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Short communication

Optimization of the oligonucleotide ligation assay for the detection of nevirapine resistance mutations in Zimbabwean Human Immunodeficiency Virus type-1 subtype C

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An oligonucleotide ligation assay (OLA) designed to detect Human Immunodeficiency Virus type-1 (HIV)-drug-resistance to the nevirapine (NVP) selected mutations K103N, Y181C, V106M and G190A was used to evaluate 200 archived dried blood spots (DBS) from infected infants participating in the Zimbabwean Early Infant Diagnosis (EID) Program. Consensus sequencing of specimens with indeterminate OLA results was performed to identify genetic sequence polymorphisms that appeared to compromise performance of the OLA. When consistent patterns of polymorphisms were observed the probes were redesigned, and DBS specimens with indeterminate OLA results were retested with the new Zimbabwe-specific (ZW) probes. OLA results obtained in Zimbabwe were compared to repeat testing in a US reference laboratory. 188/200 (94%) DBS yielded polymerase chain reaction (PCR) amplification of HIV *pol*. ZW probes reduced indeterminate OLA results from 5.2% to 2.8% of codons evaluated ($p = 0.02$), with 98.2% concordance between results obtained in the Zimbabwean and US laboratories. Optimization of OLA probes to accommodate polymorphisms in regional HIV variants improved OLA performance, and comparison to the USA results showed successful implementation of the OLA in Zimbabwe for detection of NVP resistance mutations in DBS specimens.

In Zimbabwe, NVP has been utilized widely as prophylaxis for prevention of mother-to-child transmission of HIV (PMTCT) and/or as part of first-line antiretroviral regimens (ART) (Kuonza et al., 2010; Zimbabwe, United Nations 2008–2009; Zimbabwe, PMTCT Report, 2010). Despite numerous scientific reports of selection of nevirapine (NVP)-resistance mutations (Eshleman et al., 2005a,b; Halvas et al., 2010; Johnson et al., 2005; Martinson et al., 2007; Micek et al., 2010) data from Zimbabwean infants are sparse. These data are unavailable due primarily to the unaffordable cost of consensus sequencing, the sequencing method used most commonly to assess HIV drug resistance (HIV-DR) (Beck et al., 2002; Bennett et al., 2008).

An economical oligonucleotide ligation assay (OLA) was developed to detect mutations generally associated with HIV-DR to NVP (K103N, Y181C, V106M and G190A) and other antiretrovirals (ARVs) (Ellis et al., 2004; Wallis et al., 2005; Micek et al., 2010). Ligation confers 100% specificity to the assay, but inter- and intra-subtype genetic polymorphisms with mismatches within two bases of the ligation site of the oligonucleotide probes result in indeterminate reactions (Beck et al., 2008; Wallis et al., 2005). Therefore, to detect regional HIV polymorphisms within new geographical locations, the oligonucleotide probes should be redesigned to

Abbreviations: ARV, antiretroviral; ART, antiretroviral treatment; EID, Early Infant Diagnosis of HIV; HIV, Human Immunodeficiency Virus type-1; HIVDR, HIV drug resistance; NA, not applicable; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitors; PCR, polymerase chain reaction; pMTCT, prevention of mother-to-child transmission; PI, protease inhibitors; ZW, Zimbabwe.

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Table 1
OLA detection of NVP-resistant mutants in infants' DBS using Zimbabwe-specific probes (ZW) decreased indeterminate tests compared to "generic" HIV-1 subtype C probes.

Codon	Number (%) of specimens that OLA detected mutant, wild type, mixed or indeterminate genotype at each codon								Total number of codons tested
	100% mutant		Mixture of mutant and wild-type		100% wild type		Indeterminate OLA		
	Generic[a]	ZW[b]	Generic	ZW	Generic	ZW	Generic	ZW	
K103N	4 (2.1%)	7 (3.7%)	14 (7.5%)	17 (9.0%)	164 (87.2%)	162 (86%)	6 (3.2%)	2 (1.1%)	188
Y181C	1 (0.5%)	1 (0.5%)	21 (11.1%)	25 (13.3%)	144 (76.6%)	154 (82%)	22 (11.7%)	8 (4.2%)	188
V106M	0	NA[c]	8 (4.2%)	NA	176 (93.6%)	NA	4 (2.1%)	NA	188
G190A	0	NA	2 (1.1%)	NA	179 (95.2%)	NA	7 (3.7%)	NA	188
Totals, across all 4 codons	5 (0.7%)	8 (1.1%)	45 (6.0%)	52 (6.9%)	663 (88. 2%)	671 (89.2%)	39* (5.2%)	21* (2.8%)	752

[a] Generic OLA probes designed using HIV-1 subtype C sequences from Los Alamos database as of 2005.
[b] ZW – Zimbabwe specific OLA probes modified from generic probes based on polymorphisms prevalent in Zimbabwean specimens initially testing indeterminate. Optimized probes were tested on a subset of specimens that included all specimens with indeterminate OLA results at codons 103 and 181.
[c] NA – not applicable, as oligonucleotides probes for V106M and G190A were not redesigned; DBS specimens were only tested with generic OLA probes.
* Fisher's exact, two-tailed p value = 0.02.

accommodate HIV polymorphisms common in the target population (Edelstein et al., 1998; Beck et al., 2002, 2008; Jallow et al., 2007).

The objective of this study was to transfer the OLA to a Zimbabwean laboratory, optimize OLA reagents to detect the four NVP-resistance mutations (K103N, Y181C, V106M and G190A) in Zimbabwean infants, and validate the performance of the Zimbabwe-specific OLA reagents in the Zimbabwean laboratory for potential use in the detection of NVP resistant mutations in infants infected through MTCT.

