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Review

Performance evaluation of the two protease sequencing primers of the Trugene HIV-1 genotyping kit

Sergio García Ribas, Leo Heyndrickx, Pascale Ondoa, Katrien Fransen *

Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium

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Abstract

This article describes the performance of the two protease sequencing modules available in the Trugene HIV-1 genotyping Kit on a sample population with a high prevalence of HIV-1 non-B subtypes ($n = 110$). The relevance of the algorithm recommended by the kit was also evaluated. The results indicated a high sequencing failure rate of the PR module (34%). Forty-five percent of the failed sequences derived from non-B subtype viruses. Furthermore, no PR sequence could be obtained from any of the HIV-1 subtype A and C infected samples that were tested. In contrast, a sequence could be obtained from the entire panel using the P2 module. The data indicated that the high rate of sequencing failures of the PR module was related to both the HIV-1 non-B subtypes as well as lower levels of RNA viral load. In six out of the 73 samples for which both protease modules were successful, discrepancies between the two protease sequences were observed, which led to discordant resistance reports in two cases. The data highlight the problems and the clinical implications that may occur during resistance genotyping of clinical samples with a high prevalence of HIV-1 non-B subtypes.

Keywords: Trugene; Genotyping; HIV-1; Subtype; Protease

Contents

* Corresponding author. Tel.: +32 3 2476332; fax: +32 3 2476333.
E-mail address: kfransen@itg.be (K. Fransen).

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1. Introduction

Resistance genotyping of the human immunodeficiency virus type 1 (HIV-1) has become a standard assay for the follow-up of infected patients receiving antiretroviral therapy (ART) in industrialized countries. These assays are intended to detect the most significant HIV-1 genomic mutations that confer resistance to specific antiretroviral drugs. Resistance genotypes are used by the clinician to either adjust or continue the patient's treatment. By analogy to the HIV-1 RNA viral load assays, commercial genotyping kits have been mainly developed and validated to detect HIV-1 subtype B strains, the predominant clade present in the Western world (Perrin et al., 2003), although a major increase of non-B subtypes is taking place worldwide (Deroo et al., 2002; Chaix et al., 2003). In Belgium, the presence of HIV-1 non-B subtypes is high owing to immigration and frequent travelling abroad (Snoeck et al., 2002). In the Institute of Tropical Medicine (ITM) in Antwerp, Belgium, almost 50% of HIV-infected patients, mostly originating from sub-Saharan African countries, are infected with HIV-1 non-B subtypes. This genetic diversity may represent a problem for the present assays. Suboptimal performance of resistance testing in a population of patients mainly infected with HIV-1 non-B clades may have huge implications for the adequate follow-up of antiretroviral therapy.

Since 2004, the AIDS Reference Laboratory (ARL) of the ITM has been using the FDA approved Trugene HIV-1 Genotyping Kit from Bayer Healthcare to monitor drug resistance among HIV-1 infected patients. Determination of resistance is based on the identification of certain mutations in the *protease* and *reverse transcriptase* (*RT*) genes. Hitherto, sequencing is performed using four CLIP reactions, two covering the *protease* gene (PR and P2 module) and two covering part of the *RT* gene (RTbegin and RTmiddle). The manufacturer's guidelines stipulate that the resistance report should be based on the sequences obtained from both the PR and RT modules. The sequence obtained with the P2 module is only used if the PR module fails.

The genotyping kit was used for a year at the ITM and a high rate of sequencing failures of HIV-1 non-B subtypes was observed with the PR module. Subsequently, a systematic investigation of the P2 module and its use in preparing resistance reports was initiated, even when the PR module was successful. Parallel analysis of both PR and P2 modules sometimes revealed discrepancies resulting in ambiguous resistance reports. The present communication highlights the problems that may occur with the Trugene kit with some clinical samples principally those infected with HIV-1 non-B subtypes.

2. Materials and methods

2.1. Patients and samples

One hundred and ten plasma samples from 105 different consecutive HIV-infected patients attending the ITM clinic were retrospectively examined. The resistance genotype was requested by the clinicians as part of the pre-therapy assessment or in the context of a suspected therapy failure. There were no exclusion criteria.

