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Interlaboratory Comparison of Sequence-Specific PCR and Ligase Detection Reaction To Detect a Human Immunodeficiency Virus Type 1 Drug Resistance Mutation

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Sequence-specific PCR was used in six laboratories and a ligase detection reaction was used in one laboratory to detect the zidovudine-resistance mutation at codon 215 of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase DNA. The genotypes of 27 different clinical samples, including cultured HIV-1 isolates, peripheral blood mononuclear cells, and plasma, were correctly identified by 140 of 154 (91%) assays. The sensitivity for detecting a mutation was 96% for HIV-1 reverse transcriptase DNA clone mixtures containing 30% mutant DNA and 62% for mixtures containing 6% mutant DNA.

Human immunodeficiency virus type 1 (HIV-1) drug resistance contributes to the limited benefit of antiretroviral therapy (4, 14). Current methods for detecting HIV-1 drug resistance include phenotypic cell culture assays and genotypic assays for mutations known to confer drug resistance. Point mutation assays are useful when there is a strong correlation between a specific mutation and drug resistance. For example, drug resistance during zidovudine, didanosine, nevirapine, or lamivudine monotherapy is usually associated with reverse transcriptase (RT) DNA mutations at codons 215, 74, 181, and 184, respectively (1, 16, 17, 20).

Several assays have been developed to rapidly detect point mutations (3, 5, 6, 9, 12, 13, 15). In 1991, Larder et al. developed a nested PCR protocol for detecting zidovudine resistance mutations using oligonucleotides specific for either wild-type or mutant HIV-1 RT DNA (13). Another assay uses PCR amplification of HIV-1 RT DNA followed by a ligase detection reaction (LDR) (5). Our report describes the sensitivity and specificity of detection in six laboratories using nested PCR (Fig. 1) and one laboratory using LDR for detecting the zidovudine resistance codon 215 HIV-1 RT DNA mutation.

Samples. Each of the six laboratories using selective PCR received 41 different samples, including cultured patient peripheral blood mononuclear cells (PBMC) (10 samples), uncultured PBMC (10^6 cells, 7 samples), plasma (10 samples), and HIV-1 RT DNA clones with different proportions of mutant alleles at codon 215 (14 samples). The codon 215 genotype of these samples was confirmed by dideoxy-terminator sequencing. The laboratory using LDR joined the working group after the first round of comparisons on cultured PBMC was completed.

Sample processing. PBMC pellets and cultured PBMC were diluted to $\approx 10^6$ cells per 100 μl (≈1 μg of DNA per 20 μl) and treated with proteinase K (100 μg/ml) at 56°C for >2 h. Cell lysates were heated to 95°C for 5 to 10 min to denature the proteinase K.

Thawed plasma aliquots (200 to 1,000 μl) were dispensed into 1.5-ml tubes and centrifuged for 30 min at 12,500 × *g* or for 10 min at 125,000 × *g* at 10°C. The supernatant was discarded, leaving an invisible pellet of virions. Four hundred microliters of a guanidine thiocyanate solution or Tri-Reagent (Molecular Research Centers, Cincinnati, Ohio) was added to disrupt the pellet and inactivate RNases. RNA was extracted by using Tris-saturated phenol, chloroform (with or without isoamyl alcohol), and yeast tRNA. The upper aqueous phase was transferred to a new microcentrifuge tube, isopropanol (400 μl) was added, and the mixture was maintained at −20°C for ≥1 h. The mixture was then centrifuged at 12,500 × *g* for 15 to 20 min at 4°C, and the supernatant was removed, leaving an invisible RNA pellet. The pellet was washed three times with 75% ethanol, dried, and suspended in 25 μl of diethyl pyrocarbonate-treated H_2O.

To reverse transcribe viral RNA to cDNA, a 25-μl master mixture containing ≥200 U of murine leukemia virus RT, 25 mM deoxynucleoside triphosphate (dNTP), primer NE1 (35) (250 ng; CCTACTAACTTCTGTATGTCATTGACAGTCCA GCT), 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 10% Nonidet P-40, 0.1 M dithiothreitol, and RNasin (40 U/μl) was added to the 25-μl virus RNA sample. Reverse transcription was performed in a thermocycler: 10 min at 25°C, 30 min at 42°C, and 5 min at 95 to 99°C.

