Chapter 5

The application of Seq2Res to evaluate ultra deep pyrosequencing as a large-scale, cost-effective alternative to conventional HIV resistance genotyping

# Introduction

HIV exists in an infected individual as a complex heterogeneous population called quasispecies (Yin et al., 2012) primarily arising as a result of HIV’s high replication rate and the high error rate of the viral reverse transcriptase (Bebenek et al., 1993; Ji and Loeb, 1992; Preston et al., 1988). During the replication process, HIV develops random mutations (Bebenek et al., 1989; Bebenek et al., 1993; Berkhout et al., 2001; Roberts et al., 1988) in its genes that can result in viral resistance against one or more antiretroviral drugs (Clavel and Hance, 2004; D’Aquila et al., 2003; Kantor and Katzenstein, 2004; Sebastian and Faruki, 2004). Viral variants containing DRMs can be present at varying levels in the viral quasispecies (Devereux et al., 1999; Johnson et al., 2008; Metzner et al., 2009) with these variants emerging to dominate the viral population in response to treatment (Adje et al., 2001; Adje-Toure et al., 2003; Johnson et al., 2008; Marconi et al., 2008).

Approximately 8 million HIV infected individuals in resource-limited countries are receiving antiretroviral therapy by the end of 2012 (UNAIDS, 2012) following the scale-up of treatment programmes in 2002 (Beck et al., 2006; Ferradini et al., 2006; Gilks et al., 2006; Stringer et al., 2006). The number of HIV infected individuals with transmitted drug resistant mutations (DRMs) is increasing (Aghokeng et al., 2011; Phillips et al., 2013; Zaidi et al., 2013) with studies showing that while antiretroviral (ART) drugs increase life expectancy of infected individuals, this increase life expectancy increases the risk of transmission of drug resistant HIV variants to uninfected individuals (Zaidi et al., 2013).

The history of HIV treatment in 1980s has shown that the therapy with a single dose antiretroviral drug or a combination of drugs from a single drug class usually results in treatment failure (Kellam et al., 1994; Larder et al., 1989; Larder et al., 1991; Larder and Kemp, 1989; Larder et al., 1987). This has shown that administration of a single antiretroviral drug selects the drug resistant variants and increases the chance of rapid drug failure (Hamers et al., 2012; Jackson et al., 2000; Partaledis et al., 1995; Tisdale et al., 1993). A perfect example of this is the emergence of resistance in single dose nevirapine (NVP) programmes. NVP, an NNRTI, is prescribed to HIOV positive pregnant woman in order to prevent HIV transmission from mother to child in resource poor settings (Audureau et al., 2013; Chi et al., 2013; Shapiro et al., 2010; Stringer et al., 2010a; Zolfo et al., 2010). The drug is effective in reducing the viral transmission as has been reported in several research reports (Connor et al., 1994a; Connor et al., 1994b; Guay et al., 1999; Jackson et al., 2003; Lallemant et al., 2004). However, studies have shown that the use of single dose NVP to prevent mother to child transmission of the virus showed distinct patterns of emergence and fading of nevirapine associated resistant mutations (Coovadia et al., 2009; Eshleman et al., 2004; Eshleman et al., 2005; Eshleman et al., 2001; Flys et al., 2005; Havlir et al., 1996; Jackson et al., 2000; Loubser et al., 2006; Martinson et al., 2007; Richman et al., 1994; Tisdale et al., 1993). The persistence of NVP resistant virus in the mothers and children treated with single dose NVP (Hauser et al., 2011) compromises the treatment with subsequent NVP containing highly active antiretroviral therapy (HAART) (Arrive et al., 2007; Chi et al., 2007; Lehman et al., 2012; Martinson et al., 2007). Thus, studies have shown that the first line therapy, which is a combination of at least three fully active ART drugs from different drug classes – Non-Nucleotide Reverse Transciptase Inhibitors (NNRTIs) and Nucleotide Reverse Transcriptase Inhibitors (NRTIs) are necessary for optimum suppression of HIV from replication and resistance development (Gupta et al., 2009; Hamers et al., 2012; Robbins et al., 2003; Shafer et al., 2003; van Leeuwen et al., 2003; van Leth et al., 2004). For this, the World Health Organization (WHO) recommends drug resistance testing before prescribing ART drugs.

