAR, STAR version 2006.

HIV-1 Variant (no.)	PHY	Stanford	Rega	Geno2pheno	TherapyEdge	STAR	EuResist	NCBI05	NCBI09
Subtype B (587)	87.6	87.3	75.2	86	84.5	83.4	87.3	71.3	42.7
"Pure" non-B subtypes (13)	1.9	2.2	3.9	5.8	2.2	2.8	3.9	1.6	1
CRF02_AG (31)	4.6	4.3	3.3	4.5	4	4	4.3	3.7	0.4
CRF non-02 (16)	2.4	0.6	0.4	3.7	1.6	0.4	4.5	1.2	0.6
URF (23)	3.4	5.5	-	-	7.6	-	-	22.1	55.2
Not Determined	-	-	17.2	-	-	9.3	-	-	-

Fig. 1. Distribution of HIV-1 variants in the CoRIS cohort according to each subtyping method. PHY, phylogenetic analysis considered the gold standard method to classify HIV-1; no., number of sequences; CRF, circulating recombinant forms; URF, unique recombinant forms. The automated tools included were: Stanford HIVdb 6.0.5, Rega 2.0, Geno2pheno 3.0, TherapyEdge-HIV 2009, STAR 2006, EuResist 2009 and NCBI (including both set of references from 2005 and 2009). Stanford and TherapyEdge provided independent results for protease and reverse transcriptase, and the cases with different results in the two regions were considered as URF. For NCBI, the *pol* sequences including regions ascribed to different HIV-1 subtypes and/or CRF were considered as URF.

STAR were capable of identifying URF, but with very low sensitivities (Table 1). Rega and STAR unassigned 15 and 16 of the 23 URF, respectively, and the remaining URF were wrongly classified as pure subtypes or CRF using both tools. Nevertheless, Rega correctly showed the subtypes involved in the recombinants in 4 unassigned URF (2 URF_BF1, 1 BA1, 1 BC) and so did STAR in 4 other unassigned URF (3 URF_B02, 1 BC). On the other hand, NCBI05 correctly detected 6 URF (3 URF_02B, 1 03B, 1 BG02 and 1 BC). The URF_BC was the only one correctly identified by the three tools.

3.2. HIV-1 non-B variants identified as subtype B by automated subtyping tools

A considerable rate (1–16%) of the 83 non-B variants by phy was wrongly identified as subtype B by automated subtyping tools, with the exception of NCBI09 and Rega (Table 2). Among the 83 non-B variants, Geno2pheno identified as subtype B 16% of cases, EuResist 14%, TherapyEdge 6%, Stanford 5% and STAR and NCBI05, 1%. Therefore, Geno2pheno and EuResist presented the highest overestimation of subtype B, and NCBI and Rega the lowest.

3.3. HIV-1 variant distribution in CoRIS according to the subtyping tool

The distribution of HIV-1 subtypes and recombinants among the 670 patients from CoRIS was different according to each tool (Fig. 1). In STAR and Rega, a high rate (9 and 17%, respectively) of sequences was not assigned to any subtype/CRF. The differences between tools were especially pronounced in CRFs non-02_AG: present in 2.4% of patients according to phy, but $\leq$0.6% for STAR, Stanford and Rega, and almost fivefold for EuResist. Finally, although URF represented only 3.4% of cohort by phy, this estimation was 55.2% for NCBI09 and 22.1% for NCBI05. For TherapyEdge and Stanford, different results in PR and RT were considered as URF (7.6% and 5.5%, respectively).

4. Discussion

4.1. Reliability of automated subtyping tools

HIV-1 subtyping is of clinical importance, as previously mentioned. The correct identification of non-B variants is important due to their high prevalence in pandemics and their increasing rate in industrialized countries by population movements from areas where non-B variants are epidemic (Wensing et al., 2005; Yerly et al., 2007; Holguín et al., 2008a). In fact, a third of newly HIV-diagnosed cases in Spain are immigrants (Caro-Murillo et al., 2009). Since the HIV/AIDS pandemic tends to constantly grow in complexity (Zhang et al., 2010) by the increasing spread of CRF and URF favored by coinfections and/or superinfections, the expansion of recombinant viruses has complicated the HIV-1 subtyping. Thus, an improvement in complex recombinant variants detection is strongly recommended. Phylogeny (phy) is considered the gold-standard method for HIV subtyping. However, since it can be laborious and complex, automated tools have been developed. Although useful for subtype B identification, they present limitations (Smith et al., 2005; Gifford et al., 2006; Loveday and MacRae, 2006; Ntemgwa et al., 2008; Galán et al., 2009; Wilkinson and Engelbrecht, 2009; Yebra et al., 2010).