PCR. The first-round PCR template was either 20 μl of cell

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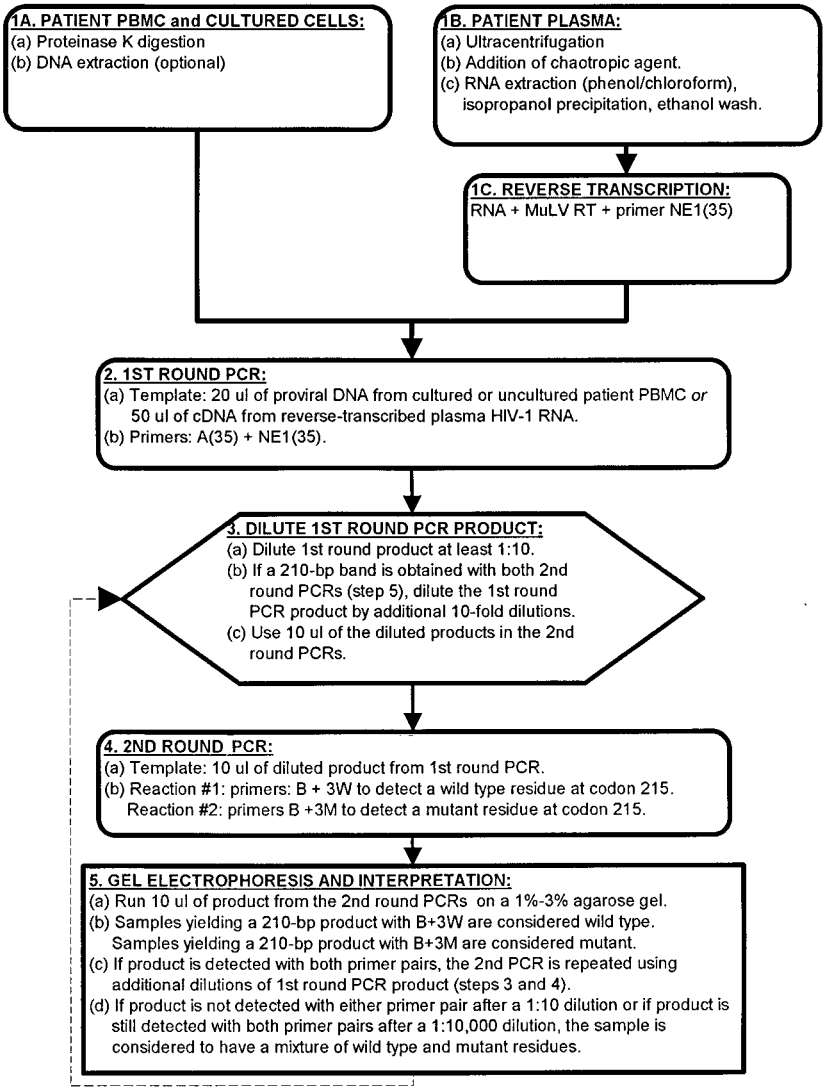

FIG. 1. Overview of sequence-specific PCR protocol for detecting zidovudine resistance mutations at codon 215 of HIV-1 reverse transcriptase DNA in clinical samples including cultured and uncultured PBMC and plasma. MuLV, murine leukemia virus.

lysate (containing proviral HIV-1 DNA) or 50 μl of cDNA from plasma HIV-1 RNA. Primers A (35) (TTGGTTGCAC TTTAAATTTTCCCATTAGTCCTATT) and NE1 (35) (250 ng each), 2.5 U of *Taq* polymerase, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris HCl (pH 8.3) were added to the template in a final volume of 100 μl. The cycling parameters were 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s (18 to 30 cycles).

The product from the first PCR served as the template for the second PCR. To detect a wild-type residue, PCR was done with 250 ng each of primers B (GGATGGAAAGGATCACC) and 3W (ATGTTTTTTGTCTGGTGTGGT; complementary to 215T; complementary nucleotides are underlined). To detect a mutation, PCR was done with 250 ng each of primers B and 3M (ATGTTTTTTGTCTGGTGTGAA; complementary to 215Y and 215F). To improve specificity, dNTPs were reduced to 0.025 mM and [$MgCl_2$] was lowered to 1.5 to 2.0 mM. Cycling parameters were 94°C for 30 s, 45 to 48°C for 30 s, and 72°C for 60 s (30 cycles).

PCR product detection and interpretation. Samples containing 10 μl of product from each second PCR were run on an agarose gel with ethidium bromide staining. Samples yielding a 210-bp product with the wild-type primer pair (B and 3W) were considered wild type. Samples yielding a 210-bp product with the mutant primer pair (B and 3M) were considered mutant. If product was detected with both primer pairs, the second PCR was repeated with successive 10-fold dilutions (1:10, 1:100, 1:1,000, or 1:10,000) of template DNA from the first PCR. If both bands were lost after a 10-fold dilution or if mixtures were still present after a 1:10,000 dilution, the sample was considered to have a mixture of wild-type and mutant residues.

Results. The three cultured PBMC samples with mutant HIV-1 RT and the seven cultured PBMC samples with wild-type HIV-1 RT were correctly identified by each of the laboratories using sequence-specific PCR (Table 1).

