Journal of Clinical Virology 32 (2005) 265–271

www.elsevier.com/locate/jcv

RNA extraction prior to HIV-1 resistance detection using Line Probe Assay (LiPA): comparison of three methods

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Received in revised form 22 July 2004; accepted 12 August 2004

Abstract

Background: Genotyping testing has been accepted as a guidance in the therapeutic management of Human Immunodeficiency virus 1 (HIV-1). However, optimization of the available routine techniques for such purpose has not been fulfilled.
Objective: To evaluate the use of three RNA extraction methods in order to be applied in the genotypic HIV-1 resistance testing by LiPA.
Study design: Comparative prospective study of three HIV-1 RNA extraction methods. Forty-eight plasma samples were tested for the determination of viral load (VL) by means of Cobas Amplicor HIV-1 Monitor™ (Roche Diagnostics. Branchburg, NJ, USA), preserving the obtained RNA extracts. RNA was also extracted using two other techniques: "SV Total RNA Isolation System" (Promega Corporation. Madison, WI, USA) and "QIAamp Viral RNA" (QIAGEN Inc., Valencia, CA, USA). The three RNA extracts were processed in parallel for the detection of HIV resistance by LiPA, and bands were recorded comparatively.
Results: Results obtained by Roche extraction method were superior, followed by those of Qiagen and Promega, in the several studied parameters. First, proportion of amplified samples (75.0% by Promega versus 95.8 by Qiagen and 97.9% by Roche for LiPA RT and 97.7% by Promega versus 100.0% by Roche and Qiagen for LiPA P); second, percentage of combined mutations patterns, and third, differences in band intensity. Thus, for LiPA RT 51.4% and 54.3% of the samples showed greater intensity after Roche and Qiagen extractions, respectively. These percentages dropped to 12.8 and 19.1 for LiPA P.
Conclusions: The outcome obtained by LiPA after RNA extraction by Roche methodology was remarkably superior to those of Promega and Qiagen. LiPA technique needs further optimization, especially the sample amplification phase of LiPA RT.

Keywords: HIV; Resistance; LiPA; RNA extraction

1. Introduction

The establishment of VL quantitation techniques in the follow-up and control of HIV-1 infected individuals is at present a fact that needs no discussion. This variable, along with CD_4 cell count, acts as an illness evolution marker, as a defining criterion of therapeutic failure and as an indicative tool for starting or switching antiretroviral treatment (Carpenter et al., 2000; DHHS Panel, 2001; Rubio et al., 2002; Weinstein et al., 2001). Several changes gradually applied to VL determination techniques have led to the detection of HIV RNA plasma levels down to 20 RNA copies/ml, offering optimization of therapeutic patient management (Ficher et al., 1999; Venturi et al., 2000).

The implementation of techniques for the detection of antiretroviral agents resistance in clinical practice has been widely strengthened due to a clearer understanding of the

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doi:10.1016/j.jcv.2004.08.007

natural history of the infection, improved infection control and the establishment of highly active antiretroviral therapy (HAART). In this sense, the use of molecular microbiological diagnosis techniques emerges as a need in which lack of standardization and validation, and relatively scarce experience in its performance may be present as limiting factors (Erali et al., 2001; Kartsonis and D'Aquila, 2000).

A critical point in the development and performance of molecular diagnosis techniques in general, and in particular in those based on polymerase chain reaction (PCR) amplification, is the extraction of genetic material in the purest conditions. This essential step constitutes the limiting phase for achieving optimum outcome, especially when HIV RNA plasma levels are close to the detectable threshold (Gómez-Cano et al., 1999; Verhofstede et al., 1996; Villahermosa et al., 2000), a situation that is becoming more frequent with HAART use.

The Clinical Microbiology laboratory is usually pressed to carry out several determinations starting from a single sample. Given personnel, cost and infrastructure restrictions affecting many laboratories, the sharing of common steps, which facilitate and improve the outcome of these techniques, is urged. In this respect the objective of the present study is to compare the output of three different HIV-1 RNA extraction methods prior to the detection of genotypic resistance to both nucleoside reverse transcriptase inhibitors (nRTIs) (LiPA RT) and to protease inhibitors (PIs) (LiPA P).

2. Materials and methods

2.1. Patients

From February to May 2002, 48 plasma samples from different patients with ≥1000 RNA copies/ml were randomly selected from VL determination requests received at our Microbiology laboratory.