Resistance testing reveals the drug resistance mutations in the HIV quasispecies. HIV with drug resistant mutations is present at varying prevalence levels in the quasispecies. The conventional HIV resistance genotyping is limited to detecting the mutations in HIV with prevalence of 20% or greater (Booth and Geretti, 2007; Liang et al., 2011; Wang et al., 2007). Ultra deep pyrosequencing (UDPS) technology has ability to detect HIV with prevalence to as low as 1% or below (Archer et al., 2009; Balduin M, 2011; Bansode et al., 2013; Dudley et al., 2012; Gilles et al., 2011; Hedskog et al., 2010; Hoffmann et al., 2007; Huse et al., 2007; Ji et al., 2012; Ji et al., 2010; Lataillade et al., 2010; Le et al., 2009; Liang et al., 2011; Wang et al., 2007).

Here, we describe the application of the computational tool Seq2Res to HIV resistance testing to a significant dataset generated using UDPS.

# Methods and Materials

The datasets used in this study had been generated as part of the CIPRA-SA study (Comprehensive International Program for Research in AIDS in South Africa) which was a prospective, unblinded, randomized controlled trial of comparing “doctor-initiative-doctor monitored” and “doctor-initiative-nurse-monitored” strategies for antiretroviral drug monitoring in resource poor setting (Sanne et al., 2010). The study population consisted of 831 HIV infected individuals with a CD4+ count less than 350-cells/mm3 or AIDS-defining illness were enrolled on the study. HIV positive mothers with previous exposure of single dose nevirapine (NVP) drug for prevention of viral transmission from mother to child (PMTCT) during their pregnancy were also included in the study.

562 patients were followed up with the remainder not included in the study for reasons such as drug toxicity, death, and withdrawal of consent or loss to follow-up. Baseline blood samples were retrieved from all 562 patients (sampled from 2005 – 2006). In this instance, baseline describes samples obtained from individuals immediately before initiation of first line antiretroviral therapy (ART). 71% of these patients received the drug combination D4T-3TC-EFV, 20% received D4T-3TC-NVP, 8% received D4T-3TC-LPV/r and 1% received D4T-3TC-NLF.

Virologic failure (VF) to the treatment was defined as decline of viral load less than 1.5 log10 from baseline to 12 weeks of treatment or two consecutive samples from a patient taken four weeks apart have viral load greater than 1000 RNA copies/ml (Sanne et al., 2010). VF to first line ART was identified in 79 patients, with 15 patients failing second-line therapy (Sanne et al., 2010). Blood samples had been retrieved for all of these individuals upon failure detection.

From all the obtained samples, the entire protease and reverse transcriptase genes of HIV had been amplified as three fragments - PR (HXB2 *pol* position 169 - 480) RT1 (HXB2 *pol* position 466 – 795) and RT2 (HXB2 pol position 796 – 1185) using HIV subtype C specific primers. Ten UDPS sequencing runs using the Roche/454 Junior (hereafter only Junior) platform had been attempted for 471 samples using MID tags to pool 48 samples per sequencing plate. Further, sequencing was attempted for 630 samples using the FLX platform (hereafter only FLX). 12 FLX runs were undertaken, dividing each plate into 8 distinct sections with 8 MID tagged samples per section for each sequencing run.

Conventional genotyping results were also available for 349 of the samples. All of the sequence data had been generated by our collaborators in the laboratory of Prof Maria Papathanasopoulos at the University of the Witwatersrand Medical School, South Africa.

Sequence data from all samples (baseline and first line VF samples) were analyzed using Seq2Res. To facilitate direct comparisons with the Sanger data the prevalence cutoff was set to 20%, consistent with the reported ability of Sanger-based sequencing to detect resistant variants to a level of 20% in the viral population (Hudelson et al., 2010; Larder et al., 1993; Leitner et al., 1993; Schuurman et al., 1999; Van Laethem et al., 1999). The presence of resistance in the UDPS genotypic data was further explored at prevalence levels of 15%, 10%, 5% and 1% of the amplified and sequenced viral population.

