CHAPTER V

DISCUSSION AND CONCLUSION

Drug resistance is a major contributing factor to the failure of antiretroviral therapy. The development of drug resistance further complicates clinical management because of the high level of cross-resistance within the drug classes. For instance, the mutations that convey high-level of cross resistance within NNRTI class e.g., K103N (multi-NNRTIs resistance), Y181C and the mutation that is crucial for subsequent development of high-level cross resistance within NRTI class e.g., Q151M for multi-nucleoside resistance (MNR),[13] T215Y/F for AZT resistance (also known as thymidine analog mutations, TAMs).[11] Resistance to AZT sequentially occurs at codon 70, 215, 41, 67 and/or 219 under the treatment pressure. Currently, AZT resistance mutation can be induced by other NRTIs and known as nucleoside analog mutations (NAMs).[12] Furthermore, 4 NAMs or more mutations approved to associate with cross-resistance to NRTIs except lamivudine. Therefore, the T215Y/F mutation is chosen as a potential marker of NAMs.

Resistance testing has recently been recommended as a helpful monitoring assay for the management of HIV infection.[93] Recently, several phenotypic and genotypic assays for drug resistance have been developed. However, they are still expensive and not widely accessible. A rapid, cheap, and less laborious assay is desperately needed. The K103N/Y181C and Q151M/T215Y/F duplex selective PCRs have been developed in this study.

The Amplification Refractory Mutation System (ARMS) has been successfully applied for analysis of genetic mutations [19] and has been modified for development of HIV drug resistance assay. To improve selectivity of ARMS, an additional mismatch was introduced into both WT and MT specific primers.[20]

We have modified the ARMS technique by introducing an additional mismatch close to the 3'-terminal nucleotide. However, better specificity was observed in only some primer sets (codon 151, 181, and 215). In an attempt to make codon 103 primer sets more specific, the primers was modified from a mutagenically-separated PCR primer as described previously by Frater et al.[106] and it showed a satisfactory specificity.

The obstacle to develop the primer of codon 215 is the difference of nucleotide specific sequence between HIV-1 subtype B and subtype A/E. In subtype B, most of the mutations at Thr 215 to Phe or Tyr are changed from ACC to TT**C** or TA**C** while most of this mutations in subtype A/E (Thailand) are changed from AC**T** to TT**T** to TA**T**. This observation explains why previously mutant specific primer by Larder et al. [20] that could identify both mutation changes in subtype B failed to detect subtype A/E 215Y mutation in Thai patients. This study indicates the limitation of primer design for use in different subtypes.

The key to develop a successful duplex selective PCR depends upon several factors such as dNTPs, magnesium, enzyme, primer concentrations, and amplification annealing temperature. However, the most important factor is the good primer design including the strength and positions of nucleotide mismatches in the primer.

Previous studies demonstrated the use of 3'-terminal mismatch primers for ARMS.[19,107] In those studies, G:T, T:G, A:C, and C:A, mismatches were extended by Taq DNA polymerase whereas A:A, T:T, C:T, and G:A mismatches were refractory to extension. This observations may explain the reasons of why the primers of codon 181 that used C:T additional mismatches and the modified primer of codon 103 from Frater et al.[106] that used G:A additional mismatches have more specificity than the first generation primers of codon 103 which used C:A additional mismatches.

In Thailand, most individuals are infected with HIV-1 subtype A/E. Much have been reported for genotypic resistance of HIV-1 in North America and Europe where HIV-1 subtype B predominates. However, little is known about the HIV-1 genotypic resistance in other part of the world, particularly in regards to other subtypes. By using duplex selective PCR assay for genotypic resistance evaluation in HIV-1 infected Thais, more than 94% showed concordant results with sequencing analysis which is the gold standard. There was significant difference of genotypic resistance between ART-naïve patients (group I) and ART-experienced of more than 6 months HIV-1 infected patients (group II) ($p < 0.01$, Chi-square test). No mutations were observed in the ART-naïve, while 23 of the 25 (92%) ART-experienced patients harbored drug-resistant mutant viruses. Our finding is quite different from a current report from Spain that revealed up to 12.7% of ART-naïve patients that carried drug-resistant mutant viruses.[108] However, the genotypic resistance in ART-experienced

Thai patients (subtype A/E) is similar to this report. The most common mutation occurred at codon 215 (76%) since most of the ART-experienced patients were treated with AZT in combination therapy and no mutation were found at codon 103 or 181 in patients who have been treated with NRTIs only. These findings suggest that HIV resistance occurs under selective pressure of antiretroviral drugs and also emphasizes that resistance testing is helpful to guide switching regimens in patients who fail therapy.

In comparison to the results generated by sequencing analysis (the gold standard assay), the duplex selective PCR showed more than 94% (33/35) concordance. Of the few discordance, the first one was 103N mutation by duplex selective PCR but 103K (wild type) by the sequencing assay. It may suggest possibility of greater sensitivity of duplex selective PCR. Since this patient was treated with EFV in his combination drugs, so the K103N could be occurred. However, it is also possible that it could be a result of mispriming or false positive. The second discordance was 215Y mutation by sequencing but was unamplifable by duplex selective PCR. It may be a result of inefficiency of the 215 mutant specific primer. The issues of minority of mutants in the samples amplifying errors from a tiny volume of blood for both duplex selective PCR and sequencing may explain such discordance. Further development is needed to improve the efficiency of the primer of this codon.

Although genotypic resistance assay by HIV genomic sequencing is currently the reference procedure that provides information on all nucleotides on the regions being sequenced, the assay is expensive and thus unsuitable for resource limited countries. With the high sensitivity (96%) and specificity (98%), as well as lower cost, these new duplex selective PCR assays are thus valid for further cost-effective HIV drug resistance surveillance study in resource limited countries (Table XI). For clinical uses in detection of NNRTIs and multi-nucleoside resistances however needs further investigation in a larger scale study.

Table XI : Comparison of duplex selective PCR with sequencing analysis

	Duplex Selective PCR	**Sequencing Analysis**
Minimal viral load cut-off	1,000 copies/mL	1,000 copies/mL
Methodology	Comparable assay	Gold standard assay
Number of mutation detection	4 codons	all
Cost[a]	500 bath	2,500 bath
Time consuming of the assay	8 hrs.	3 days
Sensitivity	96 %	Gold standard
Specificity	98 %	Gold standard

[a] This is not include the fixed cost of labor and instrument maintenance.