Emergence of Drug-Resistant HIV-1 after Intrapartum Administration of Single-Dose Nevirapine Is Substantially Underestimated

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Conventional sequence analysis detects human immunodeficiency virus (HIV)–1 drug resistance mutations in ∼40% of women shortly after they receive intrapartum single-dose nevirapine (SD-NVP). Using sensitive real-time polymerase chain reaction assays for the K103N and Y181C resistance mutations, we tested genotyped virus before and after SD-NVP in 50 South African women infected with HIV-1 subtype C. By sequence analysis, 40 women had no detectable resistance mutations, and an additional 6 women were negative for Y181C after SD-NVP. We found K103N in 16 (40%) of 40 women and Y181C in 5 (11%) of 46 women at 6–36 weeks postpartum. Clonal sequencing confirmed K103N in 5 of 5 representative samples and Y181C in 4 of 4 samples. Four of the 5 women with newly identified Y181C also had K103N. These findings indicate that resistance mutations emerged in at least 65% of the women after SD-NVP and emphasize the importance of further research to determine the clinical implications.

In resource-limited countries where antiretroviral treatment programs for HIV infections are not implemented, the perinatal administration of antiretroviral drugs has been shown to be an effective prophylaxis for the prevention of mother-to-child transmission (pMTCT) of HIV-1. Such interventions were spurred by the observations of the 1991–1993 pediatric AIDS Clinical Trials Group Protocol 076 trial, which found that mothers who received pre- and perinatal zidovudine (ZDV) were 70% less likely to have HIV-infected children than were mothers who received placebo [1]. Although effective, the pre- and perinatal ZDV therapy carried with it an expense that prohibited its widespread implementation and, thus, necessitated the exploration of alternative MTCT interventions.

The lower cost of the nonnucleoside reverse-transcriptase inhibitor (NNRTI) nevirapine (NVP) and its infrequent dosing requirement made it an attractive candidate for intrapartum pMTCT efficacy evaluations. The 1997–1999 HIV Network for Prevention Trials 012 randomized trial in Kampala, Uganda, found that the intrapartum administration of a single 200-mg dose of NVP, followed by a dose to the newborns within 72 h of delivery, was efficacious. The single-dose NVP (SD-NVP) intervention afforded a slightly lower HIV transmission risk (8.2%) at 6–8 weeks postpartum, compared with the risk after a more involved peri- and postpartum ZDV regimen (10.4%) [2]. Moreover, at 14–16 weeks postpartum, the NVP regimen had 47% greater efficacy, compared with that of the ZDV regimen.
The efficacy, simplicity, and affordability of SD-NVP for pMTCT has made it the intervention of choice in resource-restricted countries. However, 20%–44% of women who are administered SD-NVP develop detectable resistance mutations (e.g., K103N, Y181C, and G190A) to NNRTIs [3–8]. The selection of resistance mutations raises questions as to whether these viruses will compromise the efficacy of subsequent NNRTI-containing regimens. Resistance mutations are conventionally detected by sequence analysis, which, because of sensitivity limitations, cannot reliably identify HIV mutants that constitute <20% of the total viral population in a sample. Because drug-resistant mutants detected after the administration of SD-NVP are typically found at various frequencies in mixtures with wild-type (wt) virus, improved detection of drug-resistant mutants is critical for identifying NVP resistance mutations in these women. Such improvements will determine if the incidence of drug resistance after the administration of SD-NVP is underestimated and will allow for better assessments of the clinical impact of drug-resistant viruses. We report here findings from sensitive real-time polymerase chain reaction (PCR) assays for detecting the presence of the K103N and Y181C mutations in HIV-1 subtype C found in the plasma of South African women who were administered SD-NVP for pMTCT.

**STUDY POPULATION, MATERIALS, AND METHODS**

We evaluated paired plasma samples obtained before (pre-NVP) and after (post-NVP) the administration of SD-NVP to 50 women with HIV-1 subtype C. These women gave consent to participate in a South African pMTCT study examining the incidence and persistence of NVP resistance mutations in women and infants [5, 6]. The parent study was approved by the University of the Witwatersrand’s Human Research Ethics Committee (Medical). The substudy presented here was approved by the internal review board of the Centers for Disease Control and Prevention after the removal of all identifiers and the blinding of samples. None of the 50 pre-NVP samples had evidence of resistance mutations by sequence analysis (ViroSeq HIV-1 assay; Celera Diagnostics). The first available post-NVP samples with viral loads >3000 RNA copies/mL were selected from the parent cohort for testing by real-time PCR assays. The post-NVP samples were collected 6–36 weeks postpartum, with a median collection time of 8 weeks postpartum. The median viral load in the 50 women in the study population was 23,200 RNA copies/mL. Neither the post-NVP sample collection times nor the viral loads for the 50 women in the study population were significantly different from those of the parent

