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Line Probe Assay for Rapid Detection of Drug-Selected Mutations in the Human Immunodeficiency Virus Type 1 Reverse Transcriptase Gene

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Upon prolonged treatment with various antiretroviral nucleoside analogs such as 3′-azido-3′-deoxythymidine, 2′,3′-dideoxyinosine, 2′,3′-dideoxycytidine, (−)-β-L-2′,3′-dideoxy-3′-thiacytidine, and 2′,3′-didehydro-3′-deoxythymidine, selection of human immunodeficiency virus type 1 (HIV-1) strains with mutations in the reverse transcriptase (RT) gene has been reported. We designed a reverse hybridization line probe assay (LiPA) for the rapid and simultaneous characterization of the following variations in the RT gene: M41 or L41; T69, N69, A69, or D69; K70 or R70; L74 or V74; V75 or T75; M184, I184, or V184; T215, Y215, or F215; and K219, Q219, or E219. Nucleotide polymorphisms for codon L41 (TTG or CTG), T69 (ACT or ACA), V75 (GTA or GTG), T215 (ACC or ACT), and Y215 (TAC or TAT) could be detected. In addition to the codons mentioned above, several third-letter polymorphisms in the direct vicinity of the target codons (E40, E42, K43, K73, D76, Q182, Y183, D185, G213, F214, and L214) were found, and specific probes were selected. In total, 48 probes were designed and applied on the LiPA test strips and optimized with a well-characterized and representative reference panel. Plasma samples from 358 HIV-infected patients were analyzed with all 48 probes. The amino acid profiles could be deduced by LiPA hybridization in an average of 92.7% of the samples for each individual codon. When combined with changes in viral load and CD4$^+$ T-cell count, this LiPA approach proved to be useful in studying genetic resistance in follow-up samples from antiretroviral agent-treated HIV-1-infected individuals.

Reverse transcriptase (RT) inhibitors such as the nucleoside analogs 3′-azido-3′-deoxythymidine (AZT; zidovudine), 2′,3′-dideoxyinosine (ddI; didanosine), 2′,3′-dideoxycytidine (ddC; zalcitabine), (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC; lamivudine), and 2′,3′-didehydro-3′-deoxythymidine (d4T; stavudine) are the nucleosides currently approved for the treatment of advanced human immunodeficiency virus (HIV) type 1 (HIV-1) infections (2, 19). All these compounds act in a similar way, namely, as chain terminators of the RT reaction after phosphorylation by intracellular kinases (5, 20). Unfortunately, resistance to these drugs has been increasingly observed (10, 15, 17, 18, 21). Upon prolonged treatment with these nucleoside analogs, viral variants having the possibility of escaping the inhibitory effects of these antiviral agents are selected. Resistant viral strains show nucleotide changes in the RT gene (17, 18), resulting in amino acid (aa) changes that lead to gradually increasing resistance. Among these changes, aa positions 41 (M to L), 69 (T to D), 70 (K to R), 74 (L to V), 184 (M to V), and 215 (T to Y or F) are known to be of particular importance (13). Mutations at aa's 65, 67, 75, and 219, and the more recently described aa changes at codons 62, 75, 77, 116, and 151 encoding multidrug resistance (7) may also be of equal significance. A detailed overview of genotypic resistance profiles and their corresponding phenotypic resistance consequences is available (13).

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Although anti-HIV therapy with nucleoside analogs was introduced several years ago, the optimal algorithm for efficient treatment remains unclear. Currently, the best prognostic markers of survival for HIV-infected individuals undergoing antiviral treatment are obtained by monitoring the changes in viral load and CD4$^+$ T cells (14). The appearance of one or several of the RT variants during antiviral treatment (genotypic resistance) with respect to disease progression and clinical deterioration is generally not interpreted in conjunction with the parameters listed above. For example, in vitro studies have shown that the effect of AZT resistance mutations can be suppressed after the appearance of the 3TC-selected M184V mutation (1, 11, 21). However, the clinical influence of this genetic combination needs further evaluation, especially in long-term treatment studies. In order to gain a better insight into the mechanisms of genotypic drug resistance and HIV biology, a genotyping line probe assay (LiPA) was developed. This assay allows for the rapid detection of variations at those aa positions conferring resistance to antiviral drugs (see Tables 1 to 6). The principle of the assay is based on reverse hybridization of a biotinylated PCR fragment of the relevant part of the HIV RT with short, immobilized oligonucleotides (22, 23). The latter hybrid can then be detected via a biotin-streptavidin coupling with a colorimetric system. The selection of these probes is described herein, and their applicability is demonstrated by analyzing plasma samples from European and American HIV-1-infected individuals.

Genotypic resistance of the HIV-1 RT gene can be determined by means of several molecular biology applications: Southern blotting (16), primer-specific PCR (10), PCR-ligase

detection reaction (4), RNase A mismatch (6), hybridization against labeled probes (3), point mutation assay (9), and the LiPA described here. The relative merits or disadvantages of deriving genotypic data in the absence of phenotypic data (8) are still open questions. The development of rapid, sensitive, and simple genotyping and phenotyping tests can increase the understanding of that relation.