Every sequence read was tested for drug resistance using the Stanford HIV drug resistance database resistance interpretation algorithm (Liu and Shafer, 2006; Rhee et al., 2003; Shafer, 2006). The percentage of sequences that were predicted as resistant for a particular antiretroviral drug by the algorithm was calculated. Using a number of prevalence cutoffs (20%, 15%, 10%, 5% and 1%), if the percentage of sequences predicted as resistant to a drug from the total number of sequences analyzed for a sample, was greater or equal to the cutoff, the sample was predicted resistance for that drug.

As the baseline regimens of each of the individuals was known, resistance was defined as predicted resistance to one or more of the drugs in the individuals regimen. In the very rare event of an individual’s regimen not being recorded, resistance was defined as predicted resistance to one or more of the entire spectrum of drugs used as first line therapy in the study (D4T, 3TC, EFV, NVP, LPV/r or NLF).

In the case of VF, samples that failed the first line therapy, resistance was defined as predicted resistance to one or more of the drugs in the first line therapy. Similarly, individuals that failed second line therapy were predicted resistant if one or more of the drugs in the second line therapy were predicted to be resistant.

The number of samples with and without predicted resistance was obtained and statistical significance was calculated using Fisher’s exact test .

# Results

In the preliminary assessment of the sample’s sequence data from FLX and Junior, the samples in which protease (PR) or reverse transcriptase (RT) or both were not amplified were not considered for subsequent analysis. A total of 599 samples and 468 samples that were sequenced in FLX and Junior respectively from both baseline and first line VFs had both PR and RT sequences (**Table 5.1**) and were considered for analysis.

Out of the samples that were eligible for analysis, 464 samples were sequenced using both the FLX and Junior platforms, 327 samples using both the FLX and population based Sanger genotyping method and 257 samples with both the Junior and population based Sanger genotyping method (**Table 5.2**).

## Analysis of baseline samples

### Genotyping of baseline samples using the Roche/454 FLX sequencing platform

FLX sequencing was successful for baseline samples from a total of 526 patients of which 187 samples had previous ARV exposure as a result of PMTCT therapy while the remaining 339 had no previous exposure to ARVs. The eventual clinical outcome of all of these individuals was known and showed that out of the 339 no-PMTCT patients, 50 had exhibited VF while 289 exhibited virologic success (VS) during the course of follow-up. On the other hand, out of 187 PMTCT exposed patients, 25 exhibited VF and 162 exhibited VS.

The number of samples with and without predicted drug resistant HIV in the PMTCT and no-PMTCT groups at varying prevalence cutoffs (1%, 5%, 10%, 15% and 20%) that were predicted drug resistant to at least one baseline drug increased when the prevalence cutoff was decreased to 1% (**Figure 5.1**). In the no-PMTCT VF group of 50, there was only one sample exhibiting resistant virus to at least one baseline drug at prevalence level range 5% to 20% but this increased to five samples (10%) at prevalence level of 1%. Similar increments in the number of individuals predicted as resistant as the prevalence levels decreased were observed in other groups as well (**Figure 5.1**).

At the 1% prevalence cutoff in the no-PMTCT group there was a significant difference between the number of clinical viral failures predicted as resistant when compared with the number of clinical viral successes predicted as resistant (p < 0.05, Fisher’s exact test) while a similar observation was observed at the 15% prevalence cutoff in the PMTCT exposed group (**Figure 5.1**).

### Genotyping of baseline samples using the Roche/454 Junior sequencing platform

407 patients were sampled at baseline and sequenced using Junior, 250 patients had no previous ARV exposure through PMTCT and 147 patients had previous PMTCT therapy. The clinical outcome showed that out of 250 non-PMTCT patients, 40 had VF and 210 had VS. On the other hand, out of 147 PMTCT exposed patients, 21 had VF and 136 had VS (**Figure 5.2**).

