Discrepancies of HIV-1 Reverse Transcriptase Resistance Interpretation of Insertions and Deletions between Two Genotypic Algorithms

Giselle Ibetete Silva López-Lopes André Minhoto Lança

João Leandro de Paula Ferreira Luciana Oliveira Souza

Luís Fernando de Macedo Brígido

Laboratório de Retrovírus, Centro de Virologia, Instituto Adolfo Lutz, São Paulo, Brazil

Key Words

Highly active antiretroviral therapy · HIV-1 antiretroviral drugs · HIV Stanford Resistance Database · Geno2Pheno_[resistance]

Abstract

Background: Bioinformatics algorithms have been developed for the interpretation of resistance from sequence submission, which supports clinical decision making. This study evaluated divergences of the interpretation of the genotyping in two commonly used algorithms, using sequences with indels of reverse transcriptase genes. **Methods:** Sequences were obtained from virus RNA of patients failing highly active antiretroviral therapy from 2004 to 2011. Alignments were obtained using Clustal W including subtype B consensus and HXB2. Sequences with evidence of indels were submitted to the Stanford Resistance Database and to the Geno2Pheno to locate indel positioning and determine the resistance profile. **Results:** A total of 1,959 partial reverse transcriptase sequences were assessed, mostly subtype B (74%). Insertions and deletions were observed in 0.9 and 0.6% of sequences, respectively. Discordant insert positioning was assigned for most (90%) insertion sequences, with 27% discordances for deletions. Susceptibility differed for some antiretroviral

drugs, predominantly for TDF, d4T and ETV, when sequences with deletions were evaluated. **Conclusion:** Both indel positioning and its impact on drug susceptibility varies depending on the algorithm, a fact that might influence the clinical decision. Critical analysis of indel sequences with manual alignments is important, and its use alongside different algorithms may be important to better understand the outcomes of genotypic resistance prediction.

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Introduction

The HIV-1 polymerase gene is responsible for coding different enzymes which are essential for the retrovirus replicative cycle, including the protease and reverse transcriptase (RT) that are targets for most of HIV-1 antiretroviral (ARV) drugs. During reverse transcription, a lack of the proofreading properties of the enzyme, known for its relatively high error rate, is one of the factors responsible for viral diversity, an exquisite environment for the emergence of mutant viral strains. Also, the selective ef-

G.I.S. López-Lopes and A.M. Lança contributed equally to this work.

fect of the several ARV drugs composing the armamentarium of HIV-1 therapy on viral replication is essential to the development of the resistance observed in treatment failure [1]. As mutations can be transmitted [2], its impact on treatment options, both for initial and salvage therapies, constitutes an important public health issue. Over two hundred mutations have been associated with drug resistance, with complex mutation patterns in patients on highly active antiretroviral therapy (HAART) [3]. The evaluation of susceptibility to ARV drugs is of great importance in providing alternatives to salvage therapy in patients failing treatment, and genotyping tests have been employed to identify mutations that may confer resistance to ARV drugs [4]. The majority of substitutions confer cross-resistance drugs of the same class [5], such as K65R that reduces susceptibility to almost all nucleotide analogue reverse transcriptase inhibitors (NRTI) [6]. Moreover, strains exhibiting insertions of multiple nucleotides in protease and RT genes have been found in patients who have failed ARV therapy, and compensatory mutations are favored by drug-selective pressure on resistant strains replicating in a viremic patient [7]. Among the various mutations that confer reduced susceptibility, insertions and deletions, often called indels, can be observed in 0.5–2.4% of heavily treated patients [8, 9].

Along with sequence data per se, different interpretation algorithms have been developed to allow fast and concise resistance profile definition from sequence submission. Some of the genotyping algorithms are freely available, namely the HIV Stanford Resistance Database (HIVdb) [5] and the Geno2Pheno_[resistance] (G2P) [10]; these are two of the algorithms most commonly used by researchers and physicians worldwide. However, while most of the time these various systems agree, the interpretation of some sequences can be rather divergent. Several authors have made use of different criteria to establish a comparison between those different methods of resistance prediction [11–18]. However, the literature lacks a comparison of algorithms regarding when insertions or deletions of amino acids are present and how the positioning of these codons in the websites contributes to different susceptibility predictions. The aim of this study was to evaluate the divergences in the interpretation of the genotyping in two different algorithms using a routine resistance sequence dataset with insertions and deletions in the RT gene. Also, the association of the indels with other mutations was evaluated to better understand their role in patients extensively exposed to HAART.

