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Sabelle Jallow
Medical Research Council Laboratories, Banjul

Steve Kaye
Medical Research Council Laboratories, Banjul

Martin Schutten
Erasmus Medical Centre

Eleonor Brandin
Swedish Institute for Infectious Disease Control

Jan Albert
Swedish Institute for Infectious Disease Control

See next page for additional authors

Authors
Sabelle Jallow, Steve Kaye, Martin Schutten, Eleonor Brandin, Jan Albert, Samuel McConkey, Tumani Corrah, Hilton Whittle, Guido Vanham, Sarah Rowland-Jones, and Wouter Janssens

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Development and Evaluation of an Oligonucleotide Ligation Assay for Detection of Drug Resistance-Associated Mutations in the Human Immunodeficiency Virus Type 2 *pol* Gene▽

Sabelle Jallow,[1,2]* Steve Kaye,[1]† Martin Schutten,[3] Eleonor Brandin,[4,5] Jan Albert,[4,5] Samuel J. McConkey,[1]‡ Tumani Corrah,[1] Hilton Whittle,[1] Guido Vanham,[2] Sarah Rowland-Jones,[1] and Wouter Janssens[2]

Medical Research Council Laboratories, Banjul, The Gambia[1]; Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium[2]; Department of Virology, Erasmus Medical Centre, Rotterdam, The Netherlands[3]; Department of Virology, Immunology and Vaccinology, Swedish Institute for Infectious Disease Control, Solna, Sweden[4]; and Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden[5]

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Human immunodeficiency virus type 2 (HIV-2) is naturally resistant to several antiretroviral drugs, including all of the non-nucleoside reverse transcriptase inhibitors and the entry inhibitor T-20, and may have reduced susceptibility to some protease inhibitors. These resistance properties make treatment of HIV-2 patients difficult, with very limited treatment options. Therefore, early detection of resistance mutations is important for understanding treatment failures and guiding subsequent therapy decisions. With the Global Fund Initiative, a substantial number of HIV-2 patients in West Africa will receive antiretroviral therapy. Therefore, development of cheaper and more sustainable resistance assays, such as the oligonucleotide ligation assay (OLA), is a priority. In this study, we designed oligonucleotide probes to detect the Q151M mutation, associated with phenotypic resistance to zidovudine, didanosine, zalcitabine, and stavudine, and the M184V mutation, associated with phenotypic resistance to lamivudine and emtricitabine, in HIV-2. The assay was successfully developed and evaluated with 122 samples from The Gambia, Guinea Bissau, The Netherlands, and Sweden. The overall sensitivity of the assay was 98.8%, with 99.2% for Q151M and 98.4% for M184V. OLA results were compared with sequencing to give high concordances of 98.4% (Q151M) and 97.5% (M184V). OLA demonstrated a higher sensitivity for detection of minor variants as a mixture of wild-type and mutant viruses in cases when sequencing detected only the major population. In conclusion, we have developed a simple, easy-to-use, and economical assay for genotyping of drug resistance in HIV-2 that is more sustainable for use in resource-poor settings than is consensus sequencing.

Human immunodeficiency virus type 2 (HIV-2), one of the causative agents of AIDS (10), has a relatively lower prevalence than does HIV-1. HIV-2 is restricted mainly to West African countries such as Senegal, Guinea Bissau, and The Gambia (29), where its prevalence varies from 1 to 10% of the adult population (11, 17, 21, 26, 31, 35), although the prevalence can be as high as 28% in high-risk groups such as commercial sex workers in The Gambia (29). Cases of HIV-2 infection occur in countries outside of West Africa, such as Portugal and countries with former socioeconomic links to Portugal, including southwest India, Angola, Mozambique, and Brazil (2, 27, 29).

HIV-2 is naturally resistant to some currently available antiretroviral drugs, such as the non-nucleoside reverse transcriptase inhibitors (nNRTIs) and the entry inhibitor T-20, due to the presence of natural polymorphisms that confer resistance (24). In addition, HIV-2 has several natural polymorphisms associated with major and minor resistance to some protease inhibitors, notably amprenavir and nelfinavir (28). These polymorphisms make the nucleoside reverse transcriptase inhibitors (NRTIs) central to HAART regimens used in the treatment of HIV-2 infection. Thus, early detection of resistance mutations to NRTIs is very important for explaining treatment failures and guiding subsequent treatment decisions.

