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Concordance between allele-specific PCR and ultra-deep pyrosequencing for the detection of HIV-1 non-nucleoside reverse transcriptase inhibitor resistance mutations

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Abstract

Recent advances in genotyping technologies have allowed for detection of HIV-1 drug resistance mutations present at low levels. The presence and percentage of Y181C and K103N drug-resistant variants in the blood of 105 subtype C HIV-infected infants who failed single-dose nevirapine prophylaxis for HIV transmission were compared using two highly sensitive genotyping methods, allele-specific PCR (AS-PCR) and ultra-deep pyrosequencing. Significant correlations in detection between both methods were found for both Y181C (correlation coefficients of 0.94 [95% CI 0.91-0.96]) and K103N (0.89 [95% CI 0.84 – 0.92]) mutations. The majority of discordant specimens (3/5 Y181C and 8/11 K103N) had wild-type variants when population sequencing was

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used, but mutant variants were detectable at very low levels ($\leq 5\%$) with either assay. This difference is most likely due to stochastic variations in the appearance of mutant variants. Overall, both AS-PCR and ultra-deep pyrosequencing methods have proven to be sensitive and accurate, and may confidently be used where feasible.

Keywords

HIV-1 drug resistance; Allele-specific PCR; Ultra-deep sequencing; low-frequency NNRTI variants

1. Introduction

HIV-1 drug resistance monitoring relies predominantly on sequence analysis of the polymerase (*pol*) gene to detect genetic mutations known to confer resistance to particular drugs (Clavel and Hance, 2004). While dideoxynucleotide (Sanger) sequencing of PCR amplicons remains the gold standard, and is routinely employed in commercial and in-house assays, it cannot reliably detect resistant variants when present at levels below $\sim 20\%$ in a mixed virus population. Sensitive methods, such as ultra-deep pyrosequencing (UDPS) have the capacity to detect low-frequency drug-resistance mutations in anti-retroviral exposed adult and pediatric patients that cannot be detected by Sanger sequencing methods (Simen et al., 2009; Lataillade et al., 2010; Moorthy et al., 2011). Whilst significant efforts are underway to reduce cost and ease work-flow (Ji et al., 2010), at present this technology remains resource-intensive. Other methods commonly used to detect low-frequency drug-resistance variants include real-time allele-specific PCR (AS-PCR) (Palmer et al., 2006; Johnson et al., 2007; Rowley et al., 2010). AS-PCR is designed such that the terminal 3'-nucleotide of a primer is allele-specific, allowing for sequence scrutiny at a point mutation polymorphism (Newton et al., 1989) using real-time PCR apparatus. The system is advantageous in that it is fast, inexpensive and can detect mutant species at frequencies below 1% (Johnson et al., 2007), but is limited by its requirement for adequate knowledge of the extent of sequence variation at the nucleotide site of interest for its application.

K103N and Y181C are the major nucleoside reverse transcriptase inhibitors (NNRTI) mutations selected under nevirapine (NVP) prophylaxis which is still used in low resource settings as a core component of interventions to prevent mother-to-child HIV-1 transmission (pMTCT) (Eshleman and Jackson, 2002; Martinson et al., 2007). We previously showed a high prevalence of age-related NNRTI resistance mutations persisting among infants and children as a result of exposure to single-dose nevirapine (sdNVP) prior to start of antiretroviral treatment (ART) with a protease inhibitor-based regimen (Hunt et al., 2011; Moorthy et al., 2011). Some of these children were successfully switched to a NVP-containing regimen after effective control of virus replication with PI-based therapy. Importantly, a significant association between ART failure and detection of NNRTI mutations in pretreatment samples by sensitive sequencing methods highlights the importance of assessing these mutations in infants (Coovadia et al., 2010; Moorthy et al., 2011; Taylor et al., 2011). For clinical and public health purposes, these, and other (MacLeod et al., 2010; Rowley et al., 2010), studies have indicated the need to accurately quantify the prevalence of NNRTI mutations in infants and young children at the time of

treatment initiation as these mutations may predict virologic response to NNRTI-based treatment.

The purpose of this study was to compare the concordance and quantity of the Y181C and K103N mutations detected by AS-PCR and UDPS methodologies in the cohort of infants undergoing switch to nevirapine-based ART mentioned above (Hunt et al., 2011; Moorthy et al., 2011) and to assess the utility of the more rapid, and cheaper AS-PCR assays in settings where this has been optimized.