The obtained baseline blood samples were sequenced using Junior and again analyzed using Seq2Res. The observation on the number of samples with and without predicted resistant HIV showed that in the no-PMTCT VF group of 40, there was only one individual predicted resistant to at least one drug in baseline at prevalence cutoff 20% and that increased to four individuals at prevalence cutoff 1% (**Figure 5.2**). Similar increments in the number of samples with predicted resistant HIV was observed in the no-PMTCT VS group as well as in PMTCT VF and VS groups (**Figure 5.2**). The highest number of samples (16 samples) with predicted resistant HIV was observed in PMTCT VS group at a prevalence cutoff 1%. Further, in the No-PMTCT group there was a significant difference between the number of clinical viral failures predicted as resistant when compared with the number of clinical viral successes predicted as resistant (p < 0.05, Fisher’s exact test, **Figure 5.2**).

### Comparison of number of sequence reads per baseline sample generated by FLX and Junior

Sequencing had been successful on both FLX and Junior for 464 samples (**Table 5.2**). The initial analysis focused on comparing the number of sequence reads generated by each platform for each sample and identifying if ‘deeper’ sequencing coverage resulted in more accurate prediction of resistance. We saw that the FLX platform generated on average 6412 sequence reads per sample (standard deviation 2297) while the Junior platform generated an average 1903 sequence reads per sample (Standard deviation 595, **Figure 5.3**). Thus, it is clear that the FLX platform produced significantly (P-value < 2.2-16) more reads per sample than the Junior platform.

### Comparison of genotyping results between the FLX and Junior platforms on baseline samples

Baseline samples from 405 individuals had been sequenced using both the FLX and Junior platforms. 249 had no previous PMTCT therapy while 156 had previous exposure to ARVs as a result of PMTCT therapy. Of these 249 patients, 40 exhibited VF and 209 exhibited VS to the first line antiretroviral therapy regimen. Of the 146 PMTCT exposed patients, 21 exhibited VF and 135 exhibited VS in first line antiretroviral therapy (**Figure 5.4**).

Our results show that the the number of samples with and without predicted resistance in both the PMTCT and no-PMTCT groups is not significantly different between the FLX and Junior sequencing platforms at all prevalence cutoffs (**Figure 5.4**).

Thus, despite the significantly higher numbers of sequence reads generated per individual for the FLX data, both the genotyping results from both platforms were completely comparable.

### Comparison of ultra deep pyrosequencing and population based Sanger method for resistance prediction using baseline samples

In section 3.1.4 we showed that there was no significant difference between the FLX and Junior platforms for the prediction of resistance. While genotyping results from the Junior and FLX sequencing platforms are comparable between each other, this is essentially meaningless unless these results are comparable to that of the current “gold-standard” of population based Sanger genotyping method, to be used as a replacement. Thus, we compared the Junior platform (now also referred to as UDPS) results with those from the Sanger-based genotyping.

239 of 302 baseline samples were sequenced using both Junior and conventional population based Sanger genotyping technology. 128 of them had no previous PMTCT therapy exposure and 111 had previous PMTCT therapy exposure. Out of 128 no-PMTCT patients, 15 exhibited VF and 113 exhibited VS in first-line antiretroviral therapy. Similarly, out of 111 previously PMTCT exposed patients, 10 exhibited VF and 101 exhibited VS (**Figure 5.5**).

The results from the Junior platform showed identical numbers of individuals predicted as resistant at the 20%, 15% and 10% prevalence levels with now significant difference from the resistance predictions from the Sanger-based genotyping (Figure 5.5). The numbers of individuals predicted as resistant by the Junior platform increased at the lower prevalence levels (Figure 5.5), however these observations were still not significantly different from the predictions of the Sanger-based genotyping.

Thus, it appears for baseline samples at least, the UDPS resistance genotyping approaches employed here are directly comparable to that of Sanger-based resistance genotyping.