HIV is characterized by extensive genetic variability, resulting in different strains classified into groups, subtypes, subsubtypes, and circulating recombinant forms (CRFs). While the HIV-1 pandemic is recognized globally as consisting of many separate epidemics (25), with nine subtypes and 34 CRFs (http://www.hiv.lanl.gov/content/hiv-db/CRFs/CRFs.html), HIV-2 is characterized by an epidemic of only two subtypes (A and B) and five nonepidemic subtypes (C to G) (23). Only one member each of subtypes C, E, F, and G and two members of subtype D have been identified (29). While HIV-2 subtype A viruses have been documented in different locations across West Africa and other regions of the world, subtype B is geographically restricted mainly to Ivory Coast and Ghana, with a few cases documented in Europe and the Middle East (18).

The oligonucleotide ligation assay (OLA) is a point mutation assay based on the covalent joining of two adjacent, dif-

* Corresponding author. Mailing address: Institute of Tropical Medicine, Microbiology Department, Nationalestraat 155, Antwerp 2000, Belgium. Phone: 32 3 247 6328. Fax: 32 3 247 6333. E-mail: sjallow@itg.be.

† Present address: Department of Retrovirology, Imperial College London, London, United Kingdom.

‡ Present address: Department of International Health and Tropical Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland.

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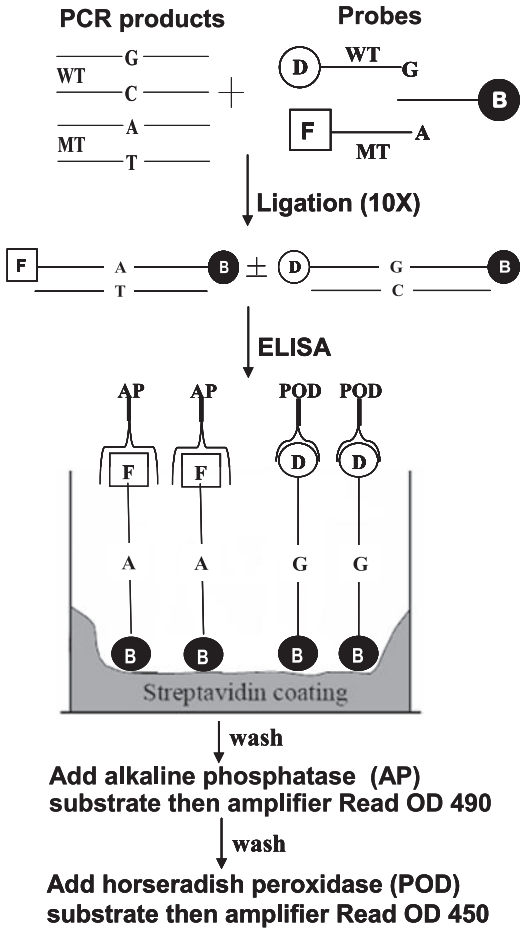


FIG. 1. OLA schema. OLA involves the generation of PCR fragments and the use of three labeled probes: digoxigenin (D) for WT, fluorescein (F) for MT, and biotin (B) for the common probe. These probes anneal to the PCR product, and the WT or MT probe is ligated to the common probe. Ligated products are captured on streptavidin-coated microtiter wells and detected by ELISA with horseradish peroxidase (POD)-labeled anti-D antibodies and alkaline phosphatase (AP)-labeled anti-F antibodies.

ferentially labeled oligonucleotide probes by a DNA ligase when they are hybridized to a cDNA template (15, 20). OLA has been used for the evaluation of the primary mutations associated with HIV-1 resistance to currently available antiretroviral drugs (4, 13, 14, 34). Each primary drug resistance point mutation of interest is analyzed with a set of three labeled oligonucleotide probes: a 5′ digoxigenin-labeled probe to detect the wild-type (WT) sequence, a 5′ fluorescein-labeled probe to detect the mutant (MT) sequence, and a 3′ biotinylated oligonucleotide probe that hybridizes on the 3′ side of the mutation site for both WT and MT targets (34). OLA is a simple assay in which the probes are annealed to a PCR fragment derived from the patient sample, the WT or MT probe is ligated to the common probe, and the ligated product is detected in an enzyme-linked immunosorbent assay (ELISA) (4, 32) (Fig. 1). Routinely, genotypic resistance testing involves sequencing of the protease and reverse transcriptase (RT) genes. However, OLA has been shown to be simple, rapid, sensitive, and economical and without the need for expensive equipment and technical expertise. Excluding the PCR costs necessary for both methods, OLA costs ~$1 for the two mutations per sample, whereas sequencing costs at least $10. Like all ligation assays, the potential disadvantage of OLA is that the presence of other mutations around the ligation site may result in failure of the assay (indeterminate result). The occurrence of indeterminate results with OLA is related to the high level of genetic variability in HIV, such that the HIV-1 OLA has been modified for some non-B subtypes (33). For HIV-2, only two subtypes, A and B, are of epidemiological importance.

