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Next generation sequencing improves detection of drug resistance mutations in infants after PMTCT failure

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ABSTRACT

Background: Next generation sequencing (NGS) allows the detection of minor variant HIV drug resistance mutations (DRMs). However data from new NGS platforms after Prevention-of-Mother-to-Child-Transmission (PMTCT) regimen failure are limited.
Objective: To compare major and minor variant HIV DRMs with Illumina MiSeq and Life Technologies Ion Personal Genome Machine (PGM) in infants infected despite a PMTCT regimen.
Study design: We conducted a cross-sectional study of NGS for detecting DRMs in infants infected despite a zidovudine (AZT) and Nevirapine (NVP) regimen, before initiation of combination antiretroviral therapy. Sequencing was performed on PCR products from plasma samples on PGM and MiSeq platforms. Bioinformatic analyses were undertaken using a codon-aware version of the Smith–Waterman mapping algorithm and a mixture multinomial error filtering statistical model.
Results: Of 15 infants, tested at a median age of 3.4 months after birth, 2 (13%) had non-nucleoside reverse transcriptase inhibitor (NNRTI) DRMs (K103N and Y181C) by bulk sequencing, whereas PGM detected 4 (26%) and MiSeq 5 (30%). NGS enabled the detection of additional minor variant DRMs in the infant with K103N. Coverage and instrument quality scores were higher with MiSeq, increasing the confidence of minor variant calls.
Conclusions: NGS followed by bioinformatic analyses detected multiple minor variant DRMs in HIV-1 RT among infants where PMTCT failed. The high coverage of MiSeq and high read quality improved the confidence of identified DRMs and may make this platform ideal for minor variant detection.

Abbreviations: DRMs, drug resistance mutations; NGS, next generation sequencing; PGM, Ion Personal Genome Machine; MiSeq, Illumina MiSeq Sequencer; PMTCT, prevention of HIV mother to child transmission; cART, combination antiretroviral therapy; LPV/r, lopinavir/ritonavir co-formulation; sdNVP, single dose nevirapine.

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1. Background

Between 2004 and 2010, a dual regimen of Zidovudine (AZT) and Nevirapine (NVP) was used to prevent mother to child transmission of HIV (PMTCT) in the Western Cape Province, South Africa [1]. During our study period (October 2006–October 2009), mothers received AZT from 28 weeks of gestation and single dose NVP (sdNVP) intra-partum, while the neonate received sdNVP, AZT for 1 week and was formula fed. The HIV transmission rate was <10% during this period [1]. In 2010, the National guidelines replaced infant sdNVP with daily NVP for the first 6 weeks of life and the PMTCT-failure rate decreased to <3% [2]. Further, in 2013 the WHO option B plus, which recommends lifelong combination antiretroviral therapy (cART) for pregnant women regardless of CD4 count or disease stage was adopted in the Western Cape in order to decrease the PTMCT failure rate even further.

Children, infected despite prophylactic antiretrovirals, are at high risk of acquiring antiretroviral drug resistance mutations (DRMs) [3]. Even low frequency non-nucleoside reverse transcriptase inhibitor (NNRTI) DRMs could affect NNRTI-containing regimen outcomes [4–7]. In South Africa, however, all children, under the age of 3 years, receive protease inhibitors (lopinavir/ritonavir [LPV/r]) in the first-line regimen [8,9]. The prevalence of minor variant DRMs to NVP, nevertheless, remains important where there is limited access to LPV/r infant formulations or where NNRTIs are required in second-line regimens.

Various investigations have employed allele-specific real-time PCR or oligonucleotide ligation assays (OLA) for detecting minor variant DRMs after NVP PMTCT exposure [6,10–12]. Despite the reported sensitivity, the utility of these methods is limited by mismatches in primer binding [13], and by a limit to the number of reactions that can be multiplexed. Next generation sequencing (NGS) offers an attractive alternative to potentially detect all DRMs across the HIV-1 *reverse transcriptase* (RT) coding region. The read lengths of modern NGS systems including Roche 454 (454 Life Sciences, Branford, CT, USA), Ion Torrent Personal Genome Machine (PGM) (Life Technologies, Carlsbad, CA, USA) (PGM) and MiSeq (Illumina, San Diego, CA, USA), also permit the study of linkage between some DRMs [14]. A recent study found good correlation of Roche 454 sequencing for K103N and Y181C, when screening PMTCT exposed children (less than 2 years of age) prior to cART initiation [15]. We conducted the first investigation, to our knowledge, comparing bulk sequencing to Ion PGM and MiSeq in investigating DRMs after PMTCT exposure.