Patients and Methods

Sequence Selection

Sequences were obtained from plasma RNA of patients failing HAART, with a viral load over 2,000 copies/ml (3.3 log₁₀), while they were attending facilities administered by the public health system of São Paulo and nearby cities, as part of routine resistance genotyping from 2004 and 2011. HIV-1 RT gene sequences were obtained using ViroSeq (Abbott Laboratories, USA) resolved on ABI 3100 Genetic Analyzer (until 2008) or TRUGENE® HIV-1 Genotyping Kit for Drug Resistance resolved on OpenGene (Siemens Healthcare Diagnostics, USA) thereafter. All sequences obtained were screened for the presence of insertions and deletions.

Multiple sequence alignments were obtained using the Clustal W algorithm embedded in BioEdit software including subtype B consensus and HXB2 reference sequences downloaded from Los Alamos National Laboratory (LANL) database (<http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html>) [19].

Sequence Submission and Indel Positioning

Sequences with evidence of indels were submitted to HIVdb version 6.2.0 (last update May 29, 2012) and G2P version 3.3 (last update September 13, 2012) to detect and locate insertion/deletion positioning, and to determine the drug susceptibility profile. Manual entering of individual mutations in HIVdb was also evaluated to verify if this would change the prediction made by HIVdb automatic submission. Moreover, a manual alignment was conducted to allow indel positioning. The determination of which would be the most probable positioning of the indels was based on the prior conservative assumption that deletions at position 67 and insertions at codon 69 are the most common changes in this genomic region associated with genetic resistance to ARV drugs [20, 21]. The amino acid composition of all sequences was evaluated to document the prevalence of resistance mutations in both groups.

Interpretation of Genotyping

HIVdb stratifies resistance in five levels (H = high-level resistance, I = intermediate resistance, L = low-level resistance, P = potential low-level resistance and S = susceptible), while G2P stratifies it in three levels (1 = resistant, 2 = intermediate and 3 = susceptible). Our paired analysis collapsed the scores 'potential low-level resistance' as 'susceptible' and the low level as 'intermediate resistance' (1 = S, P, L; 2 = L, I; 3 = H), as suggested by the HIVdb HIValg program. Differences between algorithms were evaluated as partial discordance, when the algorithms disagree at the level of resistance or between susceptible and intermediate, or as full discordance, when one algorithm scores a sequence as susceptible and the other shows a fully resistant profile. The International AIDS Society mutation list update from 2011 [5] was also used in the characterization of mutations associated with resistance.

Results

A total of 2,272 RT partial gene sequences were screened and those with available data such as age, gender, CD4, viral load and treatment regimens were selected. Also, only the first sequences from each patient were

Fig. 1. Deduced amino acids of the indel regions with positioning according to the manual alignment, to G2P and to HIVdb. Hyphens represent gaps in the alignment, while black boxes highlight the different G2P and HIVdb positioning of indels. Codon numbering is adjusted to the HXB2 reference. Samples BR185SP11* and BR577SP09* are subsequent samples of patients BR085SP10 and BR067SP07, respectively.

included, i.e. a total of 1,959 sequences. Thirty (1.4%) of these were found to contain amino acid insertions (0.9%) or deletions (0.6%) (fig. 1). Patients' characteristics according to the indel type as well as from the remaining 1,929 of nonindel cases are detailed in table 1.