In the HIV-2 RT enzyme, as is the case for HIV-1, the Q151M mutation is a multi-NRTI resistance mutation associated with phenotypic resistance to zidovudine, didanosine, zalcitabine, abacavir, and stavudine, while the M184V mutation is associated with phenotypic resistance to lamivudine (3TC) and emtricitabine (1) in HIV-2. We report the development and evaluation of an OLA for detection of the Q151M and M184V mutations associated with high-level resistance to NRTIs in HIV-2.

MATERIALS AND METHODS

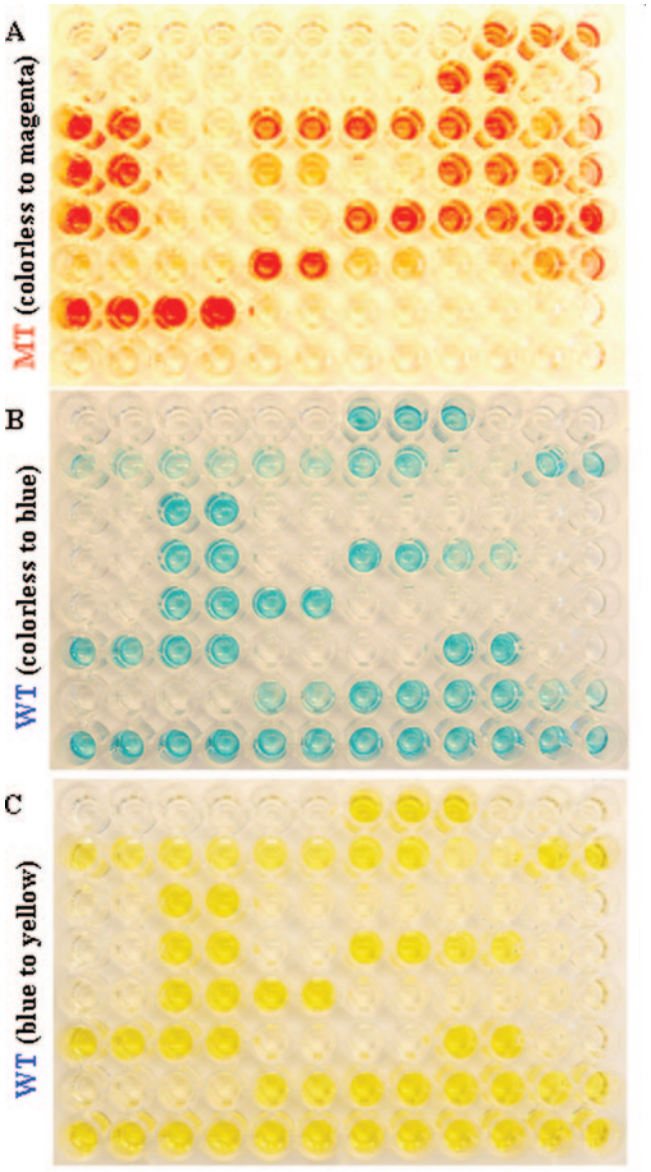
Patients. A total of 122 samples were obtained from 64 patients from the Gambia (n = 42), Guinea Bissau (n = 24), The Netherlands (n = 26), and Sweden (n = 30). The samples from the Gambia included 32 samples from eight patients treated with Combivir (AZT-3TC), as previously described (19), and 10 samples from five patients on triple therapy (AZT-3TC-Lop/r). The 24 samples from Guinea Bissau were from 23 subjects included in the Caio HIV-2 cohort (29). The 26 samples from The Netherlands were from 16 treatment-experienced HIV-2-infected patients, mostly of West African origin, who were genotyped at the Erasmus Medical Centre in Rotterdam, The Netherlands. The 30 Swedish samples were from 12 patients—6 untreated, 1 treated successfully, and 5 failing therapy—as previously described (8). These samples were selected from 20 of 23 known cases of HIV-2 in Sweden. Most of these patients were immigrants from different West African countries (8).

Nucleic acid extraction and PCR amplification. HIV-2 RNA was extracted from 140 μl of EDTA plasma from Caio samples with the QIAamp viral RNA kit (QIAGEN, Venlo, The Netherlands) or 200 μl of heparin plasma from Gambian samples by the method of Boom et al. (5). The RNA was eluted into 50 μl of nuclease-free water, and 3 μl of the eluate was reverse transcribed and amplified by Titan one-tube RT-PCR (Roche Applied Science, Lewes, United Kingdom). DNA was extracted from Caio samples with the QIAamp DNA kit (QIAGEN). The inner primers SJH21 (forward) (5′-GAAAGAAGCCCCGCA ACTTCCC, positions 1861 to 1882) and SJH22 (reverse) (5′-GAGTCTGTTA GTGCCATCGCAAAG, positions 3843 to 3820) were used. All primer positions refer to HIV-2 ROD (GenBank accession no. M15390). This PCR was performed in a final volume of 25 μl. Cycling conditions were 50°C for 45 min, reverse transcription; 94°C for 2 min, initial denaturation; 10 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 120 s, 25 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 120 s, plus 5 s/cycle, elongation; and a final elongation of 68°C for 10 min.