## Analysis of VF samples

Using the baseline samples, we showed that there was no significant difference between FLX and Junior and between UDPS and population based Sanger method. We repeated the platforms comparative analysis test using samples collected following clinical evidence of viral failure in individuals on 1st line therapy (Either a decline of < 1.5 log10 in viral load from baseline to 12 weeks of treatment or two consecutive viral loads 4 weeks apart of >1000 RNA copies/ml ).

### Resistance genotyping of samples collected from individuals at first-line VF using FLX

51 of the first line ART VF samples had been sequenced using FLX technology. 15 of these had previous ARV exposure through PMTCT while 36 had no previous exposure through PMTCT.

Genotyping using the FLX platform predicted resistance to at least one of the first line drugs at all prevalence levels in 14 out of 15 the PMTCT samples (**Figure 5.6**). On the other hand, in the no-PMTCT sample, 23 out of 36 had predicted resistance to at least one of the first line drugs at all prevalence levels while 13 had no predicted resistance (**Figure 5.6**).

The observation of the number of samples with and without predicted resistance showed that there was a significant difference between the PMTCT and no-PMTCT groups at all prevalence cutoffs. The observation also showed that the viral resistance prediction in the samples from PMTCT group was more than in no-PMTCT group at prevalence cutoffs using FLX system.

### Resistance genotyping of samples collected from individuals at VF using Junior

Out of the 36 first-line therapy failure samples sequenced using the Junior platform, 23 had no previous PMTCT therapy exposure while 13 had previous PMTCT therapy exposure. The numbers of predicted resistant and non-resistant viral samples were calculated at all prevalence cutoffs (**Figure 5.7**).

We observed that the amplified and UDPS sequenced viral population in all 13 VF samples in PMTCT group were predicted resistant when the prevalence cutoff was below 20% (**Figure 5.7**). At the 20% cutoff, 12 out of 13 PMTCT samples (92.3%) were predicted resistant (**Figure 5.7**). Similarly in no-PMTCT group, 14 out of 23 samples (60.86%) were predicted resistant at prevalence cutoffs 20% down to 5%. The number of samples with predicted resistance increased to 15 out of 23 (65%) at a prevalence cutoff of 1%. The observation of the number of samples that were predicted drug resistant showed that there were significant differences between the PMTCT and no-PMTCT at all prevalence cutoffs. The result obtained was similar to the result in VF samples sequenced using FLX, which indicated that the likelihood of predicting resistance in PMTCT group is more than in no-PMTCT group.

The number of VF samples that were predicted as resistant using FLX and Junior showed that FLX and Junior were comparable at genotyping for virologic drug resistance prediction in the sample. Therefore, in further analysis, we compared the virologic resistance prediction on the VF samples that were sequenced using both Junior and population based Sanger method to test the platform comparability.

### Comparison of the genotyping performance of the Junior platform and conventional population based Sanger genotyping method using VF samples

Genotypic data from both Junior and conventional population based Sanger genotyping method were available from 13 VF samples. Out of the 13 patients, 6 had no previous PMTCT therapy and 7 had PMTCT therapy exposure. The observation of the number of virologic samples that were predicted with or without resistant for both approaches at 20% prevalence cutoff showed that there was 100% concordance between the approaches across all clinical outcome categories between the Sanger-based and UDPS-based resistance genotyping approaches (**Figure 5.8**).

## Resistance to nevirapine is more likely to be present at baseline in individuals previously exposed to nevirapine through PMTCT

We compared the resistance predictions for PMTCT versus no-PMTCT therapy exposed individuals and identified the percentage of individuals with predicted resistance to nevirapine at baseline for conventional Sanger genotyping method, FLX and Junior (**Figure 5.9**). In all comparisons we found that the percentage of individuals with predicted resistance to nevirapine was always significantly higher in the PMTCT therapy exposed group when compared with the no-PMTCT therapy exposed group. This discordance became more evident at the ‘deeper’ prevalence cutoff (**Figure 5.9**), suggesting a large number of PMTCT-exposed individuals were harboring low-abundance NVP resistant viruses.