MATERIALS AND METHODS

Plasma sample collection. Plasma samples were taken from HIV-1-infected patients and were stored at −20°C until use. Patients were treated with AZT, ddI, ddC, d4T, 3TC, or several combinations of these drugs. The European serum samples were randomly selected. For the U.S. serum sample collection, only the first sample from a follow-up series was taken. Some of these U.S. patients were treated, while others were not.

HIV RNA preparation, cDNA synthesis, and PCR. HIV RNA and cDNA preparation was identical to that described for the hepatitis C virus (HCV) (22, 23). Briefly, 50 μl of plasma was mixed with guanidinium-phenol. After lysis and denaturation, $CHCl_3$ was added to obtain phase separation, and nucleic acids were precipitated from the aqueous phase with isopropanol and were collected by centrifugation. The RNA pellet was dissolved in a random primer solution [$pd(N)_6$; Pharmacia, Brussel, Belgium]. cDNA synthesis occurred with avian myeloblastosis virus RT (Stratagene, La Jolla, Calif.) at 42°C. The HIV RT gene was then amplified in the following mixture: 5 μl of cDNA, 4.5 μl of 10× *Taq* buffer, 0.3 μl of 25 mM (each) deoxynucleoside triphosphates, 1 μl (10 pmol) of each PCR primer, 38 μl of H_2O, and 0.2 μl (1 U) of *Taq* (Stratagene). The annealing temperature was set at 57°C, extension was at 72°C, and denaturation was at 94°C. Each step of the cycle took 1 min. The outer PCR contained 40 cycles, and the nested reaction contained 35 cycles. The annealing temperature seemed to be crucial (57°C). At 55°C, a second aspecific amplicon of approximately 1,500 bp was generated, and at 59°C the amount of specific fragment decreased markedly. Nested PCR products were analyzed on agarose gels, and only clearly visible amplification products were used in the LiPA procedure. Quantification of viral RNA was done by the HIV Monitor test (Roche, Brussels, Belgium). The detection limit in these experiments was between 150 and 200 viral copies per ml of plasma.

Primers. PCR primers outside the target regions were chosen for probe design; their sequences were based on published HIV-1 genotype B information. The amplified region located inside the nested primers covered the RT gene from codon 29 to codon 220. The primers had the indicated sequences: outer sense primer RT-9, 5′-bio-GTACAGTATTAGTAGGACCTACACCTGTC-3′; nested sense primer RT-1, 5′-bio-CCAAAAGTTAAACAATGGCCATTGACAGA-3′; nested antisense primer RT-4, 5′-bio-AGTTCATAACCCATCCAAAG-3′; and outer antisense primer RT-12, 5′-bio-ATCAGGATGGAGTTCATAACCCATCCA-3′. With the current primer combination, the corresponding RT region of the HIV-1 genotype A, C, D, and F clade could also be amplified, but with a reduced sensitivity.

In principle, any biotinylated PCR product covering the RT region from codon 29 to codon 220 can be used in this assay without restrictions to the amplicon size. However, since the assay was optimized for end-standing biotin groups, it is advisable to keep this amplicon as short as possible. The indicated PCR primers were carefully selected accordingly.

Probes. Probes were designed to cover different polymorphisms and drug-selected mutations. In principle, only those probes that discriminated between a single nucleotide variation were retained. For certain polymorphisms at the extreme ends of the probe, cross-reactivity was tolerated. Specificity was reached for each probe individually after considering the percent G+C content, probe length, final concentration, and hybridization temperature.

In total, 48 specific oligonucleotide probes, able to detect 44 sequence motifs in the HIV-1 RT gene, were selected (Tables 1 to 6). This selection can be split into four subsequent steps. (i) A total of 35 probes were based on sequence motifs present in the National Center for Biotechnology Information (NCBI; Bethesda, Md.) nucleotide sequence database. All HIV-1 genome entries were retrieved and analyzed one by one. Only those entries displaying nonambiguous sequence information in the vicinity of the codons mentioned above were retained for further interpretation. For the design of relevant probes, only those database motifs that systematically returned (highly prevalent motif) were included, while scattered mutations which were found randomly (low prevalent motif; data not shown) were excluded. On the basis of database sequences, eight probes for codon 41 (91.6% of all entries), seven for codons 69 and 70 (86.2%), two for codons 74 and 75 (90.4%), five for codon 184 (96.6%), 11 for codon 215 (94.1%), and 2 for codon 219 (88.2%) were selected. Four probes (probes 41w15, 70w8, 215w29, and 215w27) are, in fact, redundant, because they detect identical sequence motifs covered by other probes. The locations of these redundant probes are slightly different with respect to their counterpart with an identical sequence. These probes can prevent negative results which might otherwise appear as a consequence of random mutations in the probe target area and can therefore increase the sensitivity of recognition. (ii) By analyzing European and U.S. plasma samples, another eight motifs not predicted in the database appeared. The corresponding probes (probes 41w20, 41m12, 70m13, 74w9, 74m6, 74m12, 184w24, 215m49) were designed. (iii) Another four probes (probes 41m11, 215m50, 219m7, and 219m9) were optimized because their sequence motif was found in recombinant clones retrieved from plasma (see the section on the reference panel below). It is assumed that these motifs exist at an extremely low frequency in the viral quasispecies, remaining undetectable by direct detection methods, but becoming apparent after cloning. (iv) The sequence motif of probe 215m13 was generated in recombinant clones by site-directed mutagenesis (data not shown). The rationale for this was to determine whether the sequence combination of codon Y215 (TAC) can occur in combination with L214 (CTT) in vivo.