Nested PCR was performed with primers SJH23 (forward) (5′-GAGAGACC ATACAAAGARGTG, positions 1992 to 2012) and SJH24 (reverse) (5′-CTAT TGCAGGATCCATCTGTG, positions 3719 to 3699). The reaction mix contained 2.6 units of Expand high-fidelity polymerase (Roche), 0.3 μM

TABLE 1. Differentially labeled oligonucleotide probes for detection of mutations in HIV-2 associated with resistance to NRTIs

Mutation	Probe	Label	Sequence (5′ to 3′)
Q151M	WT	Digoxigenin	*dig*-TACATATATAAAGTCTTGCCACA
	MT	Fluorescein	*flu*-TACATATATAAAGTCTTGCCAAT
	Common	Biotin	RGGRTGGAAGGGATCACCAGCA-*bio*
M184V	WT	Digoxigenin	*dig*-GATGTCATTATCATTCARTAYA
	MT	Fluorescein	*flu*-GATGTCATTATCATTCARTAYG
	Common	Biotin	TGGATGATATCTTAATAGCTAGT-*bio*



D

blank	blank	blank	H_2O c	H_2O c	H_2O c	WT c	WT c	WT c	MT c	MT c	MT c
WT S1	WT S1	WT S2	WT S2	WT S3	WT S3	WT S4	WT S4	MIX S5	MIX S5	WT S6	WT S6
MT S7	MT S7	WT S8	WT S8	MT S9	MT S9	MT S10	MT S10	MT S11	MT S11	MT S12	MT S12
MT S13	MT S13	WT S14	WT S14	MT S15	MT S15	WT S16	WT S16	MIX S17	MIX S17	MT S18	MT S18
MT S19	MT S19	WT S20	WT S20	WT S21	WT S21	MT S22	MT S22	MT S23	MT S23	MT S24	MT S24
WT S25	WT S25	WT S26	WT S26	MT S27	MT S27	MT S28	MT S28	WT S29	WT S29	MT S30	MT S30
MT S31	MT S31	MT S32	MT S32	WT S33	WT S33	WT S34	WT S34	WT S35	WT S35	WT S36	WT S36
WT S37	WT S37	WT S38	WT S38	WT S39	WT S39	WT S40	WT S40	WT S41	WT S41	WT S42	WT S42

FIG. 2. OLA genotyping (visual results). (A) Samples with MT genotypes produce a deep magenta color upon addition of alkaline phosphatase substrate and amplifier, while negative samples remain colorless/clear. After washing of the plate, the addition of horseradish peroxidase substrate turns WT samples blue (B) and the addition of the amplifier turns them yellow (C), while the negative samples remain clear. (D) Final interpretation of results. The first row has the controls (C), consisting of blanks, H_2O, WT, and MT in triplicate, and subsequent rows have the samples (S) in duplicate. Samples 5 and 17 have a mixture (MIX) of WT and MT viruses (positive for magenta, blue, and yellow).

concentrations of each primer, and 200 μM concentrations of each deoxynucleoside triphosphate in Expand reaction buffer. Cycling conditions were 95°C for 3 min, initial denaturation; 10 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 105 s, 25 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 105 s, plus 5 s/cycle, elongation; and a final elongation of 72°C for 7 min to amplify the entire HIV-2 protease and RT coding region. PCR products were analyzed on 0.8% agarose gels. Samples from The Netherlands and Sweden were supplied as PCR amplicons amplified from the protease and RT coding region of *pol*.

Sequencing. PCR products were purified with the QIAquick PCR purification system (QIAGEN) and directly sequenced. Sequencing was done on both strands with primers SJH23, SJH24, JA220 (reverse) (5′-GTCTTTATYCCTGGGTAG ATTTGTG-3′, positions 3183 to 3207) (8), JA222 (forward) (5′- ACCTCCAA CTAATCCTTATAATACC, positions 2530 to 2555) (8), H2Mp4 (reverse) (5′-CCCAAATGACTAGTGCTTCTT-3′, positions 3527 to 3707) (11), and H2Mp6 (forward) (5′-AAAAGAGATCTGTGCAAAAATGG-3′, positions 2482 to 2504) (11). Sequencing was done by the VIB Genetic Service Facility (Wilrijk, Belgium) using capillary sequencers (Applied Biosystems 3730 DNA analyzer) combined with ABI PRISM BigDye Terminator cycle sequencing kits. The sequences were assembled with DNAsis software (Hitachi Software Engineering; Molecular Biology Insights), aligned with HIV-2 ROD, and then edited, translated, and analyzed with EditSeq and MegAlign (Lasergene Software; DNASTAR). The viral subtype was determined with an NCBI online program (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) and by phylogenetic analysis (DNASTAR). Multiple sequence alignments of our samples with several HIV-2 reference strains (subtypes A, B, and G) were performed with CLUSTAL, version W, of Megalign (DNASTAR). Consequently, trimmed alignment was used to construct the phylogenetic tree (DNASTAR). SIVmne (GenBank accession no. AF361745) was employed as the outgroup to root the tree.