To ascertain whether the prediction of NVP resistance in PMTCT exposed individuals correlates with the time since NVP exposure, we compared the time since NVP exposure in baseline PMTCT samples with predicted NVP resistance and those predicted as susceptible to NVP. At prevalence thresholds of 15% and below (for both FLX and Junior) we found that the prediction of NVP resistance significantlycorrelates with time since NVP exposure (**Figure 5.9**). The median number of days since PMTCT exposure was observed to be 674 days for those individuals predicted as susceptible to NVP and 172 days for those predicted as resistant.

# Discussion and Conclusions

We have analyzed 562 baseline samples and 79 first line ART viral failure (VF1) samples using UDPS (both FLX and Junior) and conventional population based Sanger genotyping method. The baseline samples were collected in 2005 – 2006. The samples were grouped as – individuals with prior exposure to ARVs through PMTCT therapy and individuals without prior exposure to ARVs.

## Junior is comparable to FLX platform

Junior is a desktop sequencing platform with a single sequencing plate (www.454.com) and has capacity of multiplex sequencing of at least 48 samples (Dudley et al., 2012). FLX has similar sequencing chemistry like Junior and is a larger sequencing platform that has up to 16 sequencing plates ([www.454.com](http://www.454.com)). Each independent sequencing plate in FLX system has a sequencing capacity of a Junior system (www.454.com).

In our study, 48 samples were sequenced using MIDs in a single sequencing plate in Junior per run, whereas, up to 64 samples were sequenced using MIDs in eight sequencing plates (8 samples per sequencing plate) in an FLX per run. The total number of sequence reads from all Junior runs (10 runs in total), each containing 48 samples in average, was obtained and divided by total samples in the Junior runs (476 samples) to get the average number of sequence reads per sample. Similarly, the total number of sequence reads from 12 FLX runs (total 89 sequencing plates) and divided by the total number of samples (637 samples) to get the average number of sequence reads per sample. We obtained that Junior produced 1903 sequence reads per sample while FLX produced 6412 sequence reads per sample. Thus, on average FLX produces almost four times the number of sequence reads per sample than Junior.

Although the FLX platform produced significantly higher number of sequence reads per sample than Junior, for the 405 samples were sequenced using both the Junior and FLX platforms there was no significant difference in the resistance prediction by both sequencing platforms at all prevalence levels (205, 15%, 10%, 5% and 1%). This would mean that Junior performs as well as FLX at drug resistance prediction.

However, genotyping per sample is more cost effective in Junior than in FLX system. The low cost genotyping in Junior has advantage over FLX for massive HIV drug resistance test in resource-limited settings like sub-Saharan African regions. On the other hand, Junior is a desktop sequencing system and requires low space while FLX is a much bigger and complex machine. In addition to that, Junior instrument price (~$1,25,000) is much cheaper than FLX (~$5,00,000) meaning that many small laboratories can afford Junior and is a major advantage in poor settings.

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## Resistance prediction is more likely in drug exposed than drug naïve HIV infected individuals

We assessed the resistance prediction in the baseline samples from individuals with previous exposure of ART through PMTCT therapy and ART naïve individuals independently using the UDPS method. 526 baseline samples (339 no-PMTCT and 187 PMTCT) and 407 baseline samples (250 no-PMTCT and 157 PMTCT) were genotyped using FLX and Junior systems respectively.

As the UDPS approach is capable of detecting minor variants as low as 1% or below (Le et al., 2009; Schmitt et al., 2012; Wang et al., 2007), we assessed the resistance prediction at “ultra deep” level of up to 1% prevalence cutoff.