Sequence Analysis

Nineteen sequences exhibited an insertion of 1, 2 or 8 amino acids at codon 69 according to manual alignment (fig. 1). Thirteen presented the usual insertion of 2 amino acids (69ins²) followed by T69S substitution in most of those sequences (92.3%). The insertions observed were seven SG (53%), three SS (23.6%), two VT (15.4%) and one CG (7.7%). Five sequences showed the presence of 1-amino acid insertion (69ins¹), mostly T (60%), and all those sequences exhibited the substitution K64N. Additionally, one sequence showed an unusual 8-amino acid insertion (69ins⁸). Along the insertion sequences eleven isolates had deletions at codon 67 ($\Delta 67$); the substitution T69G was observed in all strains and the mutations S68N or S68D in 16.2% each.

Sequences were also evaluated for resistance profiles according to the 2011 IAS [5] resistance mutation list grouped by $\Delta 67$, 69ins¹, 69ins² and 69ins⁸. Within the 69ins² group, the prevalent resistance mutations were the thymidine analogue mutations type I (TAM-I), as well as

A62V and M184 V/I. On the other hand, along the 69ins¹, the most common resistance mutations were the TAM type II (TAM-II) followed by T215F. The third group, Δ 67, had a high prevalence of both TAM-I and TAM-II, as well as L74I and M184V. The single 69ins⁸ sequence showed the TAM-I substitutions M41L, L210W and T215F, as well as the TAM-II K70R. Data of the NRTI mutations are detailed in table 2.

Comparison of Algorithms

HIVdb and G2P alignment algorithms were compared both as positioning concordance as well as indel impact on drug susceptibility. Regarding positioning, the insertions were discordant for 90% of sequences, while the discordance was 27% in samples with deletions. All 19 events of insertion were positioned at codon 69 by HIVdb, while positioning varied between 64 and 69 when using G2P. Sequences defined by HIVdb as 69SG/SS were alternatively classified as 66ins² in G2P, with the exception of one 69SS sequence defined as 67ins². Those containing 69CG/VT had its positioning set as 68ins² in G2P. No difference was observed among the 69D and 69N sequences, both being classified as 69ins¹. The three 69T insertions were positioned at 68 by G2P. Furthermore, the sequence containing the 8-amino acid insertion was positioned at codon 64. The patient with this variant (BR067SP07) had another

Table 1. Clinical and demographic characteristics of the study patients according to indel type

Demographic/clinical variables	Nonindel (n = 1,929)	67Δ (n = 11)	69ins ¹ (n = 5)	69ins ² (n = 13)	69ins ⁸ (n = 1)
Age, years	38 (30–45)	41 (19–42)	14 (11–17)	41 (17–46)	42
Gender (male)	60	71.4	40	92.3	0
CD4+ T cells, cells/mm ³	238 (117–399)	146 (28–465)	430 (155–553)	300 (112–389)	381
Nadir CD4+ T cells, cells/mm ³	120 (47–233)	38 (6–292)	428 (121–496)	149 (98–267)	381
Viral load (log ₁₀)	4.49 (4.01–4.98)	4.6 (3.8–4.7)	4.65 (4.33–5.78)	4.65 (4.27–5.02)	5.6
Exposure, weeks	348 (227–457)	409 (388–668)	379 (324–430)	398 (345–521)	168
Number of treatments	3 (2–4)	4.5 (2.5–5.0)	1 (1–2)	3 (2–4.5)	1
Initial therapy					
Mono	6.9	0	0	7.7	0
Double	34.7	54.5	100	46.2	0
HAART	58.4	45.5	0	46.2	100
Present therapy					
Mono	0.1	0	0	0	0
Double	3.4	0	60	0	0
HAART	96.5	100	40	100	100
Subtype					
B	73.7	81.8	40	61.2	100
F	7.4	9.1	–	23.1	–
C	2.6	9.1	–	7.7	–
Recombinant mosaic	15.8	–	60	7.7	–
Unknown	0.5	–	–	–	–

Results are expressed as the median with the interquartile range in parentheses or as percentage. Nonindels = From sequences without indels at RT; Δ67 = deletion at codon 67; 69ins¹, 69ins² and 69ins⁸ = insertion of 1, 2 and 8 amino acids at codon 69, respectively.

