

Blinded, Multicenter Comparison of Methods To Detect a Drug-Resistant Mutant of Human Immunodeficiency Virus Type 1 at Low Frequency

Elias K. Halvas,¹ Grace M. Aldrovandi,² Peter Balfe,³ Ingrid A. Beck,⁴ Valerie F. Boltz,⁵ John M. Coffin,⁵ Lisa M. Frenkel,⁴ J. Darren Hazelwood,⁶ Victoria A. Johnson,⁶ Mary Kearney,⁵ Andrea Kovacs,⁷ Daniel R. Kuritzkes,⁸ Karin J. Metzner,⁹ Dwight V. Nissley,¹⁰ Marek Nowicki,⁷ Sarah Palmer,⁵ Rainer Ziermann,¹¹ Richard Y. Zhao,¹² Cheryl L. Jennings,¹³ James Bremer,¹³ Don Brambilla,¹⁴ and John W. Mellors^{1*}

*University of Pittsburgh, Pittsburgh, Pennsylvania*¹; *Children's Hospital of Los Angeles, Keck School of Medicine, Los Angeles, California*²; *Columbia University, New York, New York*³; *University of Washington, Seattle, Washington*⁴; *HIV Drug Resistance Program, National Cancer Institute, Frederick, Maryland*⁵; *Birmingham VA Medical Center and University of Alabama at Birmingham, Birmingham, Alabama*⁶; *University of Southern California, Los Angeles, California*⁷; *Section of Retroviral Therapeutics, Brigham and Women's Hospital and Division of AIDS, Harvard Medical School, Boston, Massachusetts*⁸; *University of Erlangen-Nuremberg, Erlangen, Germany*⁹; *Basic Research Program, SAIC-Frederick, National Cancer Institute, Frederick, Maryland*¹⁰; *Bayer HealthCare-Diagnostics, Berkeley, California*¹¹; *University of Maryland School of Medicine, Baltimore, Maryland*¹²; *Rush Medical College, Chicago, Illinois*¹³; and *New England Research Institute, Watertown, Massachusetts*¹⁴

Received 1 March 2006/Returned for modification 14 April 2006/Accepted 25 April 2006

We determined the abilities of 10 technologies to detect and quantify a common drug-resistant mutant of human immunodeficiency virus type 1 (lysine to asparagine at codon 103 of the reverse transcriptase) using a blinded test panel containing mutant–wild-type mixtures ranging from 0.01% to 100% mutant. Two technologies, allele-specific reverse transcriptase PCR and a Ty1HRT yeast system, could quantify the mutant down to 0.1 to 0.4%. These technologies should help define the impact of low-frequency drug-resistant mutants on response to antiretroviral therapy.

The high replication rate and error-prone reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) generates a large population of genetically distinct variants (1). Variants with a fitness advantage can rapidly outcompete others (1). In the case of antiretroviral therapy, outgrowth of drug-resistant mutants can lead to treatment failure (7). After the removal of drug selection, resistant mutants often decline to undetectable levels, albeit at variable rates (2, 6). These “undetectable” mutants can rapidly reappear after reinitiation of antiretroviral therapy, but it is uncertain how often they cause treatment failure (3, 8, 12). Answering this question requires methods to accurately detect drug-resistant mutants present at a low frequency.

Standard genotyping methods provide a composite of the HIV sequences present, but they generally do not detect mutants that comprise less than 20% of the virus population (11, 18). Several assays have been developed with improved sensitivity for resistant mutants (4, 10, 13–17, 20); however, their relative performances have not been assessed. In the current study, a panel of wild-type–mutant HIV-1 mixtures was created to evaluate the performance of these assays. The mutant virus encodes the K103N mutation in HIV-1 RT that is commonly

selected for by nonnucleoside RT inhibitor (NNRTI) therapy and confers cross-resistance to all FDA-approved NNRTIs.

