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A Radiolabeled Oligonucleotide Ligation Assay Demonstrates the High Frequency of Nevirapine Resistance Mutations in HIV Type 1 Quasispecies of NVP-Treated and Untreated Mother–Infant Pairs from Uganda

RYAN M. TROYER,[1,*] MATTHEW S. LALONDE,[1,*] ERIKA FRAUNDORF,[1] KOREY R. DEMERS,[1,2] FRED KYEYUNE,[1,2] PETER MUGYENYI,[2] ASLAM SYED,[1] CHRISTOPHER C. WHALEN,[4] FRANCIS BAJUNIRWE,[2,3] and ERIC J. ARTS[1]

ABSTRACT

This study explores the levels of NVP and AZT resistance mutations in untreated, NVP- or AZT-treated mother–infant pairs in Uganda. PCR-amplified reverse transcriptase (RT) gene fragments derived from PBMC samples of 85 mothers (10 AZT treated, 35 NVP treated, and 40 untreated) and their 52 infected infants (5 AZT, 9 NVP, and 38 untreated) were classified as subtype A (59%), D (29%), C (3%), and recombinant forms (9%) by population sequencing. Only 16% of the NVP-treated infected mothers and infants harbored either the K103N or the Y181C at 6 weeks postdelivery. The majority of these samples ($n = 107$) were then analyzed using a radiolabeled oligonucleotide ligation assay (OLA) specific for K70R, K103N, and Y181C, using nonstandard bases to accommodate sequence heterogeneity. By OLA, 43% of the NVP-treated group had K103N and/or Y181C mutations in their HIV-1 population, using >0.6% cutoff based on a comparative clonal analysis of clinical isolates. Surprisingly, an equal fraction of the untreated and NVP-treated mother–infant group had the K103N mutation in their HIV-1 population in the range of 0.6–5%. These findings suggest a relatively high frequency of K103N mutation in the drug-naive, subtype A and D infected Ugandan population as compared to the very low frequency of the Y181C and K70R mutation (<0.6%). The prevalence of the K103N mutations may be related to its low fitness cost and high genetic stability. The persistence of these mutations may reduce the effectiveness of subsequent NVP use in treatment or prevention of perinatal transmission.

INTRODUCTION

Mother-to-child transmission (MTCT) of HIV in resource-limited settings has been greatly reduced by the implementation of short course antiretroviral therapy. More than 10 years ago the AIDS Clinical Trials Group Protocol 076 demonstrated that zidovudine (AZT) given to the mother pre- and intrapartum reduced MTCT by 70%.[1] In 1999 the HIV Network for Prevention Trials 012, performed in Uganda, found that a single dose of the nonnucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (NVP) resulted in a further 50% reduction in MTCT when compared to a short course of zidovudine (ZDV).[2,3] In this protocol, NVP was self-administered by the mother at the onset of labor followed by a single dose administered to the infant within 72 h of birth. Single-dose nevirapine has subsequently been demonstrated to be effective in a Ugandan community-based observational study conducted apart from the clinical trials setting.[4] This treatment regimen has wide applicability in resource-limited rural settings because it is inexpensive, effective, simple to administer, and does not

[1]Division of Infectious Diseases, Department of Medicine, Case Western Reserve University, Cleveland, Ohio 44106.
[2]Joint Clinical Research Centre, Kampala, Uganda.
[3]Department of Community Health, Mbarara University of Science and Technology, Mbarara, Uganda.
[4]Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, Ohio 44106.
*These authors contributed equally to this work.

require a complex health care infrastructure. However, NVP resistance mutations, typically identified in patients failing NVP-containing treatment regimens,[5] also emerged as a result of a single NVP dose.[6] Nevirapine resistance mutations fade over time in the absence of selection,[7,8] but could remain in that patient's virus population (or quasispecies) at a level elevated above that present in the HIV-1 quasispecies of a drug-naive patient.[9] Low level NVP resistance mutations will hamper future treatments regimens with NVP due in large part to the rapid reemergence of NVP-resistant virus.[10] The recent finding that single-dose NVP can compromise the efficacy of future NVP-containing regimens[10] has highlighted concerns about the use of single-dose NVP therapy in resource-limited settings where nevirapine is one of few drugs available for long-term therapy.[10–12]

Nevirapine resistance results from selection of one or several mutations in the HIV reverse transcriptase.[13] The two most prevalent NVP resistance mutations among all HIV-1 subtypes investigated thus far are K103N and Y181C (subtypes A, C, D)[14] (subtype B).[5] Rapid selection of NVP resistance is a consequence of the high mutation rate of the HIV reverse transcriptase combined with the long persistence (>2 weeks post-treatment in the plasma of most women) of NVP in the body[15,16] and a low genetic barrier to NVP resistance. It is likely that these mutations preexist at very low levels in the HIV quasispecies of untreated individuals,[17] but this has not been directly demonstrated. A number of studies have detected resistance in NVP-treated individuals by nucleotide sequencing and complex chromatogram analysis.[6,10,18] Studies of MTCT prevention with NVP in Uganda have found that up to 25% of mothers may develop detectable NVP resistance when evaluated at 6–8 weeks postpartum by nucleotide sequencing.[19] In addition, infants have been found to harbor NVP resistance mutations. These mutations may arise by transmission from the NVP-treated mother or selection by a single dose of NVP in the infant.[20]

Nucleotide sequencing is a rather poor method for the detection of low level polymorphisms in a DNA sequence and typically is limited to >20% mutant to wild-type nucleotide ratio in the HIV population. Genotypic assays of this kind involve computational analysis of fluorescence intensity from DNA sequencer output,[21] although cloning prior to this step allows a higher degree of sensitivity. The clinical significance of low level drug resistance coupled with the low sensitivity of modern genotyping assays has represented a deficiency in our understanding of virus adaptation and possibly a handicap in effective infection management. Thus, a number of recent investigations have employed other techniques directed to measure the low level genetic polymorphisms at specific sites. Two preferred techniques include an oligonucleotide ligation assay (OLA)[22,23] and allelic-specific polymerase chain reaction (AS-PCR).[24,25] Recent studies have now readily detected low levels of NVP-resistant HIV-1 clones in the quasispecies of NVP-treated, HIV-infected mothers of MTCT cohorts.[9,26,27] NVP resistance mutations have also been found in 2.1% of untreated mothers in one study.[28]

In this study, mothers and infants who were untreated or treated with either short course AZT during late gestation, or NVP prior to delivery, were recruited from postnatal clinics in Kampala, Uganda. The HIV-1 DNA was extracted and amplified from these mother–infant pairs and then sequenced to determine HIV-1 subtype and to detect the dominant drug-resistant mutation in the HIV-1 quasispecies (i.e., HIV-1 population infecting each patient). We also employed a modified radiolabeled OLA for the low frequency detection of the nevirapine mutations Y181C and K103N or the AZT-resistant mutation K70R in the patient's HIV-1 quasispecies. The sensitivity of the OLA was tested using various template controls containing specific mutations in the HIV-1 subtype A and D backgrounds. In addition, we performed HIV-1 clonal analyses from particular patient samples to verify an OLA sensitivity of 0.6% mutation frequency. By using this highly sensitive and well-defined OLA, it is quite clear that a majority of the NVP-treated mother–infant pairs harbored NVP-resistant mutations at a low frequency. However, we demonstrate here that the K103N is already present at significant levels in the HIV-1 quaispecies of drug-naive women in Uganda suggesting that the measures of NVP resistance selection can often be overestimated in NVP-treated cohorts if not appropriately controlled.

MATERIALS AND METHODS

Perinatal transmission cohort

HIV-infected Ugandan mothers and their infants were recruited to this community-based observational study as previously described in detail.[4] Briefly, 109 women who had received antiretroviral therapy to prevent perinatal HIV transmission as part of a Ugandan Ministry of Health UNICEF/Elizabeth Glazer Pediatric AIDS Foundation funded pilot program were enrolled between June and October 2000. Each woman received therapy at one of three hospitals located in Kampala, Uganda. Treatment consisted of either a single dose of 200 mg nevirapine at the onset of labor ($n = 61$) or a short course of zidovudine (AZT) with 300 mg twice daily beginning at 36 weeks of gestation continuing until delivery and 1 week postpartum ($n = 48$). Infants of mothers participating in these trials were given either a single dose of nevirapine syrup (2 mg/kg) within the first 72 h after birth or 1–2 weeks of oral AZT twice daily (5 mg/kg). Infants were tested for HIV using the Roche AMPLICOR MONITOR assay (Roche Diagnostics, Indianapolis, IN). HIV infection was detected at 6 weeks in nine infants of the NVP arm and in five infants of the AZT arm. An antiretroviral (ARV) therapy-naive group of mothers ($n = 90$) was recruited for comparison from three Kampala postnatal clinics where ARV therapy was not yet available. Blood was drawn from mothers and their infants at a mean of 38.5 weeks postpartum. HIV infection was detected in 37 infants in the untreated group. Details of the samples utilized for this study are described in the Results section. Informed consent was obtained from all mothers prior to study enrollment. The study was reviewed and approved by the Institutional Review Boards at Case Western Reserve University, Cleveland, OH, and by the AIDS Research Sub-Committee in Uganda. Additional details of this cohort are provided.[4]

Cell isolation and DNA purification

Blood (2 ml from infants and 5 ml from mothers) was collected in EDTA vacutainer tubes from the study participants at each clinic site and plasma was separated from cells by cen-

trifugation at 1500 rpm for 10 min and stored at −70°C. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque centrifugation. Proviral DNA was extracted from lysed PBMC using the Qiagen DNA extraction kit. DNA was resuspended in 50 ml of water and sequenced in Uganda (see below) or transferred to Cleveland for OLA (see below) and to measure viral DNA load on a subset of samples. HIV-1 viral RNA load was measured on a subset of plasma samples using the ultrasensitive RNA load assay from Roche Amplicor 1.5. Results pertaining to the viral RNA and DNA loads were previously published.[29] The remaining RNA was employed for bulk OLA and previously published.[29]

Nucleotide sequencing and sequence analyses

For sequencing and further analysis, we were able to amplify the RT coding region from 137 of the mother or infant PBMC isolates. Characteristics of these individuals have been previously described.[4] HIV-1 proviral DNA was extracted from the PBMCs of the infected patient using the QIAamp DNA blood kit (Qiagen). A region of HIV-1 *pol* corresponding to RT amino acids 58–311 (nucleotide 2721–3482) was amplified by nested PCR as previously described.[30] Briefly, an external PCR reaction was performed using sense primer RTS1 (5′-TAAACAATGGCCATTGACAGAAGA-3′) and antisense primer RTA9 (5′-TAAATTTAGGAGTCTTTCCCCATA-3′) followed by an internal PCR using primers RTS2 (5′-TCAAAAATTGGGCCTGAAAATCCAT-3′) and RTA8 (5′-GCTATTAAGTCTTTTGATGGGTCAT-3′). Bidirectional population nucleotide sequencing of RT was performed at the Joint Clinical Research Center using a Beckman-Coulter DNA sequencer. All sequences were manually edited and aligned using BioEdit (Tom Hall, North Carolina State University). Nucleotide sequences reported in this study have been submitted to GenBank under the accession numbers XXX–XXX (submitted but accession numbers not yet available).

A phylogenetic tree of the HIV *pol* sequences from each patient was constructed with PAUP* software.[31] The significance of the branching order was estimated by bootstrap resampling of 1000 replicates. All sequences were analyzed for evidence of intersubtype recombination using the RIP 2.0 program (available through the Los Alamos HIV Sequence Database, www.hiv.lanl.gov). All sequences were compared to subtype consensus sequences using a window size of 300 nucleotides. Sequences that yielded evidence of intersubtype recombination were subjected to further phylogenetic analyses for confirmation.

