Reviewer's report

Title: DR_SEQAN: a PC/Windows-based software to evaluate drug resistance using human immunodeficiency virus type 1 genotypes

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Reviewer: Klaus Korn

Reviewer's report:

General
This manuscript describes a new software for the interpretation of genotypic data for HIV-1 drug resistance to protease and RT inhibitors. It has some advantages compared to other already available systems, especially the implementation of a large bibliography on published drug resistance data and the consideration of recent publications that are not (yet) incorporated into other systems. Another feature that I liked in comparison to other systems (which the authors do not feel worth pointing out) is that if a sequence does not cover all relevant amino acid positions, the missing positions are easily visible in the output.

However, there are also a number of disadvantages compared to other system. Some of these concern mistakes in the current version of the software that I think will be not too difficult to overcome. Others are more structural, especially with regard to the way how information is incorporated and the reliability of sources of information is judged. Furthermore, some features that other systems have like information on the viral subtype (like in HIVDB or geno2pheno) or the presentation of a complete alignment with a reference sequence (like in geno2pheno) are not part of this interpretation system.

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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

1) The way in which ambiguities in the nucleotide sequence that influence the amino acid sequence are handled is inadequate and there is a serious defect in the software concerning this problem: The authors state in the summary that “When mixtures of nucleotides are detected at positions relevant for drug resistance (i.e. T215X in the RT or V82X in the PR), the program allows the user to intervene by selecting the proper mutation to be considered by the program’s drug resistance interpretation algorithm.” However, an interpretation software like this is not the right place to do this type of editing. A sequence to be loaded into the software should have been checked for ambiguities beforehand with a software which allows judgement of the chromatograms (or other type of raw data). If ambiguities remain after this step, it has to be assumed that the sample under investigation contains viruses with different nucleotides and – if the amino acid sequence is affected – also different amino acids at the respective position(s). Since any nucleotide that shows up significantly in the chromatograms will represent at least 20 % of the virus population in conventional sequencing of plasma-derived PCR products, it is prudent to assume the “worst case”, i.e. to choose the resistant variant (or if more than one deviation from the wildtype amino acid sequence is possible the most resistant variant) for the interpretation. An output like “V82X” is not useful to the user because he does not know what is behind it if he cannot look at the original chromatograms simultaneously. And the window in which to select the “proper mutation” is not useful either, which can be nicely illustrated with the very polymorphic position 82 in the protease: What one can choose there is one of either the wildtype amino acid V or mutant amino acids A, F, I, L, S or T. Apart from the fact that it will be quite difficult to locate the codon in the sequence, what if a mixture of for example A and T is encoded or a mixture with a mutation not listed here (e.g. V/M) ? Furthermore, one can choose for example “T” even if the ambiguous codon encodes only “V” and “A”. And, finally, this is the big bug in the software, if one chooses the answer...
“yes” to the question “Sequence heterogeneities have been found at relevant positions for resistance to Protease Inhibitors. Do you want to revise the sequence at those positions?” one can select the appropriate mutant amino acid at the heterogeneous positions, but any other homogeneous resistance mutations present in the protease will be set to wildtype for the purpose of interpretation. That is, the mutation appears in the list of mutations, but it is not considered for interpretation. The same is true if I choose to edit in the RT. This was really frustrating and it took me quite some time to find out what went wrong. Thus, the only strategy that makes sense in my opinion is not to display an “X” at those positions but rather to display all amino acids that can be derived from the sequence of the respective codon (maybe the “X” only if there are more than 4 possibilities, which would indicate bad sequence quality) and then use the “worst case” for prediction of drug resistance (for example in the above mentioned mixture of V82A/T the “A” in the Indinavir, Ritonavir and Lopinavir prediction and the “T” in the Tipranavir prediction (for the other PI it would not matter, because V82A and V82T are treated identically).

Furthermore, the problem of resistant minorities and the limitations in their detection by conventional sequencing should be addressed in the manuscript in this context. Instead, one could omit the sentence describing that if ambiguities in the nucleotide sequence do not affect the amino acid encoded, only this amino acid is displayed. This seems rather trivial to me.

