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 transmission to a newborn child from an infected mother during delivery or at breast-feeding has been concerned since it was reported first in mid 1980s (Mofenson, 1997; Wiktor et al., 1997). The only available antiretroviral drug in that time was zidovudine (AZT). A study conducted on a group of pregnant women that were given AZT 14 weeks before child birth and an another group of pregnant women that were not given AZT showed that the risk of transmission of HIV virus from infected mother to child during birth was significantly reduced by two-third (Connor et al., 1994). Another study that began in 1997 in Ugandan HIV infected pregnant women compared the efficacy of zidovudine and nevirapine (NVP) antiretroviral drug to prevent the viral transmission from mother to child (Guay et al., 1999). The study showed that NVP reduced the risk of transmission better than AZT (Guay et al., 1999; Jackson et al., 2003). A study in Thailand showed that single dose of NVP to both mother and newborn child could further reduce the transmission in the child (Lallemant et al., 2004).

NVP is an NNRTI drug, which has longer half-life than AZT in the infected individuals both mother and a child. This makes it more efficacious and a better drug than AZT (Mirochnick et al., 1998). The drug is still used in resource poor countries to prevent mother to child transmission of HIV virus (Audureau et al., 2013; Chi et al., 2013; Shapiro et al., 2010; Stringer et al., 2010; Zolfo et al., 2010).

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 (NVPR) HIV variants (Eshleman et al., 2005b; Eshleman et al., 2001; Havlir et al., 1996; Jackson et al., 2000; Richman et al., 1994; Tisdale et al., 1993). The NVP resistant mutation Y181C develops rapid within seven days, followed by slow development of K103N NVP resistant mutation in 6-8 weeks (Eshleman et al., 2004), which then persists as the most common NVP resistant mutation (Coovadia et al., 2009; Flys et al., 2005; Jackson et al., 2000; Loubser et al., 2006; Martinson et al., 2007).

The persistence of NVP resistant mutations in the mothers and children treated with single dose NVP (Hauser et al., 2011) has compromised the subsequent NVP containing highly active antiretroviral therapy (HAART) (Arrive et al., 2007; Chi et al., 2007; Lehman et al., 2012; Martinson et al., 2007). The frequency of HIV variants with NVP resistant mutations declines slowly at post single dose NVP (Loubser et al., 2006) therapy and they exist as minor variants that are not detected by the conventional population based sequencing method (Palmer et al., 2005). Other technique like allele specific real time polymerase chain reaction is able to detect the rare mutation in the viral population (Coovadia et al., 2009; Rowley et al., 2010) but is limited to mutations in the targeted codons only (Lang et al., 2011).

Roche/454 high throughput sequencing technology (HTS) has shown the ability to sequence minor HIV variants (less than 1%) in the viral population (Avidor et al., 2013). In this chapter, we analyzed HTS data from single dose NVP exposed individuals at baseline (before antiretroviral therapy) and after first line antiretroviral therapy to compare HTS and conventional sequencing platforms, and to know the persistence of NVP resistant mutations in the samples at ultra deep prevalence level.

# 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 for all samples were analyzed drug resistance using Seq2Res computational tool. The demultiplexing of sequences was done using primer and MID sequences with primer tolerance of 3 and MID tolerance of 2. The demultiplexed sequence reads were quality trimmed using QTrim with default parameters – mean quality of reads was 20, minimum read length was 50 and the trimming was from 3’ end of the sequence reads. The quality cleaned sequence reads were mapped to the HXB2 *pol* reference sequence using RAMICS and only those sequences that satisfied the optimal full length criteria (optimal full length – sequence reads that cover first DRM position to the last DRM position) were selected for subsequent analysis. The mapped sequences in fastm files were tested for drug resistance using locally installed sierra. The prevalence of resistant sequence reads to a drug was calculated as a number of sequences that were predicted resistance to the drug. The viral population in a sample was called as resistant to a drug if the prevalence of amplified sequence reads that are resistant to the drug was greater or equal to the prevalence cutoff. While the conventional 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 viral sequence reads in the sample at the same prevalence cutoff. For the high throughput sequence reads, we calculated the drug resistance of the amplified resistant viral sequenced reads at 20%, 15%, 10%, 5% and 1% prevalence cutoffs.