Radiolabeled OLA

Our modified radiolabeled oligonucleotide ligation assay was adapted from the method of Beck and Frenkel[32] and has been described in detail (M.S. Lalonde and E.J. Arts, unpublished observations) (see Fig. 2). Briefly, PCR products were generated (see above) and purified using the Qiagen PCR Purification Kit. Purified PCR products were resolved on agarose and the DNA concentration was estimated by comparison to the Bio-Rad Low Mass DNA Ladder using Kodak 1D gel analysis software. Upstream oligonucleotide primers were radiolabeled by incubating 100 pmol primer at 37°C for 10 min in a 100 μl reaction containing 40 units Invitrogen T4 polynucleotide kinase (PNK), 100 pmol of 3000 Ci/mmol [γ-^{32} P]ATP (Perkin-Elmer), and 20 μl 5× T4 PNK forward buffer (Invitrogen). Reactions were then incubated at 65°C for 10 min, placed on ice, and extracted with phenol/chloroform/isoamyl alcohol (25:24:1, Roche) and eluted from G-25 Sephadex columns (Amersham). The ligation reactions were carried out using both 5 ng and 25 ng template PCR product for each sample. The ligation reaction mixture also contained 1.5 pmol ^{32}P-radiolabeled oligonucleotide, 2.5 units Ampligase DNA ligase (Epicentre), 20 mM Tris–HCl (pH 8.3), 0.07% Triton X-100, 0.8 mM DTT, 20 mM KCl, 8.3 mM $MgCl_2$, and 0.83 mM NAD in 12 μl total volume. Reactions were subjected to 30 cycles of 93°C for 30 s and 37°C for 4 min, then stopped by the addition of 10 μl buffer containing 0.1 M EDTA, 0.1% Triton X-100, 25% formamide, 0.025% bromophenol blue and 0.025% xylene cyanol. Ligation products were then separated from unligated oligos by electrophoresis on a denaturing 10% polyacrylamide gel. Gels were dried under vacuum at 80°C and visualized by autoradiography and phosphorimaging.

OLAs were performed on 5 and 25 ng of patient PCR products with oligonucleotide sets specific for wild-type or mutant sequences at position 70, 103, and 181. The amount of ligated product was quantified by phosphorimaging analyses and then related back to ligation products from HIV-1 clonal DNA of varying quantities (0.25–50 ng) and known to contain specific mutations or wild-type sequence. To establish these quantitation controls, PCR products from patient samples MTA308 and MTA333 (AZT-resistant, site 70), MTN207 and MTN207 (for K103N, see Table 1), and MTN121 and INN131 (for Y181C, see Table 1) were PCR amplified, cloned into pCR2.1-TOPO vectors, and then sequenced. K70R-containing subtype A and D clones were obtained from samples of a previous study on drug resistance in Uganda.[30]

Clonal OLA analysis

PCR products were generated as above for nucleotide sequencing using the TOPO cloning technique.[33] Individual colonies (100–500 X-gal blue colonies for each patient) were then grown in 3 ml liquid LB medium with 100 μg/ml ampicillin at 37°C for 16 h. Pools were generated by combining 150 μl of 20 different cultures, each representing a single clone from the HIV proviral quasispecies (see Fig. 5). Pooled plasmid DNA was isolated using a Qiagen plasmid purification kit. PCR amplification of *pol*-RT was then performed as described above for nucleotide sequencing using the pooled plasmid DNA as a template. PCR products were then diluted and OLA was performed as described above. When OLA analysis of any 20-clone pool resulted in a reading of >1% mutant (a positive clone would be expected to yield a result of ~5% mutant), all the individual clones from that pool were then subjected to separate OLA analyses to confirm the presence of individual clones producing a positive OLA result in the pool. All OLA mutant-positive clones were subjected to nucleotide sequencing to confirm the presence of NVP resistance mutations.

Quantitative PCR for PBMC HIV-1 DNA load

Real time PCR primers were generated as described previously.[34] HIV-1 strong stop (SS) primers LTR1: 5′-GCCTCAATAAAGCTTGCCTGA-3′ and LTR2: 5′-CTGAGGGAT-

TABLE 1. IDENTIFICATIONS OF NEVIRAPINE RESISTANCE MUTATIONS IN THE INFECTED MOTHER OR INFANT SAMPLE

Patient	*Subtype in RT*	*NVP resistance by sequencing*[a]	*NVP resistance by OLA*[b]	*Percent in quasispecies by OLA (K103N%, Y181C%)*	*Site 103 mutant codon detected*
MTU001	A	None	K103N	1.0%	AAC
MTU029	A	None	K103N	1.3%	AAT
INU036	A/D	None	K103N	0.6%	AAT
MTU037	A	None	K103N	0.7%	AAC
MTA039	A/D/A	None	K103N	1.3%	AAT
MTU047	A	None	K103N	0.9%	AAT
INU048	A	None	K103N	0.9%	AAT
MTU051	D	None	K103N	1.3%	AAT
MTU053	D	None	K103N	1.1%	AAT
INU058	A	None	K103N	1.8%	AAT
MTU059	A	None	K103N	1.3%	AAT
INU060	A	None	K103N	1.3%	AAT
MTU065	D	None	K103N, Y181C	2.9%, 0.6%	AAT
INU066	D	None	K103N	3.9%	AAT
MTU067	A	None	K103N	3.3%	AAT
MTU069	A	None	K103N	3.7%	AAT
MTA071	D	None	Y181C	0.0%,[c] 0.7%	AAA
MTA077	A/D/A	None	K103N	2.9%	AAT
MTU081	A	None	K103N	3.5%	AAT
INA094	D	None	K103N	0.8%	AAC
INU100	D	None	K103N	0.6%	AAC
MTU103	A	None	K103N	3.2%	AAC
INU104	A	None	K103N	2.9%	AAC
MTN121	D	None	K103N, Y181C	0.9%, 0.6%	AAC
INU129	A	None	K103N	2.1%	AAT
INN131	A	Y181C	K103N, Y181C	8.0%, 61.1%	AAT
INN133	A	Y181C	K103N	3.5%	AAC
MTN148	A	K103N	K103N	98.5%	AAT
INN149	A	Y181C	K103N, Y181C	86.5%, 99.9%	AAT
MTN164	C	None	K103N, Y181C	1.3%, 52.3%	AAT
MTN206	A	None	K103N	1.2%	AAT
MTN207	A	K103N	K103N	44.6%	AAC
MTN208	D	None	K103N, Y181C	1.3%, 63.5%	AAT
MTN209	D	None	K103N, Y181C	2.0%, 5.6%	AAT
MTN210	D	Y181C	K103N, Y181C	2.2%, 92.5%	AAT
MTN213	A	None	K103N	0.6%	AAC
MTN218	D	Y181C	K103N, Y181C	42.2%, 2.7%	AAT
MTN219	D	None	K103N, Y181C	0.7%, 29.8%	AAT
MTN224	A	None	K103N, Y181C	0.6%, 0.9%	AAT
MTN226	A	None	K103N	1.1%	AAT
MTN228	D	None	K103N, Y181C	2.2%, 3.8%	AAT

[a]An NVP mutation was detected by sequencing only if the mutation was >30% above background on the chromatogram.
[b]An NVP mutation was detected by OLA if the mutation was >0.5%.
[c]Sample MTA071 harbored no detectable mutation at site 103.

CTCTAGTTACCAGAGTCA-3′ generated an 80-bp amplicon to which probe LTRprobe 6FAM-AACAGACGGGCACA-CACTACTTGAAGCA-TAMRA bound. Reactions contained 5 μl PBMC DNA extract, 12.5 μl 2× buffer (Applied Biosystems; product #4302237), 3 μM each primer and 2 μM LTR-probe in 25 μl. Reactions are subjected to 50°C for 2 min, then 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data were collected on ABI 7700 Sequence Detector and SDS 1.9 software (Applied Biosystems). All copy numbers from samples were derived from real time PCR of the standard control of the pHIV-1 pbs ($10–10^7$ copies). Based on the variation in viral load estimates (published estimates of ±0.5 log) and in the reduced primer efficiency for non-subtype B samples, we have estimated a range of 10-fold for DNA viral loads. The primers and probes employed in this assay were developed for detection and quantitation of ssDNA from subtype B and the sequence variation in the primers-probe with the subtype A and D samples can be as high as 15%.

RESULTS

Clinical characteristics on analyzed patient samples

All mothers and infants were recruited in postnatal clinics in Kampala, Uganda at a mean of 8.7 weeks of infant age in the NVP- or AZT-treated cohort and a mean of 38.5 weeks of infant age in the untreated cohort.[4] For infants less than the 20 weeks of age, the perinatal transmission rate was 16% for both the NVP- and AZT-treated mother–infant pairs and 48% in the absence of any NVP or AZT treatment[4]. In this study, we initially screened for AZT and NVP resistance mutations by performing sequencing on PCR-amplified DNA from PBMC extractions of 86 HIV-infected mothers (MT) and 51 HIV-infected infants (IN). HIV-infected mothers were from untreated (MTU, $n = 41$), AZT-treated (MTA, $n = 10$), or NVP-treated cohorts (MTN, $n = 35$). Fewer samples from HIV-infected infants were available from these pairs due to the transmission rates and prevention by NVP and AZT intervention [37, 5, and 9 HIV-infected infants from untreated (INU), AZT-(INA), and NVP-treated cohorts (INN), respectively]. Radiolabeled OLA analyses were performed on a subset of 107 samples to estimate the levels NVP and AZT resistance mutations in the infecting HIV-1 quasispecies. Clinical characteristics of the naive and treated mothers–infants have been previously described.[4] Although more patients samples are available in this study than others, there is still insufficient numbers to provide statistically significant differences in NVP resistance mutations among specific groups (e.g., with a specific subtype from a mother versus another subtype from infant) but still enough power to describe differences between treatment arms (e.g., lack of treatment versus NVP treatment to prevent perinatal transmission).

Subtype analyses of the infected mothers and infants

A 762-nucleotide segment of RT (amino acids 58–311) was PCR amplified from PBMC DNA of HIV-infected mother–infant pairs. HIV-1 subtype was determined by aligning these DNA sequences to published HIV-1 sequences and contructing a phylogenetic tree. A neighbor-joining phylogenic tree with all the HIV-1 sequences from the HIV-infected mothers as well as from mother–infant paired sequences (sequential numbers for infected pairs) was trimmed in Fig. 1 to show a representative subset of the HIV-1 RT sequences in this cohort. As expected, the majority of sequences clustered with either subtype A ($n = 81$; 59%) or D ($n = 39$; 29%). A few HIV-1 RT sequences were identified as subtype C ($n = 4$; 3%). Thirteen of the HIV-1 RT sequences appeared to be intersubtype recombinants (9%). These sequences were analyzed using the recombination identification program (RIP). The subtypes involved in the recombined RT sequence and the approximate position of the breakpoints are provided in the supplementary table. As an example, a 300-nt bootscanning window revealed that the MT039 sequence was an A/D/A HIV-1 recombinant with probable breakpoints at approximately nt 2925 and another at nt 3274, whereas the mother–infant pair MTU107 and INU108 harbored an A/D HIV-1 isolate with a breakpoint at approximately nt 3237. None of the intersubtype recombinants had either breakpoints matching known circulating recombinant forms (CRFs) or those that suggest classification of new CRF.

Overall, there were 51 infected infants transmitted from 86 infected mothers in the untreated, AZT- and NVP-treated cohorts but we found no evidence for preferential perinatal transmission of specific subtypes (50, 3, and 24 mothers infected with subtype A, C, and D transmitted to 29, 1, and 15 infected infants, respectively). In addition, AZT or NVP did not appear to preferentially block transmission of specific subtypes.