2.2. Laboratory tests

2.2.1. HIV-1 RNA viral load

The HIV-1 RNA viral load was assessed with the COBAS Ampliprep/COBAS Amplicor HIV-1 Monitor Test V1.5 (Roche Molecular Systems, Branchburg, NJ, USA) according to the manufacturer's instructions. Two different protocols were used. The lower detection limits of this test are 50 ($\log_{10}$ 1.70; ultrasensitive procedure) or 400 ($\log_{10}$ 2.60; standard procedure) RNA copies/ml.

2.2.2. Resistance genotype

The resistance genotyping was performed with the Trugene HIV-1 Genotyping Kit (Revision A, 2003-09) and the OpenGene DNA sequencing system (Bayer Healthcare, NY, USA) as explained elsewhere (Grant et al., 2003). Version 8 of the resistance guidelines was used to prepare the resistance reports. Briefly, 140 μl of plasma was used to extract viral RNA (QIAGEN, Hilden, Germany). A single round RT-PCR was performed to amplify a fragment of 1.3 kb in the *pol* gene. Subsequently the amplicon was sequenced (CLIP reaction) using four modules, two in the *protease*, PR and P2, covering codons 10–99 and two in the *RT* gene, RTbegin and RTmiddle, covering codons 38–247. The P2 is included as an alternative module in case of failure of the PR module. Because a high rate of sequencing failures was observed with the PR module, results from both the PR and the P2 modules were used when preparing the resistance reports.

2.2.3. HIV-1 genetic subtypes

Genetic subtyping of the samples was performed in the *RT* gene using the subtype HIV-1 RT prototype 1 library (2004) (Geneobjects 3.2, 2002/12/17).

2.3. Statistical analysis

Samples with a viral load higher than the upper detection limit of the assay were assigned a value of $\log_{10}$ 6.00. The normality of data distribution was examined by the Kolmogrov–Smirnov test. Differences in the rate of the protease sequencing failures between HIV-1 subtypes were evaluated with the χ^2-test. Viral loads between two groups of samples were compared using Student's *t*-test. A one-way ANOVA was used when more than two groups were involved. The level of significance was set to ≤ 0.05.

3. Results

3.1. RNA viral load and HIV-1 subtypes

All the samples had a detectable viral load with a mean value of $\log_{10}$ 4.44 RNA copies/ml (range: $\log_{10}$ 2.70–6.00). Two samples had an RNA viral load lower than $\log_{10}$ 3.00 (Table 1). All the HIV-1 strains *RT* gene sequences could be classified into nine group M subtypes and three circulating recombinant forms

Table 1
HIV-1 subtypes and viral loads of the tested sample population

HIV-1 subtype	*N*	*N*2 (%)	Mean VL (log_{10} RNA copies/ml)	Range VL (log_{10} RNA copies/ml)
A	13	13 (100)	3.77	2.76–4.72
B	60	5 (8)	4.72	3.14–6.00
C	6	6 (100)	4.32	3.76–5.99
D	1	0 (0)	3.40	3.40
F	4	1 (25)	3.58	3.05–4.50
G	8	3 (38)	4.29	2.70–5.59
H	1	0 (0)	4.01	4.01
J	2	1 (50)	4.51	3.43–5.58
K	2	1 (50)	4.03	3.40–4.66
CRF01_AE	6	4 (67)	4.09	3.24–5.73
CRF02_AG	5	3 (60)	4.68	3.33–6.00
CRF06_cpx	2	0 (0)	5.01	4.76–5.26
Total	110	37 (34)	4.44	2.70–6.00

N is the total number of samples sequenced; *N*2 is the number of failed samples with the PR module.

(Table 1). Forty-five percent of the sequenced samples were classified as non-B.