If a viral population (amplified and sequenced population) in a sample is resistant to at least one drug in the baseline drug regimen then the drug was called as resistant at a defined prevalence cutoff, the sample is counted as resistant. If a patient’s baseline 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 FLX and Junior platforms, 327 samples in FLX and conventional method and 257 samples in Junior and conventional 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 had virologic failure and 289 had virologic success. On the other hand, out of 187 PMTCT exposed patients, 25 had virologic failure and 162 had virologic success.

The obtained baseline blood samples were sequenced using Roche/454 FLX technology and analyzed using Seq2Res. The observation showed that the number of samples with viral sequence reads that are resistant to at least one baseline drug increased when the prevalence cutoff is decreased to 1% (**Figure 5.1**). In the no PMTCT virologic failure group of 50, the number of samples with at least one baseline drug resistance was one from prevalence level 20% to 5% but increased to five samples (10% of the group) at prevalence 1%. Similarly, in the no PMTCT virologic success group of 289 individuals, the number of samples with at least one baseline drug resistance increased from one at 20% prevalence cutoff to four at 1% prevalence cutoff. In the PMTCT virologic failure group of 25, the sequenced viral sample from two individual showed resistant to at least one baseline drug at 20%, which increased to five individuals (20%) at 1% prevalence cutoff and in the PMTCT virologic success group of 162, at prevalence of 20% there were four individuals, which increase to 18 individuals at prevalence cutoff 1% (**Figure 5.1**). Significant difference was observed at 1% prevalence cutoff in no PMTCT group and 15% prevalence cutoffs in PMTCT group using two-tailed T test method at p-value 0.05 (**Figure 5.1**). The significant difference observed at the 1% prevalence cutoff in no PMTCT showed that the likelihood of prediction of drug resistance at 1% is higher than in the PMTCT group while the likelihood of prediction of 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 showed that in the no PMTCT virologic failure group of 40, one individual has viral sequenced population, resistant to at least one drug in baseline at prevalence cutoff 20%, which increased to four individuals at prevalence cutoff 1% while in no PMTCT virologic success group of 210, the number of individuals with viral sequenced population, resistant to at least one drug in baseline was one at prevalence cutoff 20% which increased to five individuals at cutoff 1%. In the PMTCT virologic failure group of 21, two individuals had sequence reads from viral population resistant to at least one baseline drug at 20% prevalence cutoff, which increased to thee individuals at 1% prevalence cutoff. Similarly in the PMTCT virologic success group of 136, four individual had sequence reads from viral population resistant to at least one baseline drug at 20% prevalence cutoff, which increased to 16 individuals at 1% prevalence cutoff (**Figure 5.2**). 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**). Thus, 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 had virologic failure and 209 had virologic success to the first line antiretroviral therapy regimen. Of the 146 PMTCT exposed patients, 21 had virologic failure and 135 had virologic success in first line antiretroviral therapy (**Figure 5.4**).

The drug resistance prediction for the sequenced viral population in the samples and analyzed using Seq2Res showed that at 20% and 15% prevalence cutoffs there were no difference in between the platforms for any of the clinical outcome categories. At lower prevalence cutoffs (10%, 5% and 1%), the number of samples with sequenced viral population, resistant to at least one baseline drug increased as the prevalence decreased.. At the 10% and 5% cutoffs, FLX predicted one more resistant sample than Junior in the PMTCT virologic success group, while at the 1% cutoff, Junior predicted two samples more than FLX (Junior 5, FLX 3) in no PMTCT virologic success group (**Figure 5.4**).

At all prevalence cutoffs, there was no significant difference observed using a two tailed T test, between the numbers of 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 high throughput and conventional 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 is more likely to be used. In the subsequent analysis we have used Junior platform as HTS approach.

We, thus, compared HTS (Junior platform) and conventional method for their sensitivity at drug resistance test because the HTS, although cost less, cannot be used if it is not comparable to the “gold standard’ conventional method.

