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Comparison of Laboratory Methods for Analysis of Non-nucleoside Reverse Transcriptase Inhibitor Resistance in Ugandan Infants

Jessica D. Church,[1] Wei Huang,[2] Neil Parkin,[2] Natalia Marlowe,[3] Laura A. Guay,[1] Saad B. Omer,[4] Philippa Musoke,[5] J. Brooks Jackson,[1] and Susan H. Eshleman[1]

Abstract

Detailed comparisons of HIV drug resistance assays are needed to identify the most useful assays for research studies, and to facilitate comparison of results from studies that use different methods. We analyzed non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance in 40 HIV-infected Ugandan infants who had received nevirapine (NVP)-based prophylaxis using the following assays: an FDA-cleared HIV genotyping assay (the ViroSeq HIV-1 Genotyping System v2.0), a commercially available HIV genotyping assay (GeneSeq HIV), a commercially available HIV phenotyping assay (PhenoSense HIV), and a sensitive point mutation assay (LigAmp). ViroSeq and GeneSeq HIV results (NVP resistance yes/no) were similar for 38 (95%) of 40 samples. In 6 (15%) of 40 samples, GeneSeq HIV detected mutations in minor subpopulations that were not detected by ViroSeq, which identified two additional infants with NVP resistance. LigAmp detected low-level mutations in 12 samples that were not detected by ViroSeq; however, LigAmp testing identified only one additional infant with NVP resistance. GeneSeq HIV and PhenoSense HIV determinations of susceptibility differed for specific NNRTIs in 12 (31%) of the 39 samples containing mixtures at relevant mutation positions. PhenoSense HIV did not detect any infants with NVP resistance who were not identified with GeneSeq HIV testing. In this setting, population sequencing-based methods (ViroSeq and GeneSeq HIV) were the most informative and had concordant results for 95% of the samples. LigAmp was useful for the detection and quantification of minority variants. PhenoSense HIV provided a direct and quantitative measure of NNRTI susceptibility.

Introduction

In resource-limited settings, nevirapine (NVP)-based regimens are often used for prevention of mother-to-child transmission of HIV-1 (pMTCT).[1,2] Use of these regimens is associated with the emergence of NVP-resistant variants in many infants who are HIV infected despite prophylaxis,[3–6] which can compromise future treatment with a non-nucleoside reverse transcriptase inhibitor (NNRTI)-containing regimen.[7] Recent studies in adults suggest that the presence of minority variants may be associated with a poor response to subsequent antiretroviral therapy.[8–11] On-going studies are evaluating whether selection of minority variants with NVP resistance mutations in children who were HIV infected despite NVP-based prophylaxis compromises their subsequent treatment with an NNRTI-containing regimen. Cost and other factors limit the availability of resistance testing in resource-limited settings. However, analysis of resistance in HIV-infected infants who received these regimens is possible in the setting of clinical trials; this information is needed to evaluate different pMTCT regimens to inform public health policy.

In this report, we compared the performance of four assays for the detection of NNRTI resistance in infants who received either single-dose NVP (sdNVP) or an extended NVP regimen for pMTCT. The comparison included two population sequencing-based genotyping assays (the ViroSeq HIV-1 Genotyping System and the GeneSeq HIV assay), a sensitive point mutation assay (LigAmp), and a phenotypic resistance assay (the PhenoSense HIV assay). Reproducibility of the ViroSeq,[12] GeneSeq HIV (unpublished data, Monogram Biosciences), and PhenoSense HIV[13,14] assays has been demonstrated previously using standard test conditions, and

[1]Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.
[2]Monogram Biosciences, South San Francisco, California 94080.
[3]Celera, Alameda, California 94502.
[4]Johns Hopkins University School of Public Health, Baltimore, Maryland 21205.
[5]Makerere University, Kampala, Uganda.

