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) due to high replication rate and high error rate of reverse transcriptase (Bebenek et al., 1993; Ji and Loeb, 1992; Preston et al., 1988). During the replication process, HIV develops random mutations [Berkhout, 2001 #1505][Bebenek, 1993 #1152][Bebenek, 1989 #982][Roberts, 1988 #306] in its proteins that can provide the virus resistance against the antiretroviral drugs [D’Aquila, 2003 #415;Clavel, 2004 #314;Kantor, 2004 #514;Sebastian, 2004 #206]. HIV with mutations at different codon positions of a protein is present at varying prevalence levels in the quasispecies [Johnson, 2008 #387][Metzner, 2009 #1573][Devereux, 1999 #1508]. In order to prevent HIV from replicating and destroying the immune cells, antiretroviral drugs have been developed that bind the specific viral protein (Tantillo et al., 1994) or host protein [Dorr, 2005 #1158;Westby, 2005 #1157;Westby, 2007 #254] and inhibit its function. But drug resistant mutations change three-dimensional structure of the proteins and prevent drug binding [Clavel, 2004 #314].

Approximately 8 million HIV infected individuals in resource-limited countries are receiving antiretroviral therapy by the end of 2012 (UNAIDS, 2012) after the scale-up of the treatment in 2002 (Beck et al., 2006; Ferradini et al., 2006; Gilks et al., 2006; Stringer et al., 2006). Despite the scaled-up treatment, the number of HIV infected individuals with transmitted drug resistant mutations (DRMs) in the treatment-introduced regions is increasing (Aghokeng et al., 2011; Phillips et al., 2013; Zaidi et al., 2013). A study shows that the life saving cocktail of antiretroviral (ART) drugs increase life expectancy of the infected individuals that in turn increases the risk of transmission of drug resistant HIV variants to uninfected individuals (Zaidi et al., 2013).

While mutations may be present at different codon positions of a protein in HIV in the quasispecies, an antiretroviral drug or a drug class cannot act against all the viruses (Johnson et al., 2008; Shafer and Schapiro, 2008) as shown by the failing treatment with single drug or drugs from same class in late 1980s (Kellam et al., 1994; Larder et al., 1989; Larder et al., 1991; Larder and Kemp, 1989; Larder et al., 1987). Therefore, administration of a single antiretroviral drug increases the chance of rapid drug failure (Hamers et al., 2012). This is support by resistance development against a single dose antiretroviral drug nevirapine (NVP). NVP is a non-nucleotide reverse transcriptase drug that is prescribed for an infected pregnant woman 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 and 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, 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 (Coovadia et al., 2009; Eshleman et al., 2004; 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, at least a combination of three fully active ART drugs from different drug classes – Non-Nucleotide Reverse Transciptase Inhibitors (NNRTIs), Nucleotide Reverse Transcriptase Inhibitors (NRTIs) and Protease Inhibitors (PI) are necessary for optimum suppression of HIV from replication and resistance development (Hamers et al., 2012). 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 below 1% (Bansode et al., 2013; Dudley et al., 2012; Gilles et al., 2011; Hoffmann et al., 2007; Huse et al., 2007; Ji et al., 2012; Ji et al., 2010; Liang et al., 2011; Wang et al., 2007). But UDPS generates up to a million reads, each up to 1000 bases length (www.454.com). The computational tool Seq2Res can be used to analyze drug resistant mutations in the large sequence data.

# 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 like drug toxicity, death, withdrawn of consent or lost 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 were amplified using HIV subtype C specific primers. Ten HTS 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 were analyzed for drug resistance using Seq2Res computational tool. As various studies have suggested that the population based Sanger method is able to detect the sequence reads at prevalence greater or equal to 20% (Hudelson et al., 2010; Larder et al., 1993; Leitner et al., 1993; Schuurman et al., 1999; Van Laethem et al., 1999), we calculated the drug resistance of the consensus viral sequence read in a sample at the same prevalence cutoff. In the UDPS generated sequence reads of a sample, we calculated the drug resistance at 20%, 15%, 10%, 5% and 1% prevalence cutoffs.

