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Oligonucleotide Ligation Assay for Detection of Mutations Associated with Reverse Transcriptase and Protease Inhibitor Resistance in Non-B Subtypes and Recombinant Forms of Human Immunodeficiency Virus Type 1

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The oligonucleotide ligation assay is a genotypic assay for the detection of resistance-associated mutations to reverse transcriptase and protease inhibitors in human immunodeficiency virus type 1 subtype B. This assay has been modified and developed for non-B subtypes and recombinant strains and has been evaluated with sequencing, resulting in a more sensitive assay than sequencing for non-B subtypes.

Methods to identify resistance mutations in the human immunodeficiency virus type 1 (HIV-1) *pol* gene are needed since the use of protease (PR) and/or reverse transcriptase (RT) inhibitors may increase the prevalence of resistance mutations (9, 11). DNA sequencing is the most commonly used method to evaluate the presence of drug resistance mutations, but its high cost and equipment requirements make it unsuitable when economic resources are limited (1, 8).

The oligonucleotide ligation assay (OLA) is a genotypic assay which has been used to identify point mutations in DNA for a variety of diseases (3, 5) and to detect drug resistance-associated mutations in HIV-1 subtype B (1, 7, 8, 15). This is the most prevalent subtype in developed countries, although non-B subtypes and recombinant forms (RFs) are dramatically increasing in developing countries, where antiretroviral therapy is starting to be accessible (13), as well as in several areas of the developed world (9, 13).

In this work, we describe the development of OLA for the detection of point mutations associated with high-level resistance to PR and RT inhibitors in HIV-1 non-B subtypes and RFs, and we evaluate OLA and sequencing methods for resistance-associated mutation detection in non-B and subtype B strains.

RNAs extracted from plasmas of 66 HIV-1 specimens (22 non-B subtypes and 44 subtype B strains) were selected. The distribution of the 22 non-B subtypes, according to their *pol* genes, was as follows: (i) three subtype G strains and one each of subtypes A_1, C, and F_1; (ii) 10 circulating recombinant forms (CRFs) (nine CRF02_AG and one CRF14_BG strain); and (iii) two GKU strains and one each of subtypes AG, UA_2, and UAJ (U, unknown fragment). Five non-B strains included in this study were characterized in our laboratory by full-length genome sequencing (4).

To carry out RT-PCR, 5 to 15 μl of RNA extracted from plasma was reverse transcribed following purification and sequencing with an automated fluorescence sequencer (Applied Biosystems). Reaction conditions, oligonucleotides, and the thermocycling profile were described previously (14).

The OLA is based on the covalent joining of two adjacent oligonucleotides by a DNA ligase when they are hybridized to a cDNA target. A set of three oligonucleotides was designed for the detection of the following mutations: K103N, Q151M, Y181C, M184V, and T215Y in RT and D30N, V82A, and L90M in PR. Table 1 shows the drugs to which these mutations appear to confer resistance, the amino acid substitutions detected, and the oligonucleotide sequences. The 5′ end of the common oligonucleotide is phosphorylated and designed to anneal to the complementary region of the *pol* gene adjacent to the 3′ end of the wild-type/mutant oligonucleotide. Ligation between wild-type/mutant and common oligonucleotides occurs when both are annealed to the complementary strand of the PCR product. The procedure and reaction conditions were described by Villahermosa et al. (15), with the following modifications: the ligation reaction included 2 μl of the PCR product, 10 μl of distilled H_2O containing 0.1% Triton X-100, and 10 μl of a reaction mixture of 20% 10× ligase buffer with 200 nmol Tris, pH 8, 100 mM NAD (Sigma), 2.5% 1 M KCl (Sigma), 57.5% distilled H_2O containing 0.1% Triton X-100, 3 U of thermostable ligase (Epicenter Technologies), and 5 pmol of each ligation oligonucleotide. The McNemar test (RSIGMA; Babel statistical computer program) was used to assess significant differences between percentages.

To perform OLA with non-B strains, we performed sequence alignment and analysis and designed specific oligonucleotides for positions 181 and 184 of RT and for codon 90 of PR, obtaining a significant improvement over the use of standard subtype B oligonucleotides (data not shown).