Viral stocks were generated by CaPO₄ transfection of 293T cells (9) with the infectious plasmid clones of HIV-1_{LAI} encoding wild-type 103K (AAA) or mutant 103N (AAC) RT (19). The HIV-1 RNA concentrations of stocks were determined (Amplicor HIV-1 Monitor Assay version 1.0; Roche Molecular Systems, Branchburg, NJ). Viral mixtures were generated by adding appropriate amounts of mutant and wild-type virus stocks, as determined by the HIV-1 RNA concentration, to yield final mutant fractions of 0, 0.01, 0.1, 0.4, 1, 2, 5, 10, 25, 50, and 100%. Each of the viral mixtures was then spiked into 25 ml of HIV-seronegative human plasma from a single donor to obtain a final HIV-1 RNA concentration of $\sim 1 \times 10^6$ RNA copies/ml. The mean (\pm standard deviation) HIV-1 RNA in the mixtures was $5.4 \times 10^5 \pm 9.2 \times 10^4$ copies/ml (each mixture was tested in triplicate), which is within twofold of the expected concentration.

A broad solicitation of interest in testing the panel was sent to academic, government, and industry laboratories working on the detection of minor drug-resistant variants. Thirteen laboratories were selected with the goal of maximizing the number of technologies evaluated. The technologies included pyrosequencing of PCR products (15), real-time allele-specific RT-PCR (ASPCR) (10, 13, 17), oligonucleotide ligation-based assays (OLA) (4), a Ty1/HIV-1 RT hybrid system (TyHRT)

* Corresponding author. Mailing address: Division of Infectious Diseases, Department of Medicine, University of Pittsburgh, S818 Scaife Hall, 3550 Terrace St., Pittsburgh, PA 15261. Phone: (412) 383-7963. Fax: (412) 687-7982. E-mail: Mellors@msx.dept-med.pitt.edu.

TABLE 1. Detection and quantification limits of different technologies for the K103N drug-resistant HIV-1 variant

Expected % 103N	Actual % mutant (103N) ^a																
	ASPCR ^e			TyHRT	Pyro Seq ^f	SGS	ViroSeq ^g			TRUGENE ^h			OLA ⁱ		LiPA ^j	Cloning ScDNA ^k	Cloning McDNA ^l
	Lab 1	Lab 2	Lab 3				Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2			
0 ^b	<0.043	0.006	0	<0.3	0	<2.5	0	0	0	0	0	0	2-5	K103	K103	0	0
0.01	<0.04	0.009	0	<0.3	0	<3	0	0	0	0	0	0	<2	K103	K103	0	ND ^d
0.1	<0.041	0.11	0	<0.3	0	<3.1	0	0	0	0	0	0	<2	K103	K103	0	ND
0.4	0.69	0.48	0	0.3	0	<5.6	0	0	0	0	0	0	<2	K103	K103	0	ND
1	1.02	1.3	0	3	0	<6.3	0	0	0	0	0	0	<2	K103	K103	0	4
2	3.11	3.7	<1	3	6.8	5.9	0	2-5	0	0	0	0	2-5	Mix	Mix	0	ND
5	5.59	5	0	4	0.9	1.9	0	0	0	8	10.5	Mix^c	5	K103	Mix	0	16
10	9.06	11	0	7	5.9	6.9	10	10	6.2	15	16.5	Mix	10	Mix	Mix	12	4
25	27.8	31	15	19	17.9	31.8	30	40	24.3	30	32.5	25	25	Mix	Mix	20	20
50	53.86	72	30	65	37.3	48.1	50	65	53.3	46	57	50	50	Mix	Mix	58.3	40
100	>99.97	127	100	100	89.7	>97	100	100	100	87	100	100	>95	N103	N103	100	100

^a For each assay, the mutant detection limit is shown in boldface and the quantification limit is in italics.

^b 0% 103N/100% wild type.

^c Mix, mixture of wild type and mutant detected.

^d ND, not determined.

^e For ASPCR, laboratories 1, 2, and 3 used different methods.

^f Pyro Seq, pyrosequencing.

^g ViroSeq v2.0 platform from Applied Biosystems.

^h TRUGENE platform from Bayer Diagnostics.

ⁱ For OLA, samples assayed by laboratory 1 were spiked with a plasmid standard and thus were quantified.

^j VERSANT HIV-1 Resistance assay (LiPA) (Bayer Healthcare-Diagnostics).