At a defined prevalence cutoff, if the percentage of viral sequence reads predicted as resistant to at least one drug in the baseline regimen is greater or equal to the cutoff value then the viral population in a sample is predicted as resistant. If a patient’s baseline drug regimen was not known then resistance to at least one of the possible baseline drugs was taken.

# 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 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 in FLX and Junior platforms, 327 samples in FLX and population based Sanger genotyping method and 257 samples in Junior and population based Sanger genotyping method (**Table 5.2**).

## 3.1. Analysis of baseline samples

### 3.1.1. 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 exhibited virologic failure and 289 exhibited virologic success. On the other hand, out of 187 PMTCT exposed patients, 25 exhibited virologic failure and 162 exhibited virologic success.

The observation of the number of samples with and without predicted drug resistant HIV at varying prevalence cutoffs (1%, 5%, 10%, 15% and 20%) showed that the number of samples with viral sequence reads 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 increased to five samples (10% ) at prevalence 1%. Similar increment of the number of resistant samples was observed in other groups as well (**Figure 5.1**). Significant difference was observed at 1% prevalence cutoff in no-PMTCT group and 15% prevalence cutoffs in PMTCT group (**Figure 5.1**). The observed significant difference showed that the likelihood of prediction of drug resistance at 1% is higher than in the PMTCT group while the likelihood of prediction of drug resistance at 15% prevalence cutoff in the PMTCT group is higher than in the no-PMTCT group.

### 3.1.2. 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 PMTCT therapy 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. In 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 with HIV that is predicted resistant to at least one drug in baseline at prevalence cutoff 20% and that increased to four individuals at prevalence cutoff 1%. Similar increment of the number of samples with predicted resistant HIV was observed in 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 prevalence cutoff 1%. The significant difference was observed at prevalence cutoff of 1% in no-PMTCT group (**Figure 5.2**), which showed that the likelihood of prediction of resistance at 1% in this group is higher than in PMTCT group.

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

Sequencing had been successful on both HTS 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 sensitive prediction of resistance. We saw that 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.

## 3.1.4 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 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**).

The number of samples with and without predicted drug resistant viral population, analyzed using Seq2Res showed that at 20% and 15% prevalence cutoffs there were no difference in resistance prediction in between the platforms for any of the clinical outcome categories. At lower prevalence cutoffs, the number of samples with predicted resistant HIV decreased. At 10% and 5% cutoffs, we observed one sample with predicted resistant HIV more in FLX than Junior in the PMTCT virologic success group, while at 1% cutoff, we observed two samples with predicted resistant HIV more in Junior than in FLX (Junior 5, FLX 3) in no-PMTCT virologic success group (**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, FLX and Junior were comparable at HIV genotyping for drug resistance test.

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

As our observation showed that there was no significant difference (using two tailed T test method) between the FLX and Junior platforms at drug resistance prediction, its more likely that the low cost Junior platform will be used. In the subsequent analysis we have used Junior platform as HTS approach.

We, thus, compared HTS (Junior platform) and population based Sanger genotyping method for their sensitivity at drug resistance test because the HTS, although costs less, may not be used if it is not comparable to the “gold standard’ population based Sanger method.

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 HTS 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**).

The statistical analysis on our observation of the number of samples with and without predicted resistance call showed that there was no significant difference observed at the prevalence cutoff 20% between the HTS and population based Sanger method (**Figure 5.5**). This showed that HTS was comparable to the conventional population based Sanger genotyping method for HIV genotyping and drug resistance testing.

## 3.2 Analysis on virologic failure samples

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

#### 3.2.1 Resistance genotyping of samples collected from individuals at 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 PMTCT samples. 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 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.

#### 3.2.2 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 HTS sequenced viral population in all 13 VF samples in PMTCT group were predicted resistant to at least one drug in the regimen below 20% prevalence cutoff. At 20% cutoff, 12 out of 13 samples were predicted resistant (Figure 5.7). 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 resistant using FLX and Junior showed that both the HTS 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.

#### 3.2.3: 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**)

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

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 HTS (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.

