JOURNAL OF CLINICAL MICROBIOLOGY, June 2002, p. 2257–2259 Vol. 40, No. 6
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.6.2257–2259.2002
Copyright © 2002, American Society for Microbiology. All Rights Reserved.

Comparative Study of Two Methods for RNA Extraction Prior to Detection of Resistance to Human Immunodeficiency Virus Type 1 with the Line Probe Assay

Jose María Eiros,[1]* Cristina Labayru,[2] Beatriz Hernández,[1] Raúl Ortiz de Lejarazu,[1] and Antonio Rodríguez Torres[1]

Microbiology Service, University Hospital of Valladolid,[1] and Microbiology Service, University Hospital "Rio Hortega,"[2] Valladolid, Spain

Received 1 November 2001/Returned for modification 5 January 2002/Accepted 26 March 2002

We evaluated two methods from Roche and Promega for RNA extraction prior to the genotypic detection of human immunodeficiency virus type 1 resistance by line probe assay (LiPA). Fifty plasma RNA extracts were processed in parallel by LiPA. Results obtained by the Roche method were superior in the proportion of amplified samples, the percentage of mutated samples, and band intensity.

From February to May 2001, 50 plasma samples from different patients were randomly selected from viral load (VL) determination requests that had ≥1,000 RNA copies/ml.

All samples were processed for VL analysis by means of PCR after previous retrotranscription (reverse transcriptase [RT]-PCR) (Cobas Amplicor HIV-1 Monitor; Roche Diagnostics, Branchburg, N.J.) in its ultrasensitive version with a threshold of 50 RNA copies/ml. This version includes ultracentrifugation at 23,600 × g of 500 μl of each plasma sample at 2 to 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 ≥1,000 RNA copies/ml was also extracted by using the SV Total RNA Isolation System (Promega Corporation, Madison, Wis.). This method is based on a lysis-centrifugation process followed by a column filtration through a silica membrane in an RNase-free environment starting with 125 μl of plasma.

RNA extracts obtained by the two methods were tested in parallel for the detection of genotypic resistance by means of the commercial line probe assay (LiPA) (INNO-LiPA HIV-1 RT and INNO-LiPA HIV-1 Protease; Innogenetics, Ghent, Belgium).

Briefly, LiPA is based on a post-RT-PCR hybridization that takes place on nitrocellulose strips onto which specific oligonucleotide probes are fixed in parallel lines. RT-PCR was carried out following the manufacturer's instructions by using the RT-PCR Access kit (Promega Corporation), except that the primers used were those included in the LiPA Amplification kit. This assay allows the study of wild-type and mutant sequences at codons 41, 69, 70, 74, 184, and 215 of the RT gene (LiPA RT) and at codons 30, 46, 48, 50, 54, 82, 84, and 90 of the protease (P) gene (LiPA P). Mutations in these positions have been reported as associated with resistance to nucleoside RT inhibitors and to protease inhibitors. cDNA synthesis and PCR with biotinylated primers were performed as described by Stuyver et al. (13). Hybridization was performed according to the manufacturer's instructions. Briefly, biotinylated DNA was hybridized with specific oligonucleotide probes immobilized in parallel lines on membrane-based strips. After hybridization, streptavidin labeled with alkaline phosphatase was added and bound to biotinylated hybrids. Incubation with a chromogen resulted in a purple-brown precipitate visible to the naked eye.

Comparative reading of strip bands was done subjectively. Extraction by Promega was taken as reference. We adopted a triple strategy. First, the human immunodeficiency virus (HIV) control band intensity was checked. Second, the intensities of the rest of the bands were checked, and finally, the appearance of different bands by each extraction method was evaluated. Our findings were classified into four groups: (i) equal intensity, when all bands were similar; (ii) intensity 1+, when the HIV control band was slightly darker by the Roche method with the same number of bands; (iii) intensity 2+, when the HIV control band and the rest of the bands were markedly darker, with the same number of bands, or an extra band was observed by the Roche method; (iv) intensity 3+, when band coloring was markedly superior by the Roche method and more than one extra band appeared. When the Roche extraction strip showed lower intensity than the Promega strip, a similar assessment was done using negative figures.