This study compared the sensitivity of seven widely used HIV-1 subtyping tools [Stanford, Geno2pheno, Rega, NCBI (2005 and 2009 versions), EuResist, STAR and TherapyEdge] with respect to phy in the identification of subtypes and recombinants in a large and representative Spanish HIV-infected Cohort (CoRIS). To our knowledge, this work includes the highest numbers of tools, HIV-1 sequences and different CRFs among the studies which compare results of automated tools with phylogeny (Table 3).

We have shown that the sensitivity of certain automated subtyping tools (especially EuResist and Geno2pheno) in large cohorts of HIV-1 infected patients can be very high if subtype B is predominant, as in our cohort. This good sensitivity is due to the overrepresentation of subtype B in the databases because of its dominance in the HIV epidemic in developed countries, where more *pol* sequences are routinely available for resistance testing (Table 3). However, we observed important failures in the subtype B identification for specific tools. Most of the automated subtyping tools provided, to a greater or lesser extent, false subtype B among the non-B sequences, overestimating the rate of subtype B isolates in a given population. In more detail, between 10 and 15% of non-B sequences were identified as subtype B when using Geno2pheno, EuResist, and the separate analysis of PR and RT by Stanford and TherapyEdge.

For pure non-B subtypes identification, the best tools were also Geno2pheno and EuResist (sensitivity 100%). Previous reports have also described specific limitations in pure non-B subtypes identification using online subtyping tools. For instance, subtype D was usually underestimated using Rega (Gifford et al., 2006; Holguín et al., 2008c) but overestimated using Geno2pheno, Stanford (Galán et al., 2009) and TherapyEdge due to its confusion with subtype B especially in the PR, as our study confirmed. The misclassification of subtype D is of special relevance because this variant seems to be more pathogenic than others (Vasan et al., 2006; Baeten et al., 2007; Sacktor et al., 2009). An inadequate detection of subtype G by STAR (Gifford et al., 2006), subtype J by Rega (Gifford et al., 2006; Holguín et al., 2008c) and subtype A by Stanford (Gifford et al., 2006) has also been published.

Focusing on recombinant variants, our data demonstrate that the sensitivity of the automated tools decreases drastically when there is an increase in non-B recombinants other than CRF01_AE

Table 3
Studies of the limitations of automated subtyping tools which include sequences of HIV-1 non-B variants.

Author	Country	Number of HIV-1 sequences analyzed (number of different variants)	Viral region	Subtyping method included										
				Phy	Stanford	Rega	Geno2pheno	Therapy Edge	STAR	EuResist	NCBI	jpHMM	LANL	Virco
Present study	Spain	83 non-B sequences (5 subtypes, 12 CRF) 587 subtype B	*Pol*	√	√	√	√	√	√	√	√			
Yebra et al. (2010)	Spain	128 non-B (6 subtypes, 11 CRF) 226 subtype B	*Pol*		√	√	√			√	√			
Wilkinson and Engelbrecht (2009)	South Africa	10 non-B (3 subtypes, 7 URF) 1 subtype B	Nearly full genome	√		√						√		
Galán et al. (2009)	Spain	56 non-B (5 subtypes, 8 CRF, 11 URF, 3 U) 14 subtype B	*Pol*		√	√	√				√			√
Holguín et al. (2008c)	Spain	277 PR/171 RT non-B (8 subtypes, 9 CRF) 33 subtype B	*Pol*		√	√					√			
Holguín et al. (2008d)	Spain	5 non-B (1 CRF, 4 URF)	Nearly full genome	√	√	√					√			
Ntemgwa et al. (2008)	Canada	4 sequences of the same URF	*Pol*	√	√	√	√		√			√		√
Loveday and MacRae (2006)	UK	1,002 non-B and B	*Pol*		√	√			√		√		√	
Gifford et al. (2006)	UK	10,503 PR/10,476 RT non-B and B	*Pol*		√	√			√					
Smith et al. (2005)	UK	81 non-B (5 subtypes, 2 CRF) 19 subtype B	*Env*	√							√		√	

UK, United Kingdom; PR, protease; RT, reverse transcriptase; Pol, region including complete PR and partial RT; U, unclassified; CRF, circulating recombinant form; URF, unique recombinant form; Ref, corresponding reference; Phy, phylogenetic analysis (the gold standard method to classify HIV variants); jpHMM, jumping profile Hidden Markov Model (available at: http://jphmm.gobics.de); LANL, Los Alamos National Laboratory RIP or BLAST tools (available at: http://www.hiv.lanl.gov). The "tick" symbol indicates the inclusion of the corresponding subtyping method in each study.

