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Brief Report

Evaluation of Two Commercial Kits for the Detection of Genotypic Drug Resistance on a Panel of HIV Type 1 Subtypes A Through J

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Summary: We compared the two commercially available sequencing kits for HIV-1 drug resistance testing, the ViroSeq Genotyping System (Applied Biosystems, Foster City, CA, U.S.A.) and the TRUGENE HIV-1 Genotyping Kit (Visible Genetics, Inc., Toronto, Ontario, Canada), with our in-house genotyping system. Fifteen viral isolates from African patients (6 treated and 9 untreated) covering a panel of HIV-1 subtypes A through J and 7 plasma samples from Belgian and African patients (2 treated and 5 untreated) were tested. All the samples could be amplified and sequenced by the three systems; however, for all systems, alternative amplification/sequencing primers had to be used for some samples belonging to subtype B as well as to other subtypes. The consensus sequence was partially derived from only one strand for the in-house system and for the ViroSeq Genotyping System. The TRUGENE HIV-1 Genotyping Kit scored the highest number of ambiguities, followed by the ViroSeq Genotyping System and the in-house system. For 11 samples, these differences in reporting mixtures affected 14 resistance-related positions, which altered the interpretation toward protease inhibitors for 2 samples when using version 1.2 RetroGram software (Virology Networks, Utrecht, The Netherlands). All three systems were able to sequence diluted samples with a viral load down to 10^3 or 10^4 RNA copies/ml. Our data therefore suggest that the performance of amplification and sequencing primers must be improved to allow fast and reliable resistance testing for all HIV-1 subtypes. **Key Words:** Resistance testing—Genotyping—HIV-1 subtypes—AJ270543–AJ270564.

Drug resistance is a major limitation to antiretroviral treatment; therefore drug resistance testing is increasingly important for patient clinical management. Guidelines recommending resistance testing for particular indications have been published (1–4). Among the current methods for the detection of drug resistance, genotypic assays are faster, easier to perform, and less expensive than phenotypic assays. Current genotypic resistance assays have been designed and optimized for subtype B strains, the most prevalent subtype in countries manufacturing the current tests. Non-subtype B strains are increasingly being spread worldwide, and the need to test genotypic drug resistance for patients infected with HIV-1 strains other than subtype B is becoming important.

In the current study, we analyzed the performance of two commercially available sequencing kits, the ViroSeq Genotyping System (Applied Biosystems, Foster City, CA, U.S.A.) and the TRUGENE HIV-1 Genotyping Kit

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(Visible Genetics, Inc., Toronto, Ontario, Canada), in parallel with our in-house genotyping system (5) on a panel of 15 HIV-1 virus stocks and seven patient plasma samples belonging to subtypes A through J.

METHODS

The 15 isolates (indicated as MP) were obtained from peripheral blood mononuclear cells of African patients or patients living in France who became infected with HIV after overseas deployment (6 treated and 9 drug-naive patients) (6,7), and the seven plasma samples (indicated as P99-) were obtained from the University Hospitals, Leuven, Belgium (2 treated and 5 drug-naive patients). The virus stocks of the 15 isolates had a 50% of tissue culture infectious dose titer ranging from 4000 to 50,000 units/ml on peripheral blood mononuclear cells. The plasma viral load from the patient samples measured using the Quantiplex HIV-1 RNA 3.0 Assay (Chiron Diagnostics, Emeryville, CA, U.S.A.) ranged from 4.1 to 5.4 $\log_{10}$ RNA copies/ml. The subtype of all samples was determined by phylogenetic analysis (neighbor joining using PHYLIP software (8); 1000 bootstrap replicates) of the *pol* sequence that was obtained with our in-house amplification and sequencing system. All sequences were also verified for recombination using SimPlot for Windows software (9).