Table 2. Resistance mutations associated with NRTIs in the different groups of indels according to the 'IAS Resistance Update 2011' [5]

	n	M41L	A62V	K65R	<i>D67N</i>	<i>K70R</i>	<i>L74I/V</i>	<i>V75I/M</i>	<i>F77L</i>	<i>Y115F</i>	<i>F116Y</i>	<i>Q151M</i>	<i>M184I/V</i>	L210W	T215F/Y	<i>K219E/Q</i>
Control	1,929	33	4	2	30	20	4/4	2/7	3	1	2	2	1/62	24	12/37	
Δ67	11	36		9		73	45/0		9		9	9	0/91	18	46/36	80/0
69ins ¹	5	20			40	100	0/20						0/20		40/0	0/100
69ins ²	13	69	46				12/12	0/31	15				23/54	54	17/75	0/8
69ins ⁸	1	100				100								100	100/0	

Results are expressed as percentage. TAM-I are given in bold, while TAM-II are given in italics.

genotype test performed 2 years later that showed the persistence of this insertion, as well one other case with subsequent sample evaluation; these are included in figure 1. Regarding the deletions, all 7 sequences exhibiting the wild-type S68 (according to manual alignment) were classified as Δ67 in both HIVdb and G2P, while the two S68N mutations had their deletion positioned at codon 69 by HIVdb and 67 by G2P. Moreover, the remaining two sequences exhibiting S68D were classified as Δ70 by G2P, while HIVdb positioned the deletion at 68 and 69 codons (fig. 1). The manual entering of mutations either by typing

or by using the drop-down menu offered as HIVdb Mutation List option did not differ from the susceptibility results at automatic sequence analysis. However, some differences were seen at positioning, when the drop-down menu was used, where some uncommon changes exhibited at automatic analysis were not available for selection, e.g. BR11SP151's K70d (deletion). This could be solved by manually typing in those mutations. Both alternatives did not change the susceptibility profile.

The prediction of susceptibility to each drug of the two algorithms was evaluated separately for sequences har-

boring insertions or deletions. The exhibited partial and full discordance between algorithms is detailed in table 3, with some discordance observed for all ARV drugs. Partial or full discordance was observed for a new generation of non-NRTI drugs, ETV (37% for insertions and 73% for deletions), as for NRTI drugs, especially d4T and TDF. Additionally, there was full discordance in 2 (10%) insertion samples (ddI and ETR). Full discordance was more common among sequences with deletions (36%). Using different combinations of HIVdb classifications (e.g. considering low as susceptible or potential-low as intermediate at G2P) did not show important differences in algorithm comparisons. It is of note that all full discordant sequences were resistant at HIVdb and susceptible at G2P.

Discussion

Several computational algorithms of the interpretation of the genotyping of HIV-1 viral strains have been developed to better understand and characterize resistance in clinical samples. Alongside patients' treatment history, the genotypic algorithms may be employed to predict resistance to ARV drugs and help physicians with a fast and reliable method of determining the most adequate HAART option for each individualized salvage regimen. However, differences between the construction and the in vivo and in vitro resistance information used for each algorithm development may have an impact on phenotypic prediction and may lead to different resistance profiles. Two of the most frequently used algorithms available, HIVdb and G2P, were selected for comparison. Our cohort includes mostly heavily treated patients, with a median of 7 years on ARV treatment, in many of whom treatment was initiated in the 1990s. From all sequences screened for indels, deletions (0.6%) and insertions (0.9%) among patients failing HAART were present in a prevalence that did not differ from the findings of Tamalet et al. [8], Winters and Merigan [7] and Harrigan et al. [9].