Reference panel. Selected PCR products, amplified without 5′ biotin primers, were cloned into the pretreated *Eco*RV site of the pGEMT vector (Promega Corp. Benelux, Leiden, The Netherlands). Recombinant clones were selected after α-complementation and restriction fragment length analysis and were then sequenced with plasmid primers and internal HIV RT primers. Biotinylated fragments were directly sequenced with the ABI Prism dye terminator protocol (Perkin-Elmer, ABD, Foster City, Calif.) by using the amplification primers. Alternatively, nested PCR was carried out with analogs of the RT-4 and RT-1 primers, in which the biotin group was replaced with the T7 and SP6 primer sequences, respectively. These amplicons were than sequenced with an SP6 and T7 dye primer procedure.

LiPA strip preparation. Optimized probes were provided enzymatically with a poly(T) tail by using terminal deoxynucleotidyl transferase (Pharmacia) under standard reaction conditions and were purified via precipitation. Probe pellets were dissolved in standard saline citrate buffer and were applied as horizontal parallel lines on a membrane strip. Control lines for amplification (probes 5′-CCACAGGGATGGAAAG-3′ and 5′-GATCTGACTTAGAAATAG-3′) and conjugate incubation (biotinylated DNA) were applied alongside. After fixation of the probes, membranes were sliced into 4-mm strips.

LiPA test performance. Equal amounts (10 μl) of biotinylated amplification products and denaturation mixture (0.4 N NaOH and 0.1% sodium dodecyl sulfate) were mixed, followed by an incubation at room temperature for 5 min. Following this denaturation step, 2 ml of prewarmed hybridization buffer (containing Tris-HCl, sodium dodecyl sulfate, and standard saline citrate) was added together with a membrane strip, and hybridization was carried out at 39°C for 30 min. The hybridization mixture was then replaced with a stringent wash buffer, and washing occurred first at room temperature for 5 min and then at 39°C for another 10 min. Buffers were then replaced for the streptavidin-alkaline phosphatase conjugate incubations. After 30 min of incubation at room temperature, the conjugate was rinsed away and was replaced with the substrate components for alkaline phosphatase, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich, Bornem, Belgium). After a 30-min incubation at room temperature, the probes in which hybridization occurred became visible as a purple-brown precipitate at these positions.

Nucleotide sequence accession numbers. Sequence information was submitted to GenBank and is available under accession numbers L78133 to L78157 (for the 18 PCR fragments cloned in pGEMT, accession numbers L78139 to L78156, respectively; for the 7 PCR fragments amplified from plasma virus, accession numbers L78133 to L78138 and L78157, respectively).

RESULTS

The HIV-1 RT gene and reference panel PCR. A total of 96% of the European and U.S. HIV-1-positive plasma samples, stored appropriately (at −20°C) without repeated freezing-thawing cycles, were positive by PCR (data not shown). During the probe selection procedure, a total of 25 PCR fragments with the desired target polymorphisms and mutations were retained as a reference panel and were eventually cloned in pGEMT, and both strands were sequenced. Biotinylated PCR products from this panel were used to test and optimize repeatedly the selected probes for specificity and sensitivity.

Probe specificity and sensitivity. The 48 selected probes were applied separately on LiPA strips, and each strip was incubated with a biotinylated PCR fragment generated from the reference panel or directly from virus in plasma (Fig. 1a to f). The reactivities of these probes were concordant with the nucleotide sequences. At the current stage of the ongoing probe development, cross-reactivities were observed only for probe 41w19 (Fig. 1a, lane 9) and probe 70m3 (Fig. 1b, lane 8), with extremely rare sequence motifs 41m12 (prevalence less than 0.3%) and 70m16 (not experimentally found), respectively.