2. Methods

2.1. Specimens from children infected with HIV

Plasma samples were collected between April 2005 and July 2007 as part of a randomized clinical trial designed to preserve NVP as a component of antiretroviral treatment for sdNVP-exposed children infected with HIV less than 24 months of age (Coovadia et al., 2010). Re-exposure to NVP as part of a treatment regimen was studied in the infants who controlled viral replication on a protease inhibitor, lopinavir (LPV)-based regimen (Coovadia et al., 2010; Kuhn et al., 2012). Children were enrolled at one clinical care center in Johannesburg, South Africa (Rahima Moosa Mother and Child Hospitals). From the 341 children enrolled in the clinical trial, pre-treatment plasma specimens were collected from 254 children. In 141 children, two closely-spaced pre-treatment specimens were collected prior to starting therapy. The earlier (or only available, n=254) timepoint samples were used for AS-PCR performed at the National Institute for Communicable Diseases in Johannesburg, South Africa (GH, LM) and the later (n=141) samples were processed for ultra-deep sequencing at Johns Hopkins University School of Medicine, Baltimore, USA (DP). The time difference between these two pre-treatment collection dates ranged from 0 to 90 days, with a median of 7 days.

2.2. Ethics Statement

Signed informed consent was obtained from the children's caregivers and the study was approved by the Institutional Review Boards of the University of the Witwatersrand (Neverest II, 040912) and Columbia University.

2.3. Genotyping of the polymerase gene

Sequencing of the *pol* gene was done as previously described using an in-house assay certified by the Virology Quality Assessment Program (VQA) (Pillay et al., 2008; Hunt et al., 2011). The procedure involves generation of a nested PCR amplicon spanning the entire protease and p66 and p51 regions of the reverse transcriptase genes. Genotypic resistance was defined as the presence of resistance mutations associated with impaired drug susceptibility, using the Stanford Genotypic Resistance Interpretation Algorithm (<http://hivdb.stanford.edu/>) and the December 2011 International AIDS Society drug resistance mutation list (Johnson et al., 2011). Phylogenetic analysis of nucleic sequences was performed using MEGA 5.05.

2.4. AS-PCR for K103N and Y181C

PCR products were tested for K103N and Y181C by AS-PCR as part of a previously published study (Hunt et al., 2011). Analysis of synthetic plasmid mixtures and treatment-naïve samples confirmed a detection cut-off of 0.2% for the minor variant of K103N (Loubser et al., 2006) and 0.35% for the Y181C assay (Hunt et al., 2011). All positive samples were retested twice and only those positive on all three repeats were considered true positives. Unusual polymorphisms at the 103 position (K103R and K103T) were shown not to compromise the K103N assay, but polymorphisms at the 181 position (Y181F/S) produced false positive results so the one sample with this polymorphism was excluded from analysis.

2.5. Genotyping of the polymerase gene by ultra-deep sequencing

Previously published ultra-deep sequencing data (Moorthy et al., 2011) were used in this comparison. Briefly, viral RNA was extracted from 140µl plasma using column-based nucleic acid isolation (Qiagen, Valencia, CA) and cDNA synthesized using subtype C gene specific primers. Four single-round PCR reactions were performed from cDNA for amplification of RT regions encompassing amino acid positions 98-108 and 181-190. Sequences generated by UDPS were analyzed using GS Amplicon Variant Analyzer software (Simen et al., 2009). A threshold level of 1% was set as the lower limit of detection of drug resistance mutations by UDPS (Moorthy et al., 2011).

2.6. Statistical analyses

Inter-rater agreements were calculated using Cohen's kappa coefficient (STATA v11, StataCorp, College Station, USA). Pearson correlation coefficients were performed using GraphPad InStat v3.06, San Diego, USA.

3. Results

3.1. Available datasets and assay cut-offs

Plasma samples from HIV-1 infected infants <2 years of age who had been exposed to sdNVP for pMTCT were genotyped using three different methods in two laboratories. Both laboratories performed population-based sequencing to assess the increased sensitivity afforded by AS-PCR (Hunt et al., 2011) and UDPS (Moorthy et al., 2011). Due to low volumes, samples from two different time-points, which were on average 7 days apart, were used for the AS-PCR and UDPS. For this study we made use of these available datasets to directly compare AS-PCR and UDPS for detection of Y181C and K103N, the major mutations selected following sdNVP.