2.2. Methods

All samples were processed for VL analysis by means of PCR after previous reverse transcription (RT-PCR) (Cobas Amplicor HIV-1 Monitor™; Roche Diagnostics, Branchburg, NJ, USA) in its ultrasensitive version with a threshold of 50 RNA copies/ml. This version includes ultracentrifugation at 23,600 × *g* of the 500 μl of plasma at 2–8 °C for 60 min prior to the viral particle lysis. RNA is extracted by adding a chaotropic agent (guanidinium-thiocyanate) followed by RNA precipitation with ethanol. After VL determination all RNA extracts were preserved at −80 °C.

RNA from samples with a VL of ≥1000 RNA copies/ml was also extracted using two additional techniques, "SV Total RNA Isolation System" (Promega Corporation, Madison, WI, USA) and "QIAamp Viral RNA" (QIAGEN Inc., Valencia, CA, USA). Both methods are based on a lysis–centrifugation process followed by a column filtration through a silica membrane in a RNase-free environment. The starting plasma volumes were 125 and 140 μl, respectively.

RNA extracts obtained by the two methods along with those obtained for VL determination were tested in parallel for the detection of genotypic resistance by means of the commercial assay Line Probe Assay (LiPA) (VERSANT® HIV-1 RT Resistance Assay, VERSANT® HIV-1 Protease Resistance Assay, Bayer Corporation. Tarrytown, NY, USA).

Briefly, LiPA™ is based on a post-PCR hybridization that takes place on nitrocellulose strips onto which specific oligonucleotide probes are fixed in parallel lines. This assay allows the study of possible mutations at codons 41, 69, 70, 74, 184 and 215 of reverse transcriptase (RT) gene (LiPA RT) and at codons 30, 46, 48, 50, 54, 82, 84 and 90 of protease gene (LiPA P). Mutations in these positions have been reported as associated to nRTIs and PIs resistance, respectively.

Comparative reading of the strip bands was done subjectively. As the LiPA manufacturer in Spain recommends RNA extraction by Promega, this was taken as reference, quoting the results of Roche and Qiagen extractions in comparison to that. For this effect we adopted a triple strategy. First, HIV control band intensity was checked. Second, the intensities of the rest of bands were checked, and finally, the appearance of different bands by each extraction method was evaluated. Our findings were classified in four groups (Table 1): (1) "Equal intensity", when all bands were similar; (2) "Intensity 1+", when the HIV control band was slightly darker by Roche and Qiagen methods with the same number of bands; (3) "Intensity 2+", when the HIV control band and the rest of bands were markedly darker, with the same number of bands, or an extra band was observed by Roche or Qiagen methods; (4) "Intensity 3+", when band coloring was markedly superior by Roche or Qiagen methods and more than one extra band appeared. When the Roche or Qiagen extraction strip showed lower intensity than the Promega strip, similar criteria were applied using negative figures. We also compared the extraction after Roche method versus Qiagen one.

Mutations were interpreted following the manufacturer's instructions (VERSANT® HIV-1 RT, 2001; VERSANT® HIV-1 P, 2001) and according to the Medscape Guide to Antiretroviral Resistance Mutations (http://hiv.medscape.com/updates/quickguide) and to the "International AIDS Society—USA Panel" recommendations (Hirsch et al., 2000).

Table 1
Adopted criteria for the classification of findings after band comparative reading

Intensity classification	Band intensity	Band number
Equal	Equal	Equal
1+	Slightly superior	Equal
2+	Markedly superior	Equal or 1 extra band
3+	Markedly superior	More than one extra band

2.3. Statistical analysis

A descriptive study of all findings was carried out using the statistical program SPSS 9.0 for Windows. "Ji square" tests were applied to evaluate the possible relation between the different HIV RNA extraction methods and the outcome of PCR amplification as well as the presence of wild-type or mutated HIV variants. Finally, possible discrepant LiPA results, both at the band intensity level and at that of differences in mutations detected, were studied according to the RNA extraction method used.

3. Results

Fourty-eight plasma samples were randomly selected from all plasma samples received for VL determination from February to May 2002 whose VL was ≥1000 RNA copies/ml. The mean was 59,926.7 RNA copies/ml, and the median was 80,250.0 RNA copies/ml (range: 1300 to >100,000 RNA copies/ml).