Drug resistance genotyping of the infected mothers and infants

Each RT sequence was then analyzed for the presence of possible mutations conferring drug resistance. Drug resistance mutations were identified by manual inspection of the chromatograms and by analyzing the sequences with the Stanford Resistance Surveillance v1.0 beta (http://hivdb6.stanford.edu). None of the MT or IN sequences harbored any resistance mutations to nucleoside RT inhibitors including AZT. In a previous study,[35] AZT-resistant mutations did not emerge in pregnant mothers treated with AZT for 10.5 weeks at the end of gestation. The lack of dominant AZT resistance mutations in our MTCT cohort was not surprising since any inability of AZT to prevent perinatal transmission does not appear to be associated with a dominant AZT-resistant mutation in the infected newborn or mother.

In contrast, dominant NVP-resistant mutations were observed in 4 of 35 (11%) NVP-treated mothers and 3 of 9 (33%) infected infant samples while no dominant NVP resistance mutations were detected in untreated and AZT-treated mothers ($n = 51$) or infants ($n = 42$). The NVP resistance mutations were either K103N or Y181C, and not less frequent mutations, e.g. 101P, 106A, 188L, 190A, or 230L mutations (Table 1). These percentages of NVP-treated, HIV-infected mothers and infants carrying NVP resistance mutations are lower than those previously reported by Eshleman *et al.*[8,19] in an Ugandan cohort (25% in mothers and 46% in infants at 6–8 weeks postdelivery). However, it is important to note that the study herein was performed with PBMC DNA as opposed to plasma RNA.[8,19] Recent studies have suggested that detection of NVP resistance mutations by nucleotide sequencing (which generally has a detection limit of $>20\%$ of the HIV-1 population) may underestimate the true percentage of NVP-treated mothers and infants harboring low-level NVP resistance mutations.[9,26,27] However, these studies have not carefully examined the level of resistance mutations in the HIV-1 quasispecies of untreated individuals. To address these issues in both ARV-treated and untreated mother–infant pairs, we modified an OLA[36] to employ radiolabeled primers for the low level detection and quantification of NVP- and AZT-resistant mutations.

Optimization and assessment of the radiolabeled OLA for detection of low-frequency NVP- and AZT-resistant mutations

Figure 2 provides a schematic of the OLA employed to detect low level NVP (K103N or Y181C) or AZT (K70R) mutations. AZT resistance can emerge with the appearance of several mutations (e.g., M41L, D76N, K70R, T215Y, K219E) but we selected for the K70R AZT-resistant mutation because it emerges early during AZT monotherapy.[37] Furthermore, previous reports[38] and our data suggest that unlike NVP-resistant

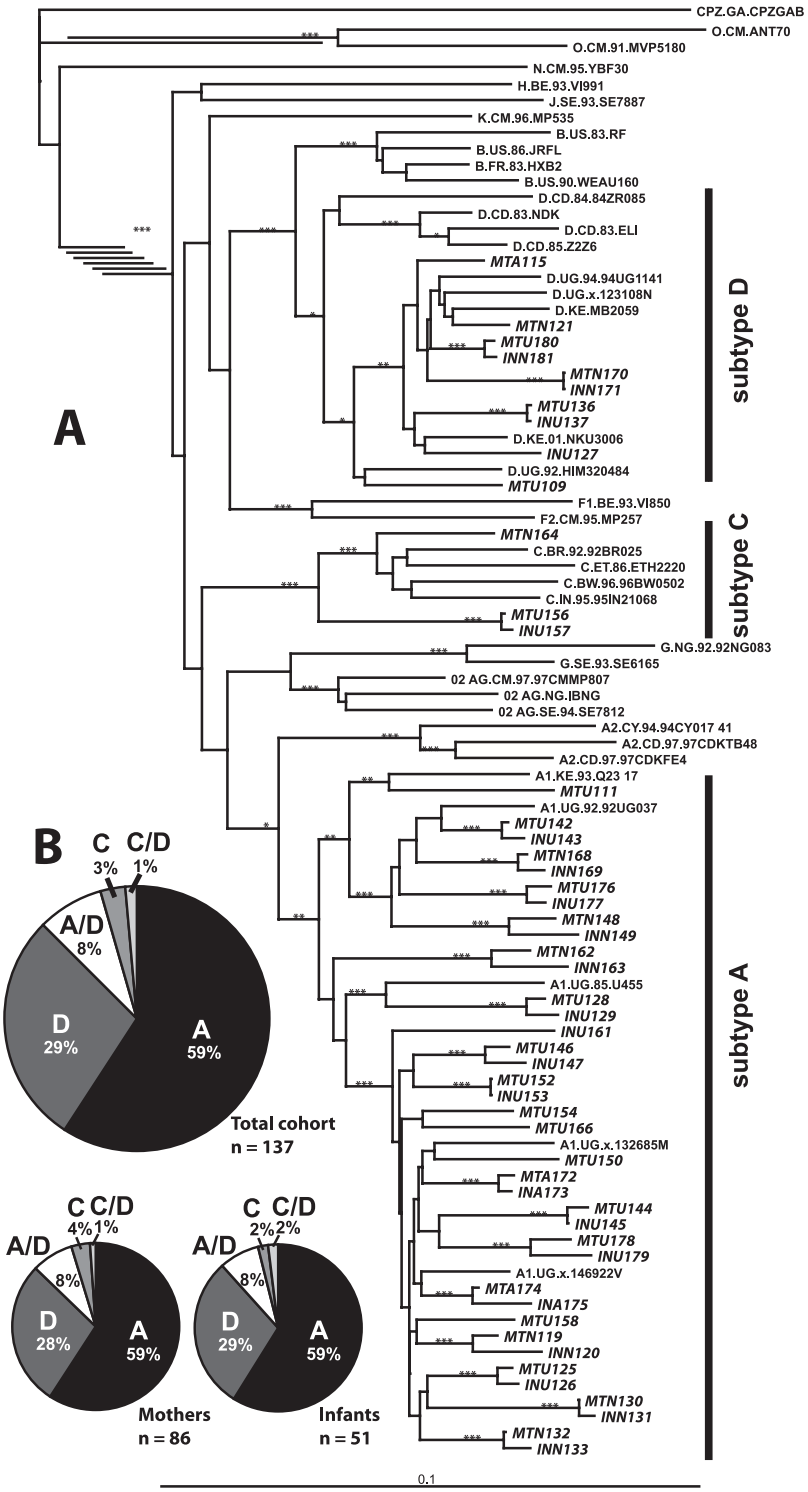


FIG. 1. Phylogenetic tree of HIV-1 RT sequences (**A**) and the proportion of HIV-1 subtypes (**B**) in the HIV-1-infected mother–infant cohort from Kampala, Uganda. (**A**) A neighbor-joining phylogenetic tree based on a 839 nt RT sequence (nt 2721–3560) from 85 mother and 51 infant samples. The samples are labeled by mother (MT) or infant (IN) followed by treatment status (U for untreated, A for AZT treated, and N for NVP treated) and patient number. Sequential mother and infant patient numbers indicate a transmission pair. For example, MTN170 and INN171 are sequences from an NVP-treated HIV-infected mother and her NVP-treated infected infant. RT genetic subtypes A, C, and D are indicated. Bootstrap resampling values of 90–100% and 70–90% are represented by ** and *, respectively. All sequences have been submitted to GenBank (see Materials and Methods for accession numbers) and accession numbers for reference sequences are available upon request. (**B**) The subtype data for this cohort. The pie chart on the top contains the percentages of subtypes and recombinant forms for the entire cohort and on the bottom, the pie charts for mothers and infants independently.

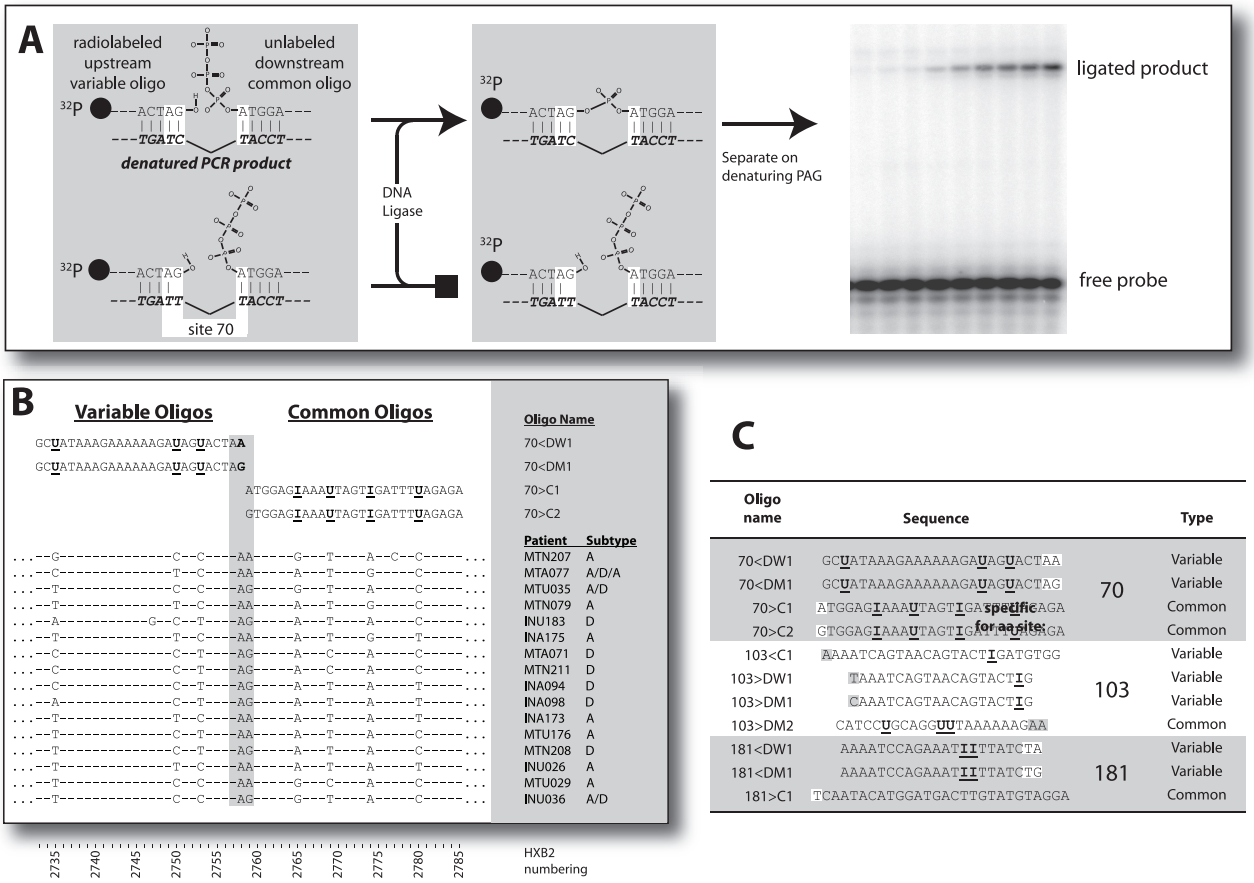


FIG. 2. Schematic of the radiolabeled oligonucleotide ligation assay. (**A**) An illustration of the reaction catalyzed by the cycling DNA ligase. In the top flow chart of this panel, the radiolabeled upstream oligonucleotide (70 < DM1) has a discriminating base at the 3′ end that base pairs with only the mutant nucleotide on the template (i.e., AZT-resistant codon K70R). The upstream oligonucleotide simply anneals to the template and acts to create a single-stranded break in a double-stranded DNA segment. If there is a mismatch within 2 nt of this single-stranded break, ligation cannot occur. In contrast, complementarity at the discriminating base in the upstream oligonucleotide will permit ligation. The free radiolabeled probe can be discriminated from the ligated product on a 10% denaturing polyacrylamide gel. (**B**) An example of the downstream and upstream oligonucleotides employed for detection of the wild-type and mutant codon 70 sequence. Sample subtype A and D HIV-1 RT sequences are aligned around site 70 at the bottom of this panel. The uracils were introduced to maintain base stacking interactions at sites of heterogeneity in the HIV-1 template and to base pair with the deoxyadenosine and deoxyguanine bases (in the "–" sequence that pairs with the OLA oligonucleotides). When diversity in a position was limited to only A and G in the "+" sequence, deoxyinosine was employed because it can base pair with deoxycytosine and deoxythymidine (in the "–" strand). (**C**) All of the OLA oligonucleotides used in this study and the position of the nonstandard bases. Site 70, 103, and 181 codons are highlighted in each oligonucleotide.