Four hundred seventy-seven codons of the HIV-1 *pol* gene were analyzed, including 156 codons from non-B and RF samples and 321 from subtype B samples. Four hundred four (84.7%) of the 477 codons analyzed were coincident by OLA

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TABLE 1. Oligonucleotides used in OLA to detect mutations in HIV-1 *pol* associated with resistance to RT and PR inhibitors

Drug(s) to which oligonucleotide confers high or intermediate resistance in HIV-1[a]	Codon	Amino acid substitution	Genotype detected[b]	Sequence (5′→3′)[c]
Nevirapine, delavirdine, and efavirenz	103		WT	dig-ACATCCCGCAGGGTTAAAAAAGAA
		K103N	Mutant	f-ACATCCCGCAGGGTTAAAAAAGAAC
			Common	p-AAAATCAGTAACAGTACTGGATGTGGGT-bio
Zidovudine, stavudine, abacavir, and didanosine	151		WT	dig-CAGTACAATGTGCTTCCACA
		Q151M	Mutant	f-CAGTACAATGTGCTTCCAAT
			Common	p-GGGATGGAAAGATCAC-bio
Nevirapine and delavirdine	181		WT	dig-ACAAAATCCAGACATAGTTATCTA
		Y181C	Mutant	f-ACAAAATCCAGACATAGTTATCTG
			Common	p-TCAATACAGTGGATGATTTGTATGTA-bio
			Common	p-CCAATACATGGATGATTTGTATGTA-bio
Lamivudine and emtricitabine	184		WT	dig-AGACATAGTTATCTATCAATACA
			WT	dig-AGACATAGTTATCTATCAATA**T**A
		M184V	Mutant	f-AGACATAGTTATCTATCAATACG
		M184V	**Mutant**	f-AGACATAGTTATCTATCAATA**T**G
			Common	p-TGGATGATTTGTATGTA GGATC-bio
Zidovudine and stavudine	215		WT	dig-CAACATCTGTTGAGGTGGGGATTTAC
		T215Y	Mutant	f-CAACATCTGTTGAGGTGGGGATTTTA
			Common	p-CACACCAGACAAAAAACATCAGAA-bio
Nelfinavir	30		WT	dig-TATTAGATACAGGAGCAGATG
		D30N	Mutant	f-TATTAGATACAGGAGCAGATA
			Common	p-ATACAGTATTAGAAGAAATGAAT-bio
Ritonavir, indinavir, lopinavir, nelfinavir, amprenavir, and atazanavir	82		WT	dig-TATTAGTAGGACCTACACCTGT
		V82A	Mutant	f-TATTAGTAGGACCTACACCTGC
			Common	p-CAACATAATTGGAAGAAATCTGT-bio
Saquinavir, nelfinavir, indinavir, ritonavir, amprenavir, lopinavir, and Atazanavir	90		WT	dig-CAACATAATTGGAAGAAATCTGT
			WT	dig-CAACATAATTGGAAGAAA**YR**T**R**T
		L90M	Mutant	f-CAACATAATTGGAAGAAATCTGA
		L90M	**Mutant**	f-CAACATAATTGGAAGAAA**YR**T**R**A
			Common	p-TGACTCAGATTGGTTGCACTTT-bio

[a] Resistances are based on the Stanford database.
[b] WT, wild-type. Oligonucleotides in bold were designed for non-B subtypes and RF samples.
[c] Bases comprising the codons of interest are underlined. dig, digoxigenin; f, fluorescein; p, phosphate; bio, biotin.

TABLE 2. Summary of genotype results for 477 codons associated with resistance to RT and PR inhibitors by OLA and sequencing of HIV-1 samples

