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Short communication

Evaluation of the automatic editing tool RECall for HIV-1 *pol* and V3 loop sequences

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Genotypic drug resistance testing is routine practice in HIV-1 clinical care. The visual interpretation of sequencing electropherograms is labour-intensive and subject to intra- and inter-assay variability because decisions are based on operators' judgments. In this study the performance of the automatic editing tool RECall was compared to the current standard of editing manually and editing using the tool ViroSeq. Using RECall a consensus sequence could be generated for 97% of the V3 loop and for 79% of the *pol* experiments. By comparison, using manual editing a consensus sequence could be reached for 87% of the V3 and 87% of the *pol* experiments. Using ViroSeq, a consensus sequence was generated for 68% of the *pol* experiments. On a predefined dataset, manual editing displayed the highest probability to accurately assign mixtures (0.91 vs. 0.88 by ViroSeq vs. 0.76 by RECall) and the lowest probability to inaccurately assign a mixture to a pure base call (0.002 vs. 0.019 by ViroSeq vs. 0.002 by RECall). As differences in base calling have little impact on drug resistance interpretation and hands-on-time could be substantially reduced, RECall could be a valuable tool for the standardization and acceleration of the editing process.

The number of people living with human immunodeficiency virus type 1 (HIV-1) still continues to grow due to the high incidence of new HIV-1 diagnoses each year and the success of antiretroviral therapy (ART) (UNAIDS, 2010). Genotypic drug resistance testing is strongly recommended for the selection of optimal ART in HIV-1 infected patients and has contributed to this success (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2011; Vandamme et al., 2011). Standard tests involve the genotyping of the protease (PR) and reverse transcriptase (RT) region of *pol* in order to detect amino acid (AA) mutations that are known to cause resistance to PR inhibitors (PIs), nucleoside RT inhibitors (NRTIs) and non-NRTIs (NNRTIs). They should be performed in HIV-1 diagnosed patients entering clinical care and in treated patients failing virologically or interrupting NNRTIs inappropriately (Vandamme et al., 2011). During the last several years, drugs that target the CCR5 coreceptor, integrase and glycoprotein gp41 have been approved for clinical use. Genotyping of the regions that encode the V3 loop within glycoprotein gp120 (coreceptor testing), the heptad repeats within gp41 and the integrase is also recommended when the respective drugs are part of a failing regimen. In addition, coreceptor testing should be performed whenever the use of a CCR5 inhibitor is considered (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2011; Vandamme et al., 2011).

Therefore, laboratories are confronted with an increasing burden of drug resistance testing. Most genotypic drug resistance tests consist of the amplification and population-based sequencing of the target viral genes. As the amplicons are often too long to cover with one sequencing primer and as bi-directional coverage is recommended for drug resistance related positions, consensus nucleotide (NT) sequences are generated based upon the assembly of multiple segment electropherograms. The visual interpretation of the electropherograms is not straightforward as the "true" sequence within each patient is unknown, each infection is characterized by a swarm of viral variants, and a large number of positions could potentially contribute to drug resistance. The high genetic diversity of HIV-1,

Abbreviations: AA, amino acid; ART, antiretroviral therapy; *env*, envelope gene; EQA, external quality assessment; FPR, false positive rate; HIV-1, human immunodeficiency virus type 1; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NT, nucleotide; PI, protease inhibitor; *pol*, polymerase gene; PR, protease; /r, ritonavir boosted; RT, reverse transcriptase.

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Table 1
Panel composition.

	# Clinical samples		# EQA samples
	pol	V3	*pol*
Subtype A	15	11	3
Subtype B	56	49	15
Subtype C	14	12	14
Subtype G	20	18	2
Other subtypes	22	19	6
VL < 1000 cop/ml	18	15	0
VL > 1000 cop/ml	109	94	40
Therapy naive	78	70	–
Therapy experienced	41	32	–
Therapy unknown	8	7	–
Total	127	109	40

Subtypes were determined by submitting the manually edited *pol* consensus sequences to the REGA HIV-1 Subtyping Tool Version 2.0 (http://dbpartners.stanford.edu/RegaSubtyping/) (Abecasis et al., 2010). #, number; EQA, external quality assessment; VL, viral load; cop/ml, RNA copies/ml.

especially in *env*, results in a cascade of substitutions, deletions and insertions that is reflected by multiple mixed NT in population-based sequences. This makes the editing difficult and sometimes impossible when electropherograms are too noisy.

As the manual editing process is time consuming, prolongs the turn-around-time, and is subject to the operator's judgement – resulting into sometimes inconsistent scoring – the automated sequence analysis tool RECall was developed at the British Columbia Centre for Excellence in HIV/AIDS Research to accelerate and standardize the editing of electropherograms (Woods et al., 2012). In this study the performance of RECall was compared to our current standard of manual editing and to an additional automatic base calling software: ViroSeq® HIV-1 Genotyping System Software v2.8. These editing strategies were evaluated by assessing the ability to generate consensus sequences, the concordance at NT and AA calling and the concordance at drug resistance interpretation.

