Chapter 5

The application of Seq2Res to evaluate high-throughput sequencing 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 (Berkhout et al., 2001)(Bebenek et al., 1993)(Bebenek et al., 1989)(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 (Johnson et al., 2008)(Metzner et al., 2009)(Devereux et al., 1999) 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., 2010; 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, in general, the use of single dose NVP to prevent mother to child transmission of the virus has resulted to the rapid development of NVP resistant HIV variants in those receiving NVP through PMTCT (Coovadia et al., 2009; Eshleman et al., 2004b; Eshleman et al., 2005b; 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, 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 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). Virologic failure 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 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 Roche/454 FLX platform. 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 virologic failure 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 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 (Rhee et al., 2003; Shafer, 2006) (Liu and Shafer, 2006). The percentage of sequences that were predicted as resistant for a particular antiretroviral drug by the algorithm were calculated. Using a number of prevalence cutoffs (20%, 15%, 10%, 5% and 1%), if the percentage of sequences predicted as resistant to a drug 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 virologic failure, 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 two-tailed T test method at pvalue 0.05.

# 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 from FLX and 468 samples from Junior from both baseline and first line virologic failures 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**).

## 3.1. Analysis of baseline samples

### Genotyping of baseline samples using the Roche/454 FLX 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 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 virologic failure while 289 exhibited virologic success during the course of follow-up. On the other hand, out of 187 PMTCT exposed patients, 25 exhibited virologic failure and 162 exhibited virologic success.

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 virologic failure 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 platform

407 patients were sampled at baseline and sequenced using Roche/454 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 virologic failure and 210 had virologic success. On the other hand, out of 147 PMTCT exposed patients, 21 had virologic failure and 136 had virologic success (**Figure 5.2**).

The obtained baseline blood samples were sequenced using Roche/454 Junior sequencing technology and again analyzed using Seq2Res. The observation on the number of samples with and without predicted resistant HIV showed that in the no-PMTCT virologic failure 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 virologic success group as well as in PMTCT virologic failure and virologic success groups (**Figure 5.2**). The highest number of samples with predicted resistant HIV was observed in PMTCT virologic success 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 UDPS platforms 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 6034 sequence reads per sample (standard deviation 2297) while the Junior platform generated an average 1532 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 Roche/454 FLX and Junior platforms on baseline samples

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

Across all of the various categories there was no difference between the FLX and Junior platforms in the prediction of resistance at both the 20% and 15% prevalence level (Figure 5.4). At lower prevalence cutoffs, the number of samples with predicted resistant HIV increased with minor differences in the observations of the number of samples predicted as resistant between the data generated using the Junior and FLX platforms (**Figure 5.4**).

At all prevalence cutoffs, there was no significant difference observed between the numbers of predicted resistant and non-resistant samples sequenced using FLX and Junior (**Figure 5.4**). Thus, despite the significantly higher numbers of sequences generated per individual for the FLX data, both platforms were comparable for HIV resistance genotyping.

### Comparison of high throughput and population based Sanger method for resistance prediction using baseline samples

Our observation 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, if tne UDPS is to be used as a replacement for conventional genotyping it is imperative to compare the UDPS approaches with the current “gold standard”, population based Sanger genotyping. Thus, we compared the Junior platform UDPS results with those from the Sanger-based genotyping.

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

The predicted resistance call for each individual sequenced using the UDPS at various prevalence thresholds (20%, 15%, 10%, 5% and 1%) was compiled and compared with the genotypic resistance call from the consensus sequences generated by population based Sanger genotyping method (**Figure 5.5**).

At 20% prevalence cutoff, there was no significant difference observed between the UDPS and population based Sanger method (**Figure 5.5**). Thus, UDPS is comparable to the conventional population based Sanger genotyping method for HIV genotyping and drug resistance testing.

Junior UDPS showed stable number of individuals with predicted resistance from 20% down to 10% prevalence cutoff and then increased from 10% to 1% prevalence cutoff. The highest number of individuals with predicted resistance (11 individuals) was observed in PMTCT virologic success group.

## Analysis of virologic failure samples

Using the baseline samples, we showed that there was no significant difference between the UDPS methods – FLX and Junior and between UDPS and population based Sanger method. We repeated the platforms comparative analysis test using the virologic failure samples.