Performance characteristics. Since this assay works with any biotinylated PCR fragment, the limitation of the technique is

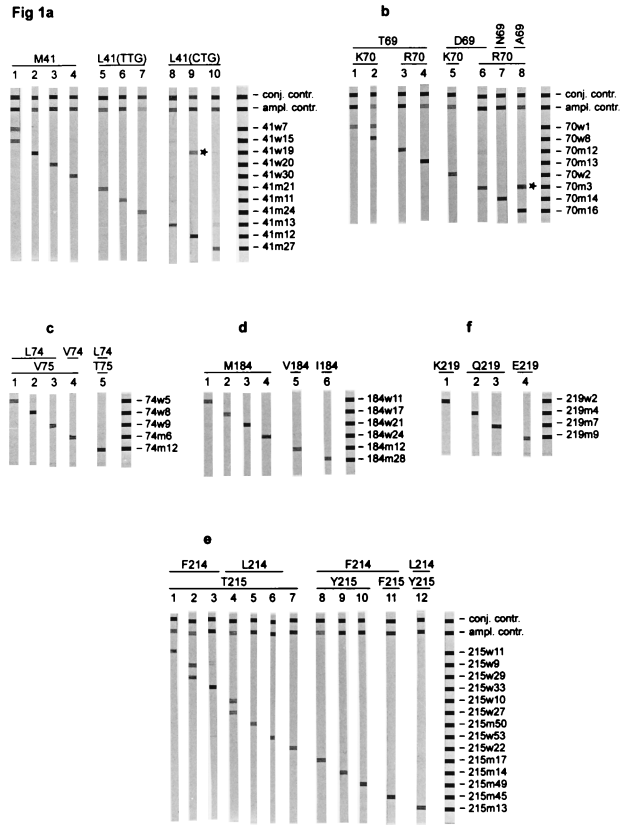

FIG. 1. Reactivities of the selected probes, immobilized on LiPA strips, with reference material. (a) Probes for codon 41; (b) probes for codons 69 and 70; (c) probes for codons 74 and 75; (d) probes for codon 184; (e) probes for codon 215; (f) probes for codon 219. The position of each probe on the membrane strip is shown at the right of each panel. The sequences of the relevant parts of the selected probes are given in Tables 1 to 6. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Tables 1 to 6. For several probes, multiple reference panel possibilities are available, but only one relevant accession number is given each time. *, cross-reactive probes. On top of the strips, the aa's at the relevant codon, as derived from the probe reactivity, are indicated.

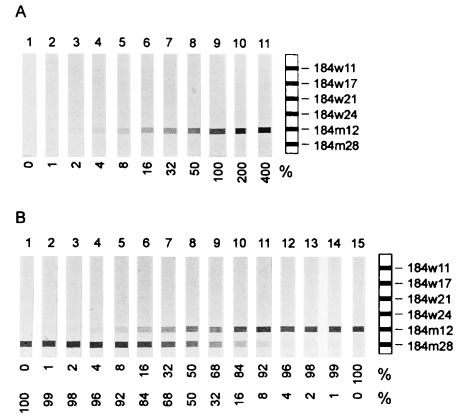

FIG. 2. Performance characteristics of the LiPA for codons 184V and 184I. (A) Strips were incubated with an increasing amount of biotinylated material from reference panel clone L78142, the amount of material is indicated beneath each strip (100% is considered the standard amount of amplicons used in routine diagnostic tests). The positions of the probes on the strips are indicated on the right; names correspond to the sequences in Table 4. (B) Artificial mixture of reference panel clones L78142 and L78148. The amount of materials used to obtain these reactivities is indicated.

only dependent on the variability of the virus and on the sensitivity of the RT-PCR procedure. We used as an RNA input standard the detection limit in the Amplicor (Roche) procedure, which was set at 150 to 200 viral copies per ml of plasma. Our in-house RT-PCR methodology was able to amplify samples containing less than 1,000 copies per ml, which consequently resulted in fully interpretable LiPA results (see Fig. 3 for data for patient B).

Figure 2 shows some further characteristics of the sensitivity of detection. We prepared two amplification products at comparable concentrations (100% in Fig. 2 corresponds to approximately 250 ng of the PCR fragment) from the reference panel (L78142 and L78148). These samples were reactive with the probes for codons 184V and 184I, respectively. Figure 2A shows the sensitivity of detection in a dilution experiment. A weak but clearly distinguishable signal is already present at a concentration of 4% (10 ng) to 8% (20 ng) of the normal amount of amplicons used in LiPA experiments. This signal increases with an increasing amount of material, but no aspecific reaction occurred, even in heavily overloaded settings (Fig. 2A, lane 11; 1 μg). In a second experiment (Fig. 2B), mixtures of both amplicons were prepared. Again in this experiment, amplicons were clearly visible and were specifically reactive at a concentration of 4% or higher. Comparable results were obtained for codons at other positions (data not shown).

Analysis of European and U.S. serum samples. The results obtained with these 48 probes with 306 European and 52 U.S. plasma samples from HIV-1-infected individuals are summarized in Tables 1 to 6. Because of the inclusion of probes for motifs not reported in the NCBI database, the percentage of motif recognition in these plasma panels is generally higher than could be predicted from the database. At each relevant codon, between 82.4% (codon 219 in European samples) and 100% (codon 41 in U.S. samples) of the variants could be assigned unambiguously (average, 92.7%). In several plasma samples ($n = 38$), we found sequence motifs which were not predicted from the database. This was particularly apparent at codon 74 (74w9) and at codon 41 (41w20).