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

The resistance call for each individual at various prevalence thresholds (20%, 15%, 10%, 5% and 1%) was compiled and compared with the genotyping calls from the consensus sequences (**Figure 5.5**).

On the other hand, resistance calls on the numbers of samples sequenced using conventional method was calculated at only at 20% prevalence cutoff.

Our observation showed at the prevalence cutoff 20%, there was a minimal difference of one or two samples with viral population called as resistant using HTS and conventional method; and that there was no significant difference observed at the prevalence cutoff (**Figure 5.5**). This showed that HTS was comparable to the conventional 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 conventional method. We repeated the test using the virologic failure samples to confirm the above result.

#### 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 resistance identified (**Figure 5.6**).

The observation showed that there was a significant difference (p-value < 0.05 using two tailed T test method) at all prevalence cutoffs and that indicated that it was more likely to predict the viral samples as resistant in PMTCT group than in no PMTCT group.

#### 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 all 13 VF samples in PMTCT group had the sequenced viral population predicted to be resistant to at least one drug in the regimen below 20% prevalence cutoff. At 20% cutoff, 12 out of 13 samples with the sequenced viral population were predicted resistant (Figure 5.7). The observation showed that there were significant differences (p-value > 0.05) at all prevalence cutoffs using two tailed T test. The result obtained was similar to the result in VF samples sequenced using Roche/454 FLX, which indicated that the likelihood of predicting resistance in PMTCT group in more than in non PMTCT group.

The observations in VF samples using FLX and Junior showed that they were comparable to each other at virologic drug resistance prediction in the samples. This result was in correlation to the result in baseline samples using FLX and Junior platforms. Therefore, in further analysis, we compared the virologic resistance prediction commonly sequenced using Junior and conventional method to test their comparability.

#### 3.2.3: Comparison of the genotyping performance of the Roche/454 Junior platform and conventional genotyping 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. At all prevalence levels in the HTS there was 100% concordance between the resistance calls for both 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 in whom resistance to nevirapine at baseline was predicted (**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 genotyping methods. The baseline samples were collected in 2005 – 2006. The samples were grouped into two categories – those with prior experience to PMTCT treatment and those without prior experience to PMTCT treatment.

Because the conventional genotyping method has the limitation that its unable to sequence the DNA in the population less than 20% (Hudelson et al., 2010; Larder et al., 1993; Leitner et al., 1993; Schuurman et al., 1999; Van Laethem et al., 1999), the Roche/454 HTS technologies that were able to sequence the DNA less or equal to one percent (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), were expected to be used as an alternative method. There are two Roche/454 HTS technologies – Junior and FLX. In our initial analysis, we compared 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 calls of individuals for which sequencing had been successful on both the Junior and FLX platforms.

The result of the comparison of the resistance calls of individuals sequenced 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. However, such an approach is not

Although the HTS method can sequence to ‘deeper’ level, its sensitivity had to be comparable to the conventional genotyping method. Therefore we also compared the HTS and conventional 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 genotyping method.

**HEADING**

Besides comparing HTS and conventional genotyping 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, 2011 #1539] and Lehman et al [Lehman, 2012 #1631] 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, 2007 #1632] and recommended to undergo first line ART after not less than 12 months from PMTCT exposure, Boltz et al [Boltz, 2011 #1539] 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.

Arrive, E, Newell, ML, Ekouevi, DK, Chaix, ML, Thiebaut, R, Masquelier, B, Leroy, V, Perre, PV, Rouzioux, C, Dabis, F (2007) Prevalence of resistance to nevirapine in mothers and children after single-dose exposure to prevent vertical transmission of HIV-1: a meta-analysis. *Int J Epidemiol* **36**: 1009-1021.

Audureau, E, Kahn, JG, Besson, MH, Saba, J, Ladner, J (2013) Scaling up prevention of mother-to-child HIV transmission programs in sub-Saharan African countries: a multilevel assessment of site-, program- and country-level determinants of performance. *BMC Public Health* **13**: 286.