The two uncultured PBMC samples with mutant HIV-1 RT were correctly identified by each laboratory using sequence-specific PCR and by the laboratory using LDR. One mutant sample (sample 13) was found to have a mixture of wild-type

TABLE 1. Interlaboratory comparison of sequence-specific PCR and LDR for detecting a mutation at codon 215 of HIV-1 RT DNA in clinical specimens[a]

Sample type	Sample no.	Patient condition and treatment	Codon 215 genotype[b]	Result of sequence-specific PCR at laboratory:						LDR assay result
				A	B	C	D	E	F	
Cultured PBMC	1		ACC	WT	WT	WT	WT	WT	WT	
	2		TAC	Mut	Mut	Mut	Mut	Mut	Mut	
	3		TAC	Mut	Mut	Mut	Mut	Mut	Mut	
	4		ACC	WT	WT	WT	WT	WT	WT	
	5		ACC	WT	WT	WT	WT	WT	WT	
	6		ACC	WT	WT	WT	WT	WT	WT	
	7		ACC	WT	WT	WT	WT	WT	WT	
	8		TTC	Mut	Mut	Mut	Mut	Mut	Mut	
	9		ACC	WT	WT	WT	WT	WT	WT	
	10		ACC	WT	WT	WT	WT	WT	WT	
Uncultured PBMC	11	HIV^+, untreated	ACC	WT	WT	WT	WT	–	WT	WT
	12	HIV^+, untreated	ACC	WT	–	–	WT	WT	WT	WT
	13	ARC, AZT for 24 mo	TCC/TAC[c]	Mix	Mix	Mut	Mix	Mix	Mix	Mix
	14	ARC, AZT for 21 mo	TAC	Mix	Mix	Mut	Mut	Mix	Mut	Mix
	15	HIV^+, untreated	ACC	WT	–	WT	WT	WT	WT	WT
	16	HIV^-	–	–	–	–	Mut	–	–	–
	17	HIV^+, untreated	ACC	WT	–	WT	WT	WT	WT	WT
Plasma	18	AIDS, AZT for 6 mo	TAC	Mut	Mix	Mut	Mix	Mix	Mut	Mix
	19	ARC, AZT for 21 mo	TAC	Mut	Mut	Mut	Mut	–	WT	Mut
	20	ARC, AZT for 24 mo	TAC	Mut	Mut	Mut	Mut	Mut	Mut	WT
	21	HIV^+, untreated	ACC	WT	WT	WT	WT	WT	WT	WT
	22	HIV^+, untreated	ACC	WT	WT	–	WT	WT	WT	WT
	23	HIV^+, AZT for 18 mo	TAC	Mut	Mut	Mut	–	Mut	–	Mut
	24	HIV^+, AZT for 14 mo	ACC	WT	WT	WT	WT	WT	WT	WT
	25	HIV^+, untreated	ACC	WT	WT	WT	–	WT	WT	WT
	26	HIV^+, AZT for 8 mo	ACC	WT	WT	WT	WT	–	–	WT
	27	HIV^+, AZT for 6 mo	ACC	WT	WT	WT	WT	WT	WT	WT

[a] HIV^+, HIV seropositive; HIV^-, HIV seronegative; AZT, zidovudine; ARC, AIDS-related complex; WT, wild type; Mut, mutant; Mix, mixture of wild type and mutant; –, DNA not detected following both PCR amplifications.

[b] ACC codes for threonine, TAC codes for tyrosine, TTC codes for phenylalanine, and TCC codes for serine.

[c] The sequence chromatogram showed that the C peak in the middle position was greater than the A peak in that position, suggesting that TCC was present at a greater concentration than TAC.

and mutant genotypes by six of the seven laboratories; the other mutant sample (sample 14) was found to have a mixture by four of the seven laboratories. The four wild-type PBMC samples were correctly identified by 23 of 28 assays: three laboratories did not detect DNA from five samples (Table 1). One laboratory obtained a false-positive mutant result on a PBMC sample from an HIV-1-seronegative person (sample 16), illustrating the risk of PCR contamination associated with nested PCR.

The four mutant plasma HIV-1 strains were identified correctly by 23 of 28 assays: three laboratories did not detect cDNA in four samples; one laboratory incorrectly identified a mutant sample as wild-type. One mutant sample (sample 18) was found to have a mixture of wild-type and mutant genotypes by four of the seven laboratories; the other three mutant samples (samples 19, 20, and 23) were not reported as having mixtures. The six wild-type plasma samples were identified correctly by 38 of 42 assays; cDNA was not detected in four assays (Table 1).