Analysis of follow-up samples from patients. We selected follow-up samples from three patients and analyzed the viral genotype with the 48 LiPA probes. The LiPA genotyping result together with the viral load and $CD4^+$ T-cell count are presented in Fig. 3. For all three patients, a wild-type virus (i.e., M41-T69-K70-L74-V75-M184-F214-T219-K219) strain was found in the sample collected before antiretroviral treatment. Only codon positions where changes occurred after treatment are presented in Fig. 3.

From patient A, 11 plasma samples were analyzed, with the first sample being collected 2 weeks before the start of therapy. LiPA revealed that before treatment, in a T215 context (all variants having threonine at codon 215), two variants at codon position 213 were predominantly present (GGG and GGA detected by probes 215w11 and 215w9/215w29, respectively). From week 50 to week 81, a mixture of T215 and Y215 could be detected. Both mutants with variations at codon 213 were also represented among the selected mutants with resistance genotypes (probes 215m17 and 215m14 were both positive). From week 94 onward, only Y215 mutant virus could be detected. A nearly identical genoconversion at codon 41 was observed, with the detection of mixtures (M41 and L41) from week 81 to week 111; from week 126 onward, only L41 could be found (data not shown). $CD4^+$ T-cell values were highly variable, but a continuous decrease was apparent ($P = 0.019$;

TABLE 1. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 38 to 43

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motifs in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 191/m = 25)[b]	Europe (n = 306)	United States (n = 52)	RP (n = 25)[c]		
	TGTACAGAAATGGAAAAG	CTEMEK						
41w7	----------	----	122 (62.9)	237	35	11	a, 1	L78149
41w15[f]	----------	-----	118	230	38	9	a, 1	L78149
41w19	-----G------	----	5 (2.6)	10	2	2	a, 2	L78156
41w20	--G--------A	----	0	6	0	1	a, 3	L78157
41w30	--------G---	----	1 (0.5)	8	6	1	a, 4	L78154
41m21	------T--------	--L--	18 (9.4)	37	7	2	a, 5	L78136
41m11	--GT--------	-L--	0	0	0	1	a, 6	L78140
41m24	---T-----G--	-L-E	12 (6.3)	1	2	1	a, 7	L78144
41m13	---C--------	-L--	14 (7.3)	21	3	1	a, 8	L78139
41m12	--GC--------	-L--	0	1	0	1	a, 9	L78155
41m27	---C-----G--	-L-E	3 (1.6)	0	1	1	a, 10	L78137
Total (%)[g]			175 (91.6)	95.1	100	88		

[a] Consensus sequence covering the vicinity of the aa motifs conferring drug resistance; the nucleotide consensus presented does not provide the exact probe sequence, but only that region that represents the required specificity motif indicated by the consensus aa.
[b] Prevalence of the indicated motifs in the NCBI nucleic acid sequence database. n, total amount of consensus and variant sequences that were retrieved; m, amount of different motifs present in the n amount of sequences.
[c] RP, reference panel indicating the number of times a certain motif was cloned.
[d] The indicated number corresponds to a lane in Fig. 1.
[e] Accession number indicating the location of the corresponding reference panel clone in the NCBI sequence database.
[f] Redundant probe having the same sequence motif as the previous probe, but with a slightly different location compared to the consensus sequence.
[g] The total percentage for European and U.S. samples is not the sum of the probe reactivities, but a result of the complete interpretations for these codons. This is due to the fact that some sera showed mixed (wild type and mutant) reactivities.

linear regression analysis). Viral load also decreased initially. However, the direct response to the treatment might have been missed in this follow-up series, since the first sample after the start of the treatment was obtained at 32 weeks. From then on, the viral load increased.

Patient B was treated with combination AZT-3TC therapy from week 2 onward. At week 10, a mixture of M184 and V184 could be detected. From week 14 on, only V184 was present. $CD4^+$ T-cell counts increased nearly 2.5-fold, with the highest level found at week 10. Viral load decreased spectacularly by 3 log units. From week 10 onward, however, a slight but steady increase was noted. The decrease in $CD4^+$ T cells and the increase in viral load coincided with the appearance of the V184 motif. AZT resistance codons were not yet apparent by week 23.

Patient C was followed for 55 weeks. AZT treatment started at week 10, followed by a supplemental ddC treatment from week 20 onward. The first sample was found to be reactive with probe 215w9/w29 (F214 T215 = TTTACC), but trace amounts of reactivity with 215w53 (L214 T215 = TTAACC) could be detected as well, indicating the presence of at least two variants at that time. From week 19 onward, the codon L214 (TTA)

TABLE 2. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 68 to 72

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motifs in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 354/m = 32)[b]	Europe (n = 306)	United States (n = 52)	RP (n = 25)[c]		
	AGTACTAAATGGAGA	STKWR						
70w1	----------	-----	224 (63.3)	230	39	13	b, 1, 2	L78147
70w8[f]	----------	----	208	210	38	11	b, 2	L78144
70m12	-------G-------	--R--	37 (10.5)	46	6	4	b, 3	L78148
70m13	-----A-G-------	--R--	0	0	1	2	b, 4	L78133
70w2	---GA----------	-D---	25 (7.1)	4	4	2	b, 5	L78136
70m3	GA--G-------	DR--	10 (2.8)	3	1	0	b, 6	Pending
70m14	----A--G----	-NR-	7 (2.0)	4	5	2	b, 7	L78154
70m16	---G---G----	-AR-	2 (0.6)	0	0	1	b, 8	L78150
Total (%)[g]			305 (86.2)	91.8	94.2	96		

[a] See note *a* to Table 1.
[b] See note *b* to Table 1.
[c] See note *c* to Table 1.
[d] See note *d* to Table 1.
[e] See note *e* to Table 1.
[f] See note *f* to Table 1.
[g] See note *g* to Table 1.