The indel groups, separated by the number of amino acids inserted, and the nonindel (control) group were compared. With exception of 69ins¹, these groups were highly homogeneous regarding demographic, laboratory and treatment data (table 1). However, the 69ins¹ group was mostly comprised of children (80% under 18 years old), a population with immunological, virological and therapeutic peculiarities (e.g. ARV options available to children and adolescents were more limited at that time, and dual NRTI therapy was used for longer periods of time). Among insertion sequences, a high het-

Table 3. NRTI and NNRTI drugs susceptibility algorithms prediction agreement between Stanford HIVdb (version 6.2.0) and Geno2Pheno (version 3.3)

ARV	Stanford HIVdb and geno2pheno discordances			
	insertions (n = 19)		deletions (n = 11)	
	partial	full	partial	full
ZDV	0%	0%	18%	0%
ddI	16%	5%	0%	0%
d4T	37%	0%	36%	27%
3TC	32%	0%	9%	0%
ABC	5%	0%	27%	0%
TDF	26%	0%	73%	0%
NVP	11%	0%	0%	9%
EFV	21%	0%	36%	9%
ETR	32%	5%	73%	0%

Partial = partial discordant; full = full discordant. Partial discordance was considered when the level of resistance disagreed, as one algorithm classified as intermediate resistance, while the other classified as resistant or susceptible. Full discordant refers to full disagreement, as susceptible versus fully resistant.

erogeneity of both substitutions and insertions was observed, where 1, 2 or 8 amino acids were identified. Also, the genotypic resistances to NRTI differed among the sequences harboring one and two insertions. The resistance mutations exhibited by 69ins² sequences were in agreement with the IAS 2011 mutation list regarding the 69 insertion complex [5]. Interestingly, recently HIVdb [22] has included in the 69 insertion comments as separate observations regarding 69 single and double inserts, where the former are often accompanied by TAM-II, while the latter are followed by TAM-I. Our data agree with HIVdb and suggest that a distinct mutation pattern may be associated with either a single or a double insertion, with potential impact on drug susceptibility. K64N, a rare mutation found by Rakik et al. [23] on a 69ins¹ sequence, was also observed on all our sequences harboring single insertions. A distinct insertion of 8 amino acids in this same region (69ins⁸) was detected in one case. A second sample, obtained 2 years after the first genotyping and a subsequent change of drug therapy, confirmed the presence of the long insertion, along some substitutions in the nearby positions (fig. 1). Large insertions between codons 64 and 70 are rather uncommon, although insertions/duplications of up to 15 amino acids have already been described [9, 24]. Furthermore, a very similar substitution to the one observed in our patient was found by Van der Hoek et al. [25], who suggested

that this large insertion would be the result of both the duplication of the virus nucleotides and the insertion of fifteen nucleotides from the human genomic DNA. No sequence of our patient's virus prior to HAART was available to investigate if the insertion emerged during or prior to therapy.

Susceptibility to ARV drugs is the most important aspect of resistance algorithm interpretation. To evaluate the impact of indels, both HIVdb and G2P were compared to identify interpretation mismatches for each different drug. We found a low algorithm prediction agreement for many drugs, both NRTI and non-NRTI in sequences harboring insertions, mainly to d4T, ETR, TDF and 3TC. An even higher degree of discordance was observed in $\Delta 67$ sequences for d4T, TDF, EFV and ETR. It is of note that a quarter of the $\Delta 67$ sequences were considered susceptible to d4T by G2P, while they were considered fully resistant according to HIVdb. The differences between indel positioning and susceptibility to ARV in both HIVdb and G2P reveal a lack of homogeneity in the way in which these algorithms handle the different indel strains. In fact, the HIVdb algorithm considers the mutation individually and by default treats any insertion or deletion between positions 66 and 71 as $\Delta 67$ and 69ins, respectively, and the impact of each individual mutations is unclear. G2P, although identifies the indels, does not elucidate how each mutation is taken into account in determining the degree of resistance. We

artificially changed the sequence by creating or removing an indel, and observed the way in which the algorithm interprets other mutations in the same sequence changes, such as T215Y/F, by scoring from polymorphism to resistance mutation (data not shown). These differences in the alignment and the interpretation of the genotyping of both algorithms can be fundamental in explaining why a high divergence between algorithms was observed for each drug. Therefore, in the absence of a phenotypic resistance test, a critical analysis of the sequence, along with the treatment history of the patient might be required for the proper interpretation of resistance prediction, especially in the presence of indels. The use of different algorithms simultaneously, along with a manual alignment of the sequences, might help identify what could be the most probable correct position of the change, and whether it corroborates or not with the automatic prediction available at those websites.

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