Construction of reference plasmids. Reference plasmids with the WT and MT sequences, for both the Q151 and M184 mutations, were generated to serve as controls. Previously documented Gambian *pol* sequence samples (19), DTS 3-12 (WT 151 and WT 184) and DTS 3-18 (MT 151 and MT 184), were amplified, purified, and inserted into TA plasmid vectors (TOPO TA cloning kit and TOP10 cells; Invitrogen, Merelbeke, Belgium). The resulting plasmids, named p12 and p18 for the WT and MT plasmids, respectively, were transformed into competent *Escherichia coli* cells and amplified. The plasmid DNA was extracted and purified with the QIAGEN plasmid mini purification kit (QIAGEN), sequenced, and analyzed by OLA.

Oligonucleotide probes. An alignment of the RT region of HIV-2 reference strain sequences, mostly subtypes A and B, retrieved from GenBank, was used to design the Q151M and M184V probes. The probes were designed to detect all HIV-2 subtypes, especially the relevant subtypes A and B. The probes were differentially labeled to simultaneously detect both MT and WT sequences (Table 1) in a single assay.

OLA. The OLA protocol and reaction conditions used were as previously described (4, 13), with the following modifications: 2 μl of *pol* amplicon, 0.167 U of Ampligase DNA ligase (Epicenter Technologies, Cambridge, United Kingdom), 0.333 pmol for all Q151M probes, and 0.5 pmol for all M184V probes (Table 1). All patient samples were tested in duplicate, and the controls were tested in triplicate. The controls consisted of blanked wells, water only, 100% WT plasmid control (p12), and 100% MT plasmid control (p18) on the first row of the 96-well plate (Fig. 2). Cutoff values for the optical readings were established as follows: for WT probes, mean optical density (OD) (negative control) + 6 standard deviations (negative control); for mutant probes, mean OD (negative control) + 9 standard deviations (negative control). Fluorescein, used to label the mutant probe, is rapidly degraded by sunlight and sensitive to high temperatures (30). Therefore, the absorbance readings for the mutant probe were subjected to a more stringent cutoff value.

In addition to the OD readings, visual results from the ELISA were recorded and used together with the OD readings to ensure accurate cutoff values and efficiency of the assay when used under field conditions (no spectrophotometer). Samples were defined as MT only (magenta color/positive OD_{490}), WT only (yellow color/positive OD_{450}), a mixture of MT and WT (magenta plus yellow

TABLE 2. Comparison of OLA with consensus sequencing for the Q151M mutation

Interpretation	Genotyping result[a]		No. of samples	%
	Sequencing	OLA		
Concordance	WT (CAR)	WT (CAR)	101	96.72
	MT (ATG)	MT (ATG)	17	
	Mix (ATG and CAR)	Mix (ATG and CAR)	0	
Detection of minor variants by OLA	WT (CAR)	MT (ATG)	0	1.64
	MT (ATG)	Mix (ATG and CAR)	1	
	WT (CAR)	Mix (ATG and CAR)	1	
Alternate mutation	Q151L (CTG)	Mix (ATG and CAR)	1	0.82
False negative	MT (ATG)	WT (CAR)	0	0
Indeterminate result	WT (CAR)	Indet	1	0.82
	MT (ATG)	Indet	0	
Total			122	100

[a] WT, Q = CAR = CAA and CAG; MT, M = ATG; Mix, mixture of WT and MT viruses; Indet, indeterminate (neither WT nor MT).

color/positive OD_{490} and positive OD_{450}), and indeterminate (no color change/negative OD_{490} and negative OD_{450}) (Fig. 2).

Nucleotide sequence accession numbers. The HIV-2 nucleotide sequences generated in this study have been submitted to the EMBL, GenBank, and DDBJ sequence databases under accession numbers AM233873 to AM233900 and AM408175 to AM408208.