Genotyping of the HIV-1 *pol* region and V3 loop was carried out on 127 and 109 plasma samples respectively, taken from patients attending the University Hospitals in Leuven. The samples were genotyped as part of the patient's clinical follow-up during the period of August 2009–August 2010 or as part of validation experiments (clinical samples). Additionally, 40 samples were included for which genotyping of the HIV-1 *pol* region was performed as part of an external quality assessment (EQA) program during the period of 2005–2012 (EQA samples).

The study panel consisted of a broad spectrum of subtypes and viral loads, above and below the viral load cut-off of 1000 RNA copies/ml for which drug resistance testing should be performed (Table 1) (DHHS guidelines, 2011). ViroSeq HIV-1 Genotyping System version 2 (Abbott, Wavre, Belgium) or an in-house method were used to generate *pol* sequences, covering the entire PR gene and the 5′ end of the RT gene (respectively up to NT 949 and 985 in RT) (Maes et al., 2004). For the V3 loop, an in-house developed PCR was performed and the amplicons then sequenced with the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Life Technologies, Gent, Belgium) (Van Laethem et al., 2005). All sequencing reactions were run on an ABI 3100 or ABI3130xl Genetic Analyzer (Life Technologies, Gent, Belgium). In the manual analysis, SeqScape version 2.6 (Life Technologies, Gent, Belgium) was used to assemble the segment electropherograms. The operator inspected each position, then trimmed and edited wherever necessary. Single coverage was allowed in some instances when the quality of the electropherograms was satisfactory in that particular region. In the automatic RECall analysis, the segment electropherograms were submitted and consensus sequences were generated, using the default settings (mixture cut-off: 13%, POL1200: base range: 1–1200 NT, V3: base range: 1–105 NT, single coverage: none) and without manual review thereafter. In the automatic ViroSeq analysis, the segment electropherograms were assembled and edited automatically, but trimmed manually if needed. For the clinical dataset, the *pol* and V3 regions were analysed manually and also automatically using RECall. For the *pol* EQA dataset, ViroSeq was used in addition to the manual and Recall methods.

RECall was able to generate a consensus sequence for 97% of the V3 loop (106/109) and for 79% of the *pol* (132/167) experiments (Table 2), whereas manual editing was successful in respectively 87% (95/109) and 87% (146/167) of the experiments. ViroSeq could generate a consensus sequence in 68% (27/40) of the included *pol* experiments. The electropherograms of *pol* and V3 sequences with discordant outcomes were subsequently manually reviewed in SeqScape. Fourteen V3 samples and 21 *pol* samples for which no consensus NT sequence was generated in the manual analysis displayed a high background noise, deemed unacceptable by the operator (Table 2). RECall passed 86% of these V3 samples (12/14) and 38% of these *pol* samples (8/21). The tool created these consensus sequences as secondary peaks below the preset mixture cut-off and mixtures observed in less than 50% of the segments were ignored. ViroSeq passed 29% (2/7) of the *pol* EQA sequences that were not accepted in the manual analysis. For one V3 sample, RECall failed to generate a consensus sequence in contrast to the manual analysis because the segments were incorrectly aligned which resulted in a stop codon. The automatic RECall analysis displayed a lower success rate for *pol* sequences than the manual approach, as single coverage was not accepted in the default setting of RECall. This had fewer implications for V3 samples in our study as in these experiments 4 primers were used and only 7 for the much larger *pol* sequences. ViroSeq failed to generate a consensus sequence for 24% (8/33) of the manual, successfully edited samples, mostly due to an incorrect alignment of the segment electropherograms.

For subsequent analysis, the clinical and EQA dataset were considered separately. In the clinical dataset, for 74% (94/127) of the *pol* and 86% (94/109) of the V3 samples, consensus NT sequences were available, generated by both manual editing and RECall (Table 3). In total, 46% (43/94) of the PR, 17% (16/94) of the RT and 64% (60/94) of the V3 sequences were fully concordant at the NT level. At the AA

Table 2
Performance in generating consensus *pol* and V3 sequences for HIV-1 samples by the different editing approaches.

	pol						V3		
	RECall		Total	ViroSeq		Total	RECall		Total
	Result	No result		Result	No result		Result	No result	
Manual									
Result	124	22	146	25	8	33	94	1	95
No result	8	13	21	2	5	7	12	2	14
Total	132	35	167	27	13	40	106	3	109

'Result' means that a consensus sequence could be generated. 'No result' means that no consensus sequence could be generated.