Interpretation of the mutations found was done following the manufacturer's instructions and according to the Medscape Guide to Antiretroviral Resistance Mutations (http://hiv.medscape.com/updates/quickguide) and to the International AIDS Society-USA Panel (6).

A descriptive study of all findings was carried out using the statistical program SPSS 9.0 for Windows. "Ji-square" tests were applied to study the possible relations between the different variables studied.

PCR amplification results of the extracts obtained by each method were different for LiPA RT and for LiPA P, the differences being statistically significant in both cases ($P < 0.001$). For LiPA RT a successful amplification was achieved after Promega extraction in 33 of 50 samples (66.0%; the 95% con-

* Corresponding author. Mailing address: Microbiology, Sixth Floor, Faculty of Medicine, Avda. Ramón y Cajal s/n, 47005 Valladolid, Spain. Phone: 34-983 423063. Fax: 34-983 423066. E-mail: eiros@med.uva.es.

Downloaded from jcm.asm.org by on December 3, 2007

TABLE 1. Discrepancies in results observed for LiPA RT according to the RNA extraction method in the absolute frequency of each mutation (column subtotals) and in combined mutation patterns (total by rows)

Presence of mutation and absolute frequency (%) by Roche (next-to-last row) and by Promega (last row)											Pattern frequency (%)	
L41(T)	L41(C)	R70	D69	D69R70	N69R70	V74	V184	Y215(18)	Y215(19)	F215	Roche	Promega
√							√				1 (100)	
	√	√					√	√	√		1 (100)	
	√	√					√					1 (100)
	√					√	√	√			1 (100)	
		√					√	√			1 (100)	
		√					√			√	1 (100)	
		√						√				1 (100)
		√								√		1 (100)
			√								1 (100)	
						√	√	√			3 (100)	2 (66.7)
							√	√			1 (50)	2 (100)
								√				1 (100)
1 (100)	2 (66.7)	3 (50)	1 (100)			4 (100)	9 (90)	7 (70)	1 (100)	1 (50)		
	1 (33.3)	3 (50)				2 (50)	5 (50)	7 (70)		1 (50)		

fidence interval [CI] was 51.2 to 78.8) and in 49 of 50 samples after Roche extraction (98%; 95% CI, 89.3 to 99.9). For LiPA P a successful amplification was obtained in 38 of 50 Promega extracts (76.0%; 95% CI, 61.8 to 86.9) and in 100.0% of Roche extracts (95% CI, 92.8 to 100.0).

Comparative analysis of the results obtained by the two extraction methods led to discrepancies in findings at three different levels. First, in three samples the final LiPA interpretation was "wild type" for the Promega extract but "mutant" for the corresponding Roche extract. This happened in two LiPA RT determinations and in one LiPA P determination. Second, 13 samples that did not amplify after Promega extraction were interpreted as "mutant" after successful amplification of the corresponding Roche extracts. This happened in four samples for both LiPA RT and LiPA P, in seven samples for LiPA RT alone, and in two samples only for LiPA P. Of the 16 samples included in the previous two levels, 10 (62.5%) had VL levels of <50,000 RNA copies/ml, but no statistical significance was observed. Third, from the group of samples successfully amplified and in which resistance mutations were present after extraction by both methodologies, disagreements could be documented both in the absolute frequency of appearance of individual mutations and in the patterns of combined mutations observed. Tables 1 and 2 present the disagreements observed for both LiPA RT and LiPA P, taking into account the outcome of the RNA extraction method; findings related to absolute mutation frequency (column subtotals) and to different combined mutation patterns (totals by rows) are shown. A tick (√) marks which individual and combined mutations were present in each RNA extraction method; this is considered in the percentage calculation, which is shown in parentheses.