Avidor, B, Girshengorn, S, Matus, N, Talio, H, Achsanov, S, Zeldis, I, Fratty, IS, Katchman, E, Brosh-Nissimov, T, Hassin, D, Alon, D, Bentwich, Z, Yust, I, Amit, S, Forer, R, Vulih Shultsman, I, Turner, D (2013) Evaluation of a benchtop HIV ultradeep pyrosequencing drug resistance assay in the clinical laboratory. *J Clin Microbiol* **51**: 880-886.

Chi, BH, Sinkala, M, Stringer, EM, Cantrell, RA, Mtonga, V, Bulterys, M, Zulu, I, Kankasa, C, Wilfert, C, Weidle, PJ, Vermund, SH, Stringer, JS (2007) Early clinical and immune response to NNRTI-based antiretroviral therapy among women with prior exposure to single-dose nevirapine. *AIDS* **21**: 957-964.

Chi, BH, Stringer, JS, Moodley, D (2013) Antiretroviral drug regimens to prevent mother-to-child transmission of HIV: a review of scientific, program, and policy advances for sub-Saharan Africa. *Curr HIV/AIDS Rep* **10**: 124-133.

Clavel, F, Race, E, Mammano, F (2000) HIV drug resistance and viral fitness. *Adv Pharmacol* **49**: 41-66.

Collins, JA, Thompson, MG, Paintsil, E, Ricketts, M, Gedzior, J, Alexander, L (2004) Competitive fitness of nevirapine-resistant human immunodeficiency virus type 1 mutants. *J Virol* **78**: 603-611.

Connor, EM, Sperling, RS, Gelber, R, Kiselev, P, Scott, G, O'Sullivan, MJ, VanDyke, R, Bey, M, Shearer, W, Jacobson, RL, et al. (1994) Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. *N Engl J Med* **331**: 1173-1180.

Coovadia, A, Hunt, G, Abrams, EJ, Sherman, G, Meyers, T, Barry, G, Malan, E, Marais, B, Stehlau, R, Ledwaba, J, Hammer, SM, Morris, L, Kuhn, L (2009) Persistent minority K103N mutations among women exposed to single-dose nevirapine and virologic response to nonnucleoside reverse-transcriptase inhibitor-based therapy. *Clin Infect Dis* **48**: 462-472.

Deeks, SG, Hoh, R, Neilands, TB, Liegler, T, Aweeka, F, Petropoulos, CJ, Grant, RM, Martin, JN (2005) Interruption of treatment with individual therapeutic drug classes in adults with multidrug-resistant HIV-1 infection. *J Infect Dis* **192**: 1537-1544.

Delobel, P, Saliou, A, Nicot, F, Dubois, M, Trancart, S, Tangre, P, Aboulker, JP, Taburet, AM, Molina, JM, Massip, P, Marchou, B, Izopet, J (2011) Minor HIV-1 variants with the K103N resistance mutation during intermittent efavirenz-containing antiretroviral therapy and virological failure. *PLoS One* **6**: e21655.

Dudley, DM, Chin, EN, Bimber, BN, Sanabani, SS, Tarosso, LF, Costa, PR, Sauer, MM, Kallas, EG, O'Connor, DH (2012) Low-cost ultra-wide genotyping using Roche/454 pyrosequencing for surveillance of HIV drug resistance. *PLoS One* **7**: e36494.

Eshleman, SH, Guay, LA, Mwatha, A, Cunningham, SP, Brown, ER, Musoke, P, Mmiro, F, Jackson, JB (2004) Comparison of nevirapine (NVP) resistance in Ugandan women 7 days vs. 6-8 weeks after single-dose nvp prophylaxis: HIVNET 012. *AIDS Res Hum Retroviruses* **20**: 595-599.

Eshleman, SH, Hoover, DR, Chen, S, Hudelson, SE, Guay, LA, Mwatha, A, Fiscus, SA, Mmiro, F, Musoke, P, Jackson, JB (2005a) Resistance after single-dose nevirapine prophylaxis emerges in a high proportion of Malawian newborns. *AIDS* **19**: 2167-2169.