The conventional population based Sanger genotyping method produces a single nucleotide sequence for a viral population and it can only detect mutations at positions of the sequence if they are present in the viral population at 20% or greater prevalence (Hudelson et al., 2010; Larder et al., 1993; Leitner et al., 1993; Schuurman et al., 1999; Van Laethem et al., 1999). In ultra deep method, the viral population of a blood sample is PCR amplified and the viral nucleotide sequences are sequenced using Roche/454 HTS producing up to 100,000 sequences reads (by Roche/454 Junior) or up to 1000000 sequence reads (by Roche/454 FLX) (www.454.com). The large number of sequence reads in ultra deep pyrosequencing may contain sequence reads from HIV that are present in the viral population below 1% prevalence (Avidor et al., 2013; Dudley et al., 2012; Garcia-Diaz et al., 2013; Hedskog et al., 2010; Hoffmann et al., 2007; Lataillade et al., 2010; Le et al., 2009; Li et al., 2011; Loman et al., 2012; Paredes et al., 2010; Simen et al., 2009a). Due to this higher sensitivity of ultra deep HTS method than conventional Sanger method, we expect HTS technology will be used as an alternative method for therapeutic HIV genotyping. Thus in our initial analysis, we compared Roche/454 systems - FLX and Junior for their sensitivity at genotyping at predicting drug resistance.

The Junior and FLX platforms do not differ in their chemistry but differ on the basis of number of reads output. This correlated with our observation that the FLX platform generated significantly higher number of sequence reads than the Junior platform. Thus, it is plausible to suggest that the generation of more sequence reads for a sample may sequence the amplified viral population to a ‘deeper’ level, thereby detecting low-abundance resistant variants. Our initial analysis, therefore, involved comparison of the resistance genotyping of individuals from both the Junior and FLX platforms.

The result of the comparison of the resistance genotyping of individuals by both Junior and FLX platforms showed that there was no significant difference between the HTS platforms at all prevalence levels. A similar observation was made in the comparison of resistance genotyping of first-line failure samples using both the FLX and Junior platforms. Thus it appears that, despite the lower number of reads generated by the Junior platform, it is sufficiently accurate for HIV resistance genotyping (above 1%) even when as many as 48 samples are pooled together on a single plate. The cost savings generated by employing such an approach means that resistance genotyping using the Junior platform is a viable option for large-scale genotyping in resource poor settings [Dudley, 2012 #1209]. However, such an approach is not

Although the HTS method can sequence to ‘deeper’ level, its sensitivity had to be comparable to the conventional population based Sanger genotyping method method. Therefore we also compared the HTS and population based Sanger methods at HIV genotyping at resistance prediction using the samples (baseline and VF separately) sequenced using both the methods. For fairness, the comparison was done at the 20% prevalence cutoff. The result using both the baseline and VF samples showed that HTS was comparable to the conventional population based Sanger genotyping method method.

**HEADING**

Besides comparing HTS and conventional population based Sanger genotyping method methods, we also analyzed the prevalence of NVP resistance. NVP is a NNRTI drug given to pregnant women as a prophylaxis for prevention from mother to child transmission (PMTCT) of HIV virus (Eshleman et al., 2005a; Eshleman et al., 2005b; Guay et al., 1999; Jackson et al., 2000; Marseille et al., 1999; Musoke et al., 1999). The PMTCT exposed individuals had obtained NVP treatment 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). The first NVP resistant mutation to develop was Y181C, which slowly faded out, and another mutation K103N emerged out as the dominant mutation within 6-8 weeks (Eshleman et al., 2004). These data correlated well with our analysis using conventional consensus method and HTS method at prevalence cutoff 20% that showed the number of previous PMTCT experienced individuals with resistance call to NVP was significantly higher than the number of individuals without previous PMTCT experience. This also indicated that the likelihood of developing resistance to NVP was higher in PMTCT experienced individuals than the individuals without PMTCT experience.

In the presence of an antiretroviral drugs, the virus that has drug resistant mutations to the drugs has high viral fitness while the wild type virus has low viral fitness (Clavel et al., 2000; Collins et al., 2004). In the absence of the drugs, the viral fitness of the resistant variants decreases while viral fitness of the wild type variants increases (Clavel et al., 2000; Deeks et al., 2005; Paquet et al., 2011; Rosenbloom et al., 2012). Thus the wild type HIV virus replicates and their prevalence increases while prevalence of resistant variants decreases. When the antiretroviral drugs are reintroduced, the resistant virus explodes in the viral population leading to virologic failure while the wild type virus exists in low abundance (Delobel et al., 2011; Le et al., 2009; Li et al., 2011; Paredes et al., 2010; Simen et al., 2009b).