9 of 339 (2.6%) no-PMTCT samples (5 viral failures and 4 exhibited viral successes) genotyped using FLX were predicted as resistant to one or more of the drugs in the individual’s 1st line regimen while 23 of 187 (12.2%) PMTCT samples (5 viral failures and 18 viral successes) were predicted as resistant to at least one drug. Similarly, genotyping using Junior showed that 9 of 250 (3.6%) no-PMTCT samples (4 exhibited VF and 5 exhibited VS) and 19 of 157 (12.10%) PMTCT samples (3 exhibited VF and 16 exhibited VS) were predicted resistant. Thus, the number of baseline samples from the PMTCT group that would be expected to experience drug resistance to at least one drug was over four times than the baseline samples from no-PMTCT group. Moorthy et al (Moorthy et al., 2011) supported our observation showing that 33% of 124 children exposed to single dose nevirapine were harboring the drug resistant mutations after median 9 months of age. According to the authors, 61% of 96 children had archived nevirapine resistant HIV variants in their body cells. The authors assessed the nevirapine resistant mutations up to 1% or lower prevalence level. Simen et al (Simen et al., 2009) studied the prevalence of drug resistant mutations in ART naïve individuals by conventional Sanger genotyping and UDPS methods in an independent research. The authors showed that 10.1% and 21.3% of 258 ART naïve individuals harbored at least one drug resistant mutation as detected by conventional Sanger genotyping and UDPS methods respectively.

## First line therapy failure correlates to historical antiretroviral drug use

We studied the resistance prediction in first line antiretroviral treatment failure samples using UDPS genotypic data. Resistant prediction in first line VF samples from PMTCT and no-PMTCT were done independently. In our study, resistance prediction with FLX genotypic data showed that 14 of 15 (93.33%) of the first line VF samples that were ART experienced through PMTCT therapy and 23 of 36 (63.88%) ART naïve first line VF samples were predicted resistant. Similarly, 13 or 13 (100%) ART experienced first line VF samples and 15 of 23 (65.22%) ART naïve first line VF samples were predicted resistance. The observation showed that the frequency of first line VF in single dose nevirapine exposed individuals in PMTCT therapy was significantly higher than drug naïve individuals. Lockman et al (Lockman et al., 2007) had also studied the response of nevirapine based first line ART on the samples exposed to the drug through PMTCT therapy. They observed that 5% of 106 women that received placebo and 18.4% of 112 women that received single dose nevirapine experienced first line VF in the first six month of the initiation of the ART treatment. 41.7% of 60 nevirapine-experienced women starting first line ART also experienced VF. In another study, Chi et al (Chi et al., 2007) studied the association of exposure of nevirapine through PMTCT with CD4 count; death and VF of the nevirapine based first line ART. In their analysis of the hazard risk of first line VF, they obtained that patients with exposure to nevirapine had higher risk for clinical VF than drug naïve patients.

## Ultra deep pyrosequencing is more sensitive than Conventional Sanger genotyping method

Conventional Sanger genotyping method has been the “gold standard” method for genotypic HIV drug resistance test (Izopet et al., 2002; Woods et al., 2012). The method produces a single consensus sequence for HIV quasispecies in a sample. In a nucleotide position with mixture of nucleotides in the consensus sequence, an ambiguous base that represents the nucleotides with 20% or greater prevalence in the viral population (Hudelson et al., 2010; Larder et al., 1993; Leitner et al., 1993; Schuurman et al., 1999; Van Laethem et al., 1999). However, it is an expensive and labor-intensive method (Ji et al., 2010). Thus, an alternative method that overcomes the limitations of conventional method is essential.

Ultra deep pyrosequencing is a cost effective method and has massive parallel sequencing ability that generates hundreds of thousands of sequence reads per sample (Dudley et al., 2012; Ji et al., 2010).

At prevalence cutoff 20% for the samples (baseline and first line VF samples) genotyped using UDPS, we compared the number of samples that were predicted resistant to the number of samples predicted resistant using convention Sanger genotyping method. We found that there were no significance observed in the number of samples that were predicted resistant in VF and VS from both PMTCT and no-PMTCT groups. In addition, the number of samples predicted as harboring resistant viral variants increased while assessing at 1% prevalence cutoff using UDPS genotypic data.