TABLE 3. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 72 to 77

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motifs in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 364/m = 20)[b]	Europe (n = 306)	United States (n = 52)	RP (n = 25)[c]		
	AGAAAATTAGTAGATTTC	RKLVDF						
74w5	----------	-----	320 (87.9)	264	48	16	c, 1	L78150
74w8	----------C---	-----	9 (2.5)	34	1	2	c, 2	L78147
74w9	-----G-----G---	-----	0	17	3	2	c, 3	L78137
74m6	------G--------	--V--	0	5	0	3	c, 4	L78149
74m12	------AC-------	--T--	0	1	1	1	c, 5	L78136
Total (%)[f]			329 (90.4)	93.5	98.1	96		

[a] See note *a* to Table 1.
[b] See note *b* to Table 1.
[c] See note *c* to Table 1.
[d] See note *d* to Table 1.
[e] See note *e* to Table 1.
[f] See note *g* to Table 1.

motif became more important. At week 42, the first sign of genotypic resistance could be detected by the presence of a F214 Y215 motif (TTTTAC). Finally, at week 55, only the F214 Y215 motif could be detected. The L214 (TTA) motif disappeared completely. At week 42, a mixture (K and R) at codon 70 was present, but at week 55, only R70 could be detected. Also at week 55, a mixture of codon 219 motifs (K and E) was found (data not shown). The patient's $CD4^+$ T-cell count increased initially, with a maximal effect during AZT monotherapy peaking at week 21. From then on, a continuous decrease was observed. However, 10 weeks of AZT treatment did not result in a drop in viral load, since the values at weeks 16 and 19 were nearly unchanged. It was only after start of the combination therapy (week 20) that the viral load dropped by 1.67 logs. The rise in $CD4^+$ T-cell count may be the consequence of the drug itself and not drug-induced protection (12). Phenotypic testing should be performed to determine whether the L214 T215 motif in this specific genetic background confers a certain level of resistance.

DISCUSSION

By adapting the previously designed HCV genotyping LiPA technology (22, 23) for the HIV RT gene, the LiPA format permits the rapid and simultaneous detection of wild-type and drug-selected variants associated with the genotypic resistance for AZT, ddI, ddC, d4T, and 3TC. The combination of the selected probes provides information about the genetic constitution of the RT gene in the vicinity of codons 41, 69, 70, 74, 75, 184, 215, and 219 at the nucleotide level and, hence, also at the deduced protein level. Essentially, the biotinylated RT PCR product is hybridized against immobilized specific oligonucleotides (Tables 1 to 6) which are directed against the indicated codon variabilities. Following this reverse hybridization, the oligonucleotide-biotinylated PCR strand is recognized by the streptavidin-alkaline phosphate conjugate, which then in turn converts the alkaline phosphate substrate into a purple-brown precipitate.

Using this assay, we studied the specificities and reactivities of 48 probes covering six different regions. This combination

TABLE 4. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 182 to 185

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motifs in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 322/m = 12)[b]	Europe (n = 306)	United States (n = 52)	RP (n = 25)[c]		
	CAATACATGGAT	QYMD						
184w11	----------	----	285 (88.5)	267	46	18	d, 1	L78147
184w17	--G---------	----	16 (5.0)	9	4	3	3, 2	L78137
184w21	-----T------	----	6 (1.9)	4	2	1	d, 3	L78145
184w24	----------C	----	0	1	0	1	d, 4	L78144
184m12	------G-----	--V-	1 (0.3)	8	0	1	d, 5	L78142
184m28	--------A---	--I-	3 (0.9)	0	0	1	d, 6	L78148
Total (%)[f]			311 (96.6)	93.8	98.1	100		

[a] See note *a* to Table 1.
[b] See note *b* to Table 1.
[c] See note *c* to Table 1.
[d] See note *d* to Table 1.
[e] See note *e* to Table 1.
[f] See note *g* to Table 1.