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**Figure 1.**  
*A*, Schematic of the polymerase chain reaction (PCR) assay.  
*B*, Examples of amplification curves generated by a mutant-positive sample and a wild-type (wt) sample in comparison with that of the total copy amplification for both samples. CF, curve fit; RFU, relative fluorescence units; ΔC<sub>T</sub> cutoff, experimentally determined threshold cycle cutoff for the mutation-specific reaction relative to the total viral population amplification cycle at threshold crossing (ΔC<sub>T</sub>). A mutation-specific amplification that crosses the threshold before reaching the cutoff indicates the presence of the mutation.
cohort, which had a median collection time of 7 weeks and a median viral load of 19,000 RNA copies/mL. Follow-up samples and subsequent clinical data are not available for the women in the parent cohort. Ten post-NVP samples that had detectable K103N by sequence analysis were used as experimental positive controls in the K103N real-time PCR assay (see below). Samples from the remaining 40 women, who, subsequent to the administration of SD-NVP, had no detectable resistance mutations by conventional sequence analysis, were used to assess previously undocumented emergence of the K103N mutation.

Samples from all 40 women and from an additional 6 women from the K103N-positive control group, who were negative for the Y181C mutation by sequencing analysis, were tested using the real-time PCR assay for the Y181C mutation. A total of 12 post-NVP samples with known Y181C mutations were used as positive controls. These included 4 samples with the K103N mutation and 8 other samples derived from the parent cohort.

HIV-1 RNA was isolated using the Ultrasens Virus Kit (Qiagen). For the real-time PCR assay, a 759-bp region of viral RNA reverse transcriptase (nt 59–817) was first amplified by reverse transcription (RT)–PCR using the primers C-RTgen2F (5′-AGG TTA AAC AAT GGC CAT TGA CAG AAG) and C-RTgen2R (5′-CTG GGT AAA TCT GAC TTG CCC A). The RT-PCR conditions were 39°C for 1 h and then denaturing at 95°C for 5 min. The PCR conditions were 40 cycles of melting at 94°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 1 min.

To identify drug-resistant mutants present at low frequencies, we developed a sensitive real-time fluorescence PCR assay [9, 10] for HIV-1 subtype C that recognized both K103N codons (AAC and AAT). The assay utilized 2 separate PCRs per sample, each performed in duplicate (figure 1A). The first reaction amplified the total viral population (wt and mutant), using the com.2F primer (5′-CTT CTT GGA AGT TCA ATT AGG AAT ACC; start at reverse transcriptase nt 258) and the 103/com.3R reverse primer (5′-CAT TGT TTA TAC TAG GTA TGG TGA ATG C; end at reverse transcriptase nt 412). The total copy reactions provided the reference amplification signals against which mutation-specific reactions were compared. The mutation-specific primer designs were assessed against plasmid clones from patients with wt HIV-1 subtype C and from patients with both K103N resistance mutation codons. This evaluation revealed that a mixture of 3 primers—C-103N.1F (5′-CCC AGT AGG a/gTT AAA a/gAA GGA C), C-103N.2F (5′-CCC Ag/tC a/gGG GTT a/gAA AGA GGA C), and C-103NT.3F (5′-CCC AGC AGG a/gTT AAA Aa/c/gA GGA T)—showed high specificity for both K103N codons and compensated for template variability in the primer binding site. The 3 mutation-specific primers were mixed at the experimentally determined ratios of 47% C-103N.1F, 33% C-103N.2F, and 20% C-103NT.3F, to equilibrate the differences in primer avidities and minimize cross-interference. The K103N-specific test also used 103/com.3R as the reverse primer.