2) The way in which the manuscript and the software handle the interpretation of PI resistance suffers from two serious drawbacks:
Firstly, there is no mentioning of the concept of boosting PI levels with low-dose ritonavir and the consequences this may have for resistance interpretation throughout the whole manuscript. In my opinion, a new (and hopefully superior) software for interpretation of HIV drug resistance must not ignore this development and should present an interpretation also for boosted PI, although I am aware of the problem that – apart from lopinavir, which is only available with ritonavir boosting - data published in peer-reviewed papers are quite scarce. But clearly, a cutoff for lopinavir resistance of 1.7-fold reduced susceptibility as used in table 3 is inadequate (the FDA label states a cutoff of 10-fold reduced susceptibility) and the other PI cutoffs (apart from the one for nelfinavir) are at least questionable.
Secondly, a number of the rules presented for PI resistance are in my opinion inconsistent and will lead to substantial overestimation of resistance for unboosted and even more for boosted PI therapy. For example, the combination of mutations L63P+I93L and I54V+L90M are both interpreted as causing “partial resistance” to Indinavir. Whereas the second combination of mutations will definitely have an impact on the indinavir phenotype, the effect of the first combination on the phenotype is negligible and this combination of mutations is also frequently seen in untreated patients. Similarly, for ritonavir, both L63P+V77I and M46I+L90M are interpreted as causing “partial resistance”, which is again o.k. for the second pair of mutations but not for the first one. Furthermore, for Amprenavir, both L10I/V and L90M are interpreted as causing “partial resistance”. In this case, none of these mutations – if present alone – has a substantial influence on the amprenavir phenotype. But whereas I would consider an isolated L90M as an early indicator of resistance development, which is very rare in untreated patients, L10I and L10V alone are again relatively frequent polymorphisms in untreated patients. The authors argue that “the most serious errors to be avoided are those occurring when a resistant virus is predicted to be sensitive” and that therefore, their rules for partial PI resistance are acceptable. Principally, this argument is true, but if exaggerated, it may cause more harm than good, because patients may be withheld active drugs because of erroneous assumptions of resistance. Furthermore, this argument is valid for all drugs and therefore, it is somewhat astonishing that for the RT inhibitors (particularly the nucleoside analogues) the rules chosen by the authors often predict lower levels of resistance than other interpretation systems, quite contrary to the situation for the PI.

3) On page 6 and 7, the authors list some advantages which they claim their system has compared to others. Among these, it is stated that “only DR_SEQAN warns on the presence of antagonistic mutations that could have an impact on drug resistance”. While it is true that the specific “warning” feature is unique to DR_SEQAN, the authors do not mention that other
systems do also incorporate antagonistic effects. This is most obvious for the Stanford system (HIVDB), but also some of the ANRS rules have incorporated antagonisms. And whereas with DR_SEQAN, the warning is always the same regardless of the number of antagonistic mutations, this is handled more sophisticated in the Stanford system where the antagonistic mutations are each assigned negative values, such that the antagonistic effect is increased if more than one antagonistic mutation is present. This passage needs some clarification and a better presentation of the features of other systems.

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

1) The term “render” is used in two instances: at the end of page 3 and in the third line from the bottom on page 8. In both instances, I would replace it by “lead to” or “leads to”, respectively.
2) Line 6 on page 7 reads “such as W88G, E89K or E89G could decrease AZT susceptibility”, but should read “such as W88G, E89K or E89G could decrease AZT resistance”.
3) In the fourth paragraph on page 8, lines 4 and 5, it should read “average -fold increase of IC50” rather than “average IC50”.
4) In table 1, the classification “low-level resistance” in HIVDB is missing.
5) In the legend to table 3, the term “clones” should be replaced by “isolates” (as in table 2).