PCR amplification results of the extracts obtained by each method were different for LiPA RT and for LiPA P, being statistically significant in all cases (p = 0.000) (Table 2). For LiPA RT successful amplification was achieved after Promega extraction in 36 out of 48 samples (75.0%; 95% CI, 60.4–86.6), in all 48 samples after Roche extraction (100.0%; 95% CI, 92.6–100.0) and in 46/48 samples after Qiagen extraction (95.8%; 95% CI, 85.7–99.5). Twelve samples were not amplified after Promega extraction; the VL values were 1300 RNA copies/ml in one sample, and >50,000 RNA copies/ml in the rest. For the two samples that were not amplified after Qiagen extraction, VL levels were >100,000 RNA copies/ml. For LiPA P a successful amplification was obtained in 47/48 Promega extracts (97.9%; 95% CI, 88.9–99.9) and in 100.0% of Roche and Qiagen extracts (95% CI, 92.6–100.0). The VL level of the only sample that was not amplified, after Promega extraction was >100,000 RNA copies/ml.

Comparative analysis of the results obtained by all extraction methods led to discrepancies in findings at three different levels. First, disagreements were obtained in the results according to mutations detection. Thus, for LiPA RT in six samples the final LiPA interpretation was "Wild type" for Promega and Roche extracts but "Mutant" for the corresponding Qiagen extract. Also, for one sample the genotype was recorded as "Mutant" for Promega and Roche extracts but "Wild type" for the corresponding Qiagen extract. This same situation was also detected seven times for LiPA P. Second, varying results were obtained as for sample amplification outcome. Ten samples that did not amplify after Promega extraction were interpreted as "Mutant" after successful amplification of the corresponding Roche and Qiagen extracts for LiPA RT. Third, from the group of samples successfully amplified and in which resistance mutations were present after extraction by all three methodologies, disagreements could be documented both in the absolute frequency of appearance of individual mutations (Table 3) and in the patterns of combined mutations observed (Tables 4 and 5).

The analysis of the differences in the band coloring intensity observed for each sampler for LiPA RT (Table 6), was done in three ways. First, Roche was compared to Promega revealing that 15 samples (42.9%) had the same intensity. In 18 cases, Roche band intensity was superior, being classified as "Intensity 1+", "2+" and "3+" in six cases (51.3%), respectively. In two occasions Roche band intensity was lower ("Intensity 1−"). Second, Qiagen results were compared to those of Promega showing that intensity was equal in 14 samples (40.0%) and superior in 19, distributed as follows: three (8.6%) "Intensity 1+", seven (20.0%) "Intensity 2+" and nine (27.7%) "Intensity 3+". In two cases, Qiagen showed lower intensity than Promega. Finally, Roche results were compared to Qiagen ones. Intensity was found equal in 23

Table 2
Successful amplification results according to the extraction method

	LiPA RT	LiPA P
Extraction method	Proportion of successfully amplified samples*	
Promega	75.0 (60.4–86.4)	97.9 (88.9–99.9)
Roche	100.0 (92.6–100.0)	100.0 (92.6–100.0)
Qiagen	95.8 (85.7–99.5)	100.0 (92.6–100.0)

* Results are given in percentage. 95% CI is shown in parentheses.

Table 3
Discrepancies in absolute frequency of the mutations detected by LiPA RT and LiPA P, according to the RNA extraction method*

Mutation	Promega extraction	Roche extraction	Qiagen extraction
LiPA RT	N = 23**	N = 34	N = 36
L41	11 (47.8)	18 (52.9)	22 (61.1)
R70	2 (8.7)	3 (8.8)	3 (8.3)
K70	–	1 (2.9)	2 (5.6)
V74	5 (21.7)	9 (26.5)	8 (22.2)
L74	–	–	1 (2.8)
V184	11 (47.8)	20 (58.8)	19 (52.8)
Y215	19 (82.6)	25 (73.5)	24 (66.7)
F215	2 (8.7)	2 (5.9)	4 (11.1)
LiPA P	N = 25	N = 27	N = 22
N30	1 (4.0)	2 (7.4)	1 (4.5)
I46	9 (36)	9 (33.3)	6 (27.3)
V48	2 (8.0)	2 (7.4)	2 (9.1)
V54	7 (28.0)	7 (25.9)	6 (27.3)
A54	1 (4.0)	2 (7.4)	2 (9.1)
V84	4 (16.0)	5 (18.5)	4 (18.2)
F82	2 (8.0)	2 (7.4)	2 (9.1)
F82V84	1 (4.0)	1 (3.7)	–
A82	12 (48.0)	14 (51.9)	7 (31.8)
T82	1 (4.0)	1 (3.7)	1 (4.5)
T82V84	1 (4.0)	1 (3.7)	–
M90	13 (52.0)	14 (51.9)	11 (50.0)

* Percentages are shown in parentheses.
** N, number of mutant samples for the corresponding extraction method.

