Genotypic drug resistance interpretation algorithms display high levels of discordance when applied to non-B strains from HIV-1 naive and treated patients

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Abstract

Genotypic drug resistance interpretation algorithms have been developed on patients infected with HIV-1 subtype B to interpret complex patterns of mutations. As non-B strains are characterised by the natural presence of several resistance-related mutations, we examined to what extent this might result in interalgorithm discordances in naive and treated patients. We compared the prediction by three algorithms (ANRS, Stanford and Rega) of drug susceptibilities to diverse HIV-1 strains from 272 naive and 156 treated patients. In naive patients, higher levels of interalgorithm discordance were observed for predictions of protease inhibitor (0.60–39%) than for predictions of reverse transcriptase inhibitor susceptibility (0–4%). The main reason for discordant protease inhibitor interpretation was the presence of resistance mutations that were natural protease polymorphisms. In contrast, in the treated patients, more interalgorithm discordances were observed for predictions of reverse transcriptase inhibitor (5–48%) than protease inhibitor susceptibilities (10–31%). Discordances were related to disagreement between the intermediate and susceptible scores, the intermediate and resistant scores and the interpretations of complex mutation patterns, related to cross-resistance and antagonistic interactions.

Introduction

International guidelines have recommended HIV drug resistance testing for the selection of optimal antiretroviral (ARV) therapy (Hirsch *et al.*, 2003) and resistance testing is now available as a routine tool for the care and management of HIV disease (Shafer, 2002). Genotypic resistance tests are more commonly used than phenotypic tests because of their easier implementation, lower cost and shorter turnaround time. However, genotypic mutation patterns, especially those related to cross-resistance and antagonistic interactions, require expert interpretation for a meaningful application in a clinical context. Several rules-based algorithms have been developed by correlating genotypic patterns with clinical data and/or by combining genotypic with phenotypic data and most of them are accessible through the web (Parkin *et al.*, 2002), but they can produce conflicting predictions of the therapeutic response (De Luca *et al.*, 2003; Sturmer *et al.*, 2003; Zazzi *et al.*, 2004; Ravela *et al.*, 2003).

Phylogenetic analyses revealed that HIV-1 can be divided into three groups: M (Major), N (New) and O (Outlier). Group M, which accounts for most infections worldwide, is further subdivided into nine subtypes (A–D, F–H, J–K) and 16 Circulating Recombinant Forms (CRF01–16) (Robertson *et al.*, 2000). Subtype B isolates predominate in North America and Europe, but represent only a limited proportion of infections worldwide (Peeters *et al.*, 2003). Nonetheless, genotypic drug resistance interpretation algorithms have been based mainly on results obtained from subtype B-infected patients. However, minor mutations occur as natural variants in non-B strains (Pieniazek *et al.*, 2000; Vergne *et al.*, 2000; Kantor & Katzenstein, 2003), and certain subtypes can select specific mutations under drug pressure (Gomes *et al.*, 2002; Loemba *et al.*, 2002; Brenner *et al.*, 2003; Grossman *et al.*, 2004). Antiretroviral drugs have now been introduced in developing countries where non-B variants predominate. The frequency of non-B isolates is also increasing in industrialized countries. It is thus necessary to

study to what extent the different genotypic drug resistance interpretation algorithms can predict treatment efficiency in patients infected with a non-B HIV-1 strain. We therefore compared three different algorithms for a large number of non-B treatment-naive and treated patients to examine the influence of pre-existing polymorphisms on predictions of drug susceptibilities and the subsequent choice of therapy.

Materials and methods

Patients

Samples from 428 HIV-1-infected patients were studied: 306 Africans attending hospitals in Cameroon ($n = 147$), Senegal ($n = 91$), Democratic Republic of Congo (DRC) ($n = 38$) and Gabon ($n = 30$); and 122 patients (46 Africans and 76 Europeans) attending the University Hospital in Montpellier, France (Vergne *et al.*, 2002, 2003). Of these 428 patients, 272 were antiretroviral (ARV) drug-naive and 156 were treated. Patients received different combinations of ARV drugs: nucleoside reverse transcriptase inhibitor (NRTI) only ($n = 29$; 18.6%), non-nucleoside reverse transcriptase inhibitor (NNRTI) only ($n = 16$; 10.2%), NRTI+protease inhibitor (PI) ($n = 49$; 31.4%), NRTI+NNRTI ($n = 20$; 12.8%), PI+NRTI+NNRTI ($n = 26$; 16.7%). The exact treatment regimen was unknown for 10.3% of the treated patients.