RESULTS

The OLA was successfully developed for both the Q151M and M184V mutations. Analysis of the 122 RT sequences showed that 121/122 patients were HIV-2 subtype A and 1/122 was subtype B (data not shown). The probes were optimized to give sensitivities of detection of 99.2% for the Q151M mutation (Table 2) and 98.3% for the M184V mutation (Table 3). Sensitivity (number of positive samples × 100/total number of samples) is defined as the ability of the probes to detect positive results (WT and/or MT).

For the Q151M mutation, sequence analysis gave 103 WT viruses, 18 MT viruses, and 1 sample with an alternate mutation (Q151L); OLA identified 101 WT viruses, 17 MT viruses, 3 samples with a mixture of WT and MT viruses, and 1 indeterminate result (Table 4). For the M184V mutation, the sequenced results indicated 72 WT viruses, 47 MT viruses, 2 samples with a mixture of WT and MT viruses (M184V and M184I), and 1 sample with an alternate mutation (M184I); OLA showed 70 WT viruses, 46 MT viruses, 4 samples with a mixture of WT and MT viruses, and 2 indeterminate results (Table 5).

TABLE 3. Comparison of OLA with consensus sequencing for the M184V mutation

Interpretation	Genotyping result[a]		No. of samples	%
	Sequencing	OLA		
Concordance	WT (ATG)	WT (ATG)	70	95.90
	MT (GTG)	MT (GTG)	45	
	Mix (GTG and ATG)	Mix (GTG and ATG)	2	
Detection of minor variants by OLA	WT (ATG)	MT (GTG)	0	1.64
	MT (GTG)	Mix (GTG and ATG)	1	
	WT (ATG)	Mix (ATG and ATG)	1	
Alternate mutation	M184I (ATA)	WT (ATG)	1	0.82
False negative	MT (GTG)	WT (ATG)	0	0
Indeterminate result	WT (ATG)	Indet	2	1.64
	MT (GTG)	Indet	0	
Total			122	100

[a] WT, M = ATG; MT, V = GTG; Mix, mixture of WT and MT viruses; Indet, indeterminate (neither WT nor MT).

TABLE 4. Sequencing and OLA results for the Q151M mutation

Country	No. of patients	No. of samples	No. of samples with indicated genotype[a]							
			Sequencing				OLA			
			WT	MT	Mix	Other	WT	MT	Mix	Indet
The Gambia	13	42	38	4	0	0	38	4	0	0
Guinea Bissau	23	24	22	2	0	0	22	2	0	0
The Netherlands	16	26	17	9	0	0	16	9	0	1
Sweden	12	30	26	3	0	1	25	2	3	0
Total	64	122	103	18	0	1	101	17	3	1

[a] Mix, mixture of WT and MT viruses; Other, other mutation apart from Q151M; Indet, indeterminate (neither WT nor MT).

Comparison of OLA with consensus sequencing gave a high overall concordance of 96.3%, with 96.7% for the Q151M mutation and 95.9% for the M184V mutation. Concordance was defined as the same result by both OLA and sequencing (Tables 2 and 3).

In 6.6% of the samples, OLA detected variants that were not detected by sequencing. OLA genotyped seven of eight samples as a mixture of WT and MT viruses that were identified as MT (five of seven) and WT (two of seven) by sequencing and one of eight as a WT virus that sequencing identified as an alternate mutation, M184I (Table 6 and 7).

In some cases, alternate mutations were observed at the target codon. These alternate mutations were identified by sequencing, which reports the major population, but not by OLA, whose probes were not designed to detect these mutations. However, OLA was able to report the presence of the minor WT and MT populations that were present in addition to the major population with an alternate mutation (Tables 6 and 7).

Ligation assays are sensitive to sequence variation, such that the presence of other mutations, within two bases of the ligation site, results in failure of the assay (i.e., an indeterminate

TABLE 5. Sequencing and OLA results for the M184V mutation

Country	No. of patients	No. of samples	No. of samples with indicated genotype[a]							
			Sequencing				OLA			
			WT	MT	Mix	Other	WT	MT	Mix	Indet
The Gambia	13	42	20	19	2	1	21	18	3	0
Guinea Bissau	23	24	24	0	0	0	23	0	0	1
The Netherlands	16	26	11	15	0	0	11	15	0	0
Sweden	12	30	17	13	0	0	15	13	1	1
Total	64	122	72	47	2	1	70	46	4	2

[a] Mix, mixture of WT and MT viruses; Other, other mutation apart from M184V; Indet, indeterminate (neither WT nor MT).