### Resistance genotyping of samples collected from individuals at 1st line virologic failure using FLX

51 of the first line ART virologic failure samples had been sequenced using Roche/454 high throughput 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 and that indicated 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 virologic failure using Junior

Out of the 36 1st line failure samples sequenced using the Junior platform, 23 had no previous PMTCT exposure while 13 had previous PMTCT 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 virologic failure 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 showed that there were significant differences (p-value < 0.05) at all prevalence cutoffs. The result obtained was similar to the result in virologic failure samples sequenced using Roche/454 FLX, which indicated that the likelihood of predicting resistance in PMTCT group in more than in no-PMTCT group.

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

### Comparison of the genotyping performance of the Roche/454 Junior platform and conventional population based Sanger genotyping method using virologic failure samples

Results from both genotyping approaches were available for 13 individuals’ virologic failure samples. Out of the 13 patients, 6 had no previous PMTCT therapy and 7 had PMTCT therapy. The observation of the number of virologic samples that were predicted resistant or non resistant for both approaches at 20% prevalence cutoff showed that there was 100% concordance between the approaches across all clinical outcome categories (**Figure 5.8**)

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

For each sequencing platform we compared the resistance predictions for PMTCT versus no-PMTCT exposed individuals and identified the percentage of individuals with predicted resistance to nevirapine at baseline (**Figure 5.9**). In all comparisons we found that the percentage of individuals with predicted resistance to nevirapine was always significantly higher (p < 0.05) in the PMTCT group when compared with the no-PMTCT 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 find that the prediction of NVP resistance significantlycorrelates (p < 0.05) with time since NVP exposure. 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 virologic failure samples using UDPS (both Roche/454 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 and individuals without prior exposure to ARVs.

## Roche/454 Junior is comparable to Roche/454 FLX platform

Roche/454 Junior is a desktop sequencing platform with a single sequencing plate and it is recommended that maximum of eight samples can be sequenced in the plate. Roche/454 FLX, which has similar sequencing chemistry like Junior, is a larger sequencing platform than the latter, with eight sequencing plates and each plate can contain eight samples. This would mean that FLX is almost eight times the Junior platform and can output eight times sequence reads than the latter. This is supported by our observation on the number of sequence reads per sample that was sequenced on both Junior and FLX. On average of 6034 sequence reads per sample were output from FLX whereas Junior output on average 1532 sequence reads per sample.

In general, higher number of sequence reads in FLX than Junior would mean that FLX is more sensitive and better at drug resistance prediction. 464 samples were sequenced in both Junior and FLX. We observed that there was no significant difference in the number of the samples with predicted resistance and it showed that Junior performs as well as FLX at sensitivity and drug resistance prediction.

However, genotyping in Junior is more cost effective 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.

## Prevalence of drug resistant mutations is higher in drug exposed individuals

187 PMTCT (25 exhibited virologic failure and 162 exhibited virologic success) and 339 no-PMTCT (50 exhibited virologic failure and 289 exhibited virologic success) baseline samples were genotyped using FLX UDPS system. The number of baseline samples that were predicted resistant increased as the prevalence cutoff decreased from 20% to 1% in virologic failure and virologic success groups after subsequent first line therapy in both PMTCT and no-PMTCT. The statistical significance was observed at prevalence cutoff 1% in no-PMTCT group indicating that resistant prediction in no-PMTCT group required going down to 1% prevalence. The statistical significance at prevalence cutoff 1% was also observed in baseline samples genotyped using Junior system. But in PMTCT group, the statistical significance was observed at prevalence cutoff 15% indicating that resistant prediction in PMTCT group required going down to just 15% prevalence. This indicates that prevalence of HIV drug resistant mutations in drug exposed individuals through PMTCT is higher than in antiretroviral naïve individuals.

Previous studies have shown that drug associated resistant mutations are present at high prevalence after drug exposure. Martinson et al (Martinson et al., 2007) showed that children born from HIV infected mothers exposed to single dose nevirapine through PMTCT had the drug associated mutations Y181C (75%), K103N (25%), and Y188C (12%) in their first visit. Eshleman et al (Eshleman et al., 2005b) had also showed that in a trial conducted in Uganda, nevirapine related mutations was found at 69.2% after 6-8 weeks of single dose nevirapine exposure. Similarly, Loubser et al (Loubser et al., 2006) showed that HIV infected mothers in PMTCT therapy had over 65.4% of K103N mutation after 6 weeks of the drug exposure, which decreased to minor level in 12 months. Thus, in our study, statistical significance at resistance prediction at prevalence cutoff 15% in individuals exposed to nevirapine through PMTCT also indicated that the prevalence of the drug related mutation was in declining phase.