Sequences

Viral RNA or proviral DNA was extracted from plasma or peripheral blood mononuclear cells (PBMC) using the QIAamp Viral RNA kit or the QIAamp Blood and Tissue kit (Qiagen, Courtaboeuf, France), respectively. RNA was transcribed into cDNA with the reverse primer IN3. DNA or cDNA was amplified by a nested PCR using the Expand High Fidelity PCR system (Roche, Meylan, France) with outer primers G25REV and IN3 and inner primers AV150 and polM4 (5′-CTATTAGCTGCCCCATCTACATA-3′) (Vandamme *et al.*, 1998; Vergne *et al.*, 2000). The amplified fragments, encompassing protease (99 amino acids) and RT (310 amino acids), were purified with a QIAquick Gel Extraction kit (Qiagen) and directly sequenced using a BigDye® Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Courtaboeuf, France).

Phylogenetic analyses

Genetic subtypes were determined by phylogenetic tree analysis. The new nucleotide sequences and sequences from reference strains representing the different genetic subtypes were aligned with the CLUSTAL W program, using the protein sequences as a guide (Los Alamos National Laboratory, 2003). Phylogenetic trees were constructed with the neighbor-joining method and the reliability of branching orders obtained with the bootstrap approach was implemented by CLUSTAL W (Saitou & Nei, 1987). The *pol* sequences were further investigated by bootscan and similarity analyses using SIMPLOT software to determine whether they were recombinant or classified into a known CRF (CRF01–CRF15) (Ray, 2003).

Genotypic drug resistance interpretation algorithms

Amino acid sequences were analysed for mutations associated with a reduced susceptibility to antiretroviral drugs by comparing the new *pol* sequences to a subtype B consensus sequence derived from an alignment of subtype B sequences maintained at the Los Alamos HIV Sequence Database (Los Alamos National Laboratory, 2003).

Drug susceptibilities and treatment efficiencies to PIs, NRTIs, and NNRTIs were predicted using three different algorithms: Rega 5.5 (2002), Stanford database algorithm (HIVDB) (2003.08), and French national guidelines (ANRS 2002.3), implemented at the Stanford website in 2003 (Van Laethem *et al.*, 2002; Shafer, 2003; Rhee *et al.*, 2003). Each algorithm reports its results differently. For the ANRS and Rega algorithms, viruses are scored on three levels of resistance: susceptible (S), intermediate resistant (I) and resistant (R). The HIVDB algorithm defines five levels of drug resistance: susceptible, potential low-level resistance, low-level resistance, intermediate resistance and high-level resistance. In this study a normalized comparison was established ('SIR option'). Susceptible and potential low-level resistance were considered to be susceptible (S), low-level resistance to be intermediate resistant (I) and intermediate resistance and high-level resistance to be resistant (R). Interpretations were considered concordant for a particular drug when the three algorithms assigned the same level of resistance (S, I or R) to a given sequence. Interpretations were considered discordant for a particular drug when at least one of the three algorithms assigned a different level of resistance (all possible combinations of SSI, SSR, IIS, IIR, RRS, RRI and SIR). In addition, we analysed the interpretation discordances using a two-level system where the intermediate resistance level alternatively was considered susceptible or resistant (all possible combinations of SSR and SRR).

Statistical analyses

Statistical analysis was performed using the SAS software package version 8. One-way ANOVA with Tukey confidence intervals was used to determine whether the number of discordances was drug-dependent or subtype-dependent, or both. Frequencies of mutations were compared using Fisher's exact test.

Results

Phylogenetic analyses of the protease and RT sequences

In the treatment-naive population, the 272 samples represented the following HIV-1 variants in decreasing order of importance: CRF02_AG ($n = 122$, 44.9%), A ($n = 24$, 8.8%), G ($n = 23$, 8.5%), B ($n = 21$, 7.7%), F ($n = 17$, 6.2%), D ($n = 16$, 5.9%), C ($n = 14$, 5.1%), CRF11_cpx ($n = 10$, 3.7%), CRF01_AE ($n = 8$, 2.9%), CRF06_cpx ($n = 5$, 1.8%), CRF13_cpx ($n = 5$, 1.8%), J ($n = 4$, 1.5%), CRF05_DF ($n = 2$, 0.8%) and K ($n = 1$, 0.4%). In the treated population, the 156 samples were: CRF02_AG ($n = 60$, 38.5%), B ($n = 50$, 32.1%), A ($n = 14$, 9.0%), D ($n = 8$, 5.1%), C ($n = 6$, 3.8%), F ($n = 4$, 2.6%), CRF11_cpx ($n = 4$, 2.6%), G ($n = 3$, 1.9%), CRF01_AE ($n = 2$, 1.3%), CRF13_cpx ($n = 2$, 1.3%), H ($n = 1$, 0.6%), J ($n = 1$, 0.6%) and CRF06_cpx ($n = 1$, 0.6%). More than 83% of the strains were non-B HIV-1 variants; all subtypes and five CRFs were represented. Subtype B was more frequent in the treated population, and was mainly from highly treated patients in France. Overall, CRF02_AG isolates predominated (42.5%) and the population studied represents the HIV-1 variants that circulate in West and West Central Africa.