Genotypes of the 14 plasmid HIV-1 RT mixtures were correctly identified by 76 of 98 (78%) assays (Table 2). The 100% wild-type samples were correctly identified by 32 of 35 (91%) assays; three false-positive mutant results occurred when samples with either $10^{5.7}$ or $10^{6.7}$ wild-type DNA copies were tested. Samples containing 30% mutant DNA were identified as having a mixture of mutant and wild-type DNA by 27 of 28 (96%) assays. Samples with 6 and 0.6% mutant DNA were identified as mixtures by 13 of 21 (62%) and 4 of 14 (29%) assays, respectively.

Discussion. The results obtained with cultured PBMC, uncultured PBMC, and plasma show that sequence-specific PCR and LDR are accurate and reproducible for detecting the zidovudine-resistance mutation at codon 215 of HIV-1 RT DNA in clinical samples. The 100% agreement among the laboratories testing cultured PBMC probably reflects the high concentration of mutant or wild-type genotypes in each of these samples. The slightly lower accuracy in testing uncultured PBMC and plasma (87% [61 of 70 assays] for the wild-type samples and 88% [37 of 42 assays] for the mutant samples) probably reflects the lower concentration of virus in these samples. Indeed, nearly all of the incorrect results were due to the inability to detect virus.

Plasma samples were included because plasma HIV-1 reflects the most actively replicating virus population and because drug resistance mutations often appear in plasma before PBMC (8, 10, 17, 18). Of note, only one of four mutant plasma samples and none of the cultured PBMC were reported as having mixtures. In contrast, both of the mutant uncultured PBMC samples were reported as having a mixture of wild-type and mutant genotypes by most laboratories. Mixtures may be detected because wild-type virus often persists in patients developing resistant HIV-1 while receiving zidovudine (7, 8, 10, 14, 18).

Although most clinical samples with a codon 215 mutation

TABLE 2. Interlaboratory comparison of sequence-specific PCR and LDR for detecting a mutation at codon 215 of HIV-1 RT DNA in HIV-1 clone mixtures

No. of DNA copies	% mutant	Result of sequence-specific PCR at laboratory[a]:						LDR assay result
		A	B	C	D	E	F	
$10^{2.7}$	0	WT	WT	WT	WT	WT	WT	WT
$10^{2.7}$	30	WT	Mix	Mix	Mix	Mix	Mix	Mix
$10^{3.7}$	0	WT	WT	WT	WT	WT	WT	WT
$10^{3.7}$	6	Mix	WT	—	Mix	WT	Mix	Mix
$10^{3.7}$	30	Mix	Mix	Mix	Mix	Mix	Mix	Mix
$10^{4.7}$	0	WT	WT	WT	WT	WT	WT	WT
$10^{4.7}$	0.6	WT	WT	WT	WT	Mix	Mix	WT
$10^{4.7}$	6	Mix	Mix	WT	Mix	Mix	Mix	Mix
$10^{4.7}$	30	Mix	Mix	Mix	Mix	Mix	Mix	Mix
$10^{5.7}$	0	WT	WT	WT	WT	WT	Mix	WT
$10^{5.7}$	0.6	Mix	WT	Mix	WT	Mix	Mix	WT
$10^{5.7}$	6	Mix	Mix	WT	Mix	Mix	Mix	Mix
$10^{5.7}$	30	Mix	Mix	Mix	Mix	Mix	Mix	Mix
$10^{6.7}$	0	Mix	WT	WT	WT	WT	Mix	WT

[a] WT, wild-type; Mix, mixture of wild type and mutant; —, DNA not detected following both PCR amplifications.

had a 2-bp change of ACC (threonine) to TAC (tyrosine), one sample had a 2-bp change to TTC (phenylalanine) and another had a 1-bp change to TCC (serine). The phenylalanine substitution also confers zidovudine resistance and has been observed in clinical HIV-1 strains (13). The serine substitution probably represents an intermediate stage in the transition to either tyrosine or phenylalanine.

The results for HIV-1 RT DNA clones define the sensitivity and specificity of these point mutation assays across a range of different DNA concentrations. As the proportion of the mutant genotype decreased from 30 to 6 to 0.6%, the likelihood of obtaining a mutant result decreased from 96 to 62 to 29%. Because the specificity of sequence-specific PCR can be lost if a large excess of target DNA is used in the second-round PCR (11, 12), we diluted the first-round product and repeated the second-round PCRs whenever the product was present with both sets of primer pairs (Fig. 1). The process of dilution contributed to the low number of false-positive mutant results but probably also decreased the ability to detect small proportions of the mutant genotype.

Modifications of PCR, such as using a hot start and other heat-stable polymerases, should further improve the sensitivity and specificity of sequence-specific PCR (11, 21). In addition, some point mutation assays are able to quantify the proportions of wild-type and mutant sequences within a mixture (3, 8, 9). Because point mutation assays are being used to study the timing and significance of drug resistance during antiretroviral therapy (2, 7, 8, 17–19), it has become important to determine the accuracy and reproducibility of these assays.

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