## Rebound of resistant variants in non-ART naïve individuals after first line therapy leads to virologic failure

51 individuals that exhibited virologic failure were genotyped using the FLX UDPS system. 15 of 51 had previously been exposed to ART through PMTCT and the rest were not. Resistance prediction using the genotype data of the 15 individuals with Seq2Res showed that 14 (94%) of them had predicted resistance to nevarapine and that was consistent at all prevalence cutoffs. Similarly, resistance prediction with genotype data from Junior system of the ART exposed individuals through PMTCT and that exhibited first line therapy failure showed that all (13 of 13) were predicted resistance consistently at prevalence cutoff 15% or below. This showed that the resistant HIV variants rebounded in the presence of the drugs leading to the virologic failure in the antiretroviral exposed individuals through PMTCT.

HIV variants with drug associated mutations gets selected and continue replicating in the presence of the single dose drug nevirapine (Eshleman et al., 2004a; Eshleman et al., 2005a; Martinson et al., 2007) generating more mutations. After drug interruption, the prevalence of the drug resistant variants decreases from high level to near undetectable low level (Flys et al., 2005; Loubser et al., 2006; Palmer et al., 2006). We have shown this using the UDPS genotype data from baseline samples obtained from individuals exposed to antiretroviral drug using PMTCT.

When the HIV infected individuals that were exposed to nevirapine through PMTCT underwent the first line therapy that includes nevirapine, the drug resistant viral variants emerge out in the viral population as a major variants leading to virologic failure and there were numerous researches that showed this observation (Boltz et al., 2011; Chi et al., 2007; Ciaranello et al., 2011; Coovadia et al., 2009; Lockman et al., 2007; MacLeod et al., 2010; Orrell et al., 2009).

However, adherence to the therapy is an important independent factor that needs to be considered as it could also affect the treatment outcome (Nieuwkerk and Oort, 2005). Uninterrupted treatment and timely intake of drugs helps to keep suppressing HIV virus and improve quality of life (Mannheimer et al., 2005). Nieuwkerk et al (Nieuwkerk et al., 2001) and Gifford et al (Gifford et al., 2000) showed that the HIV infected individuals who had low-level adherence to the therapy had increased HIV RNA copies per milliliter compared to high level therapy adherent HIV patients. However, a study by Bangsberg et al (Bangsberg et al., 2003) showed that the high-level adherence to the therapy do not prevent drug resistant mutation accumulation in the viral quasispecies.

In our study, we have not accounted patients’ deviation from the drug regimen and this could limit the resistance prediction in the samples from those patients.

## Ultra deep pyrosequencing is comparable to 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 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 labour intensive method (Ji et al., 2010) and that would mean it has limited use in resource-limited settings. 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 virologic failure 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 virologic failures and virologic successes from both PMTCT and no-PMTCT groups.

The comparative analysis of UDPS and conventional Sanger genotype methods by Hezhao Ji et al (Ji et al., 2011) have also 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). Le et al (Le et al., 2009) observed that UDPS was also able to detect all the drug resistant mutations that were detected by conventional Sanger genotyping method.

## Resistance prediction correlates with the time of antiretroviral exposure

The HIV infected individuals that were 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. This would mean that among the PMTCT exposed individuals, most of them had time gap of over a year before they underwent antiretroviral therapy.

A number of research publications have shown that the PMTCT treatment with NVP rapidly develops HIV variants with NVP resistant mutations (Eshleman et al., 2001; Hudelson et al., 2010; Jackson et al., 2000; Palmer et al., 2006). On interruption of NVP, the drug related resistant mutations decreases persistently to below 1% after one year (Arrive et al., 2007; Eshleman et al., 2001; Hauser et al., 2011; Loubser et al., 2006; Palmer et al., 2006). This correlated well with our observation that PMTCT treated individuals were predicted susceptible after 674 median days of treatment and were predicted resistant after 172 median days of treatment.

## Low-cost HIV drug resistant test using Roche/454 Junior system in resource limited settings

We showed that genotypic HIV drug resistance test using conventional Sanger genotyping method is comparable to using ultra deep pyrosequencing. Roche/454 FLX and Junior are two widely used UDPS systems. We have also showed that genotypic HIV drug resistance test using FLX and Junior systems are comparable. However, since genotyping cost per sample is much cheaper in Junior than in FLX system. This would mean that Roche/454 Junior system is an alternative method for low-cost HIV drug resistant test in high HIV prevalent resource limited settings.

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