Genotypic drug resistance interpretation of patient sequences by three algorithms

The results of the analysis of patient amino acid sequences with three different interpretation algorithms are presented in Table 1. The majority of sequences from treatment-naive patients were interpreted as susceptible by all algorithms. Rega 5.5 scored more sequences as intermediate resistant towards PIs, whereas hardly any sequence was scored intermediate resistant by ANRS. In the treated patient population, the majority of sequences were still scored as susceptible. However, the number of intermediate resistant and resistant scores increased for all algorithms. Again, Rega 5.5 more frequently scored intermediate resistant for PIs, and also for RTIs. For 37.8% of the patients failing therapy, no resistance-associated mutations were detected. A more detailed analysis of their clinical history revealed that this was mainly due to poor observance or interruption of ARV treatment due to major side effects or financial constraints.

Comparison of three genotypic drug resistance interpretation algorithms in ARV treatment-naive patients

Subsequently, the interalgorithm discordances were investigated more in detail (Tables 2 and 3). The proportion of sequences displaying discordant interpretations was higher for the PI (0.6–39%) than for the RTI susceptibility scores (0–4%). For PIs, 39% of the sequences were discordant for ritonavir, 38% for indinavir, 19% for nelfinavir, 12% for amprenavir, 12% for saquinavir, and only 0.6% for lopinavir (Table 2). In an overall PI drug comparison the differences were significant ($P < 0.0001$). Lopinavir displayed significantly less discordance, whereas ritonavir and indinavir displayed significantly higher discordance in a pairwise comparison with the other PIs. For ritonavir and indinavir, subtypes A and F displayed significantly more discordance than B. For amprenavir, subtype F and for saquinavir, F, K and CRF05 displayed significantly more discordance than B. As only a limited number of sequences were available for subtypes K (one strain) and CRF05 (two strains), care should be taken not to overvalue the results of the analysis of these subtypes. When the intermediate score was assigned to the susceptible score, the discordances decreased for PIs and the three tested algorithms had a very good agreement on PIs. Only subtype CRF13 still displayed significantly more discordance than subtype B for ritonavir and indinavir. When the intermediate level was considered resistant, similar results as in the three-level comparison were obtained, involving the same subtypes. This indicates that the main problem was associated with the distinction between intermediate and susceptible.

For RTIs, interalgorithm discordances were low overall, but were higher for NNRTIs (2.2–4%) than for NRTIs (0–1.8%) (Table 3). In an overall RTI drug comparison the discordances were significant ($P < 0.0001$). Nevirapine displayed a significantly higher level of discordance (4%) than the other RTIs in a pairwise analysis. No discordances were observed for lamivudine and didanosine. For all of the RTIs, except zalcitabine, the proportion of discordances did not differ significantly between the subtypes. Only CRF11 revealed significantly higher levels of discordances for zalcitabine than subtype B. The subanalysis, with only two susceptibility scores, showed that for most tested RTIs, except zidovudine, nevirapine and delavirdine, the discordances were related to interalgorithm disagreement between the intermediate and susceptible scores.

Comparison of three genotypic drug resistance interpretation algorithms in ARV-treated patients

In contrast to the treatment-naive patient population, the proportion of interalgorithm discordances for treated patients displayed a bigger range for the RTI (5–48%) than for the PI susceptibility scores (10–31%). For PIs, 31% of the strains were discordant for ritonavir, 27% for indinavir, 22% for nelfinavir, 20% for amprenavir, 10% for saquinavir and 25% for lopinavir (Table 4). Differences were significant

Table 1. Genotypic drug resistance interpretation of protease and reverse transcriptase sequences obtained from treatment-naive and treated patients