TABLE 6. Discordant results between OLA and consensus sequencing for the Q151M mutation

Sample	Result by[a]:		Interpretation
	Sequencing	OLA	
B25 (p18 Apr-00)	ATG (M)	ATG and CAR (Q and M [mix])	OLA detects WT minor population
B26 (p18 Jun-00)	CTG (L)	ATG and CAR (Q and M [mix])	OLA detects WT and MT minor populations; sequencing detects alternate MT (Q151L)
B28 (p18-01)	CAG (Q)	ATG and CAR (Q and M [mix])	OLA detects MT minor population
S26 (02-7282)	MWR (K, I, M, Q, and/or L)	— (indeterminate)	OLA fails to detect both WT and MT

[a] MWR are degenerate bases: M, A and C; W, A and T; R, A and G. MWR translates to a mixture of any of the following codons encoding for AAA and AAG (K), ATA (I), ATG (M), CAA and CAG (Q) and CTA and CTG (L).

result) (4). Of the 244 codons analyzed, we observed a total of three indeterminate results, one of three for Q151M and two of three for M184V. For the Q151M mutation, alignment of the samples shows the presence of other bases at the ligation site for sample S26 (02-7282), which had the sequence of degenerate bases MWR (M, A + C; W, A + T; R, A + G) at codon 151 (Fig. 3). MWR translates to a mixture of any of the following codons coding for lysine (AAA and AAG), isoleucine (ATA), methionine (ATG), glutamine (CAA and CAG), and leucine (CTA and CTG). However, the indeterminate result of OLA for sample S26 (02-7282) suggests that the base combinations resulting in lysine, isoleucine, and/or leucine are most likely present (Tables 6 and 7). For the M184V mutation, analysis of the sequence alignment for samples B30 (Pat24) and Caio 29 did not show additional changes around the ligation site that would account for the indeterminate results observed (Fig. 3).

DISCUSSION

The HIV-2 epidemic is restricted mainly to West Africa (29), where antiretroviral drugs have not been widely available. However, this situation is changing rapidly with the recent initiation of the Global Fund, which provides funding for the treatment of HIV-infected individuals. Therefore, in the next few years it is likely that HIV-2 drug-resistant strains will emerge in developing countries, as has been observed for HIV-1. Hence, the development of economical resistance assays is crucial for the effective management of HIV-2 patients on antiretroviral therapy, especially in resource-poor settings.

We have developed, optimized, and evaluated an OLA to detect the mutations Q151M and M184V, which are associated with phenotypic drug resistance in HIV-2 (1, 24). This assay is rapid, economical, highly sensitive, and specific for the detection of resistance mutations in HIV-2.

In this study, a total of 244 codons were evaluated in samples from 64 patients from different geographical regions to ensure that a representative panel of the HIV-2 epidemic was used. Though our probes were designed to work for all HIV-2 subtypes, most of the samples available for the evaluation of this assay were HIV-2 subtype A, with only one subtype B sample.

OLA has been reported to be a highly sensitive assay that allows the detection of variants representing as little as 5% of the total viral population within a sample (4, 13). This high sensitivity reduces the potential for false-negative detection of resistance, when mutant viruses are present at low levels (<20%), such as when resistant viruses are first emerging or when selective pressure by a drug has been removed and mutants are supplanted by fitter WT viruses (4). The overall sensitivity of detection of the assay was 98.8% for the two mutations tested, with 99.2% for the Q151M mutation and 98.4% for the M184V mutation. The documented higher sensitivity of OLA for detecting minor variants was observed in this study (4, 13, 15, 34). OLA detected mutations, which were not detected by sequencing, in 6.6% of the samples (Tables 6 and 7). The presence of the mutant variants as a mixture of MT and WT signal indicates that they represent only a proportion of the quasispecies, i.e., that they are minor variants. It is unlikely that they represent false-positive OLA results, because subsequent samples from these patients showed the presence of the mutant sequences by both OLA and sequencing (Tables 6 and 7). Thus, OLA appears to have a lower limit of detection of minor variants.

TABLE 7. Discordant results between OLA and consensus sequencing for the M184V mutation

Sample	Result by:		Interpretation
	Sequencing	OLA	
B1 (Pat1)	ATG (M)	ATG and GTG (M and V [mix])	OLA detects MT minor population
DTS 2-9	GTG (V)	ATG and GTG (M and V [mix])	OLA detects WT minor population
TTT 10.2	ATA (I)	ATG and GTG (M and V [mix])	OLA detects WT and MT minor populations; sequencing detects alternate MT (M184I)
DTS 5-22A	ATA (I)	ATG and GTG (M and V [mix])	OLA detects WT and MT minor populations; sequencing detects alternate MT (M184I)
DTS 2-10	ATA (I)	ATG (M [WT])	OLA detects WT minor population, but sequencing detects alternate MT (M184I)
B30 (Pat24)	ATG (M)	— (indeterminate)	OLA fails to detect both WT and MT
Caio 29	ATG (M)	— (indeterminate)	OLA fails to detect both WT and MT