reproducibility of the LigAmp assay has been demonstrated in a previous study.[15] The LigAmp assay has also been validated by comparing results to those obtained with a yeast-based phenotypic selection assay (TyHRT) assay[16] and an allele-specific polymerase chain reaction (PCR) assay.[17]

These four assays have different advantages for analysis of antiretroviral drug resistance. HIV genotyping assays based on population (bulk) sequencing, such as ViroSeq and GeneSeq HIV, provide information on all relevant mutations, but may not detect mutations in viral variants that are present at low levels.[18,19] Phenotypic resistance assays, such as PhenoSense HIV, are more expensive and generally less sensitive than genotyping assays. However, in some cases of very high-level resistance, shifts in phenotypic susceptibility can be detected when the resistant variant is present at levels below the sensitivity limit of population sequence-based genotypic assays. Phenotypic assays do provide quantitative information about drug susceptibility without requiring knowledge of resistance mutations, and in some cases, may reveal unexpected reductions in susceptibility in the absence of known resistance mutations.[20–23] Because genotypic and phenotypic resistance assays provide complementary information about drug resistance, use of both assays may be beneficial in certain settings (e.g., in patients with complex antiretroviral treatment histories, in subjects with non-subtype B HIV, or in settings in which use of novel antiretroviral drugs is being considered).[23,24] Point mutation assays, such as LigAmp,[15,18] can complement population sequencing-based assays by detecting and quantifying specific mutations present at low levels. However, point-mutation assays typically probe only one mutation at a time and may require different primer sets for analysis of different HIV subtypes. The ViroSeq assay can be performed in-house, and PCR products remaining after analysis can be used for research applications, such as analysis with LigAmp. This is an advantage for pediatric studies in which sample volumes may be limited. The GeneSeq HIV and PhenoSense HIV assays are performed centrally at Monogram Biosciences.

Materials and Methods

Samples used for analysis

Infant plasma samples collected at 6–8 weeks of age were obtained from a clinical trial in Uganda (the Ugandan component of the SWEN study).[2] We analyzed samples from 49 infants who received either sdNVP or sdNVP plus extended daily NVP up to 6 weeks of age for pMTCT. Because limited plasma was available for analysis, the ViroSeq assay was performed using 0.1 ml plasma (the standard test volume is 0.5 ml), the GeneSeq HIV and PhenoSense HIV assays were performed together using 0.1 ml plasma (the standard test volume is 1 ml), and the LigAmp assay was performed using PCR products remaining after the ViroSeq testing. ViroSeq results were obtained for all 49 samples (25 subtype A, 13 subtype D, 1 subtype C, and 10 intersubtype recombinant). The LigAmp assay was performed for 44 of those samples (the remaining five samples either had insufficient PCR products remaining for analysis or had HIV subtypes other than A and D in the region analyzed). Forty-two of those 44 samples had plasma remaining for analysis in the GeneSeq HIV and PhenoSense HIV assays; GeneSeq HIV results were obtained for all 40 samples and PhenoSense HIV results were obtained for 39 of the 40 samples. Failure to obtain results with one sample was due to low level replication. The final sample set used for analysis included the 40 samples with results from the ViroSeq, GeneSeq HIV, and LigAmp assays (22 subtype A, 9 subtype D, and 9 intersubtype recombinant), 39 of which also had PhenoSense HIV results. Thirty of these 40 samples had viral load results (median: 875,302 HIV RNA copies/ml; range: 3,977 to 20,900,000 HIV RNA copies/ml).