	ANRS 2002.3			Stanford HIVDB 2003.08			Rega 5.5 2002		
	S	I	R	S	I	R	S	I	R
Treatment-naive									
PI									
Amprenavir	272			266	6		245	27	
Indinavir	267	1	4	267	5		172	96	4
Lopinavir	272			270	2		272		
Nelfinavir	271		1	266	3	6	220	51	1
Ritonavir	272			267	5		172	96	4
Saquinavir	271		1	271		1	242	30	
RTI									
Lamivudine	272			272			272		
Abacavir	272			269	3		272		
Zidovudine	272			267	2	3	269	2	1
Stavudine	271		1	268	3	1	271		1
Zalcitabine	272			271	1		272		
Didanosine	272			272			272		
Tenofovir	272			269	3		272		
Delavirdine	268		4	266	2	4	259	2	11
Efavirenz	270		2	268	2	2	265	5	2
Nevirapine	270		2	267	3	2	261	2	9
Treated									
PI									
Amprenavir	138		18	116	8	32	110	24	22
Indinavir	116	3	37	117	4	35	80	36	40
Lopinavir	141	15		117	13	26	136	15	5
Nelfinavir	115	5	36	111	2	43	87	28	41
Ritonavir	122	6	28	117	4	35	80	36	40
Saquinavir	120	1	35	118	2	36	107	13	36
RTI									
Lamivudine	101	2	53	95	5	56	90	16	50
Abacavir	114	28	14	68	26	62	78	41	37
Zidovudine	90		66	84	6	66	86	7	63
Stavudine	93		63	86	11	59	102	16	38
Zalcitabine	137	2	17	73	34	49	86	46	24
Didanosine	107	2	47	73	37	46	88	54	14
Tenofovir	109	4	43	92	12	52	107	41	8
Delavirdine	123		33	124	4	28	115	4	37
Efavirenz	118		38	118	4	34	116	11	29
Nevirapine	118		38	118	1	37	111	2	43

PI, protease inhibitors; RTI, reverse transcriptase inhibitors; S, number of sequences that were interpreted as susceptible (S) by the respective algorithm for a particular drug; I, number of sequences that were interpreted as intermediate resistant (I) by the respective algorithm for a particular drug; R, number of sequences that were interpreted as resistant (R) by the respective algorithm for a particular drug.

when an overall PI drug comparison was performed ($P < 0.001$). Saquinavir displayed a significantly lower level of discordance when compared to ritonavir, indinavir and lopinavir in a pairwise analysis but not when compared to nelfinavir and amprenavir. For indinavir and amprenavir, subtype F displayed significantly higher levels of discordances than subtype B. For saquinavir, this was subtypes D, F and H. When the intermediate score was assigned to the susceptible score, the proportions of discordances in the treatment group decreased, though not as significantly as for the treatment-naive samples. These subanalyses showed that the main problem was still associated with the distinction between intermediate resistant and susceptible, but that the disagreement on intermediate resistant and resistant also made a substantial contribution to the overall level of discordances, especially for amprenavir and lopinavir. Subtype H and subtypes G and CRF13 displayed significantly higher levels of discordances than B in the I = S subanalysis for nelfinavir and saquinavir scores, respectively. As only a limited number of sequences were included in the analysis of subtypes H, G and CRF13 , the results regarding these subtypes should be interpreted with care.

Table 2. Interalgorithm discordances (%) between genotypic drug resistance interpretations of protease sequences obtained from treatment-naive patients infected with B and non-B HIV-1 subtypes

	Total analysis including 3 susceptibility scores (S, I, R)		Subanalysis including 2 susceptibility scores (S, R)			
			I score assigned to S score (I = S)		I score assigned to R score (I = R)	
PI	Discordances (%)*,†	Subtypes ‡,§	Discordances (%)†	Subtypes ‡,§	Discordances (%)*,†	Subtypes ‡,§
Ritonavir	**39**	+ (A, F)	1.5	+ (CRF13)	**37**	+ (A, F)
Indinavir	**38**	+ (A, F)	1.5	+ (CRF13)	**36**	+ (A, F)
Nelfinavir	19	+	0.70	–	19	+
Amprenavir	12	+ (F)	0	–	12	+ (F)
Saquinavir	12	+ (F, K, CRF05)	0.40	–	11	+ (F, K, CRF05)
Lopinavir	*0.60*	–	0	–	*0.70*	–

*Proportions of sequences that displayed discordances were significantly different when performing an overall drug comparison ($P < 0.0001$).
†Proportions of discordances that did not differ significantly from each other in a pairwise analysis are displayed in an identical font style (bold, regular or italic).
‡+, indicates that the proportion of discordances differed significantly between all subtypes for a respective drug ($P < 0.05$); –, indicates that the proportion of discordances did not differ significantly between all subtypes for respective drug.
§Subtypes that displayed significantly more discordances than subtype B for the susceptibility scoring to the respective drug are in brackets.