Table 4
Discrepancies in the combined mutations patterns detected by LiPA RT according the extraction method*

Mutation patterns	Promega extraction $N = 23$**	Roche extraction $N = 34$	Qiagen extraction $N = 36$
L41	–	–	1 (2.8)
L41–Y215	5 (21.7)	6 (17.6)	6 (16.7)
L41–F215	–	1 (2.9)	1 (2.8)
L41–V184–Y215	–	4 (11.8)	4 (11.1)
L41–V184–F215	1 (4.3)	–	1 (2.8)
L41–V74–Y215	2 (8.7)	2 (5.9)	1 (2.8)
L41–V74–V184–Y215	–	2 (5.9)	4 (11.1)
L41–L74–V184	–	–	1 (2.8)
L41–R70–V184–Y215	2 (8.7)	2 (5.9)	–
L41–R70–V74–V184–Y215	–	–	1 (2.8)
L41–K70–Y215	–	–	1 (2.8)
L41–K70–V74–Y215	–	–	1 (2.8)
R70	–	1 (2.9)	1 (2.8)
R70–V184	–	–	1 (2.8)
K70–Y215	–	1 (2.9)	–
V74	–	–	1 (2.8)
V74–Y215	2 (8.7)	2 (5.9)	–
V74–V184–Y215	1 (4.3)	3 (8.8)	–
V184	2 (8.7)	6 (17.6)	4 (11.1)
V184–Y215	2 (21.7)	2 (5.9)	2 (5.6)
V184–F215	–	–	1 (2.8)
Y215	2 (8.7)	1 (2.9)	3 (8.3)

* Percentages are shown in parentheses.
** *N*: number of mutant samples for the corresponding extraction method.

Table 5
Discrepancies in the combined mutations patterns detected by LiPA P according the extraction method*

Mutation patterns	Promega extraction $N = 25$**	Roche extraction $N = 27$	Qiagen extraction $N = 22$
N30	–	1 (3.7)	1 (4.5)
N30–A82	1 (4.0)	1 (3.7)	–
I46	1 (4.0)	2 (7.4)	1 (4.5)
I46–V54	1 (4.0)	–	–
I46–V54–M90	1 (4.0)	1 (3.7)	–
I46–F82–M90	1 (4.0)	1 (3.7)	2 (9.1)
I46–F82–F82V84–M90	1 (4.0)	1 (3.7)	–
I46–A82–M90	1 (4.0)	–	–
I46–A82–T82–T82V84	1 (4.0)	1 (3.7)	–
I46–T82	–	–	1 (4.5)
I46–M90	2 (8.0)	2 (7.4)	2 (9.1)
V48–V54	1 (4.0)	1 (3.7)	–
V48–V54–A82	–	1 (3.7)	1 (4.5)
V48–A54–A82	1 (4.0)	–	1 (4.5)
V54–A54	–	1 (3.7)	–
V54–A54–M90	–	1 (3.7)	1 (4.5)
V54–V84–A82–M90	–	3 (11.1)	–
V54–A82	3 (12.0)	3 (11.1)	3 (13.6)
V54–M90	1 (4.0)	–	1 (4.5)
V84	–	–	3 (13.6)
V84–A82	1 (4.0)	–	–
V84–A82–M90	6 (24.0)	6 (22.2)	–
V84–M90	1 (4.0)	1 (3.7)	1 (4.5)
T82–M90	–	–	2 (9.1)
M90	1 (4.0)	–	2 (9.1)

* Percentages are shown in parentheses.
** *N*: number of mutant samples for the corresponding extraction method.

samples (48.9%) and superior in 20 (42.5%), being classified as “Intensity 1+” in eight cases (17.1%), “Intensity 2+” in 10 (21.3%), “Intensity 3+” in two (4.2%) and “Intensity 1−” in four samples (8.5%).