Analysis with the ViroSeq system

Infant plasma samples were analyzed with the ViroSeq HIV-1 Genotyping System v2.0 and ViroSeq Software version 2.6 (Celera, Alameda, CA). Bidirectional sequence data were obtained at all positions of NVP resistance mutations for 38 of the 40 samples included in this assay comparison sub-study. The resulting *pol* sequences were used for HIV subtype determination, as previously described.[25]

Analysis with the LigAmp assay

PCR products generated from samples with subtype A and D HIV in the ViroSeq system were analyzed with the LigAmp assay for K103N (AAC), Y181C (TGT), and G190A (GCA), as previously described.[3] The LigAmp assay includes a mutation-specific oligonucleotide ligation step followed by a universal real-time PCR detection step. Different pairs of oligonucleotides were used for samples with different HIV *pol* region subtypes (A or D). The following assay cutoffs were used: 0.5% for K103N, 1% for Y181C, and 0.5% for G190A. For correlation studies in which log transformation was required, results below the assay cutoff were assigned a value of 0.01%.

Analysis with the GeneSeq HIV assay and the PhenoSense HIV assay

The PhenoSense HIV and GeneSeq HIV assays were performed at a commercial laboratory (Monogram Biosciences, Inc., South San Francisco, CA; ordered as the PhenoSenseGT assay package).[13] In both assays, the *pol* region is amplified from a test sample and is transferred into a resistance test vector. In the GeneSeq HIV assay, vector pools are sequenced to determine the HIV genotype. In the PhenoSense HIV assay, recombinant virus generated from the vector pools is used to infect cells in the presence of varying concentrations of a drug. The amount of drug needed to inhibit viral replication of the test vector by 50% (IC_{50}) is then compared to the IC_{50} of a reference strain; this ratio (IC_{50} test vector/IC_{50} reference) is referred to as the fold change in IC_{50}. The PhenoSense HIV assay provides results of susceptibility testing for all FDA-cleared antiretroviral drugs that target HIV protease and HIV reverse transcriptase. Testing was performed prior to approval of the NNRTI, etravirine; results for etravirine are not included. The following biological cutoffs were used to define reduced susceptibility: fold-change $IC_{50} > 4.5$ for NVP, >6.2 for delavirdine (DLV), and >3 for efavirenz (EFV).[26]

Results

Comparison of the ViroSeq and LigAmp assays

We first compared the results of two assays performed at Johns Hopkins University: ViroSeq and LigAmp. LigAmp reactions were performed for detection of K103N, Y181C, and G190A; testing was performed in duplicate, and results were averaged. The correlation of individual log transformed

LigAmp results (paired data from duplicate testing) was $R^2 = 0.99$ for K103N, 0.93 for Y181C, and 0.99 for G190A (overall: $R^2 = 0.97$). In the LigAmp assay, a total of 45 mutations were detected in the 40 samples (14 K103N, 22 Y181C, 9 G190A). Thirty (67%) of the 45 mutations were detected by both ViroSeq and LigAmp. Three (6.7%) of the 45 mutations were detected by ViroSeq only and 12 (26.6%) of the 45 mutations were detected by LigAmp only (Table 1). In all three cases in which mutations were detected by ViroSeq only, the resistance mutation was encoded by an alternate codon (AAT for K103N and TGC for Y181C).

LigAmp detected 12 mutations (one in each of 12 samples) that were not detected by ViroSeq, including two K103N mutations, four Y181C mutations, and six G190A mutations. Eleven of the 12 samples had at least one NVP resistance mutation detected by ViroSeq; therefore, the detection of an additional mutation by LigAmp changed the resistance interpretation in only one sample (a sample with Y181C at a level of 1.4% of the viral population). The 12 mutations detected by LigAmp only were present at very low levels (median: 1.65%, range: 0.7–5.9%, Table 1). Only one of the 12 mutations was detected by GeneSeq HIV (a G190A mutation present at 3.2%, see below). Overall, ViroSeq detected 12 (85.7%) of the 14 K103N mutations and 18 (81.8%) of the 22 Y181C mutations, but detected only three (33%) of the nine G190A mutations. The median level of the 30 mutations detected by ViroSeq was 10.5% (range: 1.6–100%).