TABLE 5. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 212 to 218

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motif in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 321/m = 36)[b]	Europe (n = 306)	United States (n = 52)	RP (n = 25)[c]		
	TGGGGATTTACCACACCAGAC	WGFTTPD						
215w11	G----------	----	9 (2.8)	15	3	2	e, 1	L78146
215w9	----------	-----	142 (44.2)	178	24	3	e, 2	L78141
215w29[f]	----------	----	142	105	16	3	e, 2	L78141
215w33	-----C------	----	9 (2.8)	8	4	1	e, 3	L78154
215w10	C----------	L---	25 (7.8)	10	0	2	e, 4	L78150
215w27[f]	------C-----	--L-	25	14	0	2	e, 4	L78150
215m50	--GC--------	-L--	0	0	0	1	e, 5	L78145
215w53	-----A------	-L--	1 (0.3)	1	3	1	e, 6	L78138
215w22	--T---------	----	3 (0.9)	10	2	1	e, 7	L78134
215m17	------TA----	--Y-	88 (27.4)	50	12	7	e, 8	L78144
215m14	--G---TA----	--Y-	24 (7.5)	24	1	1	e, 9	L78149
215m49	--G---TAT---	--Y-	0	2	0	2	e, 10	L78148
215m45	---TT------	-F--	1 (0.3)	16	0	1	e, 11	L78135
215m13	---C--TA----	-LY-	0	0	0	2	e, 12	L78155
Total (%)[g]			302 (94.1)	92.8	90.4	96		

[a] See note *a* to Table 1.
[b] See note *b* to Table 1.
[c] See note *c* to Table 1.
[d] See note *d* to Table 1.
[e] See note *e* to Table 1.
[f] See note *f* to Table 1.
[g] See note *g* to Table 1.

should allow for the reliable detection of most of the genetic resistance-related codon combinations observed to date. Mutations occasionally occurring in the vicinity of the target codons, which were not taken into consideration during probe design, may eventually prevent hybridization of the probes to a particular target region. This problem is partially solved by the redundancy of probes at the most important codons. Results obtained with 358 HIV-1-infected plasma samples showed that, depending on the codon position under investigation, between 82.4 and 100% of the combinations could be detected (average, 92.7%). It is important to mention here that the assay was developed for the detection of resistance of HIV-1 genotype B, which is found predominantly in Europe and the United States, and only limited information is available about the outcome of this assay with other HIV-1 genotypes. Since the amplification primer combination is more or less universal for all the HIV-1 isolates, some of the indeterminate results may well be due to the presence of non-genotype B virus strains.

Because of the large numbers of variables that need to be included in the selection of specific probes (temperature of hybridization, ionic strength of hybridization buffer, length of the probe, G+C content, strand polarity), some of the probes might occasionally show weak cross-reactivity with related but hitherto unreported sequences. In our experience, this has never influenced the interpretation at the deduced aa level. In the current selection of probes, all except two (41w19 and 70m3) were retained on the basis of 100% specificity: as soon as one nucleotide differs in the probe area, hybridization is abolished. Further ongoing fine-tuning of these probes can

TABLE 6. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 217 to 220

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motifs in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 204/m = 12)[b]	Europe (n = 34)	United States (n = 52)	RP (n = 26)[c]		
	CCAGACAAAAAA	PDKK						
219w2	----------	----	179 (87.7)	26	42	18	f, 1	L78144
219m4	------C-----	--Q-	1 (0.5)	2	4	2	f, 2	L78135
219m7	-----TC-----	--Q-	0	0	0	1	f, 3	L78133
219m9	------G-----	--E-	0	0	0	1	f, 4	Pending
Total (%)[f]			179 (88.2)	82.4	82.7	84.6		

[a] See note *a* to Table 1.
[b] See note *b* to Table 1.
[c] See note *c* to Table 1.
[d] See note *d* to Table 1.
[e] See note *e* to Table 1.
[f] See note *g* to Table 1.