mutations, AZT-resistant mutations rarely emerge in mother–infants receiving short course AZT treatment. Thus, it is likely that AZT resistance mutations including K70R will still be less than our limits of detection for low-frequency polymorphisms. In contrast, the NVP-resistant mutations K103N and Y181C rapidly emerge in HIV-infected individuals failing NVP-containing treatment regimens[39] and in NVP-treated, HIV-infected mother–infant cohorts.[9,19]

For detecting the Y181C or K70R wild-type/mutant amino acid, an upstream oligonucleotide harbors the wild-type/mutated nucleotide and a ^{32}P label at the 5′ end (Fig. 2). The downstream oligonucleotide anneals to the template region just downstream of the mutation (Fig. 2). If the 3′ terminal base of the upstream primer can pair with the template base (e.g., the wild-type 181Y codon in the PCR product), the upstream and downstream oligonucleotides will be ligated together following addition of the thermostable DNA ligase (Fig. 2A). The radiolabeled ligated product (43–53 nt) and the free upstream oligonucleotide (22–26 nt) are then separated and quantified by denaturing PAGE and phosphorimaging (Fig. 2A, Materials and Methods). For K103N detection, the upstream oligonucleotide has the discriminating nucleotide at the 3′ end and a ^{32}P label at the 5′ end. The downstream oligonucleoide is common. We have recently described the optimization of this ligation assay by varying the cycles, template input, salt concentration, probe concentrations, and ligase input.[29]

OLA has a distinct advantage over other methods for low level mutant frequency detection (e.g., allelic specific PCR) in that the ligase reaction is only dependent on perfect complementarity within 2 nt of a single strand break in double-

stranded DNA. Outside of this 4 nt window, reduced complementarity between the oligonucleotide probe and template has relatively minor effects on ligation efficiency.[40] However, considerable sequence diversity may reduce oligonucleotide annealing and must be accommodated. OLA has been used for the detection of drug-resistant mutations in diverse subtype B HIV-1 isolates found in North America and.[22,36,41,42] In Uganda and other east African countries, subtype A, C, D, and recombinant forms cocirculate and are quite genetically distinct such that subtype B probes are not appropriate for OLA, as previously described.[43] As a result, our sets of upstream and downstream oligonucleotides were designed to accommodate the genetic diversity adjacent to the drug resistance mutations for subtypes A and D. Unnatural deoxynucleotides were incorporated into the oligonucleotide at sites that were heterogeneous in subtype A and D HIV-1 RT sequences (Fig. 2B). For example, the site 70 (*70*) upstream (<) discriminating (*D*) wild-type 1 (*W1*) and mutant 1 (*M1*) oligos (i.e., *70* < *DW1* or *70* < *DM1*) have deoxyuracil substitutions that correspond to pyrimidine T or C heterogeneity at positions 2735, 2750, and 2753 (respectively) in subtype A and D RT sequences (Fig. 2B). At RT position 2735, all four bases are observed such that deoxyuracil in the oligonucleotide could adequately base pair with A or G or simply preserve base stacking interactions. Although the resulting U–T and U–C base pairs contribute little stability via H-bonding, these pairs preserve pi ring system interaction with minimal duplex contortion such that ligase loading is not inhibited.[44] The 70 (*70*) downstream (>) common 1 (*C1*) and common 2 (*C2*) oligos (i.e., *70* > *C1* and *70* > *C2*) have two deoxyinosine substitutions at oligo positions 7 and 16. Dexoyinosine preferentially pairs with deoxycytosine, but can also adequately pair with thymidine,[45] i.e., the two bases found at sites 2765 and 2774 in the RT coding region (Fig. 2B). Thus, we could accommodate most nucleotide diversity in RT using nonstandard bases in our oligonucleotide sets.

To test the specificity and discriminating ability of this modified OLA, we employed cloned DNA harboring a wild-type or mutant sequence at position 70, 103, and 181 (see Materials and Methods) (Fig. 3). A linear increase in ligation efficiency was observed over the template input range from 0.25 to 12 ng. The amount of ligation product reached a plateau at 18, 25, and 35 ng, then decreased slightly when the template input exceeded 35 ng. The oligonucleotides used to detect the mutant C181 sequence were also employed for OLA with the wild-type template (Y181) as a measure of background ligation. In this case, minute background levels of ligation product were observed with greater than 25 ng of wild-type template (Fig. 3). Similar specific and background OLA results were obtained with oligonucleotides detecting the mutant or wild-type 181 sequence (respectively) on a mutant C181 template (Fig. 3). The specificity and discriminating ability of OLA for detection of K103N and K70R were similar to that observed at the 181 position (data not shown). Specific background ligation ratios were typically greater than 1000:1 (Fig. 3) and well below our set limits of detection. With each set of analyses described below, background ligation products with 3 and 12 ng of template were quantified and subtracted from the amount of correct ligation product (see Materials and Methods).

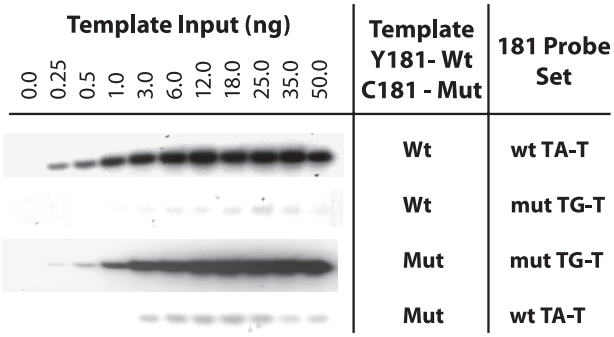


FIG. 3. Analysis of background ligation when detecting the wild-type and mutant codons at site 181. Clonal pCR2.1 vectors with HIV-1 RT nucleotide sequences from patient samples INN131 (C181 mutant; subtype A) and MTN121 (Y181 wild-type; subtype D) were PCR amplified and analyzed by OLA. The products (0–50 ng) were added to oligonucleotide ligation assays using the 181 > C1 common with the 181 < DW1 wild-type or 181 > DM1 mutant discriminating oligonucleotides. The Y181 wt template with the probe 181 < DM1 or the C181 mut template with the 181 < DW1 provides the background for these assays. For all subsequent analyses, 25 and 5 ng PCR product was probed by OLA to determine the amount of wild-type and mutant sequence. A full description of the calculations is provided in Materials and Methods.

Establishing OLA and appropriate clinical cutoffs for the detection of low-frequency NVP- and AZT-resistant mutations in the HIV-1 quasispecies

A panel of patient OLAs for the detection of K103N, Y181C, and K70R is shown in Fig. 4. For all patient sample analyses, 5 and 25 ng of the PCR-amplified DNA were used in an OLA reaction mixture with either the mutant or wild-type discriminating oligonucleotide pairs. Ligations on the control templates (0–35 ng) provided either the background or *y*-value for a plot of the specific products versus template copy number (M.S. Lalonde and E.J. Arts, unpublished observations). From the resulting exponential equations, we could calculate the proportion of mutant and wild-type sequence at codons 70, 103, and 181 within the patient sample. For mutation K103N, two nucleotide substitutions in the codon (AAA changed to AAT or AAC) can encode the aspargine. Thus, the percentage of N103 in the patient quasispecies (virus population) was the copy number of ligated mutant 1 and mutant 2 products divided by the copy number of the total ligated products (including the wt ligated products) (Fig. 4A). For mutation K70R, OLA was performed using oligonucleotides specific for two wild-type and two mutant codons at position 70 (Fig. 4C).

In nearly all samples from untreated, AZT-treated, or NVP-treated mothers or infants, we could detect K103N and sometimes Y181C, but the mutation frequency in the quasispecies was often less than 0.6%. Previous studies using allelic-specific PCR suggest a sensitivity as low as 0.01% but would require the input of at least 10,000 RNA copies into the assay from at least 250 μl of plasma (with a viral load >40,000 copies/ml).[9] Although this low percentage was also achieved by our radiolabeled OLA, we sampled, PCR amplified, and then performed OLA on approximately 10 μl DNA from PBMCs, which harbor viral

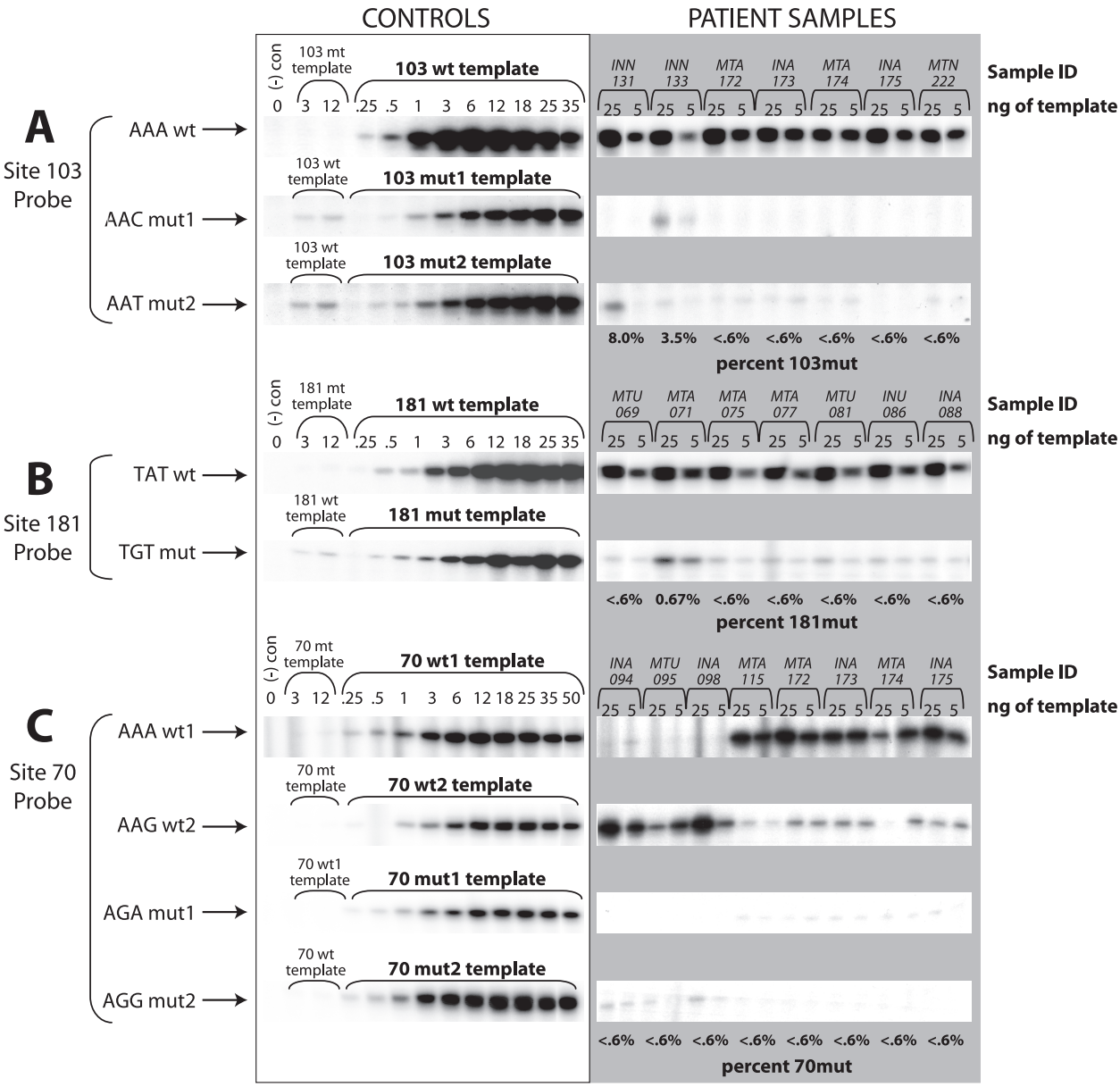


FIG. 4. Determining the percentage of K103N, Y181C, and K70R by OLA in the HIV-1 quaispecies from a subset of patient samples. OLA was performed on controls (left in the figure) and a subset of patient samples (right gray box) using the mutant and wild-type probes for site 103 (**A**), site 181 (**B**), and site 70 (**C**). The percentage of these mutations in the patient quasispecies was calculated based on the OLA standards and is provided at the bottom of each panel. It is important to note that two different transversion mutations confer the K103N change (requiring the mut1 and mut2 OLAs in panel **A**) but only one transition mutation confers the Y181C change (one mut OLA in **B**). At site 70, two wild-type codon (wt1 and wt2 in **C**) and two mutant codon (mut1 and mut2) sequences are possible for transition mutations and the K70R codon change.