In contrast to the treatment-naive patient population, the proportions of discordances obtained for NNRTIs (5–8.3%) were lower than those for NRTIs (6–48%) in the treated patient population (Table 5). Differences were significant in an overall RTI drug comparison ($P < 0.0001$). Abacavir, tenofovir, zalcitabine and didanosine displayed significantly higher levels of discordances in a pairwise comparison with zidovudine, stavudine, lamivudine, nevirapine, delavirdine and efavirenz. The proportion of discordances did not differ significantly between the subtypes for the zidovudine, stavudine and delavirdine scores. CRF06 revealed significantly higher levels of discordances for nevirapine and efavirenz than subtype B. However, as only one strain belonged to CRF06, one should take care not to overvalue this result. Subtype C was associated with significantly higher proportions of discordances for lamivudine than was subtype B. The results of the sub-analysis with only two susceptibility scores showed that the observed discordances were related to interalgorithm disagreement between the intermediate and susceptible scores, as well as between the intermediate and resistant scores.

Table 3. Interalgorithm discordances (%) between genotypic drug resistance interpretations of reverse transcriptase sequences obtained from treatment-naive patients infected with B and non-B HIV-1 subtypes

	Total analysis including 3 susceptibility scores (S, I, R)		Subanalysis including 2 susceptibility scores (S, R)			
			I score assigned to S score (I = S)		I score assigned to R score (I = R)	
NRTI	Discordances (%)*,†	Subtypes‡,§	Discordances (%)*,†	Subtypes‡,§	Discordances (%)†	Subtypes‡,§
Zidovudine	1.8	–	1.1	–	1.5	+
Abacavir	1.1	–	*0*	–	1.1	–
Tenofovir	1.1	–	*0*	–	1.1	–
Stavudine	1.1	–	*0*	–	1.1	–
Zalcitabine	0.4	+ (CRF11)	*0*	–	0.4	+ (CRF11)
Lamivudine	*0*	–	*0*	–	0	–
Didanosine	*0*	–	*0*	–	0	–
NNRTI						
Nevirapine	**4.0**	–	**2.6**	–	1.8	–
Delavirdine	3.7	–	**2.6**	–	1.5	–
Efavirenz	2.2	–	*0*	–	2.2	–

*Proportions of sequences that displayed discordances were significantly different when performing an overall drug comparison ($P < 0.0001$).
†Proportions of discordances that did not differ significantly from each other in a pairwise analysis are displayed in an identical font style (bold, regular or italic).
‡+, indicates that the proportion of discordances differed significantly between all subtypes for a respective drug ($P < 0.05$); –, indicates that the proportion of discordances did not differ significantly between all subtypes for respective drug.
§Subtypes that displayed significantly more discordances than subtype B for the susceptibility scoring to the respective drug are in brackets.

Table 4. Interalgorithm discordances (%) between genotypic drug resistance interpretations of protease sequences obtained from treated patients infected with B and non-B HIV-1 subtypes

	Total analysis including 3 susceptibility scores (S, I, R)		Subanalysis including 2 susceptibility scores (S, R)			
			I score assigned to S score (I = S)		I score assigned to R score (I = R)	
PI	Discordances (%)*,†	Subtypes‡,§	Discordances (%)*,†	Subtypes‡,§	Discordances (%)*,†	Subtypes‡,§
Ritonavir	**31**	–	8	–	**28**	–
Indinavir	**27**	+ (F)	4	–	**25**	+
Nelfinavir	**22**	+	5	+ (H)	**21**	+
Amprenavir	**20**	+ (F)	**10**	–	**17**	+ (F)
Saquinavir	10	+ (D, F, H)	2	+ (G, CRF13)	10	+ (D, F, H)
Lopinavir	**25**	+	**17**	+	**21**	+

*Proportions of sequences that displayed discordances were significantly different when performing an overall drug comparison ($P < 0.001$).
†Proportions of discordances that did not differ significantly from each other in a pairwise analysis are displayed in an identical font style (bold or underlined).
‡+, indicates that the proportion of discordances differed significantly between all subtypes for a respective drug ($P < 0.05$); –, indicates that the proportion of discordances did not differ significantly between all subtypes for respective drug.
§Subtypes that displayed significantly more discordances than subtype B for the susceptibility scoring to the respective drug are in brackets.

Prevalence of mutations and their contributions to genotypic drug resistance interpretation

For particular drugs, some non-B variants displayed significantly more discordance than subtype B. Therefore, we investigated whether certain amino acid changes that might influence the genotypic drug resistance interpretations (as recorded in Fig. 1) were more prevalent in some non-B variants. The analysis was restricted to the treatment-naive population because the analysis in the treated populations could be biased due to different treatment history in the different subtypes.