HIV variants with NVP resistance, though increased rapidly during single dose NVP treatment for PMTCT, at post treatment (a condition like drug interruption), the HIV variants with NVP resistant mutations slowly decays out and in one year their prevalence decreased to less than 5% (Arrive et al., 2007; Eshleman et al., 2001; Hauser et al., 2011; Loubser et al., 2006). Our observation also showed that the number of individuals with resistance call to NVP increased as we analyzed at decreasing prevalence cutoff using HTS, indicating the low abundance HIV variants harboring NVP resistant mutations existed in the baseline samples.

We observed that at prevalence cutoff of 1%, there were 18 of 21 baseline PMTCT experienced samples that had clinical virologic failure outcome but had no resistance call to NVP (sensitive to NVP) and 120 of 136 baseline PMTCT experienced samples that had clinical virologic success outcome but had no resistance call to NVP (sensitive to NVP) when analyzed using Junior HTS platform. We investigated if the time since PMTCT exposure corresponded to resistance or sensitive call to NVP for those individuals. We observed that 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.

Our observation on sensitive call to individuals with 674 median days since PMTCT exposure correlated well with the result obtained by Palmer et al (Palmer et al., 2006). The authors found that after one year since the PMTCT exposure, the prevalence of K103N and Y181C mutations were observed to be less than 0.7% and 0.4% respectively. Since we made resistance call if the prevalence of resistance sequence reads was greater or equal to 1%, the samples from the individuals obtained more than a year after PMTCT exposure had the prevalence of resistant sequence reads below 1% prevalence cutoff and they had sensitive call.

On the other hand, for the individuals that had resistance call for NVP had median day of 172 (approximately 6 months) since PMTCT exposure. A number of publications (Hauser et al., 2011; Lockman et al., 2007; Loubser et al., 2006; Palmer et al., 2006; Rowley et al., 2010) had shown that the prevalence of HIV variants that are resistant to NVP at six months of PMTCT exposure was higher than 20%. Therefore, the call of resistance to NVP in the individuals with 172 median days since PMTCT exposure correlated well with the results obtained by the authors.

Since the PMTCT exposed individuals were harboring NVP resistant HIV variants, we analyzed the virologic failure individuals that underwent the NNRTI containing first line antiretroviral after the ART program was rolled out.

A total of 91% of 562 individuals had undergone the first line antiretroviral therapy containing a NNRTI drug (NVP or EFV), of which the 79 individuals had clinical virologic failure. As FLX and Junior platforms were comparable to each other, we discussed the analysis and resistance prediction on virologic failure sequenced using Junior platform.

36 of the 79 first line virologic failure individuals sequence data was available using HTS Junior platform of which 13 individuals had previous PMTCT exposed. All those 13 individuals who had previous PMTCT experience and subsequent first line virologic failure had NVP resistance call at 15% and below prevalence cutoffs whereas 12 of them had NVP resistance call at 20% prevalence cutoff. This indicated that the prediction of NVP resistance in PMTCT exposed individuals and subsequent virologic failure was more likely than in non-PMTCT exposed individuals.

Our observation that individuals exposed to PMTCT had greater chance of NVP resistance call correlated with the finding by Boltz et al (Boltz et al., 2011) and Lehman et al (Lehman et al., 2012) The authors found that the presence of low abundance HIV variants with NVP resistance was associated with greater risk of virologic failure in subsequent standard care of first line antiretroviral therapy containing NVP. Although, initially the initiation of the NNRTI based first line ART before one year of PMTCT exposure was shown to be associated with greater risk of first line ART virologic failure (Chi et al., 2007) and recommended to undergo first line ART after not less than 12 months from PMTCT exposure, Boltz et al (Boltz et al., 2011) showed that there was no decrease in risk of ART virologic failure or AIDS related deaths.

As we analyzed the samples using the computational tool Seq2Res and the observed data on the prediction of resistance to viral sequence reads and drug resistance call for the viral samples correlated with the previous findings, we conclude that the computational tool was able to be used for HIV drug resistance test.

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