DNA loads of <2000–139,500 copies/ml.[46] Table 2 provides the estimated DNA viral load for 44 of 107 PBMC samples. The DNA viral load were typically 10- to 100-fold higher than the copy number needed for the estimated OLA mutant frequency. Due to limited sample volumes, we performed thorough empirical tests to determine the cut-off on clinical samples. First, we mixed known quantities of wild-type and mutant HIV-1 clonal DNA, performed OLA, and found that the OLA-derived mutant to wild-type ratios matched the actual input ratios.[29] However, this measure of sensitivity is still artificial because it does not include all of the elements present in extracted patient samples including nucleic acids and does not account for the HIV-1 sequence heterogeneity of the patient samples (not found in clones). Thus, we selected 13 clinical samples for thorough clonal OLA analysis with K103N or Y181C ranging from undetectable to 98.5% (as determined by bulk OLA; Fig. 4).

Samples with high percentages of K103N or Y181C mutations (>30% by bulk OLA) in the patient quasispecies were

TABLE 2. VIRAL DNA LOAD AND OLA MUTANT FREQUENCY IN PBMC SAMPLES

Patient sample	*DNA copies/ml ($\times 10^3$)*	*K103N (%)*	*Y181C (%)*
MTU037	4.2–42	0.7	0[a]
MTA039	51–505	1.3	0
MTU047	3.8–38	0.9	0
INU048	6.0–60	0.9	0
MTU051	40–402	1.3	0
INU058	47–467	1.8	0
MTU059	52–520	1.3	0
INU060	14,577–145,773	1.3	0
MTU065	111–1106	2.9	0.6
INU066	219–2186	3.9	0
MTU067	8.4–84	3.3	0
MTU069	21–209	3.7	0
MTA071	11–105	0	0.7
MTA077	64–645	2.9	0
MTU81	259–2588	0	0
INA094	35–354	0.8	0
INU100	3.9–39	0.6	0
MTU103	10–102	3.2	0
INN120	52–525	0	0
INU129	16–160	2.1	0
INN131	0.5–4.5	8.0	61.1
MTN148	2.8–28	98.5	98.5
MTN162	55–554	0	0
INN163	58–582	0	0
MTN164	2.1–21	1.3	52.3
MTN168	4.7–47	0	0
INN169	14–137	0	0
MTN203	5.8–58	0	0
MTN204	9.1–91	0	0
MTN206	1.6–16	1.2	0
MTN207	7.2–72	44.6	0
MTN208	22–225	1.3	63.5
MTN209	7.1–71	2.0	5.6
MTN210	5.4–54	2.2	92.5
MTN211	5.5–55	0	0
MTN213	17–173	0.6	0
MTN215	0.5–4.6	0	0
MTN216	5.7–57	0	0
MTN217	0.8–7.5	0	0
MTN223	33–333	0	0
MTN224	139–1395	0.6	0.9
MTN225	6.7–67	0	0
MTN226	3.4–34	1.1	0
MTN228	0.6–5.9	2.2	3.8

[a]0% represents less than the lower limit of OLA detection or <0.6%.

PCR amplified, cloned into the pCR2.1 vector, and then screened by DNA sequencing or OLA. For select samples harboring 3.8%, 0.6%, and 0.1% K103N mutation frequency, 200, 500, and 1000 clones (respectively) were screened by OLA. For the initial screening, 20 bacterial colonies (containing the RT clones in pCR2.1) were separately propagated and pooled prior to plasmid purification. Pools of 20 RT plasmids were then screened for >5% K103N mutation frequency by OLA, i.e., more than one K103N-positive clone represents a detection 50-fold above background. We screened 10, 25, and 50 pools of 20 clones to test the predicted 3.8, 0.6, and 0.1% K103N frequency (as performed by OLA on bulk PCR products). Positive pools were confirmed by performing OLA on each clone in the pool. At least one OLA-postitive clone was found in the pool, suggesting the presence or one more K103N-positive clone (Fig. 5). Sequencing confirmed the presence of the specific mutation in the clone. A strong direct correlation was observed between actual K103N mutants within a patient sample as compared to the estimated K103N mutant frequency determined by OLA on the bulk PCR-amplified product from the patient sample ($r > 0.97$, $p < 0.0001$; Fig. 5). We did not detect K103N in 50 pools of 20 clones (a total of 1000 clones) sampled from patient MTN217 (0.1% by OLA on the bulk PCR product). However, we did confirm the presence of 25 K103N clones by screening 500 in the MTN213 patient sample (0.6% by bulk OLA). We found a direct and significant correlation when comparing the estimated drug resistance frequency in the population (by bulk OLA) with the actual clonal frequency (by sequencing or by OLA on individual clones) (Fig. 5). The equation of the line for this relationship was $y_{\text{clonal freq.}} = 1.01 + 0.85x_{\text{bulk OLA}}$ ($r = 0.97$, $p < 0.0001$; Pearson product moment correlation). Based on these analyses and our limit of detection, we set our cut-off at 0.6% for the detection of drug resistance mutation by bulk OLA. However, it should be noted that this value can fluctuate based on the set of oligonucleotides employed for probing different drug resistance mutations.

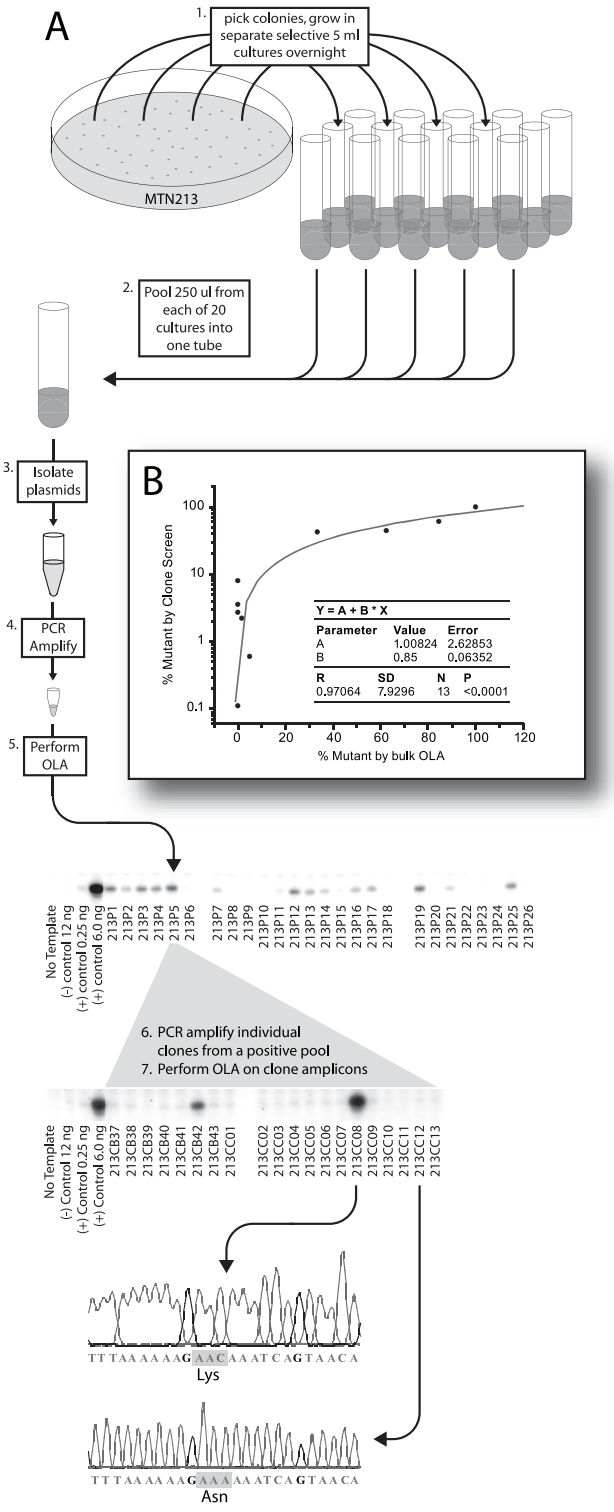
Estimating the frequency of NVP- and AZT-resistant mutations in mothers and infants of an MTCT study

Based on the detection limits, estimates of K70R, K103N, and Y181C in the quasispecies were derived from radiolabeled OLAs on 107 patient samples (48 untreated, 15 AZT treated, and 44 NVP treated) (Fig. 6). For example, patient sample INN131 harbored 7.8% K103N (AAT codon) (Fig. 4A). Use of OLA increased detection of NVP resistance mutations in NVP-treated mothers to 15 of 35 (43%) as compared to 3 of 35 (9%) identified by nucleotide sequencing (Fig. 6E). Patients in the untreated, NVP-treated, and AZT-treated arms were subdivided into three groups based on the frequency of specific mutations in the quasispecies (<0.6% or undetectable, 0.6–5% or low, >5% or moderate/high in intrapatient HIV-1 populations) (Fig. 6A–D). The K103N mutation was found at low frequencies in the mother and infant samples in NVP, AZT, or untreated mother/infant samples (Fig. 6A). For example, 24 of the 63 untreated or AZT mothers/infants (38%) had low levels of K103N mutation (0.6–5% in population) (Fig. 6A). However, none of the untreated or AZT-treated mothers and/or infants had moderate to high levels of K103N (>5% in population) in comparison to 5 of 44 (9%) NVP-treated participants (Fig. 6A). Only 2 of 63 untreated or AZT-treated participants had low to high levels of Y181C, suggesting that this mutation is rarer in the HIV-1 quasispecies (Fig. 6B). Enrichment of the Y181C mutation was more pronounced in the NVP-treated group having 9 of 44 NVP-treated participants (21%) with moderate to high levels of Y181C in the quasispecies (Fig. 6B).