^k Cloning (TOPO cloning kit; InVitrogen) from a single cDNA reaction (ScDNA); 25 clones were derived and sequenced (Big Dye Terminator kit v3.1; Applied Biosystems) from each RT-PCR product.

^l Cloning (TOPO cloning kit; InVitrogen) from multiple, independent cDNA and RT-PCR reactions (McDNA) (25 per test sample); one clone was derived and sequenced (Big Dye Terminator kit v3.1; Applied Biosystems) from each RT-PCR product.

(14), single-genome sequencing (SGS) (16), and a line probe assay (LiPA) (VERSANT HIV-RT Resistance Assay; Bayer, Berkley, CA) (20). Standard genotyping methods using FDA-cleared kits were included (HIV-1 TRUGENE [Bayer, Tarrytown, NY] and ViroSeq v2.0 [Celera Diagnostics, Alameda, CA]). In one laboratory, the RT-PCR product from the ViroSeq method was cloned (Topo Cloning; InVitrogen, Carlsbad, CA) and sequenced using the Big Dye Terminator kit (Applied Biosystems, Foster City, CA).

The nine viral mixtures (a 1.0-ml aliquot each) and two controls (100% mutant and 0% mutant) were distributed as a blinded test panel to participating laboratories. The panels were tested once because of their large size and limited supply. All results were reported on a standardized form, submitted electronically. The detection limit of an assay was defined as the lowest percent mutant detected by the assay, provided all higher-mutant fractions were detected and that the 0% mutant control was reported as negative. The quantification limit of an assay was defined as the lowest percent mutant detected, provided that all higher-mutant samples were ordered correctly. Table 1 shows the relative performances of the methods.

Of the technologies evaluated, two out of three ASPCR methods (13, 17) and the TyHRT (14) assay were the most sensitive, quantifying the mutant to 0.1 to 0.4% (Table 1). This is 10- to 100-fold more sensitive than standard HIV-1-genotyping methods. The third ASPCR method (10) was less sensitive, which may be attributable to differences in primer design or the amount of sample analyzed.

Despite the high sensitivity of ASPCR, it is limited by single-allele analysis and false negatives from nucleotide polymorphisms that can destabilize primer binding (6). An advantage of using TyHRT is the ability to directly confirm the mutant

allele by sequencing individual yeast clones and/or to identify novel NNRTI resistance mutations (reference 14 and data not shown). A shortcoming of the TyHRT assay is that resistance to nucleoside analogs and protease inhibitors cannot be assessed.

Pyrosequencing and SGS (45 sequences/mixture) had intermediate detection limits of 2% mutant (Table 1) but were not quantitative below 10% 103N. SGS has the advantage of analyzing mutation linkages without the need for cloning (16), but a high sensitivity for minor variants requires the sequencing of many genomes, e.g., 490 single genomes to detect a mutant at a 1% frequency with 99% certainty, which is currently impractical.

The TRUGENE and ViroSeq v2.0 genotyping methods quantified the 103N mutant at frequencies of 5 and 10%, respectively. The sensitivities of these products are higher than previously reported for population-based sequencing (11), which may be attributable to scrutiny of the 103 codon by the laboratories and a low background of plasmid-derived recombinant viruses. Results with other mutants may differ, and more importantly, neither method was developed or cleared for minor-variant detection.

Cloning and sequencing of RT-PCR products from a single or multiple cDNA reactions (ViroSeq) did not improve quantification of mutants over that of the standard commercial assays (11). This result was not unexpected, since only 25 clones per reaction were sequenced. Sensitivity could be improved by increasing the number of clones analyzed, but the labor and cost involved are prohibitive.

The OLA (4) and LiPA (20) assays (LiPA was used here with LiPAscan, an automated strip reader) detected the 103N mutant at frequencies between 2 and 10% (Table 1). One

laboratory performing OLA also used external standards to improve quantification but misclassified the wild-type (0%) control as having 2 to 5% 103N and thus could not detect below 10% mutant (Table 1).

Other comparative studies of genotypic assays for HIV-1 mutant detection have been performed (4, 5, 11, 15), but none has covered the breadth of technologies evaluated here. Additional panels of virus mixtures and clinical samples need to be tested to assess the potential impacts of different codons, viral heterogeneity, and operator experience on assay performance, as has been noted previously (6, 18). Nevertheless, our findings indicate that several assays are available to study the emergence, decay, and therapeutic significance of minor populations of drug-resistant HIV-1.