Comparison of the ViroSeq and GeneSeq HIV assays

We next compared results obtained with ViroSeq to results obtained with the GeneSeq HIV assay. HIV subtypes determined with the GeneSeq HIV assay matched the HIV subtypes obtained by phylogenetic analysis of *pol* region sequences generated with the ViroSeq system (see Materials and Methods). Among the 40 samples that had results from both assays, 10 (25%) of the samples had no NVP resistance mutations detected by either assay, and 24 (60%) of the samples had the same NVP resistance mutations detected by both assays. In each of the remaining six samples, GeneSeq HIV detected a single NVP resistance mutation that was not detected by ViroSeq (Table 2). Analysis of GeneSeq HIV electropherograms revealed that all six mutations were in minor viral subpopulations. In the ViroSeq electropherograms, low-level peaks consistent with the presence of these mutations were detected, but the data did not meet the ViroSeq criteria for mutation identification. In two of the six samples, no NVP resistance mutations were detected by ViroSeq, and a single NVP resistance mutation was detected by GeneSeq HIV (Table 2).

We used the data from the LigAmp assay to evaluate the sensitivity of the two genotyping assays for detection of K103N, Y181C, and G190A. Twelve samples had K103N detected by both ViroSeq and GeneSeq HIV. Ten of the 12 samples had K103N detected by LigAmp (median level of K103N = 5.2%, range: 2.6–84%). Eighteen samples had Y181C detected by both ViroSeq and GeneSeq HIV. Seventeen of the 18 samples had Y181C detected by LigAmp (median level of Y181C = 25%, range: 1.6–100%). Three samples had G190A detected by both ViroSeq and GeneSeq HIV. All three of the samples had G190A detected by LigAmp (at 2.8%, 12.9%, and 18.7%). In one sample (sample 327), LigAmp detected the Y181C mutation at 100%, while GeneSeq HIV detected the mutation as a mixture. Review of the GeneSeq HIV electropherogram confirmed that the majority of the viral population contained the Y181C mutation. The ViroSeq assay detected this mutation as an unmixed species; a minor wild-type peak was detected in the electropherogram, but it did not meet the criteria for mixture detection.

Both ViroSeq and GeneSeq HIV provide predictions of drug susceptibility using a rules-based algorithm. For 29 (72.5%) of the 40 samples tested, these predictions were the same for both assays for all three nonnucleoside reverse transcriptase inhibitors [NNRTIs: NVP, delavidine (DLV), and efavirenz (EFV); i.e., ViroSeq = none with GeneSeq HIV = no evidence of reduced susceptibility, or ViroSeq = possible/high with GeneSeq HIV = evidence of reduced susceptibility). For two samples, the different resistance predictions obtained with the two assays reflected the fact that NVP resistance mutations were detected only by the GeneSeq HIV assay (see above). For the other nine samples, the different resistance predictions reflected differences in the algorithms used to predict drug susceptibility for the mutations Y181C and K101E.

Comparison of the GeneSeq HIV and PhenoSense HIV assays

Thirty-nine (97.5%) of the 40 samples that had GeneSeq HIV, ViroSeq, and LigAmp results were successfully analyzed with the PhenoSense HIV assay; one sample failed in the PhenoSense HIV assay due to insufficient viral replication. Ten samples had no NVP resistance mutations detected by GeneSeq HIV; all 10 were susceptible to the three NNRTIs in the PhenoSense HIV assay. Twenty-nine samples had at least one NVP resistance mutation detected by GeneSeq HIV.

Table 1. Detection of K103N, Y181C, and G190A Using the ViroSeq System and the LigAmp Assay

	Assay(s) detecting the mutation of interest		
Mutation	*ViroSeq and LigAmp*	*ViroSeq only*	*LigAmp only (level of mutant)*[a]
K103N	10	2[b]	2 (0.7%, 1.7%)
Y181C	17	1[c]	4 (1.1%, 1.4%, 2.0%, 2.6%)
G190A	3	0	6 (1.2%, 1.2%, 1.6%, 3.2%, 5.5%, 5.9%)
Total	30	3	12