Eshleman, SH, Hoover, DR, Chen, S, Hudelson, SE, Guay, LA, Mwatha, A, Fiscus, SA, Mmiro, F, Musoke, P, Jackson, JB, Kumwenda, N, Taha, T (2005b) Nevirapine (NVP) resistance in women with HIV-1 subtype C, compared with subtypes A and D, after the administration of single-dose NVP. *J Infect Dis* **192**: 30-36.

Eshleman, SH, Mracna, M, Guay, LA, Deseyve, M, Cunningham, S, Mirochnick, M, Musoke, P, Fleming, T, Glenn Fowler, M, Mofenson, LM, Mmiro, F, Jackson, JB (2001) Selection and fading of resistance mutations in women and infants receiving nevirapine to prevent HIV-1 vertical transmission (HIVNET 012). *AIDS* **15**: 1951-1957.

Flys, T, Nissley, DV, Claasen, CW, Jones, D, Shi, C, Guay, LA, Musoke, P, Mmiro, F, Strathern, JN, Jackson, JB, Eshleman, JR, Eshleman, SH (2005) Sensitive drug-resistance assays reveal long-term persistence of HIV-1 variants with the K103N nevirapine (NVP) resistance mutation in some women and infants after the administration of single-dose NVP: HIVNET 012. *J Infect Dis* **192**: 24-29.

Garcia-Diaz, A, Guerrero-Ramos, A, McCormick, AL, Macartney, M, Conibear, T, Johnson, MA, Haque, T, Webster, DP (2013) Evaluation of the Roche prototype 454 HIV-1 ultradeep sequencing drug resistance assay in a routine diagnostic laboratory. *J Clin Virol* **58**: 468-473.

Guay, LA, Musoke, P, Fleming, T, Bagenda, D, Allen, M, Nakabiito, C, Sherman, J, Bakaki, P, Ducar, C, Deseyve, M, Emel, L, Mirochnick, M, Fowler, MG, Mofenson, L, Miotti, P, Dransfield, K, Bray, D, Mmiro, F, Jackson, JB (1999) Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. *Lancet* **354**: 795-802.

Hauser, A, Mugenyi, K, Kabasinguzi, R, Kuecherer, C, Harms, G, Kunz, A (2011) Emergence and persistence of minor drug-resistant HIV-1 variants in Ugandan women after nevirapine single-dose prophylaxis. *PLoS One* **6**: e20357.

Havlir, DV, Eastman, S, Gamst, A, Richman, DD (1996) Nevirapine-resistant human immunodeficiency virus: kinetics of replication and estimated prevalence in untreated patients. *J Virol* **70**: 7894-7899.

Hedskog, C, Mild, M, Jernberg, J, Sherwood, E, Bratt, G, Leitner, T, Lundeberg, J, Andersson, B, Albert, J (2010) Dynamics of HIV-1 Quasispecies during Antiviral Treatment Dissected Using Ultra-Deep Pyrosequencing. *PLoS ONE* **5**: e11345.

Hoffmann, C, Minkah, N, Leipzig, J, Wang, G, Arens, MQ, Tebas, P, Bushman, FD (2007) DNA bar coding and pyrosequencing to identify rare HIV drug resistance mutations. *Nucleic Acids Res* **35**: e91.

Hudelson, SE, McConnell, MS, Bagenda, D, Piwowar-Manning, E, Parsons, TL, Nolan, ML, Bakaki, PM, Thigpen, MC, Mubiru, M, Fowler, MG, Eshleman, SH (2010) Emergence and persistence of nevirapine resistance in breast milk after single-dose nevirapine administration. *AIDS* **24**: 557-561.

Jackson, JB, Becker-Pergola, G, Guay, LA, Musoke, P, Mracna, M, Fowler, MG, Mofenson, LM, Mirochnick, M, Mmiro, F, Eshleman, SH (2000) Identification of the K103N resistance mutation in Ugandan women receiving nevirapine to prevent HIV-1 vertical transmission. *AIDS* **14**: F111-115.

