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Citation

Published Version
doi:10.1128/JCM.00254-09

Permanent link
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Accessibility
Nevirapine Resistance in Human Immunodeficiency Virus Type 1-Positive Infants Determined Using Dried Blood Spots Stored for Up to Six Years at Room Temperature

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Received 5 February 2009/Accepted 6 February 2009

Dried blood spots that had been stored ambiently for 3 to 6 years lost approximately 1 log10 of human immunodeficiency virus type 1 (HIV-1) RNA, but the majority could still be genotyped for resistance. Nevirapine resistance was found in 7/16 (43.5%) HIV-1-positive HIVNET 024 infants at 4 to 6 weeks, but no resistance was found at other time points.

The use of single-dose nevirapine (sdNVP) has become part of standard care in the prevention of mother-to-child transmission of human immunodeficiency virus (HIV) in many parts of the world. Development of resistance to NVP in HIV-infected infants despite sdNVP treatment has been examined in some trials, but not in HIVNET 024, in which all mothers and infants received sdNVP (6). Dried blood spots (DBS) had been collected from the infants for diagnosis, and these were available for further testing of NVP resistance. Several groups have used DBS for resistance testing, using home brew assays (8, 10, 11) or the Siemens TruGene HIV-1 genotyping assay (9). However, the DBS were at least 3 years old at the time of this study.

DBS are an easy way to collect and ship specimens for diagnostic testing for HIV and preclude the necessity of a phlebotomist and maintenance of a cold chain (4, 9). Previous studies have shown that DBS that are stored at −20°C or −70°C are stable for at least 6 years (8) and that ambient storage keeps DBS stable for at least 1 year (3). To whether old DBS would work for sequencing, one group had found that DBS stored for 5 to 6 years at −20°C or −70°C could be sequenced with a home brew assay and that DBS stored ambiently 5 to 6 years could not be sequenced (8). The HIVNET 024 DBS had been stored ambiently 3 to 6 years, so we tested them to see if sequencing could still be done.

The DBS had been made from 50 μl of collected whole blood per spot on Whatman 903 cards. After drying at an ambient temperature, the cards were stored in zippered bags with desiccant at an ambient temperature in multiple countries in Africa and then in North Carolina. The UNC Institutional Review Board determined that this study fell under exemption 4 for Human Subjects Research because the samples were preexisting samples that did not have any identifying information linked to them.

DBS from 33 infants born in Tanzania (where subtypes A, C, and D are prevalent) were used for Boom extraction of total nucleic acid (2) for HIV-1 RNA quantitation with the Roche Amplicor Monitor version 1.5 microwell plate assay (standard method), and the viral loads (VLs) were compared to those found in the previous VL test, which used the same method. An entire spot was cut from the paper by using scissors and placed in 9 ml NucliSens extraction buffer (bioMérieux, Durham, NC) with 6.7 μl Roche quantitation standard for Boom extraction (2). The scissors were decontaminated with bleach, water, and ethanol between uses. At the end of the Boom extraction, 35 μl of eluate was removed from the silica beads to a new tube. Nine microliters of the eluate was removed to a second new tube, and Roche Monitor diluent was added to bring the volume up to 100 μl for VL testing. Seventeen microliters of the original eluate was used per sequencing reaction. A single negative control was included with each set of samples extracted. A total of 47 samples were tested. The samples were one or both of the first two samples that tested positive for each infant, who ranged from a few days to 15 months of age at the time of sampling. We compared the new VLs to those originally determined by the same method within 3 months of collection (except for five samples, which were originally tested for VL 1.5 to 1.8 years after collection). For the DBS samples that had been tested within 3 months, the repeat VLs were on average 1.04 log10 lower (P = 0.00002; Student’s t test; range of drop, 0.42 to 2.12 log10) than those found in prior determinations with the same DBS cards (Fig. 1). The five samples that had been tested for the first time after a 1.5- to 1.8-year delay had lower RNA levels in the second assay than in the first, with an average drop of 0.50 log10 (range, 0.047 to 0.78), although the drop was not statistically significant (P = 0.26).

Extracted RNA from DBS with >1,000 copies/ml in the most recent testing were used for TruGene genotyping (45 samples total). TruGene sequencing was successful for 31 of 45...
samples (68.9%), which represented 23 of the 31 infants (74.2%) for whom there was sufficient RNA recovery for sequencing. The samples for which sequencing was unsuccessful had VLs in the same range (1,481 to 286,766 cp/ml) as those for which sequencing was successful (1,510 to 279,470 cp/ml; \( P = 0.67 \)). The ages of the DBS were also similar for the two groups, with average ages of 4.3 years for the samples that sequenced and 4.1 years for the samples that did not sequence (\( P = 0.77 \)). Four of the samples that did not sequence were from infants from whom we obtained a sample that we could sequence at another time point, while we could not obtain sequences from eight of the infants (two had two samples that could not be sequenced). Sequences were checked for cross-contamination by making an alignment and a phylogenetic tree of the sequences, using CLC Sequence Viewer 4 (CLC Bio A/S). Subtypes of sequences were determined using the REGA HIV-1 subtyping tool (http://www.bioafrica.net/virus-genotype/html/subtypinghiv.html). The sequences that were obtained indicated that 10 infants were infected with HIV-1 subtype A, 8 were infected with subtype C, 4 were infected with subtype D, and 1 was infected with CRF10_CD.