[a]The LigAmp assay was performed in duplicate; the mean of the two results is shown in the table. The individual LigAmp results are as follows: for K103N: 0.7% (0.3%, 1%), 1.7% (1.4%, 2.0%); for Y181C: 1.1% (1.0%, 1.1%), 1.4% (0.2%, 2.5%), 2.0% (1.9%, 2.0%), 2.6% (2.8%, 2.3%); for G190A: 1.2% (1.1%, 1.2%), 1.2% (1.1%, 1.3%), 1.6% (1.9%, 1.3%), 3.2% (2.4%, 3.9%), 5.5% (6.6%, 4.4%), 5.9% (5.8%, 5.9%).

[b]Two infants had K103N encoded by the AAT codon; LigAmp was designed to detect the more common AAC codon for K103N.

[c]One infant had Y181C encoded by the TGC codon; LigAmp was designed to detect the more common TGT codon for Y181C.

Table 2. NVP Resistance Mutations Detected in Samples with Discordant ViroSeq and GeneSeq HIV Assay Results[a]

Sample	Viral load (copies/ml)	ViroSeq	GeneSeq HIV	LigAmp K103N	LigAmp Y181C	LigAmp G190A
214	3,977	None	**K101K/E**	0.0	0.0	0.0
247	NA	None	**K103K/N**	0.0[b]	0.0	0.0
327	730,049	Y181C	Y181Y/C, **Y188Y/C**	0.0	100.0	0.0
343	346,810	K103N, Y188C	**A98A/G,** K103K/N, Y188Y/C	5.4	1.1	0.0
362	4,288,194	K103N, Y181C	K103K/N, Y181Y/C, **G190G/A**	2.6	1.6	3.2[c]
441	20,927,489	K103N, Y181C	K103K/N, Y181C/**F/I/S**	3.3	33.8	5.8[d]

[a]Mutations detected by the ViroSeq and GeneSeq HIV assays are shown. Mutations that were detected by GeneSeq HIV only are shown in bold. Amino acid mixtures detected in the GeneSeq HIV assay are indicated (e.g., K101K/E). The ViroSeq report does not indicate whether resistance mutations were present as mixtures. Review of the ViroSeq electropherograms revealed that most of the NNRTI resistance mutations detected in this study were present as mixtures with wild-type HIV codons.
[b]This K103N mutation was encoded by AAT; the LigAmp assay was designed to detect the AAC codon for K103N.
[c]The LigAmp assay confirmed the presence of G190A in the test sample.
[d]G190A was not detected by ViroSeq or GeneSeq HIV in this sample.

Seventeen of those 29 samples had concordant NNRTI susceptibility results determined by GeneSeq HIV and PhenoSense HIV. For the 12 remaining samples, the susceptibility results for at least one drug were discordant (Table 3). Variability in susceptibility results in patient samples bearing similar patterns of NNRTI resistance-associated mutations is most likely related to the effects of polymorphisms in the individual patients' virus.[26,27] Most of these samples had mixtures containing resistant and wild-type variants.

Discussion

Detailed comparisons of different HIV drug resistance assays are needed to identify the most useful assay(s) for different clinical settings and to facilitate comparison of results from studies that use different methods. In our cohort of NVP-exposed infants analyzed at 6–8 weeks of age, population sequencing-based assays (ViroSeq and GeneSeq HIV) identified the majority of infants who had NVP-resistant HIV, and the results obtained with these two assays were similar for 95% of the samples tested (NVP resistance: yes/no). GeneSeq HIV did detect more NVP resistance mutations than ViroSeq. However, this changed the resistance interpretation for only two samples. Assay discordance between ViroSeq and GeneSeq could reflect test-to-test variability. In two previous studies, high variability was observed among laboratories using population sequencing-based methods for HIV genotyping.[28,29] In most perinatal studies, the volume of infant plasma available for analysis is limited, and assay procedures must often be adapted for low-volume samples. The viral load