For LiPA P (Table 6), the comparison of Roche and Qiagen to Promega rendered equal intensities in 37 (78.7%) and 31 (65.9%) samples, respectively. Intensities were found superior in six (12.8%) Roche extracts and in nine (19.1%) Qiagen ones. These intensities were classified as “1+” in four (8.5%) cases for Roche extract and in eight (17.1%) for Qiagen one, and as “2+” in two (4.3%) occasions for Roche and in one (2.1%) for Qiagen. In four Roche samples (8.5%) and in six Qiagen ones (12.8%) intensity was recorded as “1−”. The comparison of Roche vs. Qiagen, showed 40 samples (85.4%) with equal intensity, four (8.3%) with “Intensity 1+” and three (6.3%) with “Intensity 1−”.

A global comparison of the intensity differences of Roche and Qiagen extracts versus Promega ones, grouping them as equal or superior, for LiPA RT shows statistical significance ($p = 0.002$). No statistical significance was shown for LiPA P, not even after comparing Roche extract versus Qiagen one.

4. Discussion

Genotypic detection of HIV resistance by LiPA despite having known limitations (Erice et al., 2001; Ruiz and Clotet, 1999; Schmit et al., 1998; Servais et al., 2001; Wilson et al., 2000), is a relatively simple technique to perform. One of its advantages is that the information supplied is significant and useful for the clinician, as all mutations studied are highly associated to HIV resistance (Schinazi et al., 2001).

Table 6
Distribution of band intensity differences observed in LiPA RT and LiPA P according to the extraction method in samples successfully amplified by the corresponding compared methods

Band intensity	Roche/Promega $N = 35$	Qiagen/Promega $N = 35$	Roche/Qiagen $N = 47$
LiPA RT			
Equal	15 (42.9%)	14 (40.0%)	23 (48.9%)
1+	6 (17.1%)	3 (8.6%)	8 (17.1%)
2+	6 (17.1%)	7 (20.0%)	10 (21.3%)
3+	6 (17.1%)	9 (27.7%)	2 (4.2%)
1−	2 (5.8%)	2 (5.7%)	4 (8.5%)
	$N = 47$	$N = 47$	$N = 48$
LiPA P			
Equal	37 (78.7%)	32 (68.1%)	41 (85.4%)
1+	4 (8.5%)	8 (17.0%)	4 (8.3%)
2+	–	–	–
3+	2 (4.3%)	1 (2.1%)	–
1−	4 (8.5%)	6 (12.8%)	3 (6.3%)

The evidently differing results observed for the different extraction methods compared in this study highlight the importance of this step. Extraction conditions the result in any PCR reaction (Verhofstede et al., 1996; Villahermosa et al., 1998), which is an indispensable preliminary step in HIV genotypic resistance testing. Most published studies on the influence of RNA extraction refer to the subsequent VL determination (Ficher et al., 1999; Fransen et al., 1998; Venturi et al., 2000; Verhofstede et al., 1996; Villahermosa et al., 1998). In the literature we have reviewed, several references to extraction outcome applied to HIV-1 genotypic resistance detection use sequencing methodology (Kuritzkes et al., 2003; Lindström and Albert, 2003; Niubò et al., 2000; Stürmer et al., 2003) but few studies address this issue using LiPA technology (Gómez-Cano et al., 1999). The outcome of different extraction methods prior to sequencing is generally expressed as the overall amplification success rate, without further study of the influence on the detected mutations (Kuritzkes et al., 2003; Lindström and Albert, 2003; Stürmer et al., 2003).

The importance of the extraction step is again highlighted by the wide range of discrepant results observed related to successful amplification, to result interpretation as "Wild type" or "Mutant", to differences in absolute individual mutation frequency, and to combined mutation patterns for both LiPA techniques in the mutant group. This fact is especially significant because of the relevant information brought to the clinician for therapeutic patient management, particularly for those patients who have already experienced any therapeutic failure, because of the potential reduction in antiretroviral stock, as recommended by different authors in our environment (Martínez-Picado and Clotet, 1999; Moreno, 2001; Soriano, Ledesma and The Spanish Drug Resistance Panel, 2000). The little importance given to this technical aspect is somewhat surprising in such a widely studied matter as HIV resistance testing is in other aspects. Several extraction methods exist, both "in-house" and commercial, and repercussions from RNA extraction as starting material in the amplification and subsequent mutation detection are present as well. We, therefore, consider that studies such as the present comparing more extraction methods are needed to optimize the outcome of genotypic HIV resistance testing techniques.