2. Objective

To compare major and minor variant HIV DRMs with NGS *via* Illumina MiSeq and Life Technologies Ion Personal Genome Machine platforms in infants who failed a dual AZT and NVP PMTCT regimen.

3. Study design

3.1. Patients

We conducted a retrospective study in 15 HIV-infected infants, born from October 2006–October 2009, who became infected despite a regimen of maternal AZT from 28 weeks gestation, sdNVP, intrapartum, and neonatal sdNVP and 7 days of AZT.

3.2. Specimen processing, reverse transcription and cDNA quantification

Baseline plasma specimens, prior to cART, were collected and nucleic acids were extracted on the NucliSENS® Easymag® (BioMérieux, Craponne, France). Bulk sequencing was undertaken using in-house PCR and Sanger Sequencing [16]. For PGM and MiSeq deep sequencing, RNA was reverse transcribed with pentadecamers and SuperScript® III (Invitrogen, Carlsbad, CA, USA), cDNA was quantified with real-time PCR on the ABI 7900HT (Applied Biosystems, Carlsbad, USA) with a SYBR green mastermix and forward primer: RGCTCTMTTAGAYACAGGAGCAGAT (HXB2 position: 2315–2339) and reverse primer: ACTTTGATAAAACCTCCAATTCCYCC (HXB2: 2419–2394).

3.3. Library preparation and sequencing for NGS

Each cDNA sample was amplified in parallel through 14 pre- and 7 nested PCRs using Expand High Fidelity^{Plus} (Roche, Basel, Switzerland). Outer primers were: TCAGAGCAGACCAGAGCCAACAGCCCCA (HBX2: 2136–2163) and CCTACTAACTTCTGTATGTCATTGACAGTCCAGCT (HBX2: 3334–3300); Inner primers were: CTCTCTTAGACACAGGAGCAGAT (HBX2: 2317–2340); CCATTTGTCAGGATGGAGTTCATA (HBX2: 3267–3243)

3.3.1. PGM

Amplicons were gel-separated and purified prior to enzymatic shearing, adapter and barcode ligation and size selection. The Ion Xpress Plus library preparation manual, rev. M was followed for library construction. In short, the amplicons were digested using Ion Shear Enzyme II followed by barcode ligation and size selection, for an average insert size of 200 bp, using the E-Gel system (Invitrogen). Template enrichment and sequencing was performed according to manufacturer recommendations with the Ion 200 Sequencing V1 kit.

3.3.2. MiSeq

The Nextera XT DNA sample preparation manual, rev. C was followed for library construction. In short, the digestion and adapter ligation was performed simultaneously followed by the addition of indexes *via* PCR. Size selection of 300–500 bp fragments was performed using Ampure XP reagent (Beckman-Coulter). Sequencing was performed using the MiSeq Reagent Kit v2.

3.4. NGS bioinformatics

Reads were filtered using instrument quality scores and aligned to a subtype C reference sequence using a codon-aware version of the Smith–Waterman algorithm that corrects for homopolymer errors by considering both nucleotide and amino-acid homology, and directly penalizes for length miscall [17]. A Bayesian Dirichlet mixture of multinomials probabilistic model was used to distinguish sequencing error from true low-frequency variants (posterior probabilities of $\geq$99.99%) [18]. For each sample, we computed the mean of all pairwise Tamura-Nei 93 distances between reads with at least 100 overlapping base pairs to quantify nucleotide diversity [19]. We constructed a maximum likelihood tree (GTR + CAT model in FastTree), based on the first 630 nucleotides of *reverse transcriptase* (the region that was uniformly well-covered for all sequencing runs) using NGS majority consensus and Sanger bulk sequences.

Bulk sequencing was with an in-house genotyping method, with sequencing from nested PCR products, and spanning RT amino acid positions 1–262 [16]. As quality control for NGS, we performed clonal sequencing of PCR products (the same as for bulk Sanger