Sequences were also analyzed for resistance mutations by using the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu/pages/algs/HIVdb.html). NVP resistance was found in seven infants (two infected with subtype A, three infected with subtype C, and two infected with subtype D) (Table 1). Six of the seven infants with NVP resistance had a positive HIV RNA test result at birth (presumed in utero transmission), but NVP resistance was not seen in any birth samples that we tested. NVP resistance was not found in any of the other samples tested, including 9 birth samples, 2 samples at 12 weeks, and 4 samples from later time points. Overall, six of nine infants who were positive at birth and could be sequenced at 6 weeks had NVP resistance (66.7%), while only one of six infants who were HIV RNA negative at birth and could be sequenced at 6 weeks had NVP resistance (16.7%), albeit at an intermediate level.

To determine whether the samples that did not amplify were from one specific subtype or recombinant, we used the plasma samples from the mothers of the infants for whom we could not get a sequence. Of three samples tested, we found that two were subtype C and one was subtype A. Therefore, there did not appear to be a subtype bias in the samples that could not be sequenced. Also, the ability of the maternal virus to be sequenced suggests that it was not sequence differences at the primer sites that prevented successful sequencing. Other possible reasons for our inability to generate sequences from some DBS include fragmented RNA in these samples or an inhibitor of PCR. Using more-recent DBS from UNC Hospitals, we found that some samples had an inhibitor that could be overcome by using half as much of the RNA (J. A. E. Nelson, unpublished observations). We were unable to confirm the possibility of inhibition in the old samples, however.

These results for NVP resistance in infants in the HIVNET 024 trial can be compared with those seen in other trials. In the sdNVP arm of the HIVNET 012 trial, 46% of infected infants had NVP resistance at 6 to 8 weeks (5). A meta-analysis of nine

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**FIG. 1.** Correlation between VLs determined with Roche Monitor version 1.5 microwell assays within 3 months of DBS collection (first VL) and after 3 to 6 years of room temperature storage (second VL).

**TABLE 1.** Characteristics of infants with NVP resistance.

<table>
<thead>
<tr>
<th>Infant</th>
<th>Age (wk) when DBS was collected</th>
<th>Subtype</th>
<th>NVP mutation(s)</th>
<th>HIV status at birth</th>
<th>NVP resistance at birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>A</td>
<td>Y188C</td>
<td>Positive</td>
<td>No sample</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>C</td>
<td>V179T, G190A</td>
<td>Positive</td>
<td>Unable to sequence</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>C</td>
<td>Y181C</td>
<td>Positive</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>D</td>
<td>Y181C</td>
<td>Positive</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>C</td>
<td>V106 M</td>
<td>Positive</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>D</td>
<td>Y181C</td>
<td>Positive</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>A</td>
<td>K238N(^a)</td>
<td>Negative</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

\(^a\) Mutation associated with intermediate NVP resistance level (Stanford University HIV Drug Resistance Database).
sdNVP studies, including HIVNET 012, showed that just over half of HIV-positive infants had NVP resistance at 4 to 8 weeks of age after just being exposed to NVP, compared with 16.5% of HIV-positive infants exposed to NVP plus other antiretroviral drugs (1). Our overall percentage of NVP resistance in infants at 6 weeks was 43.8%, which is in line with the other studies of sdNVP alone. Most of the studies in this meta-analysis indicated that the infants had mutations different from those in their mothers, suggesting that the resistance was acquired in the infants after transmission. Our data support that hypothesis, since we saw no NVP resistance in any of the birth samples.

Data from a 6-week extended-dose NVP study indicate that, at least among infants in the sdNVP arm of the study, 70% of infants who were HIV positive at birth and 36% of infants who were HIV negative at birth had NVP resistance at 6 weeks (4a). These data are not statistically different from our results of 66.7% and 16.7%, respectively ( 1.00 and 0.61, respectively; Fisher’s exact test). These differences in rate of NVP resistance among infants indicate that the timing of sdNVP administration to the infant relative to HIV transmission is an important factor, since in utero transmission would result in higher VL when NVP was given than would intrapartum transmission.

In this study, we found that DBS that have been stored properly at room temperature (sealed in a plastic bag with desiccant) do show a drop in VL of approximately 1 log_{10} over 3 to 6 years but that if the samples had relatively high VLs usable sequences can be obtained from DBS for subtypes A, C, with the TruGene system. Our sequencing results indicate that infected infants, these spots can be used for resistance testing of Alabama at Birmingham (U01-AI-47972). NVP (Viramune) for the 48006), Johns Hopkins University (U01-AI-48005), and the University of Family Health International, contract N01-AI-45200 with the Office of AIDS Research, National Institutes of Health, U.S. Depart-

This work was supported by a grant from the National Institute of Allergy and Infectious Diseases for the International Maternal Pediatric Adolescent AIDS Clinical Trials Network (U10AI066632) and the UNC Center for AIDS Research (P30AI50410). The HIVNET 024 study was supported by the HIV Network for Prevention Trials (HIV-
NET) and sponsorship from the U.S. National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, Depart-

Infectious Diseases (NIAID), National Institutes of Health, Depart-
ment of Health and Human Services, through contract N01-AI-35173 with Family Health International, contract N01-AI-45200 with the Fred Hutchinson Cancer Research Center, subcontract N01-AI-35173-117/412 with Johns Hopkins University, Harvard University (U01-AI-48000), Johns Hopkins University (U01-AI-48005), and the University of Alabama at Birmingham (U01-AI-47972). NVP (Viramune) for the study was provided by Boehringer Ingelheim Pharmaceuticals, Inc. In addition, this work was sponsored by the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institute on Drug Abuse, National Institute of Mental Health, and the Office of AIDS Research, National Institutes of Health, U.S. Depart-
ment of Health and Human Services.

Technical assistance was provided by Wendi McIver and Mark Turner.

REFERENCES