When differences in the band coloring intensity observed were analyzed for each sample according to the extraction method, for LiPA RT 11 samples (33.3%; 95% CI, 17.9 to 51.8) had the same intensity. In 21 cases Roche band intensity was superior, being classified as intensity 1+ in 11 cases (33.3%; 95% CI, 17.9 to 51.8), as intensity 2+ in 4 cases (12.1%; 95% CI, 3.4 to 28.2), and as intensity 3+ in 6 samples (18.2%; 95% CI, 6.9 to 35.4). Only once was the Roche band intensity lower than that of Promega (intensity 1−). For LiPA P, in 31 samples (81.6%; 95% CI, 65.7 to 92.2) the band intensities for the two extraction methods were equal. In six samples intensity was superior, the distribution being as fol-

TABLE 2. Discrepancies in results observed for LiPA P according to the RNA extraction method in absolute frequency of each mutation (column subtotals) and in combined mutation patterns (total by rows)

Presence of mutation and absolute frequency (%) by Roche (next-to-last row) and by Promega (last row)													Pattern frequency (%)	
N30	I46	V48	V50	V54	A54	V84	F82	F82V84	A82	T82	T82V84	M90	Roche	Promega
	√			√										1 (100)
	√								√					1 (100)
	√											√	3 (100)	2 (66.7)
	√												3 (100)	2 (66.7)
				√	√							√	1 (100)	
				√	√									1 (100)
						√							3 (100)	2 (66.7)
									√			√	2 (100)	1 (50)
												√		1 (100)
	6 (75)			1 (33.3)	1 (50)	3 (100)			2 (66.7)			6 (100)		
	6 (75)			2 (66.7)	1 (50)	2 (66.7)			2 (66.7)			3 (50)		

Downloaded from jcm.asm.org by on December 3, 2007

lows: five samples with intensity 1+ (13.1%; 95% CI, 4.4 to 28.1) and one sample with intensity 2+ (2.6%; 95% CI, 0.1 to 13.8). No samples with intensity 3+ were found. In three cases the result was classified as intensity 1− (7.9%; 95% CI, 1.6 to 21.3). A global comparison of the intensity differences between the Roche and the Promega methods, grouping them as equal or superior (for both LiPA techniques), shows statistical significance ($P < 0.001$).

The evidently differing results observed for the two extraction methods compared in this study highlight the importance of this step. Extraction conditions the outcome of all PCRs (1, 2, 15, 17), which are likewise an indispensable prerequisite for HIV genotypic resistance testing. In the literature that we have reviewed, there are some references to extraction outcome applied to HIV sequencing for genotypic resistance detection, such as a comparison of Qiagen and Boom extractions carried out by Niubó et al. (9) and the comparative study by Shafer et al. (11) of the silica bead method versus extraction by phenol-chloroform. Most published studies on the influence of RNA extraction refer to the subsequent VL determination (4, 5, 14, 15, 17).

In our experience, the better results achieved by Roche extraction could be explained by two reasons. First, it includes an ultracentrifugation, an operation that improves the extraction yield due to the sample concentration provided (11, 17). Second, the starting sample volume is greater (500 versus 125 μl) (10, 14, 17). 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.

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 (16). Assuming this limitation and having defined reading criteria in order to compare the results obtained by the two extraction methods, once again there is a clear disagreement for LiPA RT, where band intensity was equal for the two extraction methods in only one-third of the samples.

The wide range of discrepant results observed in our study again highlights the importance of the extraction step. 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 (7, 8, 12). 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. 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.

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 (3, 6; see also http://www.msc.es/sida/asistencia/resistencias.htm), is the transcription or translation of such information to the clinical follow-up of each patient. 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.