Hezhao Ji et al (Ji et al., 2011) conducted a study of HIV surveillance on dried blood samples using UDPS and did a comparative analysis with conventional Sanger genotype method. Hezhao et al showed that UDPS and Sanger genotyping method are highly concordant at genotyping the high level HIV drug resistant mutations (99.21% and 99.51% respectively). The authors found the exceptional mutation M184I that was only detected by tagged pooled UDPS. In another study, Thuy Le et al (Le et al., 2009) found that the DRMs were present in all 22 (100%) of the patients with UDPS method while conventional Sanger method was able to detect DRMs in only 3 patients. The authors were studying the implication of resistant variants at the time of VF. While assessing the DRMs, the authors found that 90 of 247 DRMs were detected by UDPS method and 95% of the 90 DRMs were not detected by conventional Sanger genotyping method. On average, four additional mutations were detected per sample by UDPS method. Again, in another study, Liang et al (Liang et al., 2011) studied the use of UDPS to characterize the HIV-1 genetic diversity and evolution. The authors found a total of 14034 variants in HIV by UDPS method while only 3632 variants were detected by conventional Sanger method.

## Resistance prediction correlates with the time since antiretroviral exposure

The HIV infected individuals that were previously exposed to nevirapine (NVP) through PMTCT had obtained the drug at different time points in between 1999 to 2006 whereas the antiretroviral drugs were rolled out in South Africa in 2004. The individuals were enrolled for the ART treatment between 2005 and 2006. We assessed the correlation of nevirapine resistance prediction in the samples exposed to single dose nevirapine through PMTCT therapy with the time of the drug exposure.

A single dose nevirapine is highly used for pregnant mother for HIV prevention from mother to child and is recommended by the World Health Organization (WHO, 2008). However, a number of research publications have shown that the single dose nevirapine treatment rapidly develops HIV variants with nevirapine resistant mutations (Eshleman et al., 2001; Hudelson et al., 2010; Jackson et al., 2000; Palmer et al., 2006). The single dose nevirapine is provided one time to the pregnant women before labor. The effect of nevirapine decreases in those women in PMTCT therapy as the time goes on and thus nevirapine resistant viral variants decline in them (Eshleman et al., 2001; Kassaye et al., 2007). However, studies have shown the persistence of the minor nevirapine resistant variants (Flys et al., 2005; Flys et al., 2006; Loubser et al., 2006; Palmer et al., 2006; Rowley et al., 2010) and may compromise the subsequent first line therapy that contains the NNRTI drug (Boltz et al., 2011; Ciaranello et al., 2011; Jourdain et al., 2004; Lockman et al., 2007; Moorthy et al., 2011). A study by Chi et al (Chi et al., 2007) showed the time correlation (less than six months) before initiation of ART treatment as a risk factor that might produce poor treatment outcome. We then assessed the time correlation of nevirapine exposure with resistance prediction at different prevalence cutoffs. We observed that at 15% and lower prevalence cutoff, the prediction of NVP resistance significantly correlates (p < 0.05) with time since NVP exposure (**Table 5.3**). We observed that the baseline nevirapine resistant prediction in samples from individuals experiencing PMTCT therapy with long time gap (close to two years) were predicted sensitive to the drug while those with time gap of six months or less were predicted resistant to the drug. A study by Stringer et al (Stringer et al., 2010b) supported our observation on correlation between time and resistance prediction. The authors studied the prevalence of VF after nevirapine containing first line therapy in the patients experienced with single dose nevirapine. They observed that the time elapse between nevirapine exposure and initiation of nevirapine containing ART therapy were correlated. The authors observed VF in 47 of 116 (40%) of women with six or less months of time elapse, 25 of 67 (37%) of women with seven to 12 months of time elapse and 42 of 172 (24%) of women with more than 12 months of time elapse between nevirapine exposure and initiation of nevirapine containing ART therapy. Their observation showed that as the time elapsed increased, the rate of VF in nevirapine containing ART was declined. The authors concluded that risk of VF in recent drug exposed patients was high and suggested that nevirapine should not be included in the subsequent first line therapy for the drug exposed patients before 12 months of the therapy. The authors’ conclusion was highly consistent with our observation of time elapse and resistant prediction.

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