Table 3. Samples with Different Predictions for NNRTI Resistance Using the GeneSeq and PhenoSense HIV Assays

Sample	ST	GeneSeq HIV mutations[a]	GeneSeq HIV predictions[b] DLV	EFV	NVP	PhenoSense HIV results[c] (fold change IC_{50}) DLV	EFV	NVP
412	D	K101E			×	**8.1**[e]	**5.9**[e]	**16**
214	A	K101K/E[d]			×	1.6	1.3	1.4
247	A/D	K103K/N[d]	×	×	×	1.2	0.7	1.1
095	A	K103K/N, V106V/A, Y181Y/C	×	×	×	1.8	1.4	**8.4**
288	D	Y188C	×	×	×	1.5	2.4	**16**
537	D	V106A	×	×	×	5.3	2.9	**88**
229	D	K103R, Y181Y/C	×		×	4.6	0.8	**6.9**
510	A	Y181Y/C, G190G/A	×	×	×	0.7	**6.5**	>**MAX**[f]
258	A	Y181Y/C, G190G/A	×	×	×	**29**	2.1	>**MAX**[f]
327	A/D	Y181Y/C, Y188Y/C[d]	×	×	×	**41**	2.1	**140**
559	A	K103K/N, V106V/A, Y181Y/C	×	×	×	**10**	1.9	**65**
362	A	K103K/N, Y181Y/C, G190G/A[d]	×	×	×	**37**	2.7	>**MAX**[f]

[a]NNRTI resistance mutations detected by the GeneSeq HIV assay are shown; mixtures are indicated (e.g., K101K/E).
[b]DLV, delavirdine; EFV, efavirenz; NVP, nevirapine; X indicates a prediction of reduced susceptibility in the GeneSeq HIV assay.
[c]The assay cutoffs for DLV, EFV, and NVP in the PhenoSense HIV assay are 6.2-, 3-, and 4.5 fold change IC_{50}, respectively. Results that are in bold indicate reduced susceptibility (i.e., the fold change IC_{50} of the test sample is greater than the assay cutoff).
[d]ViroSeq did not detect any NNRTI resistance mutations in samples 214 and 247. ViroSeq detected only Y181C in sample 327. ViroSeq detected only K103N and Y181C in sample 362.
[e]This was the only sample for which PhenoSense HIV measured reduced susceptibility to one or more of the NNRTIs that was not predicted by GeneSeq HIV; the L283I polymorphism, which was previously reported to reduce susceptibility,[27] was also present in this sample.
[f]MAX: Fold change could not be determined because the IC_{50} was greater than the highest drug concentration tested in the assay.

for one discordant sample was low (3977 copies/ml, sample 214), and sampling bias could have caused assay discordance between ViroSeq and GeneSeq HIV for detection of K101E (Table 2). All of the other 29 samples that had viral load data available had viral loads >20,000 c/ml. However, even in samples with high viral loads, discordant results may be obtained because of assay variability or stochastic sampling of resistant viruses in samples with minority resistant populations.

In three samples (343, 362, and 441) K103N was detected at very low levels by LigAmp (2.6–5.4% of the viral population), and was also detected by ViroSeq and GeneSeq HIV. In all three cases, the ViroSeq and GeneSeq electropherograms were consistent with very low levels of the mutant strains. In validation studies, ViroSeq reliably detected mixtures present at 40% or more of the viral population, when the viral load was between 2000 and 5000 c/ml,[12] and GeneSeq HIV has been reported to reliably detect mixtures present at 10–20%.[23] However, these assays clearly detect some mutations at lower levels in some samples. In a previous study, we analyzed samples with both ViroSeq and LigAmp for K103N detection. ViroSeq detected K103N in 71.4% of samples with a LigAmp result of 5–10% and in 16.9% of samples with a LigAmp result of 1–5%.[18]