REFERENCES

1. **Boom, R. C., J. A. Sol, M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. Van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
2. **Casas, I.** 1997. Métodos de extracción de ADN y ARN aplicables a muestras clínicas: criterios de selección, p. 37–44. *In* Ministerio de Sanidad y Consumo (ed.), Teoría y práctica de la PCR en virología humana. Litofinter S.A., Madrid, Spain.
3. **The EuroGuidelines Group for HIV Resistance.** 2001. Clinical and laboratory guidelines for the use of HIV-1 drug resistance testing as a part of treatment management: recommendations for the European setting. The EuroGuidelines Group for HIV resistance. AIDS **15:**309–320.
4. **Ficher, M., W. Huber, A. Kallivroussis, P. Ott, M. Opravil, R. Lüthy, R. Weber, and R. W. Cone.** 1999. Highly sensitive methods for quantitation of human immunodeficiency virus type 1 RNA from plasma, cells, and tissues. J. Clin. Microbiol. **37:**1260–1264.
5. **Fransen, K., D. Mortier, L. Heyndrickx, C. Verhofstede, W. Janssens, and G. Van der Groen.** 1998. Isolation of HIV-1 RNA from plasma: evaluation of seven different methods for extraction (part two). J. Virol. Methods **76:**153–157.
6. **Hirsch, M. S., F. Brun-Vézinet, R. T. D'Aquila, S. M. Hammer, V. A. Johnson, D. R. Kuritzkes, C. Loveday, J. W. Mellors, B. Clotet, B. Conway, L. M. Demeter, S. Vella, D. M. Jacobsen, and D. D. Richman.** 2000. Antiretroviral drug resistance testing in adults with HIV infection. Recommendations of an International AIDS Society-USA panel. JAMA **283:**2417–2425.
7. **Martínez-Picado, J., and B. Clotet.** 1999. Mutaciones de resistencia seleccionadas durante tratamiento antirretroviral, p. 57–133. *In* Grupo E. Entheos (ed.), Guía práctica para el manejo clínico de las resistencias al VIH. Gráficas Marte, Madrid, Spain.
8. **Moreno, S.** 2001. Los estudios de resistencias son útiles para guiar el tratamiento antirretroviral. Enferm. Infecc. Microbiol. Clin. **19:**42–44.
9. **Niubó, J., W. Li, K. Henry, and A. Erice.** 2000. Recovery and analysis of human immunodeficiency virus type 1 (HIV) RNA sequences from plasma samples with low HIV RNA levels. J. Clin. Microbiol. **38:**309–312.
10. **Schockmel, G. A., S. Yerly, and L. Perrin.** 1997. Detection of low HIV-1 RNA levels in plasma. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **14:**179–183.
11. **Shafer, R. W., D. J. Levee, M. A. Winters, K. L. Richmond, D. Huang, and T. C. Merigan.** 1997. Comparison of QIAamp HCV kit spin columns, silica beads, and phenol-chloroform for recovering human immunodeficiency virus type 1 RNA from plasma. J. Clin. Microbiol. **35:**520–522.
12. **Soriano, V., E. Ledesma, and the Spanish Drug Resistance Panel.** 2000. Second Spanish Consensus on drug resistance testing in clinical practice. AIDS Rev. **2:**109–116.
13. **Stuyver, L., A. Wyseur, A. Rombout, J. Louwagie, T. Scarcez, C. Verhofstede, D. Rimland, R. F. Schinazi, and R. Rossau.** 1997. Line probe assay for rapid detection of drug-selected mutations in the human immunodeficiency virus type 1 reverse transcriptase gene. Antimicrob. Agents Chemother. **41:**284–291.
14. **Venturi, G., R. Ferruzzi, L. Romano, M. Catucci, P. E. Valesin, and M. Zazzi.** 2000. Ultrasensitive in-house transcription-competitive PCR for quantitation of HIV-1 RNA in plasma. J. Virol. Methods **87:**91–97.
15. **Verhofstede, C., K. Fransen, D. Marissens, R. Verhlest, G. Van der Groen, S. Lauwers, G. Zissis, and J. Plum.** 1996. Isolation of HIV-1 RNA from plasma: evaluation of eight different extraction methods. J. Virol. Methods **60:**155–159.
16. **Villahermosa, M. L., G. Contreras, L. Pérez-Alvarez, F. Bru, L. Medrano, E. Delgado, C. Colomo, M. Thomson, and R. Nájera.** 1998. Evaluation of mixtures of wild-type HIV-1 and HIV-1 with resistance point mutations against reverse transcriptase inhibitors. Antivir. Ther. **3:**221–227.
17. **Villahermosa, M. L., M. Thomson, E. Vázquez de Parga, M. T. Cuevas, G. Contreras, L. Pérez-Alvarez, E. Delgado, N. Manjón, L. Medrano, and R. Nájera.** 2000. Improved conditions for extraction and amplification of human immunodeficiency virus type 1 RNA from plasma samples with low viral load. J. Hum. Virol. **3:**27–34.

Downloaded from jcm.asm.org by on December 3, 2007