Jackson, JB, Musoke, P, Fleming, T, Guay, LA, Bagenda, D, Allen, M, Nakabiito, C, Sherman, J, Bakaki, P, Owor, M, Ducar, C, Deseyve, M, Mwatha, A, Emel, L, Duefield, C, Mirochnick, M, Fowler, MG, Mofenson, L, Miotti, P, Gigliotti, M, Bray, D, Mmiro, F (2003) Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: 18-month follow-up of the HIVNET 012 randomised trial. *Lancet* **362**: 859-868.

Lallemant, M, Jourdain, G, Le Coeur, S, Mary, JY, Ngo-Giang-Huong, N, Koetsawang, S, Kanshana, S, McIntosh, K, Thaineua, V (2004) Single-dose perinatal nevirapine plus standard zidovudine to prevent mother-to-child transmission of HIV-1 in Thailand. *N Engl J Med* **351**: 217-228.

Lang, AH, Drexel, H, Geller-Rhomberg, S, Stark, N, Winder, T, Geiger, K, Muendlein, A (2011) Optimized allele-specific real-time PCR assays for the detection of common mutations in KRAS and BRAF. *J Mol Diagn* **13**: 23-28.

Larder, BA, Kohli, A, Kellam, P, Kemp, SD, Kronick, M, Henfrey, RD (1993) Quantitative detection of HIV-1 drug resistance mutations by automated DNA sequencing. *Nature* **365**: 671-673.

Lataillade, M, Chiarella, J, Yang, R, Schnittman, S, Wirtz, V, Uy, J, Seekins, D, Krystal, M, Mancini, M, McGrath, D, Simen, B, Egholm, M, Kozal, M (2010) Prevalence and clinical significance of HIV drug resistance mutations by ultra-deep sequencing in antiretroviral-naive subjects in the CASTLE study. *PLoS One* **5**: e10952.

Le, T, Chiarella, J, Simen, BB, Hanczaruk, B, Egholm, M, Landry, ML, Dieckhaus, K, Rosen, MI, Kozal, MJ (2009) Low-abundance HIV drug-resistant viral variants in treatment-experienced persons correlate with historical antiretroviral use. *PLoS One* **4**: e6079.

Lehman, DA, Wamalwa, DC, McCoy, CO, Matsen, FA, Langat, A, Chohan, BH, Benki-Nugent, S, Custers-Allen, R, Bushman, FD, John-Stewart, GC, Overbaugh, J (2012) Low-frequency nevirapine resistance at multiple sites may predict treatment failure in infants on nevirapine-based treatment. *J Acquir Immune Defic Syndr* **60**: 225-233.

Leitner, T, Halapi, E, Scarlatti, G, Rossi, P, Albert, J, Fenyo, EM, Uhlen, M (1993) Analysis of heterogeneous viral populations by direct DNA sequencing. *Biotechniques* **15**: 120-127.

Li, JZ, Paredes, R, Ribaudo, HJ, Svarovskaia, ES, Metzner, KJ, Kozal, MJ, Hullsiek, KH, Balduin, M, Jakobsen, MR, Geretti, AM, Thiebaut, R, Ostergaard, L, Masquelier, B, Johnson, JA, Miller, MD, Kuritzkes, DR (2011) Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis. *JAMA* **305**: 1327-1335.

Lockman, S, Shapiro, RL, Smeaton, LM, Wester, C, Thior, I, Stevens, L, Chand, F, Makhema, J, Moffat, C, Asmelash, A, Ndase, P, Arimi, P, van Widenfelt, E, Mazhani, L, Novitsky, V, Lagakos, S, Essex, M (2007) Response to antiretroviral therapy after a single, peripartum dose of nevirapine. *N Engl J Med* **356**: 135-147.

Loman, NJ, Misra, RV, Dallman, TJ, Constantinidou, C, Gharbia, SE, Wain, J, Pallen, MJ (2012) Performance comparison of benchtop high-throughput sequencing platforms. *Nat Biotechnol* **30**: 434-439.

Loubser, S, Balfe, P, Sherman, G, Hammer, S, Kuhn, L, Morris, L (2006) Decay of K103N mutants in cellular DNA and plasma RNA after single-dose nevirapine to reduce mother-to-child HIV transmission. *AIDS* **20**: 995-1002.