The probes for both reactions were labeled on the 5′ end with FAM (6-carboxyfluorescein) and were internally quenched with QSY-7 at the nucleotide positions indicated by quotation marks. The 1P (5′-TGG GGG ATG CAT ATT”TT”TT CAG TTC CTT TAG ATG A) and 2P (5′-TGG GAG ATG CAT ATT”TT”TT CAG...
TTC CTT TAG ATG A) probes, which anneal at reverse transcriptase nt 332–365, were mixed at a ratio of 75% and 25%, respectively, and merely acted as reporters of forward primer extension in the total viral population and K103N-specific reactions. Degradation of the fluorescent probes during chain elongation removes the fluorophore from the proximity of the quencher, thereby unmasking the fluorescent signal (measured in relative fluorescence units), which accumulates with each reactive amplification cycle (figure 1B). The CT is the cycle number during which the fluorescence emission crosses the background fluorescence threshold, and it is the unit of measure used when comparing the differences in amplification levels (ΔCT) between the total viral population reaction and the mutation-specific reactions. The ΔCT value inversely reflects the approximate level of a drug-resistant HIV-1 in the sample. The ΔΔCT value indicates the change in ΔCT between the pre-NVP and post-NVP samples.

For the Y181C real-time PCR assay, evaluations of the drug-resistant and wt viruses revealed that a 2-primer mixture provided acceptable specificity and sensitivity for the detection of the drug-resistant mutant. The C-181C.1F (5′-Ga/gA CAa/c AAA ATC CAG AAA TAG Tc/tG CCT G) and C-181C.2F (5′-ACA a/c a/gA AAT CCA GAA ATA GTc/t GCT TG) primers were mixed at a ratio of 72.5% and 27.5%, respectively, and were used with the reverse primer RT181/184.1R (5′-CAG GAT GGA GTT CAT AAC CCA T). The probes for the Y181C-specific reaction, 1P (5′-FAM-TAG GAT CTG ATT”T” AGA AAT AGG GCA ACA TAG a/gAC) and 2P (5′-FAM-TAG GAT CTG ATT”T” AGA AAT AAA GCA ACA TAG a/gAC), which anneal at reverse transcriptase nt 566–599, were mixed at a ratio of 75% and 25%, respectively.

To confirm K103N-positive and Y181C-positive real-time PCR assay results, a 520-bp region of reverse transcriptase (nt 258–777) was amplified from the product of the primary RT-PCR assay and then was cloned using the pCR2.1 vector (Invitrogen) by standard methods. Up to 92 clones from each sample were screened by the real-time PCR assays. Clones that were positive by the assay underwent sequence analysis in a 396-bp region (reverse transcriptase nt 99–230) to verify the presence of K103N or Y181C.

**RESULTS**

In the determination of the K103N assay cutoff for population screening, evaluations of wt and K103N-containing plasmid clones revealed that the mutation-specific reactivity of the K103N-containing samples fell within 11 cycles of the reaction of the total viral population (ΔCT ≤11). The ΔCT cutoff of 11 cycles was >3 SDs above the mean reactivity of the mutation-specific test on wt samples and corresponded to a 0.2% mutation detection limit, which is ∼40–100 times more sensitive than sequence analysis (figure 2).

The K103N real-time PCR assay confirmed the absence of detectable K103N in all 50 pre-NVP samples (ΔCT range, 12.0–23.0 cycles; mean ΔCT, 15.9 cycles) (figure 3). The assay successfully detected K103N in all 10 post-NVP positive control samples (ΔCT range, 2.8–9.8 cycles; mean ΔCT, 6.6 cycles). Of the 40 post-NVP samples that had no detectable NVP-related

### Table 1. Results of real-time polymerase chain reaction assay and clinical data for the newly identified K103N-positive samples.

<table>
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<tr>
<th>Sample</th>
<th>Pre-NVP ΔCT, cycles</th>
<th>Post-NVP ΔCT, cycles</th>
<th>ΔΔCT, cycles</th>
<th>Weeks since NVP</th>
<th>Post-NVP VL, RNA copies/mL</th>
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<td>–5.2</td>
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<tr>
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<td>–6.4</td>
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**NOTE.** NVP, nevirapine; VL, viral load; ΔCT, difference in amplification cycles at threshold; ΔΔCT, change between the pre-NVP and post-NVP ΔCT values, in which a larger negative value indicates a greater K103N-specific reactivity, compared with baseline reactivity.

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mutations by sequence analysis, the real-time PCR assay found 16 (40%) that were positive for K103N (ΔCT range, 6.9–10.6 cycles; mean ΔCT, 8.9 cycles) (figure 3 and table 1). The ΔCT values for the newly identified K103N-positive samples were significantly lower (P < .0001, paired t test) than those of the pre-NVP samples (ΔΔCT range, −3.2 to −8.3 cycles; mean ΔΔCT, −6.0 cycles). In contrast, there was no significant difference in reactivity between the pre-NVP samples and the negative post-NVP samples (P = .61). The drug-resistant mutants were identified in samples collected throughout the entire 36-week postpartum period.