Data from 141 specimen pairs were available for comparison between AS-PCR and UDPS testing. Only samples successfully tested by both methods and from which phylogenetic analysis of standard population genotypes confirmed patient-specificity were included in the analysis of concordance between the two genotyping approaches (n=105). Thirty-six specimens were excluded from analysis for the following reasons: 12 specimens had viral loads that were too low for UDPS (<100,000 copies/mL) at the time of study (Simen et al., 2009), UDPS failed in 7 specimens, the Y181C AS-PCR was not successful in 2 specimens,

1 specimen had a false positive Y181C AS-PCR due to a Y181FS polymorphism, and phylogenetic relatedness between plasma sequences from both laboratories and UDPS consensus sequences was not confirmed in 14 specimens.

3.2. Frequency of Y181C and K103N mutant detection using different technologies

The total number of samples where the Y181C and K103N mutations were detected by population sequencing, AS-PCR and UDPS are shown in Table 1A. Given that population sequencing generally only detects mutant populations when they appear in more than 20% of the quasiespecies we chose this cut-off to categorize data from AS-PCR and UDPS as high (>20%) or low (<20%). Y181C was detected by population sequencing in 22 of 105 (21%) specimens. All of these plus an additional 6 infants were positive by the Y181C AS-PCR (mean quantitative percentage value measure (μ)=43.28%). Of these 28 AS-PCR positives (28/105, 27%), 19 were considered at a high level (20.11% – 99.97%; μ =69.99%) and 9 at low levels (0.65% - 16.03%; μ =5.65%). Slightly fewer samples, 25/105 (24%) were detected by UDPS, 16 at high levels (25.37% - 99.73%; μ =67.70%) and 9 at low levels (1.30% - 19.60%; μ =13.40%).

The K103N mutation was detected in 4 infants (4%) by population sequencing, in 9 infants (9%) by AS-PCR (4 at high levels [20.56% - 99.93%; μ =69.70%] and 5 at low levels [0.60% - 15.06%; μ =1.14%]), and in 8 specimens by UDPS (8%; 3 at high levels (31.38% - 99.60%; μ =82.60%) and 5 at low levels (1.00% - 14.62%; μ =1.80%)). Overall these data confirm the increased sensitivity of low-frequency drug resistance assays over standard genotyping. These data also confirm the higher prevalence of Y181C relative to K103N in infants who receive sdNVP for pMTCT.

A comparison of qualitative outcome (i.e. positive or negative for mutation detection) between AS-PCR and UDPS for each loci was performed using a contingency analysis for which a kappa statistic was also calculated (Table 1B). Twenty-four specimens were positive and 76 negative for Y181C in both assays, and 3 positive and 91 negative for K103N. Five samples showed discordance for Y181C and 11 for K103N between the 2 assays. The kappa statistic for detection of the Y181C mutation using AS-PCR and UDPS was determined to be 0.8739 (standard error 0.0973), with very good strength of agreement between the AS-PCR and UDPS assays. The kappa statistic for detection of K103N was 0.2962 (standard error 0.0974) with only fair agreement between the two assays.

3.3. Correlation of quantitative levels of Y181C and K103N detection between AS-PCR and UDPS

The quantitative percentage values of Y181C and K103N mutants as determined by AS-PCR and ultra-deep sequencing were compared for all 105 samples (Figure 1). The assay results were significantly correlated for detection of both mutations ($p<0.0001$), with correlation coefficients of 0.94 (95% CI 0.91-0.96) and 0.89 (95% CI 0.84 – 0.92) for Y181C and K103N respectively. Although fewer samples were positive for K103N, the trend line showed a clear correlation between data from both assays. Samples which were positive in only one assay (the discordant samples) aligned along the cut-offs for each assay

away from the trend line. The K103N showed a larger number of discordances compared to Y181C.

An analysis of quantitative percentage value of each assay plotted against the difference between the two assays showed no evidence of clustering at either end of the spectrum. This suggests that there were no systematic differences between assay values. In addition, the same analysis of each assay plotted against the time difference between each specimen collection showed no evidence of variation in mutation quantification (data not shown).

3.4. Analysis of discordance between AS-PCR and UDPS

The samples that were discordant for Y181C or K103N between the 2 technologies were further investigated (Table 2). Five specimens were discordant for Y181C and 11 for K103N. There was no overlap in 14 samples in that discordance for one mutation did not yield discordance for the other. One sample was discordant in both assays (Sample #7).