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Discretionary Revisions (which the author can choose to ignore)

Two items about which one could discuss a lot and where I disagree in a number of points with the authors are the selection of mutations considered important and the choice of cutoff values. Since a lot of pros and cons may be brought forward with respect to every single mutation and cutoff, I do not want to make this compulsory issues.

1) In the list of advantages at the top of page 7, a number of protease mutations are listed that are considered in DR_SEQAN but not in Retrogram. Whereas several of these (K20T, L33F, M36V, I54A and others) are being considered in other interpretation systems (e.g. HIVDB) and are thus not unique to DR_SEQAN, others are in my opinion of very limited importance (R8Q, Q58E). On the other hand, at least two rare, but clearly relevant primary PI mutations are missing (I50L which causes selective resistance to Atazanavir and I47A which causes selective resistance to Lopinavir). Furthermore, V82M is not considered although it may have a greater effect on PI resistance than V82I, which is considered in the amprenavir algorithm, but represents wildtype in some non-B-subtypes.

Similarly, in the RT, the mutation Q145M is incorporated into all rules, except those for Emtricitabine, based on just one paper describing this mutation. Although the data in the paper look very convincing, there has been one other instance where this group published surprising phenotypic data claiming that mutations Y181C/I cause high-level D4T resistance, which we could not confirm in our large data set. Therefore, I am quite hesitant about these data, but currently I cannot prove if they are right or wrong. On the other hand, the consideration of insertions in the RT region 67-70 is in my opinion incomplete. Thus, in one sequence with an SV insertion and another one with a 7-amino acid insertion which I tested with the software, the insertions both were not considered in the interpretation, although their effect is comparable to the SS, SG and SA insertions which are incorporated into the rules. Furthermore, I wonder why the K65R mutation is not incorporated into any 3TC rule, although it causes an about 10-15-fold reduced susceptibility, which at least as big an effect as that of the E44D/A+V118I combination, which is part of the “partial resistance” rules.

2) Concerning the cutoff values used by the authors there is some confusion around: On the one hand, cutoffs are used in the comparisons of different interpretation systems presented in tables 2 and 3. On the other hand, in the fourth paragraph on page 8, different cutoffs are presented for the classification of the information retrieved from the literature. These cutoffs are then the basis for a color-coding system. The same color-code is also used for the classification of resistance according to the rules described, which are not directly related to the cutoffs, thus creating additional confusion.
On the basis of which information have the different types of cutoffs been selected? Furthermore, it is stated on page 8 that when two or more papers report phenotypic data on the same combination of mutations, an average IC50 (it should read “an average –fold increase of IC50”, see above) is displayed. How is this average determined? Is it a mean or a median value or something else? What if the values come from different phenotypic assay systems?

3) Additionally, I want to mention the inconsistencies in the classification system: Principally, 4 classes are differentiated: green, yellow, orange and red. However, for the NNRTI, no rules for “orange” are presented. That is understandable for me, since the mutations listed for “red” mostly do cause really high levels of resistance and render the virus completely insensitive to the drug. More surprising is the fact that for all PI and also for Tenofovir, no rules for “red” are defined. What is the reason for this? Furthermore, there is some discrepancy in the nomenclature between table 1 and the text as well as the supplemental file with the rules: In table 1, “orange” is defined for DR_SEQAN as “high-level/significant resistance” and “red” as “predicted resistance levels are very high”. In the text, “orange” is “significant resistance” and “red” is “high-level resistance”. This discrepancy should be corrected.

4) Finally, as an added value to the user of the software, I would suggest to implement a printing and a storage function for the predictions generated.

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

Level of interest: An article of limited interest

Quality of written English: Acceptable

Statistical review: No

Declaration of competing interests:

I declare that I have no competing financial interests. However, since I was involved in the development of another interpretation system for HIV drug resistance that is freely available via the internet (geno2pheno, accessible at http://www.geno2pheno.org/cgi-bin/geno2pheno.pl) and since I am a member of the society supporting this service (Genafor e.V.), this may be interpreted as a "non-financial competing interest" in relation to this paper.