To confirm the results of the real-time PCR assay, a 520-bp region of reverse transcriptase in 5 newly identified K103N-positive samples (samples 1, 2, 36, 47, and 48) was amplified from the product of the primary RT-PCR and then were cloned. These samples were selected because they represented a wide range of observed ΔCTs (table 1). When clones that were positive by the real-time PCR assay were sequenced, the presence of the K103N mutation was verified (reverse-transcriptase sequences of K103N-positive clones can be found under GenBank accession nos. AY771787–AY771793). The 5 samples had the K103N mutation at the following frequencies: sample 1, 11.1%; sample 2, 8.9%; sample 36, 6.7%; sample 47, 4.4%; and sample 48, 1.1%. Sequence comparisons demonstrated that the newly identified mutants were not contaminants, because they had greater similarities with the sequences of their respective populations (98.5%–99.8% identity) than with those of the K103N mutant positive controls (<94% identity). No additional NNRTI mutations were found in the sequences of the 5 newly identified K103N-positive samples.

The Y181C assay cutoff for population screening was determined in the same manner as that of the K103N assay. Evaluations of wt and Y181C-containing plasmid clones revealed that the mutation-specific reactivity of the Y181C-containing samples fell within 9 cycles of the reaction of the total viral population (ΔCT ≤ 9). The ΔCT cutoff of 9 cycles was 3 SDs above the mean reactivity of the wt samples and corresponded to a 0.3% mutation detection limit (figure 4).

Testing of the 50 pre-NVP samples in the Y181C assay showed that all 50 samples had undetectable resistance mutations (ΔCT range, 10.9–36.2 cycles; mean ΔCT, 16.0 cycles) (figure 5). The assay successfully detected Y181C in all 12 post-NVP positive control samples (ΔCT range, 1–7.8 cycles; mean ΔCT, 4.5 cycles). Of the 46 post-NVP samples that had no detectable Y181C by sequence analysis, the real-time PCR assay found 5 (11%) that were positive for Y181C (ΔCT range, 4.9–8.7 cycles; mean ΔCT, 6.7 cycles) (figure 5 and table 2). The ΔCTs of the newly identified Y181C-positive samples were significantly lower (P < .0001, paired t test) than those of the pre-NVP samples (ΔΔCT range, −2.5 to −8.5 cycles; mean ΔΔCT, −5.8 cycles). Again, there was no significant difference in reactivity between the pre-NVP samples and the negative post-NVP samples (P = .76). All viruses with the Y181C mutation were identified in samples collected within the first 3 months postpartum.

Using the same method that was used for the K103N mutants, clonal sequencing was performed on 4 of 5 newly iden-
Table 2. Results of real-time polymerase chain reaction assay and clinical data for the newly identified Y181C-positive samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pre-NVP ΔCT, cycles</th>
<th>Post-NVP ΔCT, cycles</th>
<th>ΔΔCT, cycles</th>
<th>Weeks since NVP</th>
<th>Post-NVP VL, RNA copies/mL</th>
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</tr>
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<td>12.2</td>
<td>6.8</td>
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<td>10,000</td>
</tr>
</tbody>
</table>

NOTE. NVP, nevirapine; VL, viral load; ΔCT, difference in amplification cycles at threshold; ΔΔCT, change between the pre-NVP and post-NVP ΔCT values, in which a larger negative value indicates a greater Y181C-specific reactivity, compared with baseline reactivity.

Figure 6. Additional validation of the real-time polymerase chain reaction assay limits. A, K103N assay sensitivity and the detection limit of cloned K103N-positive sequence from sample 2. B, Y181C assay sensitivity and the detection limit of cloned Y181C-positive sequence from sample 34. ΔCT, difference in amplification cycles at threshold.
postpartum. The pre-NVP mean viral load of the women who developed resistance mutations (40,525 RNA copies/mL) was not significantly higher than that of the women in whom resistance mutations did not emerge (31,400 RNA copies/mL) ($P = .41$, paired $t$ test).