Y181C was not detected by UDPS in four specimens that were positive by AS-PCR, of which two were also positive by population genotyping of the same amplicon. The remaining two samples had AS-PCR values that were on the limit of detection for UDPS, and so were considered minor discrepancies. In these cases, the sample used for UDPS were 25 and 41 days later than those used for AS-PCR which may have contributed to the lower sensitivity of UDPS, particularly given that Y181C fades relatively quickly (Eshleman et al., 2004). One additional specimen showed minor discordance for Y181C in that the mutation was detected by UDPS at 2.62% but negative by AS-PCR, suggesting in this case the AS-PCR was less sensitive.

Of the 11 specimens discordant for K103N, three had major discrepancies. One of these (sample 3) was positive for K103N by population-sequence genotyping and AS-PCR, but the mutation was not detected by UDPS. Six samples were positive by AS-PCR but were negative by UDPS. Three of these were on the borderline of sensitivity for UDPS (1.14%, 0.60% and 0.89%). Sample 11 had an unusual polymorphism (K103T) which alternatively may have contributed to a false positive in the AS-PCR assay. K103N was detected in 5 samples by UDPS and not by AS-PCR. One of these had high levels (31.38%) that should have been detected by population-genotyping. The other four samples were 2% or less by UDPS and may reflect higher sensitivity of UDPS for K103N compared to AS-PCR in these cases. Two specimens with minor discordant K103N results had Y181C detected equally by AS-PCR and UDPS (data not shown).

Of note, two specimens with highly discrepant results for K103N each had unusual polymorphisms at position 102 (K102T in specimen #3 and K102R at specimen #5). While this site was outside of the primer binding region of the assay, these polymorphisms may have interfered with either assay.

4. Discussion

In this study we have shown that the detection of minority species of Y181C and K103N can be accurately measured by AS-PCR and UDPS. Previous studies have reported significant

correlations in frequency and copy number of K103N mutants quantified in parallel by AS-PCR and UDPS technologies in 11 adult patients treated with efavirenz (EFV) as part of the ANRS 106 trial (Delobel et al., 2011), and concordant UDPS and AS-PCR results in 20 infants exposed to sdNVP for pMTCT when resistance is present at high frequencies only (Lehman et al., 2012). Our data indicate significant correlation between the two methods to measure the quantitative percent values of each mutation in the heterogeneous viral quasispecies population present in a large cohort of infants following exposure to sdNVP. Specifically, our data were significantly correlated for detection of both mutations ($p < 0.0001$), with correlation coefficients of 0.94 and 0.89 for Y181C and K103N respectively. The kappa statistic for detection of Y181C was 0.8739 (standard error 0.0973), with very good strength of agreement between the AS-PCR and UDPS assays, and 0.2962 (standard error 0.0974) for K103N indicating only fair agreement between the two assays for the latter mutation. This statistic may have been unduly influenced by the low number of K103N positive results ($n=14$).

The majority of discordant specimens had concentrations of mutant virus that were close to the limit of detection of each assay, suggesting that detection of minor variants were due to stochastic variations in the appearance of each mutant in each of specimens tested. Five specimens showed significant levels of non-correlation, two for Y181C and three for K103N. Appropriate quality assurance procedures were followed in conducting both analyses, including gel-electrophoresis of PCR products and melting temperature interrogation of real-time PCR products. Both methods, being PCR-based, are dependent on the success of the initial PCR and optimal binding of primers to equally representative proportions of the variant sample to be tested. Polymorphisms in the sequence adjacent to the site of interest are known to cause inefficient amplification in AS-PCR, possibly leading to inaccurate rather than inconclusive results (Boltz et al., 2009). On the other hand, error rates with UDPS have been reported to vary between 1 and 50%, depending on several experimental variables including presence of homopolymers, position in sequence and size of sequence (Gilles et al., 2011). Given that codons 101 – 104 of *reverse transcriptase* consist of two 6-nucleotide adenosine strands, it is possible that sequencing and alignment errors immediately up- or downstream of codon 103 could result in incorrect amino acid assignment. Higher pyrosequencing error rates have been reported in homopolymeric sequence regions, but are usually characterized by insertions or deletions in sequence reads (Balzer et al., 2011). As such this region could be more prone to sequence error. All these factors need to be considered when designing and improving assays to detect mutations associated with HIV-1 drug resistance.