3.2. Resistance genotyping

The sequencing reaction using the P2 and RT modules was successful for the entire panel ($n = 110$). In contrast, the PR module failed to sequence 34% of the samples. Both PR and P2 sequences could be generated from 73 samples and among these, nucleotide discrepancies were found on six occasions. In two cases, this resulted in major discrepancies between the two resistance reports. These six samples belonged to four different patients who were receiving ART at the time of sampling (Table 2).

In the first case (patient 1; sample Pt1_2) the analysis of the electropherograms showed that the PR module detected a viral mixture, whereas a single virus population was detected by the P2 module (Fig. 1). The PR sequence was suggestive of a resistant profile for all protease inhibitors (PIs) while the P2 sequence indicated that the virus was sensitive to all drugs (data not shown). This result was confirmed after repeating the RNA extraction, the RT-PCR and the CLIP reaction. A previous and a later sample (Pt1_1 and Pt1_3) from the same patient were also tested. For both samples the PR module did not detect a viral mixture at certain positions compared to the P2. Nevertheless, the resistance reports for both samples were concordant.

In patient 2 (sample Pt2), the PR module detected a virus mixture at some positions while the P2 did not and vice versa (data not shown), indicating a difference in sensitivity of the protease modules towards the various virus variants. No relevant mutations could be detected in the PR sequence whereas the P2 showed three differences (Table 2). This resulted in reporting no evidence of resistance with the PR sequence, while possible resistance to indinavir (IDV), ritonavir (RTV) and amprenavir (APV) was reported with the P2 sequence.

For patients 3 and 4 (samples Pt3 and Pt4) both protease modules detected a viral mixture although the PR module missed certain positions. A concordant resistance report was generated, irrespective of the protease module used.

To investigate further the discrepancies found in sample Pt1_2, it was tested with another genotyping assay (ViroSeq HIV-1 Genotyping System, Abbott Diagnostics) as well as in

Table 2
Overview of the six samples with discordant protease sequences

Patient	Sample	Date of sample	Subtype	Viral load (log_{10})	Therapy at date of sample	Relevant protease mutations	
						PR module	P2 module
1	Pt1_1	29 April 2004	G	5.30	3TC + d4T + TDF	K20I, L33I, M36I, M46L, F53L, I54V, L63P, A71I/V, G73S, I84V, N88D, L90M	K20I, L33I, M36I, M46L, F53L, I54V, L63P, **A71I/T/V**, G73S, I84V, N88D, L90M
1	Pt1_2	25 August 2004	G	5.54	3TC + d4T + TDF	M36I, **I54V**, **A71V**, **I84V**, **N88D**, **L90M**	M36I
1	Pt1_3	10 December 2004	G	5.59	3TC + d4T + TDF	K20I, M36I	K20I, M36I
2	Pt2	07 June 2004	B	3.25	AZT + 3TC + NFV	None	**K20R**, **M36I**, **M46L**
3	Pt3	28 February 2005	B	3.53	LPV + RTV + ABC + AZT + 3TC	L10F, M36I, M46I, I54V, L63P, G73S/T, I84V, L90M	L10F, **L24I**, M36I, M46I, I54V, L63P, G73S/T, I84V, L90M
4	Pt4	06 August 2004	B	5.46	ABC + 3TC + d4T	K20I, M36I	K20I, M36I

The mutations typed in bold mark the differences recognised by the two protease sequencing modules. Samples Pt1_1, Pt1_2 and Pt1_3 belong to the same patient. The dates identify the time point at which the samples were taken.