Amplification and sequencing by the in-house protocol were performed as described previously (5) using AV150-RT2 as outer amplification primers and RVP5 and RVP3 as inner primers for the protease gene and M13-USP-A35 and M13-RSP-NE-1 [35] primers for the reverse transcriptase (RT) gene. Sequencing primers were RVP5 and RVP3 for the protease gene and M13-USP, M13-RSP, AV36, and AV44 for the RT gene (5). Alternative sequencing and polymerase chain reaction primers such as the LiPA HIV-1 RT primers RT1 and RT4 (9) nonbiotinylated and tagged with M13-USP and M13-RSP and the IN5, IN3, HP2080N, and AV4 primers (5) were used when needed. Sequencing was performed with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit on the ABI377 or ABI310, and analysis was done with ABI PRISM DNA Sequencing Analysis software, Factura, and Sequence Navigator (Applied Biosystems). Nucleotide sequences from the *pol* region amplified by the in-house procedure were submitted to GenBank and are available under accession numbers AJ270543 to AJ270564.

The ViroSeq Genotyping System (version 1) was used according to the manufacturer's recommendations with kit primers A, B, C, D, F, G, and H. Primers A and D were both provided to sequence the protease gene in the sense direction. The ViroSeq Genotyping System (version 2) with alternative amplification primers was used for samples that could not be amplified with version 1. Sequencing from the entire protease (codons 1–99) and the first part of the RT (codons 1–320) was thus obtained. The kit uses the Big Dye Terminators chemistry run on the ABI377 and processed using ABI PRISM DNA Sequencing Analysis software, Factura, and Sequence Navigator or HIV-1 Genotyping System software.

The TRUGENE HIV-1 Genotyping Kit (Visible Genetics, Inc., Toronto, Ontario, Canada) was used according to the manufacturer's recommendations with primers from the kit and an additional primer pair for the protease gene provided by Visible Genetics to be used when the regular protease primers from the kit fail. The kit uses two different dye-labeled primers for each sequencing reaction. The CLIP reaction gives a protease fragment (codons 10–99) and an RT fragment (codons 38–247). The samples are run on the Opengene System and processed using GeneObjects software and the GeneLibrarian HIV module (Visible Genetics).

RESULTS

All 15 viral isolates (3 subtype A, 2 subtype C, 3 subtype D, 1 subtype A/D, 2 subtype F, 1 subtype G, 2 subtype CRF02_AG, and 1 subtype J) and the 7 plasma samples (4 subtype B, 1 subtype C, 1 subtype D, and 1 subtype H) could be amplified and sequenced by the in-house genotyping procedure (Table 1). Primers M13-USP-A35 and M13-RSP-NE-1 [35] failed to amplify the RT gene of 4 samples, however (see Table 1). Alternative primers were used for successful amplification. Failed sequencing primers are listed in Table 1 for each sequenced sample. Although the nucleotide sequences were available in both directions only in part for 12 samples, the consensus sequences were always obtained, even though partially only in one direction (see Table 1).

Using ViroSeq, genotypic drug resistance testing could be performed on all viral and patient plasma samples. The amplification was not always successful with version 1 (see Table 1); however, these samples could successfully be amplified and sequenced with the version 2 kit. The sequencing primers are identical in both versions. As shown in Table 1, the sequencing primers that failed were primer D in 14 cases, primer A in 4 cases, primer G in 3 cases, primer F in 3 cases, and primer H in 1 case. Primer D is a backup primer in case primer A fails. Therefore, only when both fail (as for samples MP798, MP1078, and P99–48) is there a problem in obtaining the consensus sequence from both strands. For seven samples, only partial double-stranded sequencing could be accomplished (see Table 1).

Using TRUGENE, we failed to sequence the protease gene in 9 of 15 viral isolates and in three of seven plasma samples with the P1 primers included in the kit (see Table 1). For these samples, the protease gene could only be genotyped using the alternative P2 primers provided by Visible Genetics. These alternative primers are included in the current TRUGENE kit. Sequences were available for all samples in both directions, but the primers of the kit provide sequences of protease beginning at codon 4 and ending at codon 99 and sequences of RT beginning at codon 38 and ending at codon 247, resulting in a gap between the obtained protease and RT sequences and a lack of the first 10 nucleotides of the protease gene.