This study was partly supported by the following grants: PACTG Core Virology Laboratory (202PVCL03) and HD 40777 (G.M.A.); ACTG Virology Support Laboratories (V.A.J., J.W.M., and D.R.K.), subcontract of the AACTG Central Group Grant (U01AI38858); Harvard Medical School Center for AIDS Research Virology Core Laboratory (AI-60354) (D.R.K.); Deutsche Forschungsgemeinschaft (SFB 466) and Wilhelm Sander-Stiftung (grant no. 2002.079.1) (K.J.M.); National Cancer Institute, National Institutes of Health, contract no. NO1-CO-12400 (D.V.N.); P30AI27767 and the Birmingham VA Medical Center and UAB CFAR core clinic and laboratory facilities (V.A.J.); and ACTG Virology Quality Assurance Program (NO1-AI-85354).

REFERENCES

- Coffin, J. M. 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. *Science* **267**:483–489.
- Deeks, S. G., T. Wrin, T. Liegler, R. Hoh, M. Hayden, J. D. Barbour, N. S. Hellmann, C. J. Petropoulos, J. M. McCune, M. K. Hellerstein, and R. M. Grant. 2001. Virologic and immunologic consequences of discontinuing combination antiretroviral-drug therapy in HIV-infected patients with detectable viremia. *N. Engl. J. Med.* **344**:472–480.
- Delaugerre, C., M. A. Valantin, M. Mouroux, M. Bonmarchand, G. Carcelain, C. Duvivier, R. Tubiana, A. Simon, F. Bricaire, H. Agut, B. Autran, C. Katlama, and V. Calvez. 2001. Re-occurrence of HIV-1 drug mutations after treatment re-initiation following interruption in patients with multiple treatment failure. *AIDS* **15**:2189–2191.
- Edelstein, R. E., D. A. Nickerson, V. O. Tobe, L. A. Manns-Arcuino, and L. M. Frenkel. 1998. Oligonucleotide ligation assay for detecting mutations in the human immunodeficiency virus type 1 *pol* gene that are associated with resistance to zidovudine, didanosine, and lamivudine. *J. Clin. Microbiol.* **36**:569–572.
- Fontaine, E., C. Riva, M. Peeters, J. C. Schmit, E. Delaporte, K. Van Laethem, K. Van Vaerenbergh, J. Snoeck, E. Van Wijngaerden, E. De Clercq, M. Van Ranst, and A. M. Vandamme. 2001. Evaluation of two commercial kits for the detection of genotypic drug resistance on a panel of HIV type 1 subtypes A through J. *J. Acquir. Immun. Defic. Syndr.* **28**:254–258.
- Hance, A. J., V. Lemiale, J. Izopet, D. Lecossier, V. Joly, P. Massip, F. Mammano, D. Descamps, F. Brun-Vezinet, and F. Clavel. 2001. Changes in human immunodeficiency virus type 1 populations after treatment interruption in patients failing antiretroviral therapy. *J. Virol.* **75**:6410–6417.
- Hirsch, M. S., F. Brun-Vezinet, B. Clotet, B. Conway, D. R. Kuritzkes, R. T. D'Aquila, L. M. Demeter, S. M. Hammer, V. A. Johnson, C. Loveday, J. W. Mellors, D. M. Jacobsen, and D. D. Richman. 2003. Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type 1: 2003 recommendations of an International AIDS Society-USA panel. *Clin. Infect. Dis.* **37**:113–128.
- Izopet, J., C. Souyris, A. Hance, K. Sandres-Saune, M. Alvarez, C. Pasquier, F. Clavel, J. Puel, and P. Massip. 2002. Evolution of human immunodeficiency virus type 1 populations after resumption of therapy following treatment interruption and shift in resistance genotype. *J. Infect. Dis.* **185**:1506–1510.
- Julias, J. G., A. L. Ferris, P. L. Boyer, and S. H. Hughes. 2002. Replication of phenotypically mixed human immunodeficiency virus type 1 virions containing catalytically active and catalytically inactive reverse transcriptase. *J. Virol.* **75**:6537–6546.