Compared to subtype B, the major mutation M46L was more prevalent in CRF13. Minor mutations at positions 10, 20, 36, 63, 77 and 93 of the protease were significantly more often observed in subtypes A (L10I, M36I), C (K20R, M36I, I93L), D (M36I), F (L10V, K20R, M36I), G (K20I, M36I), J (M36I, L63T), CRF01 (M36I), CRF02 (K20I, M36I, I93L), CRF05

Table 5. Interalgorithm discordances (%) between genotypic drug resistance interpretations of reverse transcriptase sequences obtained from treated patients infected with B or non-B HIV-1 subtypes

	Total analysis including 3 susceptibility scores (S, I, R)		Subanalysis including 2 susceptibility scores (S, R)			
			I score assigned to S score (I = S)		I score assigned to R score (I = R)	
NRTI	Discordances (%)*,†	Subtypes‡,§	Discordances (%)*,†	Subtypes‡,§	Discordances (%)†	Subtypes‡,§
Zidovudine	6	–	3.0	–	*6.0*	+ (H)
Abacavir	**48**	+	**31**	+	**44**	+
Tenofovir	**39**	+	**32**	+	**35**	+
Stavudine	*21*	–	*18*	–	*15*	–
Zalcitabine	**44**	+	***22***	+	**38**	+
Lamivudine	10	+ (C)	4	+ (C)	*10*	+ (C)
Didanosine	**46**	+	***28***	+	**45**	+
NNRTI						
Nevirapine	5.0	+ (CRF06)	4.0	–	1.0	+ (CRF06)
Delavirdine	*8.3*	–	7.0	–	*4.0*	+ (CRF06)
Efavirenz	7.0	+ (CRF06)	6.0	–	*7.0*	+ (CRF06)

*Proportions of sequences that displayed discordances were significantly different when performing an overall drug comparison ($P < 0.0001$).
†Proportions of discordances that did not differ significantly from each other in a pairwise analysis are displayed in an identical font style (bold, regular, italic or underlined).
‡+, indicates that the proportion of discordances differed significantly between all subtypes for a respective drug ($P < 0.05$); –, indicates that the proportion of discordances did not differ significantly between all subtypes for respective drug.
§Subtypes that displayed significantly more discordances than subtype B for the susceptibility scoring to the respective drug are in brackets.