Marseille, E, Kahn, JG, Mmiro, F, Guay, L, Musoke, P, Fowler, MG, Jackson, JB (1999) Cost effectiveness of single-dose nevirapine regimen for mothers and babies to decrease vertical HIV-1 transmission in sub-Saharan Africa. *Lancet* **354**: 803-809.

Martinson, NA, Morris, L, Gray, G, Moodley, D, Pillay, V, Cohen, S, Dhlamini, P, Puren, A, Bhayroo, S, Steyn, J, McIntyre, JA (2007) Selection and persistence of viral resistance in HIV-infected children after exposure to single-dose nevirapine. *J Acquir Immune Defic Syndr* **44**: 148-153.

Mirochnick, M, Fenton, T, Gagnier, P, Pav, J, Gwynne, M, Siminski, S, Sperling, RS, Beckerman, K, Jimenez, E, Yogev, R, Spector, SA, Sullivan, JL (1998) Pharmacokinetics of nevirapine in human immunodeficiency virus type 1-infected pregnant women and their neonates. Pediatric AIDS Clinical Trials Group Protocol 250 Team. *J Infect Dis* **178**: 368-374.

Mofenson, LM (1997) Mother-child HIV-1 transmission: Timing and determinants. *Obstet Gynecol Clin North Am* **24**: 759-784.

Musoke, P, Guay, LA, Bagenda, D, Mirochnick, M, Nakabiito, C, Fleming, T, Elliott, T, Horton, S, Dransfield, K, Pav, JW, Murarka, A, Allen, M, Fowler, MG, Mofenson, L, Hom, D, Mmiro, F, Jackson, JB (1999) A phase I/II study of the safety and pharmacokinetics of nevirapine in HIV-1-infected pregnant Ugandan women and their neonates (HIVNET 006). *AIDS* **13**: 479-486.

Palmer, S, Boltz, V, Martinson, N, Maldarelli, F, Gray, G, McIntyre, J, Mellors, J, Morris, L, Coffin, J (2006) Persistence of nevirapine-resistant HIV-1 in women after single-dose nevirapine therapy for prevention of maternal-to-fetal HIV-1 transmission. *Proc Natl Acad Sci U S A* **103**: 7094-7099.

Palmer, S, Kearney, M, Maldarelli, F, Halvas, EK, Bixby, CJ, Bazmi, H, Rock, D, Falloon, J, Davey, RT, Jr., Dewar, RL, Metcalf, JA, Hammer, S, Mellors, JW, Coffin, JM (2005) Multiple, linked human immunodeficiency virus type 1 drug resistance mutations in treatment-experienced patients are missed by standard genotype analysis. *J Clin Microbiol* **43**: 406-413.

Paquet, AC, Baxter, J, Weidler, J, Lie, Y, Lawrence, J, Kim, R, Bates, M, Coakley, E, Chappey, C (2011) Differences in reversion of resistance mutations to wild-type under structured treatment interruption and related increase in replication capacity. *PLoS One* **6**: e14638.

Paredes, R, Lalama, CM, Ribaudo, HJ, Schackman, BR, Shikuma, C, Giguel, F, Meyer, WA, 3rd, Johnson, VA, Fiscus, SA, D'Aquila, RT, Gulick, RM, Kuritzkes, DR (2010) Pre-existing minority drug-resistant HIV-1 variants, adherence, and risk of antiretroviral treatment failure. *J Infect Dis* **201**: 662-671.

Richman, DD, Havlir, D, Corbeil, J, Looney, D, Ignacio, C, Spector, SA, Sullivan, J, Cheeseman, S, Barringer, K, Pauletti, D, et al. (1994) Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. *J Virol* **68**: 1660-1666.

Rosenbloom, DI, Hill, AL, Rabi, SA, Siliciano, RF, Nowak, MA (2012) Antiretroviral dynamics determines HIV evolution and predicts therapy outcome. *Nat Med* **18**: 1378-1385.

Rowley, CF, Boutwell, CL, Lee, EJ, MacLeod, IJ, Ribaudo, HJ, Essex, M, Lockman, S (2010) Ultrasensitive detection of minor drug-resistant variants for HIV after nevirapine exposure using allele-specific PCR: clinical significance. *AIDS Res Hum Retroviruses* **26**: 293-300.