In this report, LigAmp detected low-level NVP resistance mutations that were not detected by ViroSeq (12 mutations in 12 infants; only one of those mutations was detected by GeneSeq HIV). However, in 11 of those 12 infants, at least one other NVP mutation was detected by ViroSeq, so the LigAmp results did not change overall the resistance interpretation (yes/no for presence of NVP resistance). In other samples, LigAmp did not detect resistance mutations that were detected by ViroSeq (e.g., due to alternate codon use). Minority variants assays, such as LigAmp, are most useful in settings in which drug resistance mutations are likely to be present at low levels. For example, when we analyzed infants in the same cohort who were HIV infected after 6 weeks of age by breast-feeding (late-infected infants),[3] LigAmp identified NVP resistance in six of eight infants tested; ViroSeq did not detect resistance in any of those infants, reflecting the low levels of the NVP-resistant HIV variants in those samples.[3] LigAmp was also useful for analysis of the persistence of NVP resistance mutations in women and infants after sdNVP, since NVP-resistant variants often fade to levels that cannot be detected by ViroSeq within months of sdNVP exposure.[16,30] Unlike population sequencing-based genotyping assays, LigAmp also provides information on the level of mutations in the viral population. For example, we were able to use LigAmp to show that the level of NVP-resistant variants in women after sdNVP exposure is influenced by HIV subtype[31] and that repeated use of sdNVP is not associated with selection of higher levels of resistant variants.[3]

Unlike genotypic assays, the PhenoSense HIV assay provides a direct measure of drug susceptibility. We identified 12 samples with discordant results from GeneSeq HIV and PhenoSense HIV testing. In all but one case, GeneSeq HIV predicted resistance that was not detected by PhenoSense HIV. In 9 of 12 cases, the relevant mutations were present as mixtures. Previous studies have found genotypic assays to be more sensitive than phenotypic assays when resistant variants are present as mixtures.[23] Variations in phenotypic susceptibility are also seen with some drug resistance mutations,[23] which could explain detection of mutations in some samples that retain susceptibility in the PhenoSense HIV assay. Finally, some cases of genotypic–phenotypic discordance in non-subtype B samples may reflect limits in our understanding of resistance in diverse HIV strains.

This report provides a detailed comparison of four different resistance assays in a well-defined clinical cohort. In our cohort of 6-week-old infants exposed to NVP-based regimens for pMTCT, population sequencing-based methods (ViroSeq and GeneSeq HIV) were the most informative and had concordant results for 95% of the samples. LigAmp was useful for the detection and quantification of minority variants, but identified only one additional infant with NNRTI resistance. PhenoSense HIV provided a direct and quantitative measure of NNRTI susceptibility, but did not identify any additional infants with NNRTI resistance. In other settings, use of minority variants and phenotyping assays should be considered for analysis of resistance, depending on the study cohort, study design, and objectives.

Genbank Accession Numbers

The GenBank accession numbers for the 40 sequences produced by ViroSeq are EU380720–3, EU380725–6, EU380728–36, EU380738–45, EU380747–51, EU380754–64, and EU380766.

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Disclosure Statement

The following authors have commercial or other associations that might pose a conflict of interest: (1) Dr. Susan Eshleman is a co-inventor of the LigAmp assay and Johns Hopkins University has filed a patent application with the US-Patent and Trademark Office. The inventors may receive royalty payments if the patent is awarded and licensed. (2) Wei Huang and Neil Parkin are employees and stockholders of Monogram Biosciences, Inc. (provider of the GeneSeq HIV and PhenoSense HIV assays). (3) Natalia Marlowe is an employee of Celera and stockholder of Celera (manufacturer of

the ViroSeq HIV-1 Genotyping System). (4) Susan Eshleman is a member of the Clinical Advisory Board of Monogram Biosciences.

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Address correspondence to:
Susan Eshleman
Department of Pathology
The Johns Hopkins Medical Institutions
Ross Bldg. 646
720 Rutland Ave.
Baltimore, Maryland 21205

E-mail: seshlem@jhmi.edu