Genotypic results (SEQ→OLA) (*n*)[b]	No. of codons with RT amino acid mutation which confers resistance to:					No. of codons with PR amino acid mutation which confers resistance to PR inhibitors		
	NRT inhibitors[a]			NNRT inhibitors[a]				
	Q151M	M184V	T215Y	K103N	Y181C	D30N	V82A	L90M
HIV-1 non-B subtypes and RF samples								
MT (SEQ)→MT (OLA) (29)	0	7	4	12	1	0	1	4
MT (SEQ)→WT (OLA) (0)	0	0	0	0	0	0	0	0
MT (SEQ)→INDET (OLA) (3)	0	2	0	1	0	0	0	0
WT (SEQ)→WT (OLA) (103)	14	11	6	9	12	21	14	16
WT (SEQ)→MT (OLA) (5)	0	0	0	1	3	0	0	1
WT (SEQ)→INDET (OLA) (16)	1	1	2	0	3	1	7	1
Total (156)	15	21	12	23	19	22	22	22
HIV-1 subtype B samples								
MT (SEQ)→MT (OLA) (81)	10	21	8	16	7	2	5	12
MT (SEQ)→WT (OLA) (0)	0	0	0	0	0	0	0	0
MT (SEQ)→INDET (OLA) (5)	0	0	1	1	0	0	3	0
WT (SEQ)→WT (OLA) (193)	27	15	14	18	18	40	34	27
WT (SEQ)→MT (OLA) (34)	1	7	2	2	17	1	1	3
WT (SEQ)→INDET (OLA) (8)	1	1	2	1	2	0	0	1
Total (321)	39	44	27	38	44	43	43	43

[a] NRT, nucleoside reverse transcriptase; NNRT, nonnucleoside reverse transcriptase.
[b] MT, mutant; WT, wild type; INDET, indeterminate (negative for both wild-type and mutant genotypes).

and sequencing (Table 2). The greater sensitivity of the OLA method than of sequencing for the detection of minor HIV-1 species was demonstrated by our experiments. OLA detected mutant viral sequences in 39 samples (8.2%) that had been genotyped as wild type by sequencing (Table 2). Of these, 37 had mixtures of mutant and wild-type sequences, with 32 having mutations in the RT region and 5 having mutations in the PR sequence. The potential detection of minor mutant populations before they become predominant could be a useful tool for the management and preliminary orientation of antiretroviral therapy, showing a clear advantage over sequencing (1, 8, 16) and representing the first time that a genotypic assay other than the sequencing method was successfully performed for the detection of resistance-associated mutations to nucleoside and nonnucleoside RT and PR inhibitors in non-B subtypes. In fact, a nitrocellulose strip hybridization-based assay, the LiPA (Innogenetics), has been performed for the detection of resistance-associated mutations to PR and RT inhibitors, with evident limitations due to the sequence variability between non-B and B subtypes (6). The OLA, in contrast to the LiPA assay, allows the design and incorporation of new oligonucleotides into the reaction, which adapt well to the sequence variability present in non-B subtypes.

The OLA detected 32 (6.7%) indeterminate results from the 477 codons analyzed (Table 2): 19/156 (12.2%) were from non-B subtypes and RFs and 13/321 (4%) were from subtype B ($P < 0.01$). The presence of alternative mutations (T215F/C, V82I/F/S, and K103H) no longer complementary to the oligonucleotides used in the assay was the most common cause of indeterminate results. Most of these indeterminate reactions were due to mutations located within three bases of the ligation site or to the presence of two or more base changes in the region complementary to one of the ligation oligonucleotides (1, 7, 15). This is not surprising considering that polymorphisms and accessory mutations in regions of RT and also in PR are common, particularly in non-B subtypes (2). In fact, the L89M mutation of PR was present in 18 of the 22 HIV-1 non-B strains.

In conclusion, OLA is a rapid and sensitive method for identifying mutations associated with antiretroviral resistance to RT inhibitors and PR inhibitors in either HIV-1 non-B strains, RFs, or subtype B strains. In contrast to the LiPA assay, the OLA is highly adaptable since it allows the design of novel and specific oligonucleotides to detect new resistance-associated mutations as they are identified or to detect resistance-associated mutations in non-B subtype samples, where multiple genetic polymorphisms and accessory mutations are commonly found (1, 7, 16). This assay could also be used for the efficient determination and discrimination of different genetic subtypes through the design of specific oligonucleotides, as previously described for a similar genotyping approach (10, 12). Moreover, the OLA has the ability to detect drug-resistant subpopulations present in a relatively small proportion (8), which represents a big advantage compared to sequencing because this makes OLA useful for the election of initial therapeutic regimens or changes in therapy in order to prevent the accumulation of additional mutations. Finally, OLA is available from the National Institutes of Health AIDS Reagent Program for the detection of resistance-associated mutations to nucleoside RT inhibitors and nonnucleoside RT inhibitors (7), in which preparation our laboratory has actively participated (15), and to protease inhibitors (1), making this method easy and cheap to perform and offering a real and practical alternative to DNA sequencing, especially in places with limited economic resources.

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