Sanne, I, Orrell, C, Fox, MP, Conradie, F, Ive, P, Zeinecker, J, Cornell, M, Heiberg, C, Ingram, C, Panchia, R, Rassool, M, Gonin, R, Stevens, W, Truter, H, Dehlinger, M, van der Horst, C, McIntyre, J, Wood, R (2010) Nurse versus doctor management of HIV-infected patients receiving antiretroviral therapy (CIPRA-SA): a randomised non-inferiority trial. *Lancet* **376**: 33-40.

Schuurman, R, Demeter, L, Reichelderfer, P, Tijnagel, J, de Groot, T, Boucher, C (1999) Worldwide evaluation of DNA sequencing approaches for identification of drug resistance mutations in the human immunodeficiency virus type 1 reverse transcriptase. *J Clin Microbiol* **37**: 2291-2296.

Shapiro, RL, Hughes, MD, Ogwu, A, Kitch, D, Lockman, S, Moffat, C, Makhema, J, Moyo, S, Thior, I, McIntosh, K, van Widenfelt, E, Leidner, J, Powis, K, Asmelash, A, Tumbare, E, Zwerski, S, Sharma, U, Handelsman, E, Mburu, K, Jayeoba, O, Moko, E, Souda, S, Lubega, E, Akhtar, M, Wester, C, Tuomola, R, Snowden, W, Martinez-Tristani, M, Mazhani, L, Essex, M (2010) Antiretroviral regimens in pregnancy and breast-feeding in Botswana. *N Engl J Med* **362**: 2282-2294.

Simen, BB, Simons, JF, Hullsiek, KH, Novak, RM, MacArthur, RD, Baxter, JD, Huang, C, Lubeski, C, Turenchalk, GS, Braverman, MS, Desany, B, Rothberg, JM, Egholm, M (2009a) Low-Abundance Drug-Resistant Viral Variants in Chronically HIV-Infected, Antiretroviral Treatment–Naive Patients Significantly Impact Treatment Outcomes. *Journal of Infectious Diseases* **199**: 693-701.

Simen, BB, Simons, JF, Hullsiek, KH, Novak, RM, Macarthur, RD, Baxter, JD, Huang, C, Lubeski, C, Turenchalk, GS, Braverman, MS, Desany, B, Rothberg, JM, Egholm, M, Kozal, MJ (2009b) Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. *J Infect Dis* **199**: 693-701.

Stringer, EM, Ekouevi, DK, Coetzee, D, Tih, PM, Creek, TL, Stinson, K, Giganti, MJ, Welty, TK, Chintu, N, Chi, BH, Wilfert, CM, Shaffer, N, Dabis, F, Stringer, JS (2010) Coverage of nevirapine-based services to prevent mother-to-child HIV transmission in 4 African countries. *JAMA* **304**: 293-302.

Tisdale, M, Kemp, SD, Parry, NR, Larder, BA (1993) Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proc Natl Acad Sci U S A* **90**: 5653-5656.

Van Laethem, K, Van Vaerenbergh, K, Schmit, JC, Sprecher, S, Hermans, P, De Vroey, V, Schuurman, R, Harrer, T, Witvrouw, M, Van Wijngaerden, E, Stuyver, L, Van Ranst, M, Desmyter, J, De Clercq, E, Vandamme, AM (1999) Phenotypic assays and sequencing are less sensitive than point mutation assays for detection of resistance in mixed HIV-1 genotypic populations. *J Acquir Immune Defic Syndr* **22**: 107-118.

Wiktor, SZ, Ekpini, E, Nduati, RW (1997) Prevention of mother-to-child transmission of HIV-1 in Africa. *AIDS* **11 Suppl B**: S79-87.

Zolfo, M, De Weggheleire, A, Schouten, E, Lynen, L (2010) Time for "test and treat" in prevention of mother-to-child transmission programs in low- and middle-income countries. *J Acquir Immune Defic Syndr* **55**: 287-289.