and CRF02_AG, as they are absent or scarcely represented in the databases of these tools. For instance, neither STAR nor Stanford could subtype any single CRF other than 01_AE and 02_AG (Table 2), reflecting the lack of sufficient sequences in their databases. Regarding URFs, subtyping tools were inefficient in their detection. Only NCBI05, Rega and STAR were capable of identify URF, but with very low sensitivity. Our study presents a low number of non-01, non-02 CRFs ($n = 15$), due to their low prevalence in Spain, which is probably insufficient to extract a conclusion with statistical support. However, our results agree with other studies (Holguín et al., 2008c; Galán et al., 2009; Yebra et al., 2010), and the presented data could be very useful in countries where complex recombinants are more frequent.

In addition, our data reveals that the HIV-1 subtype distribution in any study cohort is different according to the automated subtyping tool used. In other words, the results of a molecular epidemiology study would change depending on the tool used and on the prevalence of non-B variants, especially recombinants (Fig. 1).

4.2. Technical differences among subtyping tools

The described discrepancies between various automated tools *vs.* phy could be explained by differences in both the subtyping methods applied and the reference datasets included. HIV-1 variants were defined by phy using the Neighbor-Joining (NJ) method. Among the automated tools assessed here, only Rega was based on phylogeny, also applying NJ combined with bootscanning. However, this tool has a threshold which prevents the assignation of a subtype/CRF when it does not obtain enough statistical support. This restricts its efficacy despite using phylogeny. It is remarkable that the highest sensitivity was obtained by tools which performed a simple BLAST search, assigning to the query sequence the subtype/CRF of the most similar reference in their databases (Geno2pheno, EuResist, TherapyEdge, Stanford and NCBI). In particular, NCBI uses a sliding-window along the query sequence and each window is compared to the references by BLAST. However, this sliding-window causes an overestimation of recombination, magnified when more reference sequences are included as we reported comparing 2005 *vs.* 2009 versions. With this method, the inclusion of multiple references which share similarity confounded the results instead of improving them. In several cases when NCBI05 assigned a specific subtype/CRF, NCBI09 provided a mixture of subtypes/CRFs with common regions. Some CRFs are very close and difficult to discriminate in the studied region and it is thus extremely difficult to obtain reliable results with systems based on Blast analysis. In contrast, STAR is a statistical method that uses position-specific scoring matrices of each subtype to perform profile subtype alignments. But, as well as Rega, there are many cases where STAR does not assign any subtype if the assignment's *P*-score does not reach the threshold. The different reference sets used by each tool are also important in the discrepancies. Meanwhile STAR and Stanford excluded any CRF different from CRF01_AE and CRF02_AG, Rega and Geno2pheno included up to CRF14_BG and TherapyEdge up to CRF19_cpx. Only NCBI09 includes references of the CRFs as they are described (at the moment of the analyses, there were 45 different CRFs).

4.3. Important considerations

Several main considerations should be considered for understanding the incorrect HIV-1 subtyping using automated subtyping tools in a given population. First, the rate of non-B variants in the study cohort; second, the rate of recombinants other than CRF01_AE and CRF02_AG among these non-B variants; third, the automated tool used for subtyping; fourth, the rate of non-B variants wrongly identified as subtype B and *vice versa*, which underestimates the

prevalence of non-B or B variants; fifth, the high rate of sequences (especially non-B) unassigned in the cases of STAR and Rega.

Thus, our data reveals that subtyping tools should be regularly updated as is done for resistance mutations before their use in routine clinical settings by increasing the number of non-B/CRF sequences to improve their detection. In light of our results, when the use of phylogenetic analysis is not available we would suggest EuResist, Geno2pheno and/or TherapyEdge as the best subtyping tools for cohorts with predominance of clade B as in our cohort. Despite the great specificity of Rega and STAR for subtype B, too many sequences were unassigned. On the other hand, in areas where non-B variants and especially recombinant forms are prevalent, none of the tools evaluated here would be sufficiently reliable. In these cases, the use of several subtyping tools instead of just one is recommended in order to compare their results. HIV-1 subtypes and predominant recombinants need to be identified with tools providing high specificities and sensitivities and subsequent phylogenetic analysis is recommended on samples that cannot be classified.

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