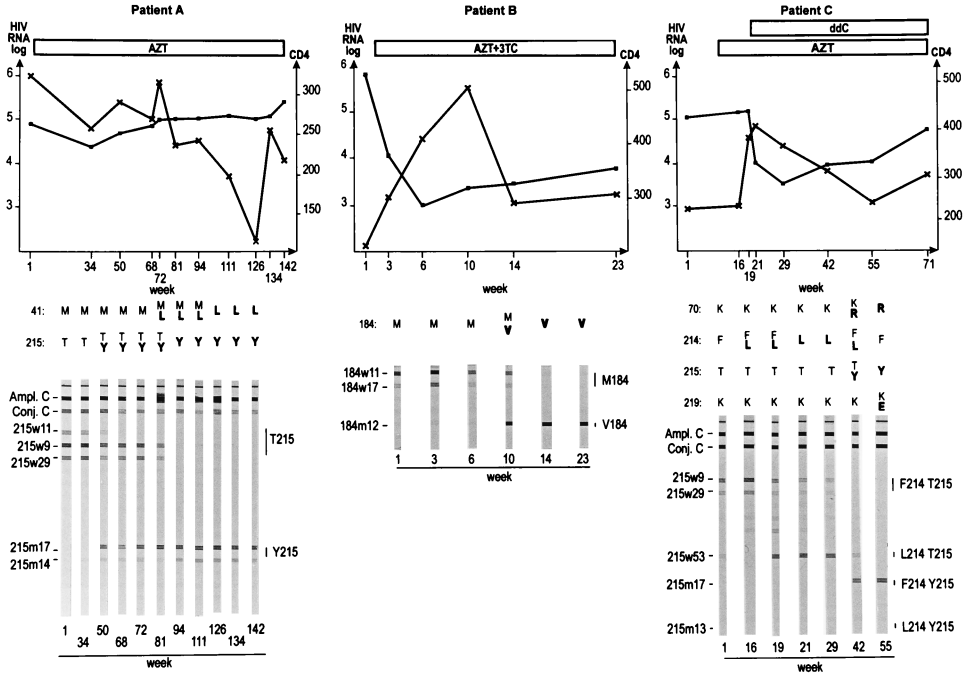

FIG. 3. Clinical and virological features detectable in follow-up samples from three patients, patients A to C. All three patients were infected with an HIV-1 strain showing the M41-T69-K70-L74-V75-M184-F214-T215-K219 genotype (wild-type pattern). Fluctuations between plasma HIV RNA copy numbers (log_{10} copies/ml) (■) and $CD4^+$ T-cell count (×) are given as a function of time in the panels on the top. The different treatment regimens and the period of treatment are also indicated. The sequences in the middle indicate the changes that appeared during the treatment period and that could be scored with the LiPA probes: for patient A at codons 41 and 215, for patient B at codon 184, and for patient C at codons 70, 214, 215, and 219. The corresponding LiPA strips for a subset of the aa changes are shown at the bottom. LiPA probes are indicated on the left, and the aa interpretation is indicated at the right of each panel. Ampl. C, amplification control; Conj. C, Conjugate control.

enhance the required specificity. For other weak cross-reactivities, the following interpretation rule is generally followed: if cross-reactivity is occurring with a cloned amplification product, this might be due to technical conditions (illustrating the necessity for stringent compliance with the described procedure); if the same result is obtained with amplification products derived from a plasma sample, the possibility of a mixture should be considered as well.

Several assays for the detection of wild-type and drug-selected mutations in the HIV RT gene have been described. These include Southern blotting (16), primer-specific PCR (10), PCR-ligase detection reaction (4), RNase A mismatch cleaving (6), point mutation assay (9), and hybridization against enzyme-labelled probes (3). The general advantage of the LiPA and other genotypic assays is the speed with which results are obtained compared to the speed with which the results of phenotypic assays are obtained. The particular advantage of our test is its multiparameter (in this particular case, multicodon) format. Moreover, the assay can easily be extended not only for the screening of the other RT codons but also for the screening of proteinase codons associated with resistance (13). As illustrated in Fig. 2, mixtures of different sequence variants can be detected easily. The detection limit for these mixtures is dependent on the sensitivity of the probes; but with amplicon concentrations ranging from 5 ng (2%) to 20 ng (8%), or an average of 10 ng (4%; Fig. 2), reliable staining patterns were observed. This is comparable to the experiences encountered during the development of the HCV genotyping LiPA (23). Our sequencing protocol has not yet provided the same degree of sensitivity in detecting such mixtures.

Accompanying polymorphisms in the vicinity of the target codons are found with a rather high prevalence in wild-type virus strain sequences, but not in mutant strain sequences. A partial list of such combinations is hereby presented: codon V74 (GTA) without polymorphism at codon 73, 75, or 76; codon V184 (GTG) without codon Q182 (CAG); and codon F215 (TTC) without F214 (TTC or TTA) or L214 (CTT). The most intriguing example is the following: L214 T215 (CTTA CC) is predicted for approximately 7.8% of the wild-type sequences. The corresponding motif, L214 Y215 (CTTTAC), apparently does not exist in virus from plasma. From the example shown in Fig. 3c, it is clear that the selection of mutants is a very flexible and complex phenomenon. In this particular case, viruses having codon F214 were replaced by a L214 viral population in the AZT monotherapy period, but upon selecting for genotypic drug resistance at codon 215, the original F214 configuration was restored. The selection for the Y215 genotype prohibits the presence of an L214 genotype. Since no evidence has yet emerged that L214 confers resistance to antiretroviral compounds, the appearance of this special mutant during AZT monotherapy period is difficult to interpret and most likely depends on the genetic background. More research will certainly be necessary to clarify this issue. This includes phenotypic resistance determinations and site-directed mutagenesis experiments for verification. These experiments are in progress.

Since antiviral treatment can result in a marked extension of life expectancy for HIV-infected individuals, it is of utmost importance to find the best drug regimen for each individual. Therefore, monitoring of the magnitude and duration of the virus load and $CD4^+$ T-cell changes is a prerequisite (14). However, knowledge concerning the genetic constitution of the virus may also be an important factor in designing optimal treatment schedules. Optimization of therapies by making

good use of available information (viral load, $CD4^+$ T-cell count, and resistance profiles) has largely remained unexploited. If this was partially due to the complexity of screening for all the mutational events by the more conventional methods, the introduction of rapid genotypic and/or phenotypic assay systems should remove one key obstacle.

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