In some instances, both K103 and Y181C mutations may have emerged in the same patient sample. Thus, we subdivided the analyses and found that 9 of 44 NVP-treated mother sam-

ples harbored moderate to high levels (>0.6%) of both mutations in their HIV-1 populations (Fig. 6D). In other words, NVP-treated patients infected with a subtype A or D virus, who harbored detectable Y181C mutations, also had the K103N mutation in their HIV-1 population. There were, however, nine additional NVP-treated patients with subtype A or D infections who harbored only the K103N mutation, suggesting again that K103N is found at higher frequencies and, in some instances, independent of NVP treatment. Only 1 of 63 untreated or AZT-treated participants harbored both K103N and Y181C NVP-resistant mutations in their quasispecies (Fig. 6D). Finally, none of the 15 AZT-treated patients had any detectable level of the AZT-resistant K70R mutation (Fig. 6C), suggesting that the K103N and Y181C NVP-resistant mutations had a lower genetic barrier and/or lower fitness cost than the AZT-resistant K70R mutation.

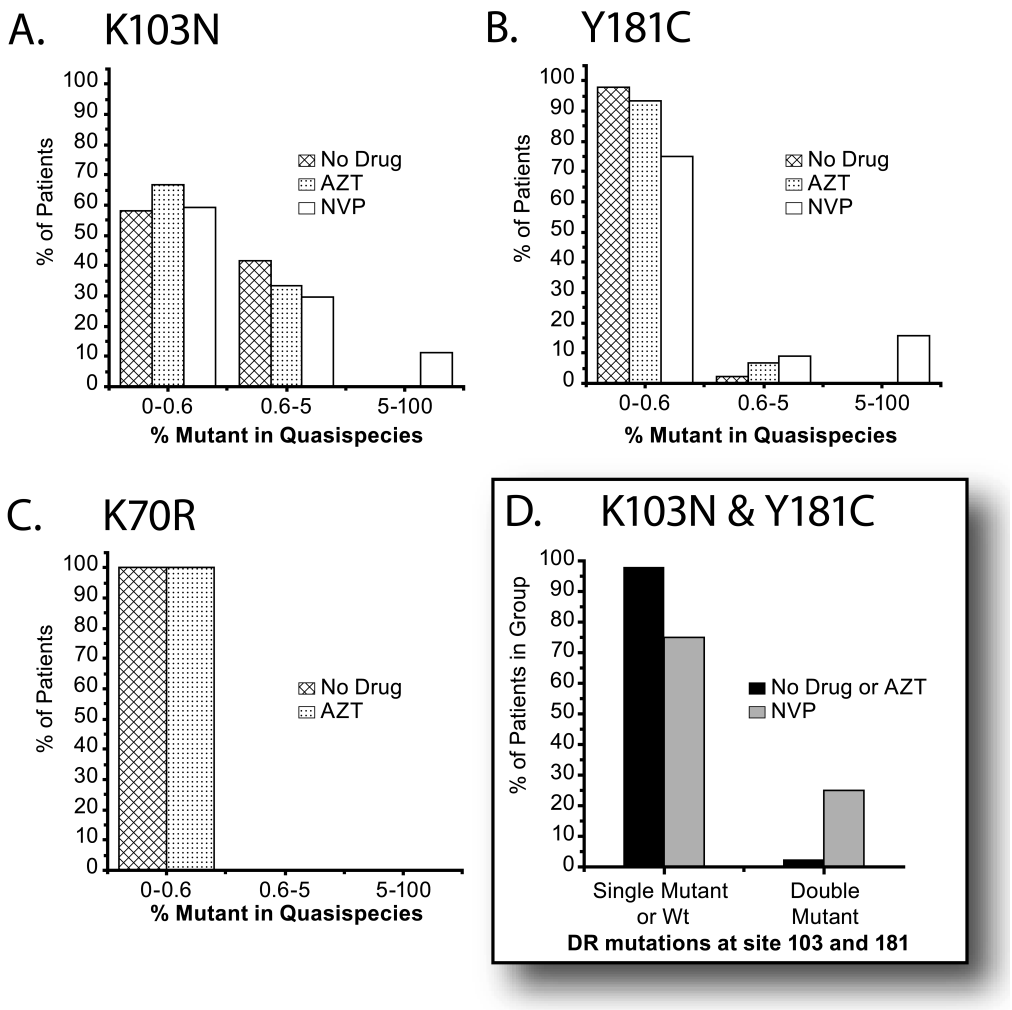
We next determined if the appearance of K103N or Y181C was associated with an infecting HIV-1 subtype. NVP-resistant mutations were present above the 0.6% detection threshold in 47% of NVP-treated, subtype D-infected participants and 38% of NVP-treated, subtype A-infected participants (Fig. 6E). While this percentage difference is minimal, there was a distinct difference between subtypes in the particular mutations present. Of seven NVP-treated subtype D-infected participants with NVP resistance, seven had detectable levels of both K103N and Y181C (Fig. 6E). In contrast, only 3 of the 10 subtype A-infected, NVP-treated participants (with NVP resistance mutations) had detectable levels of both mutations, with the remaining seven harboring K103N only (Fig. 6E). As described above, nine subtype A-infected, NVP-treated patients harbored the K103N mutation at low levels (0.6–5%) in the absence of Y181C, suggesting that this mutation may be quite common within the HIV-1 subtype A genetic background. In contrast to differences in NVP resistance mutations associated with subtype, there was an equal proportion of mother and infant samples harboring K103N, Y181C, or both mutations at low frequencies (Fig. 6E).

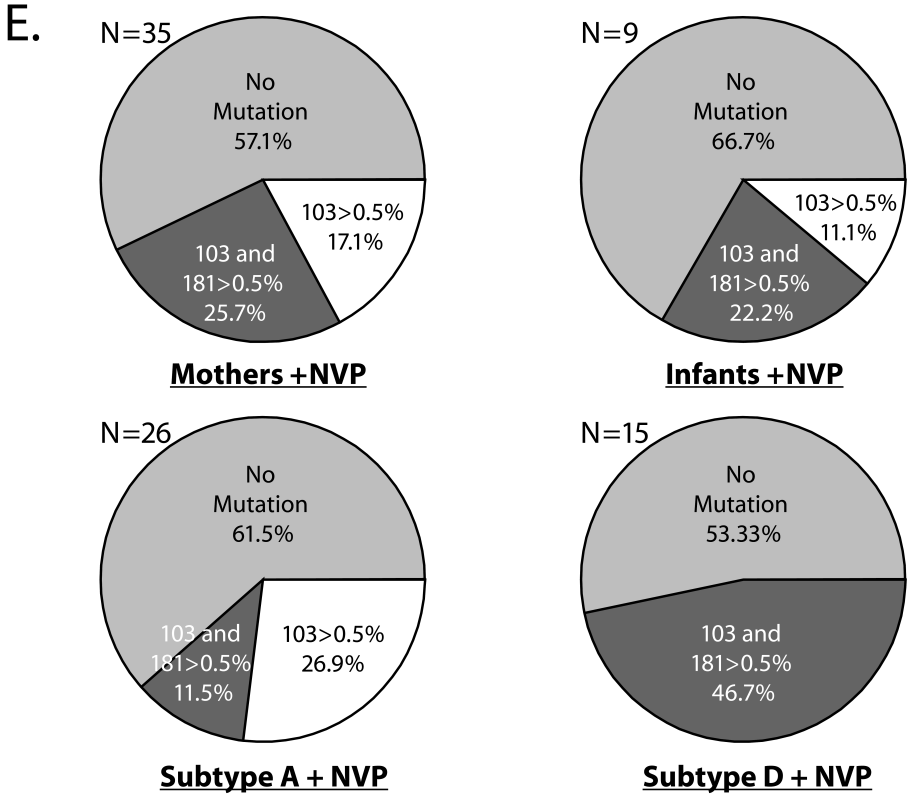


DISCUSSION

A single dose of NVP given to HIV-infected mothers during labor and to infants within the first 72 h after birth has suc-

FIG. 5. Clonal analyses of HIV-1 from patient samples to establish the sensitivity of OLA. The sensitivity of OLA to estimate low-frequency mutations in a PCR product derived from patient PBMCs was examined by using clinical samples with known frequencies of K103N and Y181C in the HIV-1 quasispecies. (**A**) A schematic of the clonal analysis procedure. PCR products of the RT fragment (839 bp) from 13 patient samples were cloned into the pCR2.1 vector. Approximately 10–20 bacterial clones were then directly screened by DNA sequencing when the mutant percentages were measured at >10% by OLA. For samples with very low mutant content by OLA, 200–1000 pCR2.1-RT clones were screened in pools of 20 bacterial colonies. (1) Each colony was grown in a separate LB-amp broth culture and then (2) 20 were combined to (3) purify the pooled plasmids. (4) Purified pooled plasmids were amplified by RT-specific primers and subjected to (5) OLA. Identification of a positive pool (>5% or at least 1 in 20 clones) led to (6) PCR amplification of the 20 individual clones followed by (7) separate OLA analyses. Positive K103N or Y181C clones from this OLA were confirmed by (8) DNA sequencing. The actual frequency of mutant K103N or Y181C clones in the HIV-1 quasispecies was then plotted against the estimated frequency determined by OLA on the bulk PCR product from that patient sample (**B**).


A. K103N
% of Patients
No Drug
AZT
NVP
0-0.6
0.6-5
5-100
% Mutant in Quasispecies
B. Y181C
% of Patients
No Drug
AZT
NVP
0-0.6
0.6-5
5-100
% Mutant in Quasispecies
C. K70R
% of Patients
No Drug
AZT
0-0.6
0.6-5
5-100
% Mutant in Quasispecies
D. K103N & Y181C
% of Patients in Group
No Drug or AZT
NVP
Single Mutant or Wt
Double Mutant
DR mutations at site 103 and 181


E.
N=35
No Mutation 57.1%
103>0.5% 17.1%
103 and 181>0.5% 25.7%
Mothers +NVP
N=9
No Mutation 66.7%
103>0.5% 11.1%
103 and 181>0.5% 22.2%
Infants +NVP
N=26
No Mutation 61.5%
103 and 181>0.5% 11.5%
103>0.5% 26.9%
Subtype A + NVP
N=15
No Mutation 53.33%
103 and 181>0.5% 46.7%
Subtype D + NVP

cessfully prevented 80–90% of perinatal HIV-1 transmissions.[4] Infection of infants does continue postnatally due to the lack of continued antiretroviral intervention in the mothers and HIV transmission from breast milk. Nonetheless, this single dose can also select for resistance in both mothers and infected infants. It is still not clear if NVP resistance facilitates and/or contributes to the remaining 10–20% of the perinatal infections.[47,48] However, previous reports suggest that different NVP resistance mutations are often selected in the NVP-treated mothers and infants.[8] Furthermore, several studies have now detected low-level NVP-resistant mutations in the HIV-1 quaispecies of both HIV-1-infected mothers and their infants.[9,26,27] Again, the impact of even low-level NVP resistance on perinatal transmission of HIV-1 has been difficult to gauge due in part to the availability of paired mother–infant samples and the laborious techniques required to screen for these low-frequency polymorphisms. Finally, the background of NVP resistance mutations in the HIV-1 quasispecies of untreated, infected mothers and their infants has not been determined.