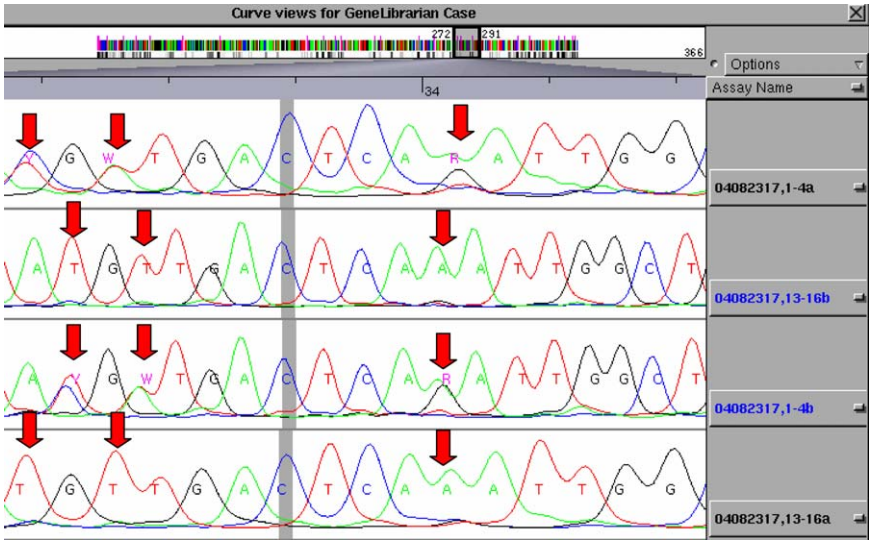

Fig. 1. Overview from a section of the *protease* gene sequenced with the PR (1–4a and b) and P2 (13–16a and b) modules in the sense (a) and anti-sense (b) direction. The arrows mark the discrepant positions between the two sequences.

an 'in-house' assay (performed by the AIDS Reference Laboratory of Ghent, Belgium; Steegen et al., 2005). The ViroSeq kit detected only the PI sensitive virus population, similarly to the P2 module. The 'in-house' assay detected a mixture of virus populations and complete resistance towards all PIs confirming the result found with the PR module (data not shown).

3.3. Resistance genotype as a function of HIV-1 subtype and HIV-1 RNA viral load

HIV-1 subtype distribution was significantly different in the group of samples that could or could not be sequenced using the PR module (Fig. 2) (χ^2, $p < 0.001$). The data suggest that HIV-1 non-B subtype samples have 7–10 times more risk of undergoing sequencing failure with the PR module as compared to HIV-1 subtype B samples (data not shown). Analysis of viral load levels indicated that the mean viral loads were significantly lower in the group of samples that could not be sequenced (mean = $\log_{10}$ 4.06) as compared to the groups that could be sequenced (mean = $\log_{10}$ 4.65) with the PR module (*t*-test, $p < 0.0001$). Comparison of HIV-1 RNA viral load between subtypes indicated that samples infected with HIV-1 subtype A samples had significantly lower viral loads (mean = $\log_{10}$ 3.77) as compared to subtype B samples (mean = $\log_{10}$ 4.71, $p < 0.0001$). This difference was not significant when other HIV-1 clades were considered. The association between lower viral load and failure to sequence with the PR module was further suggested in subtype G samples. Within the group of subtype G samples, the failed specimen had significantly lower viral loads (mean = $\log_{10}$ 3.33) as compared to the samples that were successfully sequenced (mean = $\log_{10}$ 5.26, $p < 0.0001$) (Fig. 3). These observations suggest that both the HIV-1 subtype and viral load may influence the performance of sequencing using the PR module.