The in-house genotyping system is more labor-intensive and time-consuming than the commercial kits. Nevertheless, the analyzed sequences obtained by the in-house system showed a much lower number of ambiguities, meaning that fewer sites were scored as a mixture of two (or more) nucleotides. The TRUGENE kit showed the highest number of ambiguities, followed by the ViroSeq system and then the in-house system. Com-

TABLE 1. *Performance of genotypic drug resistance testing on HIV-1 subtypes A through J by the in-house procedure and by two commercial kits: ViroSeq Genotyping System and TRUGENE HIV-1 Genotyping Kit*

		In-house system			ViroSeq Genotyping System			TRUGENE HIV-1 Genotyping Kit		
Sample code	*pol* subtype	Consensus[a]	Alternative PCR primers needed[b]	Failed sequencing primers[c]	Consensus[a]	Alternative PCR primers needed[b]	Failed sequencing primers[c]	Consensus[a]	Alternative PCR primers needed[b]	Failed sequencing primers[c]
MP 582	A	Partial SS	No	AV36	Partial SS	No	pD, pF, pG	Fully DS	Yes	P1
MP 601	A	Fully DS	No		Fully DS	No	pD	Fully DS	Yes	P1
MP 1098	A	Fully DS	Yes		Fully DS	No	pD	Fully DS	Yes	P1
MP 756	C	Fully DS	Yes		Fully DS	No	pD	Fully DS	Yes	P1
MP 1046	C	Fully DS	No		Fully DS	No	pD	Fully DS	Yes	P1
MP 954	D	Partial SS	No	AV44, RVP5	Fully DS	No		Fully DS	No	
MP 571	D	Partial SS	No	AV44	Fully DS	Yes		Fully DS	No	
MP 1263	D	Partial SS	No	AV44, RVP5	Partial SS	No	pD, pG	Fully DS	No	
MP 1144	A/D[d]	Partial SS	No	AV44, RVP5	Fully DS	No	pD	Fully DS	Yes	P1
MP 652	F	Fully DS	No		Fully DS	No	pD	Fully DS	No	
MP 798	F	Fully DS	No		Partial SS	No	pA, pD	Fully DS	No	
MP 630	G	Partial SS	Yes	M13-USP	Fully DS	No	pH	Fully DS	Yes	P1
MP 568	CRF02_AG	Partial SS	No	AV44	Partial SS	No	pD, pG	Fully DS	No	
MP 981	CRF02_AG	Partial SS	No	AV44	Fully DS	No	pD	Fully DS	Yes	P1
MP 1078	J	Partial SS	Yes	AV44, RVP5	Partial SS	No	pA, pD	Fully DS	Yes	P1
P99-83	B	Partial SS	No	RVP3	Fully DS	No		Fully DS	No	
P99-133	B	Fully DS	No		Fully DS	Yes	pD	Fully DS	No	
P99-134	B	Fully DS	No		Fully DS	No	pA	Fully DS	No	
P99-136	B	Partial SS	No	RVP5	Fully DS	No	pF	Fully DS	Yes	P1
P99-137	C	Fully DS	No		Partial SS	Yes	pF	Fully DS	Yes	P1
P99-135	D	Partial SS	No	RVP5	Fully DS	Yes		Fully DS	No	
P99-48	H	Fully DS	No		Partial SS	No	pA, pD	Fully DS	Yes	P1

[a] When the consensus was derived in part from only one strand, partial SS; when both strands could be sequenced entirely, fully DS.

[b] The alternative polymerase chain reaction (PCR) primers are described in the Methods section.

[c] Failed sequencing primers; for the ViroSeq Genotyping System kit they are called pA, pD, pF, pG, pH to avoid confusion with the subtype; for the TRUGENE HIV-1 Genotyping kit, the regular primers for protease gene are called P1.

[d] This strain was recombinant in the *pol* region, protease, and AA 1–62 of reverse transcriptase belonging to subtype A; the rest of the reverse transcriptase belonged to subtype D.

paring the consensus sequences obtained by the three systems, these differences in reporting mixtures affected 14 resistance-related positions (10) in 11 samples. The following codons were scored differently: for the protease gene, L10 (1 sample), K20 (2 samples), S37N (1 sample), L63 (2 samples), I64 (1 sample), V82 (1 sample), and L90 (1 sample); and for the RT gene, V179 (2 samples), R211 (1 sample), and K219 (1 sample). In seven cases, the ambiguity was only seen with TRUGENE; in two cases, the ambiguity was seen only with ViroSeq; in one case, the ambiguity was seen only with the in-house system; and in two cases, the ambiguity was seen with the in-house system and with ViroSeq but not with TRUGENE. The discrepancies were found in the A/D recombinant sample, in a CRF02_AG sample, in two subtype B samples, and in one subtype C, D, F, G, and H sample. Four of the samples were from treated patients.