**DISCUSSION**

Our finding of a substantial number of previously undetected viruses harboring the K103N and/or Y181C mutations in women administered SD-NVP provides a more accurate representation of the emergence of drug resistance than was previously shown. The data suggest that the emergence of drug resistance after the administration of SD-NVP occurs more often than was previously reported and highlight the importance of using assays with improved sensitivities to accurately document the presence of drug-resistant mutants. The identification of resistance mutations by real-time PCR assay in 43% of the women with previously undetected mutations, when added to present estimates of resistance mutations in $\sim 40\%$ of women as detected by sequence analysis [4–8], implies that at least 65% of SD-NVP–experienced women develop resistance mutations—a 62% increase over previous findings. This indicates that only a minority of women do not generate drug-resistant mutants and that $\geq 33\%$ of the drug-resistant mutants are not being detected by conventional sequence analysis. Because our testing accounted for only the 2 mutations most frequently associated with NVP resistance, screening by real-time PCR assay for other NVP-associated mutations (e.g., V106A and G190A) may show that the proportion of women with undetectable resistance mutations is even smaller.

The 0.2% and 0.3% detection limits reported for the K103N and Y181C assays, respectively, were derived from the reactivity of cloned clinical sequences and are a conservative level that can accommodate greater sequence diversity than was found in the samples that we tested. The assay detection limits are not expected to be achievable with all samples; nonetheless, in the testing of the diverse clinical samples with these assays, the data undoubtedly show that the sensitivity is greatly improved over that of conventional sequence analysis. This was most evident with samples 3 and 14, which were previously believed to have only wt virus. The real-time PCR assays found that these samples contained the K103N and the Y181C resistance mutations. Although the assays were validated against African HIV-1 subtype C viruses, sequence alignments indicated that they would work equally well with Asian HIV-1 subtype C viruses. However, the assays would require additional evaluation to determine their applicability to other subtypes. If needed, simple nucleotide changes can often accommodate subtype-associated polymorphisms.

Viruses with drug-resistance mutations are known to spontaneously appear as part of the viral quasispecies. However, because no resistance mutations were detected in the pre-NVP samples, any naturally occurring drug-resistant mutants would have been present at frequencies below the detection limit of the assays. By clonal sequencing, all the minority drug-resistant mutants were detected within their sample populations at frequencies of $1\%$–$12\%$. These low frequencies are below the detection limit of conventional population sequencing and, therefore, explain why they were not identified by sequence analysis. The observed incidence of the K103N and Y181C mutations present at low frequencies within the samples that were negative for the mutations by sequence analysis (40% and 11%, respectively) was comparable to the estimates of resistance mutations detectable by sequence analysis in the parent cohort (31% and 12%, respectively) [5]. This suggests that there is no significant difference in the relative proportion of drug-resistant mutants circulating at lower or higher frequencies after the administration of SD-NVP.

The emergence of the K103N mutation in a substantial proportion of the samples and its identification in a high percentage of the samples obtained >6 months after the administration of SD-NVP suggest that the K103N mutation emerges easily in HIV-1 subtype C and does not decay rapidly. Although only a few samples obtained >3 months after the administration of SD-NVP were available for testing, that K103N was observed in the majority of them suggests that this mutation is sustained in the viral population longer than the Y181C mutation. Both the frequent selection of the K103N mutation and its persistence may be explained by the low fitness cost of this mutation [11] and the extended selective pressure resulting from NVP levels that may last for several weeks [12, 13].

Although persisting drug-resistant mutants are likely to be present at low frequencies, they may, nonetheless, have the potential to affect subsequent pregnancies. Because an estimated 50% of HIV-positive infants are infected antepartum [14], exposure to even low levels of drug-resistant mutants during the prolonged gestation period may allow for the successful transmission of resistance mutations. In addition, it is not known whether persisting NVP resistance will favor the peripartum transmission of drug-resistant mutants when a subsequent regimen of SD-NVP is provided. Further research is needed to better assess all the consequences of NVP resistance for subsequent pregnancies.

The emergence of drug-resistant HIV may have important clinical implications for the women and children who are subsequently treated with antiretroviral regimens containing NNRTIs. In a recent observation from a study of Thai women who received SD-NVP [7], 62% of the SD-NVP–experienced women who had detectable NVP-associated resistance mutations by conventional sequence analysis, compared with 32% of the women who had no previous exposure to NVP, could not suppress viral loads after 6 months on the regimen. Notably, 48% of the
SD-NVP–experienced women who had no detectable resistance mutations also failed to suppress viral loads. The poorer treatment responses in the latter group, compared with those in the women who had no previous exposure to NVP, may be explained by the presence of undetected drug-resistant mutants. Therefore, there is an urgent need for studies to fully define the clinical consequences of drug-resistant mutants that are present at low frequencies. The ability of sensitive assays to identify minority drug-resistant viruses will allow for better assessments of the clinical impact of resistance mutations.

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References