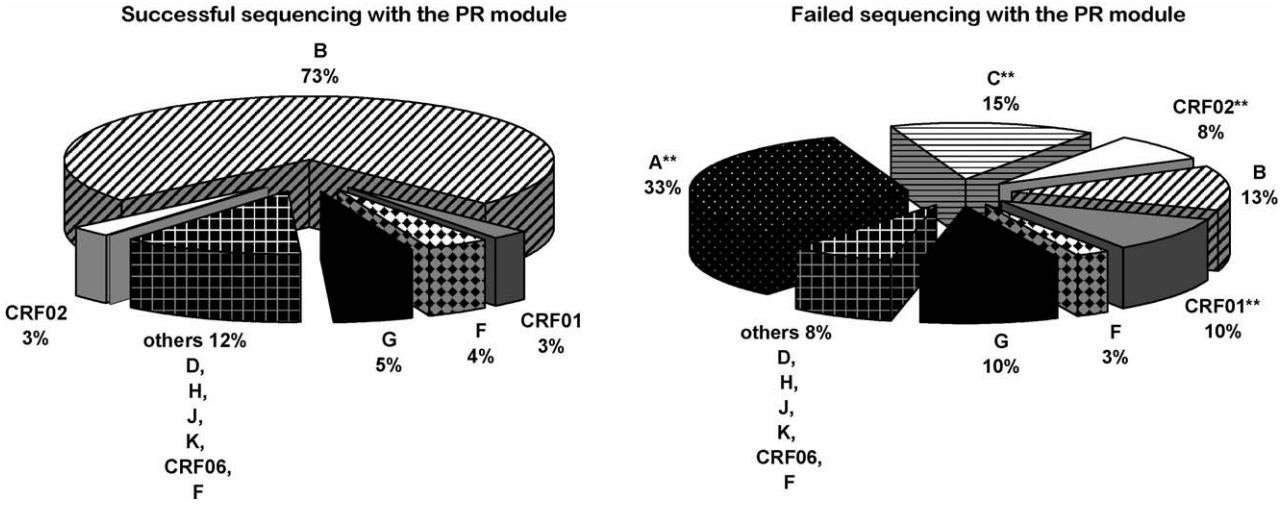

Fig. 2. Distribution of the HIV-1 subtypes as a function of the sequencing result. The pie charts represent the HIV-1 subtype distribution among samples with a successful (left) or a failed (right) PR sequences. Subtypes marked with ** are over-represented in the group of samples with a failed PR sequence (χ^2, <0.05).

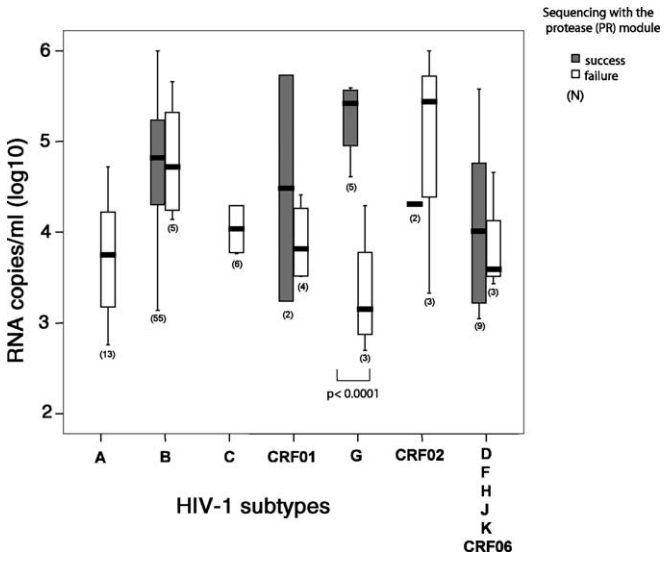

Fig. 3. Impact of the RNA viral load on the success rate of the PR module with respect to the subtype. The numbers in between brackets represent the number of samples which resulted in successful/failed sequences using the PR module.

4. Discussion

Genotyping is an important biological parameter for the detection of resistance to antiretroviral drugs. The reliability of the resistance profile generated by commercial resistance genotyping kits depends on the sensitivity of the primers used. Due to the high genetic variability of HIV-1, the success rate of amplification and subsequently the sequence reaction (Maes et al., 2004) of a given resistance genotyping kit may fluctuate. The present set of data shows that resistance genotyping of clinical samples using the Trugene kit may be problematical, especially in populations infected mainly with non-B subtypes.

Ambiguous resistance reports were related to: (1) low sensitivity of the PR module towards HIV-1 non-B subtype and specimens harbouring a low viral load; (2) discordance in scoring different viral populations with the PR and the P2 modules; (3) suboptimal algorithm for the interpretation of the resistance profiles.