The OLA for detection of NVP-associated HIV mutations was set-up in the Zimbabwean laboratory by its developers using supplies validated in Seattle, and Zimbabwean personnel were provided hands-on instructions. Following approval of the Zimbabwean Ethics Panel and Seattle Institutional Review Board, infant dried blood spots (DBS) were evaluated. All 200 DBS were from infants with documented perinatal treatment of the mother/infant pair with single-dose-NVP prophylaxis. The median interval between birth and DBS collection was 10 weeks (range 1–71 weeks, IQR: 6 and 16.5 weeks respectively).

DNA was extracted from the DBS using Chelex-100 (Beck et al., 2008) and HIV *pol* was amplified by nested polymerase chain reaction (PCR) and tested with OLA probes for NVP-associated mutations K103N, Y181C, V106M and G190A (Micek et al., 2010) designed using HIV subtype C sequences published in the Los Alamos National Laboratory (LANL) HIV database (http://www.hiv.lanl.gov/content/index) and are referred to in this paper as "generic probes" (Micek et al., 2010). The OLA conditions are described in the OLA Manual version 1.4, July 2007 (Beck et al., 2008). Mixtures of plasmids with mutant or wild-type sequence at the position of interest, combined to mutant concentrations of 0, 2, 5 or 100%, were assayed in each OLA plate. OLA reactions with optical densities (OD) $\geq$2% mutant control were considered mutant positive (Beck et al., 2002).

In total, 200 DBSs were processed for nested PCR. Amplicon was visualized in an agarose gel from 188 (94%) and 12 (6%) failed amplification. OLA using the generic subtype-C probes yielded valid results for 713 codons (94.8%) and 39 (5.2%) codons had indeterminate results, i.e., negative for both the mutant and wild type OLA reactions. Codon 181 had the highest proportion of indeterminate results (22/188; 11.7%), followed by codon 190 (3.7%), 103 (3.2%) and 106 (2.1%) (Table 1).

Thirty-eight HIV amplicons were sequenced (GenBank accession number KJ158204–KJ158241) in Seattle to identify genetic polymorphisms that were likely to interfere with the ligation of the oligonucleotide probes. When multiple Zimbabwean infants' sequences showed patterns of mismatches to the generic probes, new probes were synthesized to improve annealing to Zimbabwean HIV amplicons with polymorphisms adjacent to or within codons 103 and 181 (Table 2). Specimens evaluated with the new reagents included the 28 that tested indeterminate at codons 103 and 181 plus 26 that had yielded valid OLA results using the generic probes. The Zimbabwe-specific probes reduced the overall indeterminate rate from 5.2% to 2.8% (p = 0.02), and detected additional mutants at codons 103 (n = 6) and 181 (n = 4) (Table 1).

Using the valid results from the generic probes plus the results obtained using the Zimbabwe-specific OLA probes, the number of infants with NVP resistance mutations detected increased from 41/188 (21.8%) to 48/188 (25.5%), with a single mutation in 39/188 (20.7%) and $\geq$2 mutations in 9/188 (4.8%). Resistance mutations were detected at similar rates from 0 through 24 weeks of age; but too few infants were tested at older ages to assess whether mutants decayed (Fig. 1).

An inter-laboratory comparison of OLA results between Zimbabwe and Seattle laboratories was performed using the first 40 DBS evaluated in Zimbabwe. Concordant OLA results between the two laboratories were as follow: K103N (100%), V106M (39/40; 97.5%); Y181C (38/40; 95%), and G190A (100%). The discrepant result at codon 106 was indeterminate in Zimbabwe and a mixture of mutant and wild type in Seattle. The two specimens discrepant at codon 181 showed mixtures in Zimbabwe and indeterminate results in Seattle.

The OLA was effectively established, validated and optimized for application to clinical specimens in Zimbabwe. The high concordance of valid OLA results obtained in the Zimbabwe laboratory

Table 2
Modifications in generic HIV subtype C oligonucleotide ligation assay probes for Zimbabwean (ZW) sequences.

Generic 103 mut	fluo-ACATCCCGCAGGGTTAAAAAAGAAC
ZW specific 103 ***mut***	fluo-ACA***C***CC***A***GCAGGGTTAAAAAAGAA***Y***
Generic 103 wt	Dig-ACATCCCGCAGGGTTAAAAAAGAAR
ZW specific 103 ***wt***	Dig-ACA***C***CC***A***GCAGGGTTAAAAAAGAAR
Generic 103 common	p-AAATCAGTRACAGTACTRGATGTGGG-bio
ZW specific 103 ***com***	Unmodified
Generic 181 ***mut***	fluo-CAMAAAA***T***CCAGAAATAGTCAT***C***TG
ZW specific 181 ***mut***	fluo-CA***C***AAAA***Y***CCAGA***M***ATAGTCAT***Y***TG
Generic 181 C wt	dig-CACAAAA***T***CCAGA***A***ATAGTCAT***C***TA
ZW specific 181 ***wt***	dig-CACAAAA***Y***CCAGA***M***ATAGTCAT***Y***TA
Generic 181 common	p-***T***CAATA***C***ATGGATGA***T***TTGTATGTA-bio
ZW specific 181 ***com***	p-***Y***CAATA***T***ATGGATGACTTGTATGTA-bio

Bold italics denote modified bases to improve annealing and ligation to Zimbabwean HIV.