- Kutyavin, I. V., I. A. Afonina, A. Mills, V. V. Gorn, E. A. Lukhtanov, E. S. Belousov, M. J. Singer, D. K. Walburger, S. G. Lokhov, A. A. Gall, R. Dempsy, M. W. Reed, R. B. Meyer, and J. Hedgpeth. 2000. 3'-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res.* **28**:655–661.
- Leitner, T., E. Halapi, G. Scarlatti, P. Rossi, J. Albert, E.-M. Fenyo, and M. Uhlen. 1993. Analysis of heterogeneous viral populations by direct DNA sequencing. *BioTechniques* **15**:120–127.
- Mellors, J., S. Palmer, D. Nissley, M. Kearney, E. Halvas, C. Bixby, L. Demeter, S. Eshleman, K. Bennett, S. Hart, F. Vaida, M. Wantman, J. Coffin, and S. Hammer. 2004. Low-frequency NNRTI-resistant variants contribute to failure of Efavirenz-containing regimens, abstr. 39. Abstr. 11th Conf. Retrovir. Opportunistic Infect. Foundation for Retrovirology and Human Health, San Francisco, CA.
- Metzner, K. J., S. Bonhoeffer, M. Fischer, R. Karanicolos, K. Allers, B. Joos, R. Weber, B. Hirschel, L. G. Kostrikis, H. F. Gunthard, and the Swiss HIV Cohort Study. 2003. Emergence of minor populations of human immunodeficiency virus type 1 carrying the M184V and L90M mutations in subjects undergoing structured treatment interruptions. *J. Infect. Dis.* **188**:1433–1443.
- Nissley, D. V., E. K. Halvas, N. L. Hoppman, D. J. Garfinkel, J. W. Mellors, and J. N. Strathern. 2005. Sensitive phenotypic detection of minor drug resistant HIV-1 reverse transcriptase variants. *J. Clin. Microbiol.* **43**:5696–5704.
- O'Meara, D., K. Wilbe, T. Leitner, B. Hejdeman, J. Albert, and J. Lundeberg. 2001. Monitoring resistance to human immunodeficiency virus type 1 protease inhibitors by pyrosequencing. *J. Clin. Microbiol.* **39**:464–473.
- Palmer, S., M. Kearney, F. Maldarelli, E. K. Halvas, C. J. Bixby, H. Bazmi, D. Rock, J. Falloon, R. T. Davey, Jr., R. L. Dewar, J. A. Metcalf, S. Hammer, J. W. Mellors, and J. M. Coffin. 2005. Multiple, linked human immunodeficiency virus type 1 drug resistance mutations in treatment-experienced patients are missed by standard genotype analysis. *J. Clin. Microbiol.* **43**:406–413.
- Palmer, S., V. Boltz, F. Maldarelli, M. Kearney, E. K. Halvas, D. Rock, J. Falloon, R. T. Davey, Jr., R. L. Dewar, J. A. Metcalf, J. W. Mellors, and J. M. Coffin. 2006. Selection and persistence of non-nucleoside reverse transcriptase inhibitor-resistant HIV-1 in patients starting and stopping nonnucleoside therapy. *AIDS* **20**:701–710.
- Schuurman, R., D. Brambilla, T. de Groot, D. Huang, S. Land, J. Bremer, I. Benders, C. A. Boucher, and the ENVA Working Group. 2002. Underestimation of HIV type 1 drug resistance mutations: results from the ENVA-2 genotyping proficiency program. *AIDS Res. Hum. Retrovir.* **18**:243–248.
- Shi, C., and J. W. Mellors. 1997. A recombinant retroviral system for rapid in vivo analysis of human immunodeficiency virus type 1 susceptibility to reverse transcriptase inhibitors. *Antimicrob. Agents Chemother.* **41**:2781–2785.
- Tsongalis, G., T. Gleeson, M. Rodina, D. Anamani, J. Ross, I. Joannis, L. Tanimoto, and R. Ziermann. 2005. Comparative performance evaluation of the HIV-1 LiPA protease and reverse transcriptase resistance assay on clinical isolates. *J. Clin. Virol.* **34**:268–271.