CHAPTER 5

DISCUSSION AND CONCLUSION

HIV-1 strains are genetically diverse. Since the first report of HIV-1 genetic sequence to date the molecular pictures of HIV-1 genetic diversity in pandemic have changed from pure subtype to mixed subtype circulation. In consequences, there are many CRFs and out numbers of URFs generated. The molecular surveillance and assessment of HIV-1 diversity is important for vaccine design, development and trial design, potentially for anti-viral drug development, diagnostic procedures, and viral load monitoring assay. Moreover, the proportion of HIV-1 molecular forms is also a useful tool revealing the risk exposures, transmission link/network and ongoing recombination process of HIV-1.

Several techniques have been used for HIV-1 subtyping. The heteroduplex mobility assay (HMA) is sensitive and specific for envelope and gag genotyping. This assay is labor intensive, time consuming, limited to two small genome regions, so it is not for screening in a large-scale population. In addition, the experience is needed to interpret the results from some diverse HIV-1 strains. (17-21). Serotyping with V3-loop peptide enzyme linked immunoassay (V3-PEIA) is rapid, simple but often shows cross-reactivity (22 , 23). Subtype specific polymerase chain reaction (PCR) in some parts of HIV-1 genome is simple with high specificity but not powerful enough to catch the recombinant strains and dual infections of HIV (23). The recently developed Multi-region Hybridization Assay (MHA) family is a very powerful tool for subtype screening with high sensitivity and specificity. (24, 25, 110, 112) However, obtaining

a real time PCR machine and subtype specific probes is an expensive investment. The gold standard subtyping method is full-length genome characterization by PCR and sequencing (27, 48) but it is expensive and very low throughput. Thus, the development of a multi-region subtype specific PCR (MSSP) assay was particularly appealing. It would be as powerful as MHA for identifying subtypes and recombinant viruses circulating in Thailand and neighboring countries.

As stated in an early chapter that the objective of this study was firstly to design an assay for determination of HIV-1 subtype, inter-subtype recombinant strains, and dual infections in clinical samples in Southeast Asia where subtype B, C, and CRF01_AE co-circulate. Secondly, to use the new MSSP assay to determine the HIV-1 subtypes and recombinants among northern Thai drug users in 1999.

From the results, it was shown that the MSSP assay was designed to determine HIV-1 subtype B, C, CRF01_AE, their inter-subtype recombinant strains, and dual infections in clinical samples in Southeast Asia where these strains co-circulate. The novelty of the assay was its principle. It was to separately amplify each of eight different target regions distributed along the HIV-1 genome in a nested PCR format. The primers were designed in such a way that outer primers were universal and able to amplify all subtypes, B, C and CRF01_AE, while the inner primers were subtype specific. These outer primers located in highly conserved regions and the inner primers located in divergent regions between subtypes, B, C and CRF01_AE. These primers were positioned to incorporate multiple mutations distinguishing subtypes, well distributed along the primer and including mismatches at the 3'-end in some primers. The location of primers were designed to sit on 8 regions along HIV-1 genome that enable this assay to identify 16 HIV-1 recombinant strains found in southeast Asia,

China and India. Totally 83 primers had been designed from the database constructed from circulating HIV-1 strains in the region. These primers were designed to have the melting temperature (Tm) close to 60°C enable the assay to be done in PCR microplate format. Among them 48 selected primers were chosen. They were the primers that gave a strong positive band with a positive strain and not showing cross-reactive with a negative strain. The sizes of first round PCR amplicon ranged from 262-847 base pairs (bp), and second round PCR product ranged from 60-428 bp. The amplicons were easily visualized on ethidium bromide stained agarose gels and the interpretation was very straightforward.

Based on the evaluation of 41 DNA samples mostly from incident HIV-1 infected individuals, the specificity of the MSSP assay was 100% and the sensitivity was in a range of 73-100%. The incomplete sensitivity was due to high genetic diversity of HIV genome.

In order to evaluate the performance of the MSSP assay in field collected samples, 337 available serum samples obtained from drug users cohort in Northern Thailand in 1999 to 2000 were subjected to RT MSSP assay. From these specimens, the reactivity of RT MSSP assay was in a range of 73-93%. The numbers of typeable regions were up to 8. With respect to the number of regions and the percentage of typeable samples, up to 99.1% were typed at least for 1 region and 44.2% had all eight regions typed. Since 94% of samples had at least 4 regions typed, so a sample with 4 typeable regions was subtype assigned. On the other hand, samples with less than four reactive regions were identified as non-typeable samples. They included subtype-containing strains and non-reactive samples.