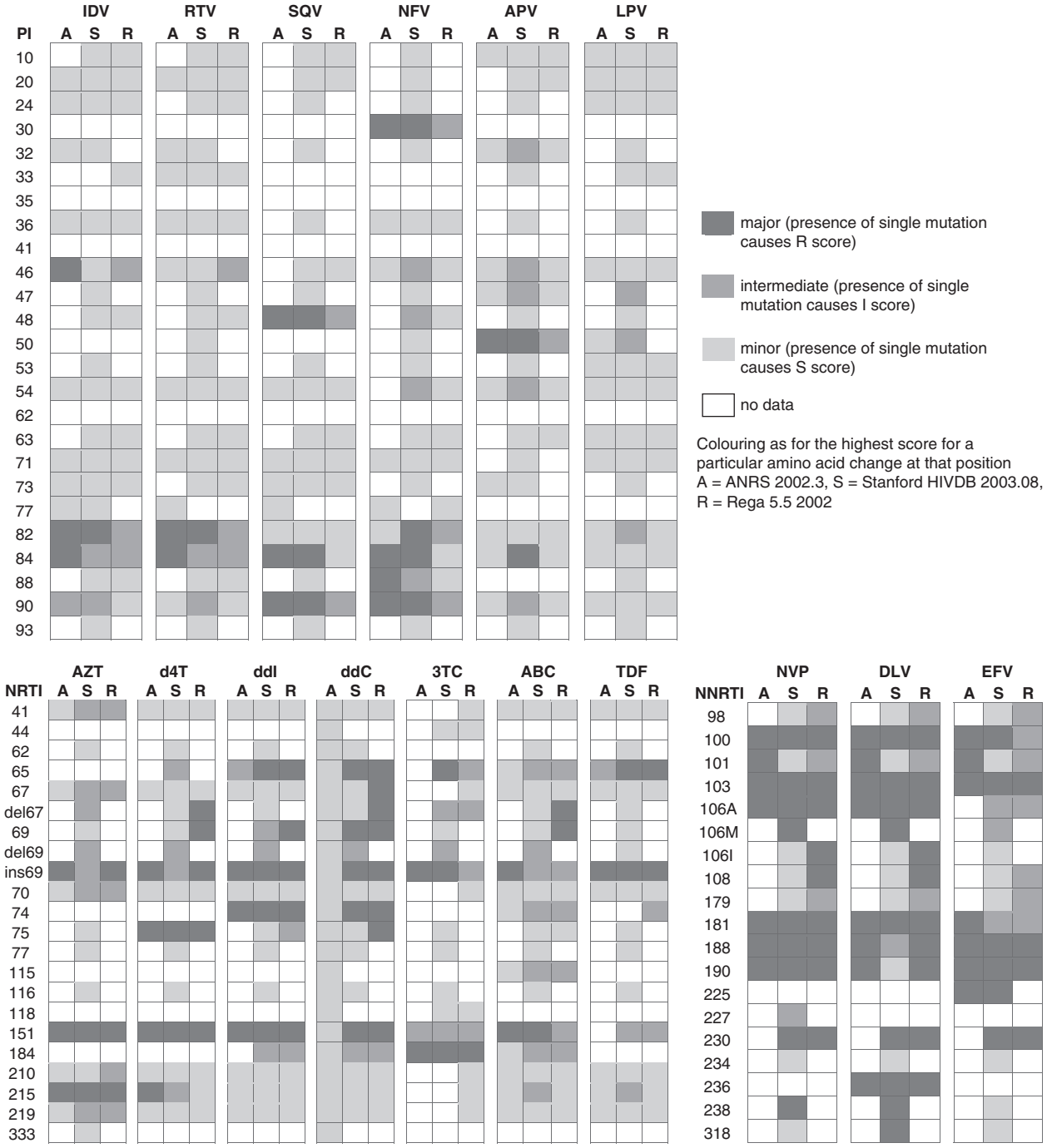

Fig. 1. Contribution of mutations to genotypic drug resistance interpretation in three different algorithms.

(L10V), CRF06 (K20I, M36I), CRF11 (L10I, L63T), CRF13 (K20I/L, M36I, L63S, V77I). The majority of non-B subtypes (A, C, F, G, J, CRF05, CRF11 and CRF13) had significantly more minor PI mutations than subtype B. The minor mutation L63P was less prevalent in subtypes A, CRF02 and CRF11 than in subtype B. V77I was less prevalent in subtypes A, F, G and CRF02. At resistance-related positions, reverse transcriptase displayed fewer differences between non-B and B. Only V118I was significantly more prevalent in subtype D, and V179I significantly more prevalent in subtype A.

Irrespective of the subtype, the presence of single mutations in treatment-naive samples that were given clearly

different scores by the algorithms could also be responsible for some of the observed discordances (e.g. Protease: M46I/L (1.8%), L90M (0.4%); RT: A98G (0.3%), K101Q (0.3%), V106I (1.9%), V108I (0.75%), V179D/I (4.9%), F227L (0.3%)).

A summary is presented in Fig. 1 of the different positions of PI and RTI mutations and their individual impact on antiretroviral drug susceptibility, as scored by the three algorithms.

Discussion

The rules of algorithms are mainly derived from *in vitro* and *in vivo* data obtained on subtype B. However, the application of these different algorithms produces discordant interpretations even on subtype B strains (Ravela *et al.*, 2003; Sturmer *et al.*, 2003; Zazzi *et al.*, 2004). Non-B subtypes are, in addition, characterised by the presence of several resistance-related mutations that occur as natural variants (Pieniazek *et al.*, 2000; Vergne *et al.*, 2000; Kantor & Katzenstein, 2003). In this study, we examined to what extent this might result in interalgorithm discordances. We showed high levels of discrepancies between the different algorithms in ARV treatment-naive and treated patients infected with either non-B or B strains.