We sequenced dilutions (10^3 and 10^4 RNA copies/ml) of samples P99–134 (subtype B) and MP1046 (subtype J) to evaluate the performance at a lower viral load. The viral load for the undiluted samples was 5.24 log RNA copies/ml for P99–134 and 8.37 log RNA copies/ml for MP1046. All dilutions could be amplified and sequenced by all three systems. The sample MP1046, with no ambiguities in the undiluted sample for the three sequencing systems, shows an ambiguity at position 46 of the protease gene at 10^3 RNA copies/ml when using ViroSeq version 1 and at 10^3 and 10^4 RNA copies/ml when using the in-house protocol, resulting in the score M46M/I instead of pure mutant M46I as was scored for the undiluted sample.

DISCUSSION

All samples were successfully amplified and sequenced by the in-house procedure and the two commercial kits, but the process was time-consuming in some cases, because alternative primers were needed for all three systems. For the in-house and ViroSeq systems,

we were not always completely confident about the results, because part of the consensus sequence was obtained in only one direction for some of the samples.

Almost all discrepancies at resistance-related positions were caused by a different scoring of mixtures that did not alter the interpretation toward particular drugs when using RetroGram version 1.2 decision support software (Virology Networks, Utrecht, The Netherlands) (12). Only two of the seven discrepancies identified with TRUGENE versus the two other systems resulted from scoring mixtures (wild-type/mutant) at positions related to primary resistance mutations in the Pro gene (V82V/F and L90L/M). The same sequences tested by the two other systems are wild-type at these two positions. For sample MP756 (discrepancy at position 82), the sequence obtained using TRUGENE resulted in the following interpretation for protease inhibitors using RetroGram version 1.2: amprenavir and saquinavir (both score A) can be used, indinavir and ritonavir (both score B) can be considered if amprenavir or saquinavir is not available, and nelfinavir (score C) can be considered if neither amprenavir, saquinavir, indinavir nor ritonavir is available, whereas there are no restrictions on the use of any protease inhibitor with the other two systems. For sample MP1144 (discrepancy at position 90 in the protease), the sequence obtained using TRUGENE resulted in the following interpretation for protease inhibitors using RetroGram version 1.2: amprenavir (score A) can be used, indinavir and ritonavir (both score B) can be considered if amprenavir is not available, and saquinavir and nelfinavir (score C) can be considered if neither amprenavir, indinavir, nor ritonavir is available, whereas there are no restrictions on the use of any protease inhibitor with the other two systems.

All three systems were able to sequence samples with low copy numbers (10^3 and 10^4 copies/ml) whether they were subtype B or non-B. For one of the samples, P99–134, there was no difference is scoring mixtures for the diluted samples compared with the undiluted samples. For the sample MP1046, ViroSeq and the in-house system scored a mixture in the Pro position (M46M/I) in the diluted samples, although all systems scored this position as mutant in the undiluted sample. These differences did not affect the resistance interpretations according to RetroGram version 1.2. These results suggest that in our samples, the different scoring in mixtures may be related to the quality of the sequence (differences in background signal) rather than to a lower sensitivity toward mixtures at a lower viral load.

Overall, the commercial kits are scoring ambiguities more often than our in-house procedures. Whether this is a result of the use of different primers, chemistry, or software is not clear. Because the samples were not cloned to verify the presence of variants at ambiguous positions, we could not verify whether the commercial systems score more ambiguities because of a lack of resolution in sequence chromatogram peaks resulting in scoring false ambiguities or if the commercial systems performed better in detecting true ambiguities.

To avoid discrepancies between different resistance assay kits and to improve the quality of the sequences, more efforts have to be made to improve genotyping assay kits and especially to improve the performance of amplification and sequencing primers so that these kits allow fast and reliable resistance testing for all HIV-1 subtypes.

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