Based on the criteria as mentioned above, 315 samples (94 %) were typed. In this cohort, HIV-1 subtype distribution was 77.4 % CRF01_AE, 12.2% CRF01_AE /B recombinant, 3.3% subtype B, 0.6% CRF01_AE/C recombinant and 7% non-typeable samples. The non-typeable samples were 54.5% CRF01_AE-containing strains, 22.7% subtype B-containing strains, 4.6% CRF01_AE /B -containing strain, 4.6% subtype C -containing strain and 13.6% non-reactive samples. This showed that the genetic diversity of this northern Thai drug user population was as complex as what was reported in Bangkok IDU cohort. (113)

In 2002, a novel simple and rapid assay based on subtype-specific PCR for subtype B, and CRF01_AE was developed for *env* region (106). The specificity of this assay was not impressive. The possible explanation for that the assay was designed to use a combination between subtype specific reverse primers and universal forward primers. In 2004, HIV-1 subtype-screening tool by nested multiplex PCR using subtype-specific primers based on subtype signature pattern (SSP) analyses was developed**.** This assay showed high sensitivity and specificity, but it was not powerful enough to catch recombinant strains because it was designed to differentiate HIV-subtype only in *gag* region. (114).

Under these circumstances the MSSP assay was superior comparing to other available subtype-specific PCR assays in terms of capability and sensitivity to use as HIV-1 subtyping tool in the region where subtype B, C and CRF01_AE co-circulate. The specimen used can be either PBMC DNA or serum/plasma RNA.

The similar discordant subtype patterns seen in this population indicated the existence of some circulating recombinant strains. They were two similar recombinant subtype patterns found in this cohort. The first group was OUR 1331 and 1332 and

they were previously characterized and termed as CRF15_01B. The second group included OUR 1969, 2275, 2475, 2476 and 2478. Among them, OUR 1969, 2275 and 2478 were further characterized by full genome amplification and sequencing. They had similar CRF01_AE/B recombinant genome structures and were classified as CRF34_01B (Tovanabutra et al, in preparation).

Moreover, the MSSP assay was able to capture dual infections. Fourteen out of 43 recombinant samples (32.6%) in this cohort gave the PCR reactivity with more than one subtype in a given genome region and they were classified as dual infections. The possible explanation for dual reactive could be (i) the presence of multiple subtypes; (ii) the presence of an atypical or unique sequence, not foretold from the overall sequences, within the viral quasi-species; or (iii) the sequence at location where the subtype-specific primer located were slightly offset due to the presence of recombinant breakpoints. However, the PCR products from a dual reactive genome region of some samples were cloned and sequenced to verify dual infections. The results revealed that some samples harbored two different HIV-1 strains and some harbored HIV-1 diverse strains.

The great advantage of this assay is the cost per test which is about 1,500 baht (approximately 38 US$ per test). Multi-region hybridization assay (MHA) is considered inexpensive (approximately 50 US$ per test), but the cost does not include an investment for a real-time PCR machine. Full-genome characterization costs 1,500 US$ per test for reagent, but this method needs a sequencer which is very expensive to purchase and maintain.

The limitation of the MSSP assay is the assay was designed for Southeast Asia, South Asia, and China where subtype B, C and CRF01_AE are co-circulating. It is not

to be used in other HIV-1 subtype epidemic regions. However, this geographic area is home to a fast growing epidemic in which millions are at risks. In addition, for other geographic regions, the principal of the assay could be used to customize a new version of the assay to fit the circulating HIV-1 strains and their recombinants.

HIV-1 genetic complexity found in this drug user cohort may not directly impact vaccine trials because most of candidate vaccine cohorts were low risk populations. However, the linkage between IDU and heterosexual epidemics in Thailand was reported. (54) Hence, the existence of these HIV- recombinants has to be taken into account.

In conclusion, the MSSP assay is a powerful tool for determining HIV-1 subtype B, C, CRF01_AE, their inter-subtype recombinant strains, and dual infections. It is a simple, cost effective and accurate genotyping tool for laboratory settings with limited resources, and is sufficiently sensitive to capture recombinant genomes and dual infections in large-scale populations. From a public health perspective HIV-1 recombinant strains and dual infections were seen more frequent among drug user populations, which must be addressed by a globally effective vaccine. Focusing interventions in IDU populations may benefit individuals, by limiting progression and maximizing the impact of therapy. It also benefits the society, by limiting the overall complexity of strains that must be covered by vaccines and therapies.