Commercial tests for HIV resistance detection should be flexible allowing the optimization of extraction methods. It is also important to have the possibility to make intermediate steps common to several virological molecular diagnosis techniques compatible and to optimize them. At the assistance level, especially due to the high number of processed samples, "in-house" RNA extraction methods are too laborious and time-consuming, although some of them offer an optimum result (Boom et al., 1990; Casas, 1997; Fransen et al., 1998; Verhofstede et al., 1996). In contrast, there are commercially techniques available at an affordable cost that offer good results requiring a reasonably small dedication in terms of time.

According to our experience, two reasons could explain the better results achieved by Roche extraction. First, the inclusion of an ultracentrifugation, which by concentrating the sample improves the extraction yield (Shafer et al., 1997; Villahermosa et al., 2000). Second, the greater starting sample volume (500 μl versus 125 μl used in Promega extraction and 140 μl in Qiagen one) (Schockmel et al., 1997; Venturi et al., 2000; Villahermosa et al., 2000). An additional advantage of this method is that VL determination should be performed prior to HIV resistance testing, and, as the Roche extraction method is used to this effect, the extract obtained serves as starting material for both techniques. Another strategy to improve HIV RNA extraction efficiency would be starting with even greater plasma volumes (up to 1.8–2 ml) (Schockmel et al., 1997; Gómez-Cano et al., 1999; Venturi et al., 2000; Villahermosa et al., 2000). In routine practice this approach is not feasible as the volume of samples received is usually just enough for the determinations requested, and even scarce for all of them or for any possible necessary repetition.

In our study, in contrast with the results of other authors (Gómez-Cano et al., 1999) the outcome of the amplification does not seem to be related to VL levels, as except one sample that had low levels (1300 RNA copies/ml) the rest of the samples that did not achieve a successful amplification had considerably high VL values.

The fact that the same extract used for LiPA RT and LiPA P rendered a noticeably greater proportion of samples that amplified after Promega extraction for LiPA P when compared to such proportion for LiPA RT, implies that the nested PCR prior to mutations detection by LiPA RT is not fully optimized. In our opinion this may be due to a better design of the nested PCR used to prepare samples before LiPA P that leads to a greater yield, and therefore the initial RNA input is less crucial. We were able to document the same issue in a previous study carried out in treatment–naive patients (Eiros et al., 2002).

Our study is limited by the absence of an objective system for band reading such as that provided by a densitometer, which other authors say offers good outcome when applied to band intensity recording (Villahermosa et al., 1998). Assuming this limitation and having defined reading criteria in order to compare the results obtained by the three extraction methods, once again there is a clear disagreement for LiPA RT, where band intensity was greater after Roche and Qiagen extraction methods in more than half of the samples, when compared to Promega one.

In our series, we have described differences in amplification according to the extraction method. From samples successfully amplified after all three extraction methods we have described differences in both the absolute frequency of single mutations and of patterns of combined mutations, as well as differences in band intensity. We are aware that the high sensitivity of the LiPA technology for detecting HIV subpopulations that represent 1–5% of the viral population (Erice al., 2001); could account for the great number

of disagreements observed at all the studied levels. Moreover, this feature hardens the assessment of our results using sequencing techniques, known to be less sensitive for detecting minor HIV variants (Erice et al., 2001; Shafer et al., 2000; Suárez et al., 2002).

The work carried out by our group faithfully reflects technical problems within the virology laboratory setting. However, we do not ignore that the critical point, as recommended by some authors (Hirsch et al., 2000; Secretaría del Plan Nacional sobre el Sida, 1997, 2000; The EuroGuidelines Group for and Resistance, 2001), is the transcription or translation of such information to the clinical follow-up of each patient, his or her therapeutic experience—past and current—being essential. This is the only way that the technical effort of those devoted to laboratory diagnosis can be efficiently applied in the optimization of therapeutic resources.

We have highlighted the importance of the technical optimization of genotypic HIV resistance testing techniques. Unfortunately, other aspects such as standardization of the techniques to avoid intra-laboratory (Shafer et al., 2000; Galli et al., 2003) and inter-laboratory variability (Demeter et al., 1998; Schuurman et al., 1999, 2002) as well as interpretation of the results, not only by the Microbiology laboratory staff (Korn et al., 2003) but also by the clinicians (Salama et al., 2003) and a consensus in such interpretations are still pending.

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