In this study, we have screened for NVP and AZT resistance mutations in 86 HIV-1-infected mothers and 51 of their infants infected by perinatal transmission. Sequence analyses revealed that only 11% of the NVP-treated infected mothers and 33% of their NVP-treated infected infants harbored dominant K103N and/or Y181C NVP-resistant mutations. This percentage of NVP resistance mutations in the NVP-treated mothers was less than the 15–32% previously reported.[10,18,19] Discrepancies between these findings are likely due to the time of sampling, which was ~1–2 days postdelivery for some studies and, on average, 6–8 weeks of the infant age for this study. In addition, this study employed PBMC DNA as opposed to plasma RNA[10,18,19] to detect these low-frequency polymorphisms. Nonetheless, we recently reported that NVP resistance mutations are more easily detected in PBMC DNA as compared to plasma RNA.[29] This may be due to incomplete converstion of RNA to DNA during the reverse transcription step and prior to PCR.[29] This difference also suggests that much of the NVP-resistant virus may have emerged in the mother, was transmitted to the infant, but did not establish infection. Regardless, we did not find a single NVP-treated, HIV-infected mother–infant pair in which both mother and infant harbored the same NVP resistance mutation by dominant sequencing. This observation was also corroborated with OLA results. Out of 28 mother–infant pairs, there were only five cases in which both mother and infant harbored the same mutations (all were 103 mutants) by the more sensitive bulk OLA method. Four of these pairs were untreated. In the remaining nevirapine-treated pair (MTN148–INN149), both mother and infant harbored high levels of K103N in the HIV-1 quasispecies. These results support previous observations suggesting that NVP resistance may not necessarily contribute to cases in which NVP failed to prevent perinatal transmission.[8]

The fading of drug-resistant clones into the HIV-1 quaispecies of an HIV-1-infected patient can now be examined by several techniques[9,26,27,49,50] and does not require the laborious and expensive DNA sequencing of many clones. In this study we employed a modified OLA to determine the percentage of NVP-treated mothers and their infants that harbored low levels of NVP-resistant clones in their HIV-1 quasispecies. OLA involves a cycling, thermostable ligase joining two oligonucleotides abutting a specific mutation site and annealed to a denatured PCR product. OLA was employed as opposed to other techniques such as allele-specific PCR or real-time PCR due to selectivity for specific mutations, higher throughput, and low background. Although allele-specific PCR is more sensitive than the standard OLA, HIV-1 sequence diversity adjacent to the mutation site affects PCR efficiency more so than the oligonucleotide ligation reaction. Ligation by the thermostable DNA ligase is dependent on Watson–Crick base pairing only within a 4 nt window at the single-stranded break of double-stranded DNA. HIV-1 sequence variation outside of this 4 nt window does not affect the specificity of the ligation reaction, whereas this same variation could reduce amplification efficiency using allelic-specific PCR and skew the estimate of mutation frequency. To further avoid the impact of sequence variability on the annealing of the OLA oligonucleotides, we optimized the upstream and downstream oligonucleotides by introducing uracil and inosine deoxynucleotides to accommodate the sequence variation adjacent to the drug resistance mutation site. These nonstandard bases did not affect the specificity or kinetics of the ligation reaction but did allow for increased detection of K70R, K103N, and Y181C mutations in both subtype A and D templates. Interestingly, we were able to detect mutations in sample MTN164, which is subtype C. Although we are confident in our findings, we hesitate to claim that our probes are optimal for all subtypes in light of previous reports.[43] We have recently designed a new set of oligonucleotides for subtype C and suspect that a combination will be required for OLA to cover group M diversity.

Through the use of radiolabeled upstream oligonucleotides, the sensitivity of the OLA was increased to that reported by the allelic-specific PCR technique. However, we placed special emphasis on determining the actual cutoffs of this OLA. The percentage of mutant detection by OLA on the bulk PCR product was compared to the identification of these mutations in the appropriate number of clones from patient samples. For an OLA reading of 0.1% K103N, we screened 1000 clones from that patient sample and were unable to identify a single K103N-positive clone. In contrast, the sample with 0.6% K103N, as determined by bulk OLA, had 25 K103N clones in 500. We set our

FIG. 6. Comparing the frequencies of NVP resistance mutations K103N and Y181C or the AZT resistance mutation K70R in untreated, NVP-treated, or AZT-treated HIV-1-infected mother–infant pairs from Uganda. The percentages of patients with detectable K103 (**A**), Y181C (**B**), K70R (**C**), and K103N + Y181C (**D**) in the HIV-1 quasispecies. Patients are subdivided into the untreated (black bar), NVP-treated (white bar), or AZT-treated (gray bar) groups. (**A–C**) The percentages of the K103N or Y181C mutant clones in HIV-1 quasispecies were subdivided as undetectable (0–0.6%), low (0.6–5%), and moderate to high (5–100%) and (**D**) was subdivided into <0.6% and >0.6% of both K103N and Y181C in the HIV-1 population of NVP-treated (hatched bar) or untreated/AZT-treated patients (dotted bar). The frequencies of NVP resistance mutations (<0.6% or >0.6%) (**E**) in NVP-treated mother and infant infections and in NVP-treated subtype A and D infections are shown.

limit of bulk OLA detection to 0.6% after the specific identification of the positive drug resistance clones in the patient population estimated at different frequencies by bulk OLA. It is important to note that this type of cutoff has not been established with other techniques employed to measure low-frequency mutations.

In this study, we sampled the archived HIV-1 population present in patient PBMC DNA. There is considerable debate as to the impact of free virus reservoir versus an archived provirus reservoir. Previous studies would suggest that the NVP-resistant virus, archived or quiescent in PBMCs, could be more difficult to eliminate and may be more problematic with any subsequent treatment with NVP. Nonetheless, virus in the plasma is more representative of the circulating virus population, of the current genetic diversity, and existing frequency of specific drug-resistant mutations.

Using OLA, NVP resistance mutations could now be detected in 43% of NVP-treated, HIV-infected mothers and 33.3% of their infants. Previous studies have also identified low level NVP-resistant mutations in the HIV-1 quasispecies of NVP-treated mothers and their infected infants.[14,20,28] However, these previous studies did not compare the NVP-resistant mutation frequencies in NVP-treated as compared to untreated HIV-infected mother–infant pairs. We found that regardless of treatment, over 35% of HIV-infected mothers and infants harbored K103N at a frequency of 0.6–5% in their quasispecies (20/48 untreated, 5/15 AZT treated, 13/44 NVP treated). NVP-treated as compared to untreated mothers–infants were more likely to have quasispecies with >5% K103N HIV-1 clones, but this was a relatively small percentage of NVP-treated patients (5/44 or 11.4% of the treated patients). The remaining 69 HIV-infected mothers and infants (regardless of treatment) had less than 0.6% K103N in their HIV-1 quasispecies. Y181C, the other dominant NVP-resistant mutation, was rarer in the quasispecies. Only one patient from the untreated group harbored low level Y181C (0.6–5%) as well as one patient from the AZT-treated group and four patients from the NVP-treated group. Sixteen percent (7/44) of NVP-treated patients had moderate levels of Y181C-containing clones in the quasispecies (>5%). Finally, none of the AZT-treated or untreated HIV-infected mothers and their infected infants harbored any K70R clones in their quasispecies. As described earlier, K70R is typically the first resistance mutation that emerges during AZT monotherapy.

The OLA results provide some insight into the genetic stability of drug resistance mutations in the quasispecies of HIV-1 subtype A and D isolates. Genetic stability may be related to a genetic resistance barrier (number of transition and transversion mutations required for the nonsynonymous codon change) as well as the fitness of HIV-1 subtype A or D clones with these mutations. In the untreated HIV-1-infected population, the K70R appears to be the least genetically stable followed by the Y181C and K103N mutations. The AZT-resistant K70R mutation significantly reduces the *in vitro* replicative fitness of subtype B HIV-1 isolates[51,52] whereas the K103N appears to have a minimal replicative fitness cost.[52–54] Considering the fitness cost of Y181C is likely somewhere between that of K70R and K103N,[52,54,55] the prevalence of these mutations in the untreated subtype A and D HIV-1 population (K70R < Y181C < K103N) reflects the *in vitro* replicative fitness/capacity of HIV-1 clones harboring these mutations (K70R < Y181C < K103N). These fitness analyses were based on an HIV-1 subtype B backbone, but recent studies by our group suggest that subtype A and D isolates harboring these resistance mutations show a similar order in replicative capacity (Nankya and E.J. Arts, unpublished data). Finally, a genetic barrier to resistance based on differential mutational pathways is unlikely since the A to G hypermutations observed with HIV-1[56] would favor the appearance of K70R or Y181C (both requiring an A to G transition mutation) over the appearance of the transversion mutations required for K103N change.

In this study, we classified 137 HIV-1 RT sequences into subtype A (59%), C (3%), and D (29%). Another 9% were intersubtype A/D, A/D/A, or C/D/C recombinants in the RT gene. As observed previously,[57] NVP resistance mutations were identified more frequently in subtype D- than in subtype A-infected Ugandans. Studies have been recently initiated in Uganda to determine if NVP resistance emerges more rapidly in subtype D- than in subtype A-infected individuals treated with NVP-containing regimens.[30] Interestingly, we observed a higher background level of K103N clones in the quasispecies of subtype A- as compared to subtype D-infected, untreated participants. Although K103N emerges in subtype A- and D-infected patients treated with NVP,[30,58] the rate of emergence during treatment has not been investigated. It is possible that the K103N mutation is more stable and fit in the RT of subtype A HIV-1 isolates than of subtype D isolates. Studies to analyze the fitness impact of K103N and Y181C mutations in subtype A, C, and D HIV-1 backbones are currently underway.

In summary, NVP treatment of HIV-1-infected mothers during labor and their infants 72 h after birth selects for NVP resistance mutations, which can be identified at 6 weeks post-treatment/delivery in both mothers (43%) and infected infants (33%). Resistance selection due to NVP treatment does not appear to be associated with transmission considering the lack of congruency with the K103N and/or Y181C in the mothers and their infected infants. However, the most significant finding of this study was that the background levels of K103N and Y181C in HIV-1 quasispecies of untreated patients were surprisingly high, i.e., 41.7% of the untreated mothers and infants had either K103N or Y181C in their HIV-1 population at greater than 0.6%. These results suggest that the "selected" NVP resistance mutations in the quasispecies of NVP-treated mother–infant pairs may have been overestimated in previous studies.