The results are in contrast with the majority of previously reported clinical studies that indicated a high efficiency of the Trugene kit (Grant et al., 2003; Kuritzkes et al., 2003; Gale et al., 2006). This is probably due to the fact that the clinical evaluations generally included none or only a small number of HIV-1 non-B subtypes (∼20–30 samples). An excellent amplification rate (100%) with the RT-PCR primers was achieved. This is in accordance with previous reports showing that the updated version of the test (Version 1.5) is successful in amplifying all viral strains (Tong et al., 2005; Grant et al., 2003). However, we observed a high failure rate of the PR sequencing module (34%). In a study from Jagodzinski and colleagues a panel of 34 HIV-1 isolates of HIV-1 group M from A–H, the failure rate of the PR was shown to be up to 61%. According to the manufacturer a relatively low frequency of failure (∼10%) had previously been observed with the PR module and was associated with polymorphic variability of HIV-1 in the area of the genome where the primers bind. This prompted Bayer to update the kit with an alternative set of protease primers (the P2 module). This module is reportedly more robust in sequencing HIV-1 non-B subtypes and/or samples with lower VL (Jagodzinski et al., 2003). However, when the P2 module is used, the genotype sequence is not obtained for the first seven codons. This is the reason why this module is not the preferred primary analytic tool in the algorithm of the kit. The resistance report is based on the analysis of the PR sequence alone and only in the event of failure is the P2 sequence considered. However, because in the present study population sequencing failures with the PR module were more frequent, the P2 sequence was used more often in determining the virus resistance profile. No sequence could be obtained for any of the tested samples belonging to subtypes A and C as well as a significant proportion of samples in subtypes B, F, G, J, K, CRF01_AE and CRF02_AG using the PR primers. Consequently, the decision was made to systematically take the P2 sequence into consideration when preparing the resistance reports. Whether the failure of the PR module was related to HIV-1 subtypes, HIV-1 viral load, or both, remains unclear. This question should be clarified in further studies.

The simultaneous analysis of both PR and P2 sequences revealed some discrepancies in scoring the different viral quasispecies. According to the manufacturer both protease modules should be able to detect any viral variant that represent at least 20% of the viral population. Nevertheless, in six out of 73 samples where both PR and P2 sequences were available, there was some divergence in the viral population detected, which occasionally led to ambiguous resistance reports. Although discordant resistance genotype profiles occurred only twice, these data raise the question of how reliable the individual PR or P2 sequences are. As far as can be ascertained, it is the first time that the reliability of this kit has been evaluated by comparing the outcome of the PR and the P2 sequences. An important issue is to determine whether resistance reports generated with the Trugene algorithm, i.e., on the basis of PR sequence alone or P2 sequence alone, may miss some crucial mutations that would have been detected if the two modules had both been successful and analysed simultaneously. An additional issue is that resistance reports will always be suboptimal in a population predominantly infected with non-B subtypes if, as claimed by the manufacturer, the PR module is considered superior to the P2. Indeed, the high failure rate of the PR module in HIV-1 non-B subtypes indicates that a significant number of the resistance reports will be generated on the basis of the P2 sequence in these populations. Analyzing the sequences produced by both protease modules simultaneously, instead of the serial procedure recommended by the manufacturer, could increase the sensitivity of detecting possible low level multiple resistant variants as seen in patient 1 (sample Pt1_2) and patient 2.

Further investigation is needed to determine whether the discrepancies between the PR and P2 modules are due to an over detection of a minor (<20%) virus subpopulation or an under detection of major (≥20%) variants within the viral quasispecies. Variants representing 20–30% of a quasispecies are difficult to characterize because their detection remains unreliable although mixtures at specific nucleotide positions can be

detected in composite sequences (Palmer et al., 2005). This may explain/apply to the failure to detect the resistant virus populations in the samples Pt1_2 and Pt2.

In conclusion, genotyping using the Trugene kit may lead to discrepant results depending on the protease module used. More importantly, following the rules as set by the manufacturer may lead to neglecting relevant drug resistant variants present within a patient. Therefore, it is recommended that the rules, indicating that the protease modules are used sequentially, should be amended so that they are used in parallel. This modification would maximize assay sensitivity as well as clinical relevance. Furthermore, it is also recommended that in future the sensitivity of primers in all commercially available genotyping assays should undergo a more thorough evaluation using a wider range of clinical samples originating from different regions of the world.

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