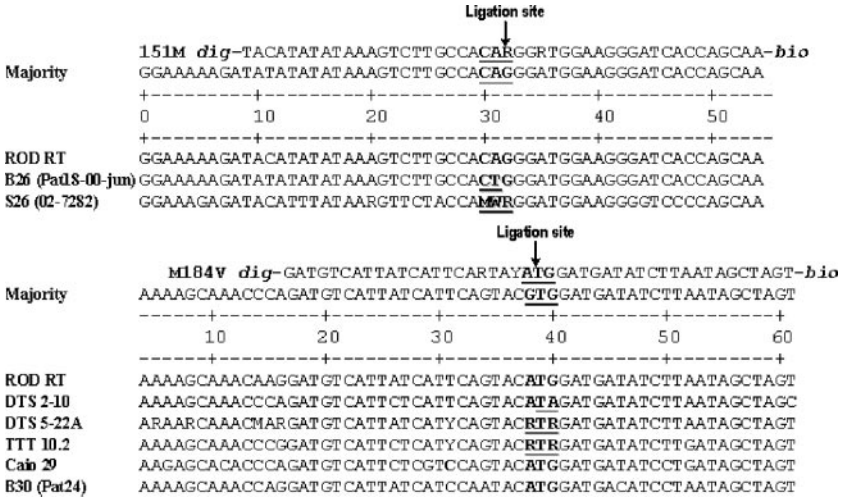


FIG. 3. Alignment of samples with alternate mutations and indeterminate results with HIV-2 ROD. Underlined codons mark positions 151 (upper alignment) and 184 (lower alignment). The degenerate codons MWR and RTR correspond to the following codon and amino acid combinations. MWR codon: AAA and AAG (K), AUA (I), AUG (M), CAA and CAG (Q), and CUA and CUG (L). RTR codon: ATA (I), AUG (M), and GTA and GTG (V).

Another attribute of OLA is its high specificity, which depends on the requirements of the DNA ligase and not the DNA annealing conditions (15, 20). The specificity of ligation between the MT/WT probe to the common probe depends on three factors: specificity of hybridization of the probes to their complementary sequences on the template, hybridization of the probes in the 5′-to-3′ orientation, and perfect base pairing at the target junctions (ligation site) of the probes. These conditions, when fulfilled, allow nonstringent annealing conditions without compromising specificity (32). However, this highly specific nature of OLA is also the cause of assay failure. The presence of mutations located within two bases of either side of the ligation site may result in failure of the DNA ligase to join the adjacent WT or MT probe to the common probe, resulting in an indeterminate result (4). Overall, three indeterminate results (1.2%) were observed in this study. In one of three samples, lack of a result was due to the present of alternate mutations at the ligation site, but for the other two, there were no changes around the target site that could explain the indeterminate results observed.

Another cause of indeterminate results is the presence of alternate mutations for which the probes were not designed. When alternate mutations are encountered, their relevance should be balanced with the cost of additional new probes. In HIV-1, Q151L is a rarely observed transitional mutation that appears to precede the emergence of Q151M (http://hivdb6.stanford.edu/). Q151L is a potential intermediate of Q151M. Also, the much lower replicative fitness of viruses bearing the Q151L variation than those with Q151M further supports the role of Q151L as a transient intermediate of the Q151M mutation (16). The M184I mutation is associated with 1,000-fold phenotypic resistance to 3TC, similar to the M184V mutation (6, 12); additionally, it was reported that M184I appears earlier (12) and is then outgrown by the 184V mutant (3, 22), which has superior RT polymerase function (4, 7, 9). It seems that both the Q151L and M184I mutations are transitional mutations with a much lower fitness. Their presence indicates the near emergence of the Q151M and the M184V mutations, respectively. Though important, these mutations are only transient and therefore do not warrant the development of additional probes to detect them.

The OLA, in addition to being simple, highly specific, and sensitive, has high throughput, allowing at least 42 samples as well as three different controls to be genotyped on a single 96-well ELISA plate. It is also highly adaptable, such that additional probes can be easily incorporated as new mutations are discovered. Though data on HIV-2 drug resistance is very limited, some genotypic HIV-2 mutations have been identified (15, 24). However, phenotypic resistance assays are necessary to determine their clinical relevance. As more phenotypic resistant HIV-2 mutations are identified, additional probes can be incorporated into the assay to detect them.

In conclusion, we have successfully developed an OLA for the detection of the mutations Q151M and M184V, which are associated with resistance to NRTIs. The economical nature of this assay and its high sensitivity, ease of use, and high concordance with sequencing make it a practical alternative to consensus sequencing that is sustainable for use in resource-poor settings.

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