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REFERENCES

1. Connor EM, Sperling RS, Gelber R, *et al.*: Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with

zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. N Engl J Med 1994;331(18):1173–1180.
2. Guay LA, Musoke P, Fleming T, *et al.*: Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. Lancet 1999;354(9181):795–802.
3. Jackson JB, Musoke P, Fleming T, *et al.*: Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: 18-month follow-up of the HIVNET 012 randomised trial. Lancet 2003;362(9387):859–868.
4. Bajunirwe F, Massaquoi I, Asiimwe S, *et al.*: Effectiveness of nevirapine and zidovudine in a pilot program for the prevention of mother-to-child transmission of HIV-1 in Uganda. Afr Health Sci 2004;4(3):146–154.
5. Richman DD, Havlir D, Corbeil J, *et al.*: Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. J Virol 1994;68(3):1660–1666.
6. Jackson JB, Becker-Pergola G, Guay LA, *et al.*: Identification of the K103N resistance mutation in Ugandan women receiving nevirapine to prevent HIV-1 vertical transmission. AIDS 2000;14(11): F111–F115.
7. Gianotti N, Galli L, Boeri E, *et al.*: In vivo dynamics of the K103N mutation following the withdrawal of non-nucleoside reverse transcriptase inhibitors in human immunodeficiency virus-infected patients. New Microbiol 2005;28(4):319–326.
8. Eshleman SH, Mracna M, Guay LA, *et al.*: Selection and fading of resistance mutations in women and infants receiving nevirapine to prevent HIV-1 vertical transmission (HIVNET 012). AIDS 2001;15(15):1951–1957.
9. Palmer S, Boltz V, Martinson N, *et al.*: Persistence of nevirapine-resistant HIV-1 in women after single-dose nevirapine therapy for prevention of maternal-to-fetal HIV-1 transmission. Proc Natl Acad Sci USA 2006;103(18):7094–7099.
10. Jourdain G, Ngo-Giang-Huong N, Le CS, *et al.*: Intrapartum exposure to nevirapine and subsequent maternal responses to nevirapine-based antiretroviral therapy. N Engl J Med 2004;351(3): 229–240.
11. Brenner BG and Wainberg MA: The role of antiretrovirals and drug resistance in vertical transmission of HIV-1 infection. Ann NY Acad Sci 2000;918:9–15.
12. Lockman S, Shapiro RL, Smeaton LM, *et al.*: Response to antiretroviral therapy after a single, peripartum dose of nevirapine. N Engl J Med 2007;356:135–147.
13. Wainberg MA: HIV resistance to nevirapine and other non-nucleoside reverse transcriptase inhibitors. J Acquir Immune Defic Syndr 2003;34(Suppl. 1):S2–S7.
14. Eshleman SH, Hoover DR, Chen S, *et al.*: Nevirapine (NVP) resistance in women with HIV-1 subtype C, compared with subtypes A and D, after the administration of single-dose NVP. J Infect Dis 2005;192(1):30–36.
15. Muro E, Droste JA, Hofstede HT, *et al.*: Nevirapine plasma concentrations are still detectable after more than 2 weeks in the majority of women receiving single-dose nevirapine: Implications for intervention studies. J Acquir Immune Defic Syndr 2005;39(4): 419–421.
16. Chaix ML, Ekouevi DK, Peytavin G, *et al.*: Impact of nevirapine (NVP) plasma concentration on selection of resistant virus in mothers who received single-dose NVP to prevent perinatal human immunodeficiency virus type 1 transmission and persistence of resistant virus in their infected children. Antimicrob Agents Chemother 2007;51(3):896–901.
17. Havlir DV, Eastman S, Gamst A, and Richman DD: Nevirapine-resistant human immunodeficiency virus: Kinetics of replication and estimated prevalence in untreated patients. J Virol 1996; 70(11):7894–7899.
18. Cunningham CK, Chaix ML, Rekacewicz C, *et al.*: Development of resistance mutations in women receiving standard antiretroviral therapy who received intrapartum nevirapine to prevent perinatal human immunodeficiency virus type 1 transmission: A substudy of pediatric AIDS clinical trials group protocol 316. J Infect Dis 2002;186(2):181–188.
19. Eshleman SH, Guay LA, Mwatha A, *et al.*: Characterization of nevirapine resistance mutations in women with subtype A vs. D HIV-1 6-8 weeks after single-dose nevirapine (HIVNET 012). J Acquir Immune Defic Syndr 2004;35(2):126–130.
20. Eshleman SH, Hoover DR, Chen S, *et al.*: Resistance after single-dose nevirapine prophylaxis emerges in a high proportion of Malawian newborns. AIDS 2005;19(18):2167–2169.
21. Church JD, Jones D, Flys T, *et al.*: Sensitivity of the ViroSeq HIV-1 genotyping system for detection of the K103N resistance mutation in HIV-1 subtypes A, C, and D. J Mol Diagn 2006;8(4):430–432.
22. Ellis GM, Mahalanabis M, Beck IA, *et al.*: Comparison of oligonucleotide ligation assay and consensus sequencing for detection of drug-resistant mutants of human immunodeficiency virus type 1 in peripheral blood mononuclear cells and plasma. J Clin Microbiol 2004;42(8):3670–3674.
23. Frenkel LM, Wagner LE, Atwood SM, Cummins TJ, and Dewhurst S: Specific, sensitive, and rapid assay for human immunodeficiency virus type 1 pol mutations associated with resistance to zidovudine and didanosine. J Clin Microbiol 1995;33(2):342–347.
24. Palmer S, Wiegand AP, Maldarelli F, *et al.*: New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 2003;41(10):4531–4536.
25. Shi C, Eshleman SH, Jones D, *et al.*: LigAmp for sensitive detection of single-nucleotide differences. Nat Methods 2004;1(2): 141–147.
26. Flys T, Nissley DV, Claasen CW, *et al.*: Sensitive drug-resistance assays reveal long-term persistence of HIV-1 variants with the K103N nevirapine (NVP) resistance mutation in some women and infants after the administration of single-dose NVP: HIVNET 012. J Infect Dis 2005;192(1):24–29.
27. Johnson JA, Li JF, Morris L, *et al.*: Emergence of drug-resistant HIV-1 after intrapartum administration of single-dose nevirapine is substantially underestimated. J Infect Dis 2005;192(1):16–23.
28. Flys TS, Chen S, Jones DC, *et al.*: Quantitative analysis of HIV-1 variants with the K103N resistance mutation after single-dose nevirapine in women with HIV-1 subtypes A, C, and D. J Acquir Immune Defic Syndr 2006;42(5):610–613.
29. Lalonde MS, Troyer RM, Syed AR, *et al.*: Sensitive oligonucleotide ligation assay for low-level detection of nevirapine resistance mutations in human immunodeficiency virus type 1 quasispecies. J Clin Microbiol 2007;45(8):2604–2615.
30. Richard N, Juntilla M, Abraha A, *et al.*: High prevalence of antiretroviral resistance in treated Ugandans infected with non-subtype B human immunodeficiency virus type 1. AIDS Res Hum Retroviruses 2004;20(4):355–364.
31. Swofford DL. PAUP*: Phylogenetic analysis using parsimony (*: and other methods). [4.0b10]. 2002. Sinauer Associates, Sunderland, MA.
32. Beck I and Frenkel LM: Genotyping kits for the detection of HIV-1 pol drug-resistance mutations by an oligonucleotide ligation assay version 1.3. NIH Research and Reference Reagent Program, 2004.
33. Shuman S: Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase. J Biol Chem 1994;269(51):32678–32684.
34. Toossi Z, Mayanja-Kizza H, Baseke J, *et al.*: Inhibition of human immunodeficiency virus-1 (HIV-1) by beta-chemokine analogues in mononuclear cells from HIV-1-infected patients with active tuberculosis. Clin Exp Immunol 2005;142(2):327–332.

35. Larbalestier N, Mullen J, O'Shea S, *et al.*: Drug resistance is uncommon in pregnant women with low viral loads taking zidovudine monotherapy to prevent perinatal HIV transmission. AIDS 2003;17(18):2665–2667.
36. Beck IA, Mahalanabis M, Pepper G, *et al.*: Rapid and sensitive oligonucleotide ligation assay for detection of mutations in human immunodeficiency virus type 1 associated with high-level resistance to protease inhibitors. J Clin Microbiol 2002;40(4): 1413–1419.
37. Boucher CA, O'Sullivan E, Mulder JW, *et al.*: Ordered appearance of zidovudine resistance mutations during treatment of 18 human immunodeficiency virus-positive subjects. J Infect Dis 1992; 165(1):105–110.
38. Eastman PS, Shapiro DE, Coombs RW, *et al.*: Maternal viral genotypic zidovudine resistance and infrequent failure of zidovudine therapy to prevent perinatal transmission of human immunodeficiency virus type 1 in pediatric AIDS Clinical Trials Group Protocol 076. J Infect Dis 1998;177(3):557–564.
39. Casado JL, Hertogs K, Ruiz L, *et al.*: Non-nucleoside reverse transcriptase inhibitor resistance among patients failing a nevirapine plus protease inhibitor-containing regimen. AIDS 2000;14(2): F1–F7.
40. Landegren U, Kaiser R, Sanders J, and Hood L: A ligase-mediated gene detection technique. Science 1988;241(4869):1077–1080.
41. Frenkel LM, McKernan J, Dinh PV, *et al.*: HIV type 1 zidovudine (ZDV) resistance in blood and uterine cervical secretions of pregnant women. AIDS Res Hum Retroviruses 2006;22(9):870–873.
42. Villahermosa ML, Beck I, Perez-Alvarez L, *et al.*: Detection and quantification of multiple drug resistance mutations in HIV-1 reverse transcriptase by an oligonucleotide ligation assay. J Hum Virol 2001;4(5):238–248.
43. Wallis CL, Mahomed I, Morris L, *et al.*: Evaluation of an oligonucleotide ligation assay for detection of mutations in HIV-1 subtype C individuals who have high level resistance to nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors. J Virol Methods 2005;125(2):99–109.
44. Holbrook SR, Cheong C, Tinoco I Jr, and Kim SH: Crystal structure of an RNA double helix incorporating a track of non-Watson-Crick base pairs. Nature 1991;353(6344):579–581.
45. Cruse WB, Aymani J, Kennard O, *et al.*: Refined crystal structure of an octanucleotide duplex with I.T. mismatched base pairs. Nucleic Acids Res 1989;17(1):55–72.
46. Lalonde MS, Troyer RM, Syed AR, *et al.*: A sensitive oligonucleotide ligation assay for low-level detection of nevirapine resistance mutations in HIV-1 quasispecies. J Clin Microbiol 2007;45: 2604–2615.
47. Niehues T, Walter H, Homeff G, Wahn V, and Schmidt B: Selective vertical transmission of HIV: Lamivudine-resistant maternal clone undetectable by conventional resistance testing. AIDS 1999; 13(17):2482–2484.
48. Johnson VA, Petropoulos CJ, Woods CR, *et al.*: Vertical transmission of multidrug-resistant human immunodeficiency virus type 1 (HIV-1) and continued evolution of drug resistance in an HIV-1-infected infant. J Infect Dis 2001;183(11):1688–1693.
49. Loubser S, Balfe P, Sherman G, *et al.*: Decay of K103N mutants in cellular DNA and plasma RNA after single-dose nevirapine to reduce mother-to-child HIV transmission. AIDS 2006;20(7): 995–1002.
50. Flys TS, Donnell D, Mwatha A, *et al.*: Persistence of K103N-containing HIV-1 variants after single-dose nevirapine for prevention of HIV-1 mother-to-child transmission. J Infect Dis 2007;195(5): 711–715.
51. Harrigan PR, Bloor S, and Larder BA: Relative replicative fitness of zidovudine-resistant human immunodeficiency virus type 1 isolates in vitro. J Virol 1998;72(5):3773–3778.
52. Collins JA, Thompson MG, Paintsil E, *et al.*: Competitive fitness of nevirapine-resistant human immunodeficiency virus type 1 mutants. J Virol 2004;78(2):603–611.
53. Koval CE, Dykes C, Wang J, Demeter LM. Relative replication fitness of efavirenz-resistant mutants of HIV-1: Correlation with frequency during clinical therapy and evidence of compensation for the reduced fitness of K103N + L100I by the nucleoside resistance mutation L74V. Virology 2006;353(1):184–192.
54. Paintsil E, Margolis A, Collins JA, and Alexander L: The contribution of HIV fitness to the evolution pattern of reverse transcriptase inhibitor resistance. J Med Virol 2006;78(4):425–430.
55. Archer RH, Dykes C, Gerondelis P, *et al.*: Mutants of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase resistant to nonnucleoside reverse transcriptase inhibitors demonstrate altered rates of RNase H cleavage that correlate with HIV-1 replication fitness in cell culture. J Virol 2000;74(18):8390–8401.
56. Cheynier R, Gratton S, Vartanian JP, Meyerhans A, and Wain-Hobson S: G → A hypermutation does not result from polymerase chain reaction. AIDS Res Hum Retroviruses 1997;13(12):985–986.
57. Eshleman SH, Becker-Pergola G, Deseyve M, *et al.*: Impact of human immunodeficiency virus type 1 (HIV-1) subtype on women receiving single-dose nevirapine prophylaxis to prevent HIV-1 vertical transmission (HIV network for prevention trials 012 study). J Infect Dis 2001;184(7):914–917.
58. Kantor R, Katzenstein DA, Efron B, *et al.*: Impact of HIV-1 subtype and antiretroviral therapy on protease and reverse transcriptase genotype: Results of a global collaboration. PLoS Med 2005; 2(4):e112.

Address reprint requests to:
Eric J. Arts
Division of Infectious Disease
Department of Medicine
Case Western Reserve University
2109 Adelbert Rd, BRB 1010
Cleveland, Ohio 44106

E-mail: eja3@case.edu