In treatment-naive patients, the highest discordances were seen in the protease gene. Minor mutations occurring naturally in non-B strains (positions 10, 20, 36, 63, 77 and 93 in protease) were responsible for the majority of discordances because combinations of them are associated with intermediate resistance by certain algorithms. In particular, the Rega algorithm, which predicts intermediate PI susceptibility when two minor mutations are present and suggests adopting alternative drug choice, contributed to the high interalgorithm discordance. Only for lopinavir was a low level of discordance observed, because in all algorithms a large number of minor mutations are required for a change in the susceptibility score. As a consequence, when the interalgorithm comparisons were made considering the intermediate level as susceptible, a better concordance was seen. It is necessary to further characterize the clinical relevance of this level in the prediction of PI susceptibility in non-B strains. Furthermore, the intermediate level is not similar for all algorithms, and has a higher score in ANRS and Stanford algorithms than in Rega. In the Rega algorithm, intermediate level just corresponds to alternative therapeutic choice. In addition, we arbitrarily classified the five levels of the Stanford algorithm in three levels, considering low-level resistance intermediate. The intermediate level classification related to minor mutations was the main source of interalgorithm discordances, with one algorithm assigning an intermediate level and the other algorithms a susceptible level. In subtype B-infected patients under therapy, minor mutations occur during the accumulation of major mutations and help compensate for the reduced fitness of mutated viruses (Nijhuis *et al.*, 1999). Their natural presence in non-B strains could thus facilitate the rapid emergence of resistant strains (Perno *et al.*, 2001). Clinical studies on the efficiency of ARVs on non-B-infected patients in Europe and Africa, however, suggest a similar efficacy of PIs after 12–18 months, although a longer follow-up may be needed to confirm this (Frater *et al.*, 2001). The M46I mutation, associated with resistance to indinavir for ANRS, intermediate resistance to nelfinavir and amprenavir for Stanford, and intermediate resistance to indinavir and ritonavir for Rega, was observed significantly more often in CRF13 strains from patients residing in regions in Africa where PIs have either not yet been introduced or have been introduced only recently, and is probably a natural polymorphism. It is necessary to elucidate the role of this mutation.

In non-B strains from naive patients, we observed NNRTI resistance substitutions at major and intermediate positions, e.g. 98, 101, 106, 108 and 179. They were not significantly specific to a particular subtype or CRF. Certain algorithms considered these substitutions major, intermediate or minor mutations (definition from Fig. 1). Their implications for NNRTI susceptibilities await further clarification.

More interpretation discordances were observed in the treated than in the naive patients. Whereas major PI mutations were selected in only 28.8% of patients, similar levels of discordances were observed for predictions of PI susceptibilities in the naive and treated population. Discordances were mainly related to interpretations of minor mutations present as natural variants in non-B subtypes. The highest level of discordance was observed for the most recent PIs, amprenavir and lopinavir, reflecting the limited knowledge on their resistance.

For RTIs, the interpretations of complex mutation patterns were the main reason for interalgorithm discordances, especially those related to cross-resistance and antagonistic interactions. These discordances were seen in patients infected with B strains as well as those infected with non-B strains. The impact of NAMs on overall NRTI susceptibilities is difficult to assess. Stavudine, didanosine and tenofovir susceptibilities are difficult to measure because biological and clinical cut-offs overlap with reproducibility cut-offs. Some clinical studies showed that NAMs can be selected by stavudine and didanosine and patients with NAM mutated virus were reported to not or only partially respond to stavudine therapy (Coakley *et al.*, 2000).

Stanford and Rega algorithms consider that the M184V mutation is also associated with intermediate resistance to other NRTIs, such as abacavir, zalcitabine and didanosine. In addition, the M184V mutation can partially reverse T215Y-mediated resistance to zidovudine. This fact was not

considered in a similar way by all algorithms (implemented by Stanford and Rega, but not by ANRS). Numerous discordances were observed to interpret tenofovir and abacavir susceptibilities (39% and 48%, respectively) but few data are available to evaluate the mutations inducing resistance in these recently introduced drugs (Brun-Vézinet *et al.*, 2003).

For NNRTIs, discordant interpretations were related to the following mutations, Y181C, Y188L, G190A, which, depending on the algorithm, were considered resistant or intermediate resistant. In addition, the natural polymorphisms A98G, V106I and V108I, often observed in non-B subtypes, were also present in the treated population and led to interpretation discrepancies.

Interpretation algorithms are regularly updated; our results were analyzed with the 2003 algorithms. Only small modifications have been added since and our data are thus still relevant on the use of these algorithms on non-B variants. The aim of this study was to test the use of these algorithms on non-B variants, not to select one algorithm as the best one, as data about clinical outcome are limited. This study shows much discordance between genotypic drug resistance interpretation algorithms when applied to non-B strains from HIV-1 naive and treated patients. It is necessary to match different algorithms for better patient care. Further retrospective and prospective clinical studies in Europe and in developing countries are necessary to elucidate the interalgorithm discordances and to determine whether some subtypes or CRFs select other mutations at positions known for subtype B or even at positions which were not documented to be associated with resistance in B strains and whether minor mutations in non-B should be differently appreciated than in subtype B. More studies on the correlation between phenotype and genotype would also help to improve algorithms and elucidate the role of minor mutations as natural polymorphisms.

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