5. Due to small sample volumes from children, this study used data from specimens collected at two different pre-treatment visits. The time-interval between these two specimens was 0-90 days with a median of 7 days. The analysis showed no impact of this time difference in specimen collection on the rates of discordance or concordance between the assays. Amongst the discordant specimens the average time delay in specimen collection was 10 days as opposed to 7 days in the concordant specimens. Eight specimens were collected more than one month apart of which only one showed minor discordance for

Y181C. Thus these time delays in specimen collection could not be responsible for variation in mutation detection.

Conclusion

The application of ultra-sensitive methods to assess the levels of minority resistant variants remains an area of debate, and consequently these assays are not routinely used in clinical practice or surveillance. The clinically relevant threshold above which their presence may affect virological outcomes is unclear. Allele-specific PCR is quick, inexpensive, and does not require sophisticated instrumentation and data interpretation, but scrutinizes only one mutation codon at a time and is sensitive to genetic variation in the primer-binding region which may produce inaccurate results (Boltz et al., 2009). Modifications in primer sequence can compensate for genetic variation in specimens analyzed. On the other hand, UDPS provides a comprehensive analysis of all codons in the region sequenced, but the methodology is highly resource-intensive and data analysis more complex. AS-PCR may prove more useful when conducting surveillance-based studies to assess the frequencies of specific mutations in response to different treatment regimens, whereas UDPS may be more applicable when comprehensive assessment of drug resistance mutations at known sites are required for clinical management purposes. This study has shown that both methods are highly sensitive, and display excellent correlation in their detection of mutations present at low frequencies.

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Highlights

- Significant correlations in detection of Y181C and K103N, using allele-specific PCR (AS-PCR) and ultra-deep pyrosequencing
- Results indicate a high degree of accuracy and sensitivity in both methods
- The application of either methodology may be feasible in certain scenarios

Quantitative Y181C mutation percentage value as measured by UDS

Figure 1. Comparison of levels of Y181C and K103N mutant genotypes as measured by AS-PCR and UDPS

Percentage of Y181C (A) and K103N (B) measured by AS-PCR and USD in 105 infant samples. The trend line shows a positive correlation for both genotypes.

Table 1A
Detection of Y181C and K103N mutations by population sequencing, allele-specific (AS-PCR) and ultra-deep sequencing (UDPS) in infants exposed to sdNVP

	Population Sequence	Method of analysis	
		Allele-Specific PCR	Ultra-Deep Sequencing
Number of samples with Y181C			
Detected/Above detection threshold	22	28	25
High Level (at 20% total quasispecies population)		19	16
Low Level (at <20% total quasispecies population)		9	9
Number of samples with K103N			
Detected/Above detection threshold	4	9	8
High Level (at 20% total quasispecies population)		4	3
Low Level (at <20% total quasispecies population)		5	5

Table 1B
Kappa statistical calculation of Y181C and K103N mutations detected by AS-PCR and UDPS

Y181C	AS-PCR Positive	AS-PCR Negative	TOTAL	K103N	AS-PCR Positive	AS-PCR Negative	TOTAL
UDPS Positive	24	1	25	UDPS Positive	3	5	8
UDPS Negative	4	76	80	UDPS Negative	6	91	97
TOTAL	28	77	105	TOTAL	9	96	105
K = 0.8739			K = 0.2962				

Table 2
Analysis of samples showing discordance between AS-PCR and UDPS

Study ID	Percent Y181C detected by AS-PCR	Population sequence genotype*	Percent Y181C detected by UDPS	Days between sample collection
<i>Major Discordance</i>				
1	36.77	Y181C	<1.00	0
2	8.98	Y181C	<1.00	25
<i>Minor Discordance</i>				
3	1.00	Y181	<1.00	41
4	0.88	Y181	<1.00	8
5	<0.35	Y181	2.62	12
Study ID	Percent K103N detected by AS-PCR	Population sequence genotype*	Percent K103N detected by UDPS	Days between sample collection
<i>Major Discordance</i>				
6	54.33	K103N	<1.00	6
7	15.06	K103	<1.00	7
8	<0.20	K103	31.38	7
<i>Minor Discordance</i>				
4	5.34	K103	<1.00	8
9	1.14	K103	<1.00	9
10	0.60	K103	<1.00	14
11	0.89 (K103T)	K103	<1.00	9
12	<0.2	K103	2.02	7
13	<0.2	K103	1.8	0
14	<0.2	K103	1.26	9
15	<0.2	K103	1	9

* Data obtained from sample used for AS-PCR.