CHAPTER VI

DISCUSSION

The emergence of viral resistant strains to antiretroviral therapy (ART) and the transmission of HIV-1 drug resistance (HIV-1 DR) variants have played role as a major and minor cause of treatment failure and limits options for alternative antiretroviral regimens, respectively. In the current situation that the use of antiretroviral drugs is widespread, HIV-1 DR screening and monitoring may be useful in guiding the choice of therapeutic regimens by identifying drugs that are unlikely to suppress viral replication. HIV-1 DR variants can be identified based on the phenotypic and/or genotypic determinations. Phenotypic assays evaluate the ability of HIV-1 replication in the presence of increasing concentrations of the antiretroviral agents whereas the genotypic assays determine the presence of drug resistance mutations in HIV-1 genome. However, due to the requirement of costly equipments, the infrastructure, and the skilled personnel of both assays, it is difficult to implement this assay to resource-limited areas.

From the systemic observation into the relationship between the phenotype and the genotype of HIV-1 variants that confer resistance to the antiviral drugs, it is evident that some specific point mutations affect HIV-1 drug-resistance (Johnson et al., 2008). Therefore, the techniques developed to detect the single nucleotide polymorphisms or point mutations may be applicable to detect HIV-1 DR as an alternative method to nucleotide sequencing (Edelstein et al., 1998; O'Meara et al.,

2001; Metzner et al., 2003, Nissley et al., 2005; Palmer et al., 2005; Tsongalis et al., 2005; Palmer et al., 2006). Multiple assays designed to detect point mutations in HIV-1 have been developed for evaluation of minor populations of drug-resistant: allele-specific PCR (ASPCR), pyrosequencing, single-genome sequencing, line probe assay and oligonucleotide ligation assay (OLA). These assays feature variable sensitivity and specificity in detecting mutations and require different laboratory equipments and technical expertise. Allele-specific PCR is considered as a highly sensitive and reproducible method for studying point mutations in viral genomes such as the study of minor HIV-1 variants harboring resistant mutations (Metzner et al., 2003; Metzner et al., 2005; Bergroth et al., 2005). ASPCR separately performs realtime PCR amplifications for the mutant and wild-type of any codon of interest, therefore, this assay is quite costly. The lower limit of detection of mutant variant is below 0.1-1%. This assay is significantly less labor-intensive and time-consuming than other techniques used for similar purposes. However, the polymorphisms that occur in the primer binding sites can significantly impair the sensitivity and accuracy of ASPCR.

Single-genome Sequencing (SGS) was developed based on limiting-dilution assays. SGS analyses HIV-1 populations by obtaining cDNA sequences derived from many single viral genomes. SGS detects minority variants that are present in at least 2% of the viral population. Although it is time and labour-consuming and requires highly skilled laboratory personnel, SGS is perfectly suited for assessing the linkage of several mutations in individual genomes, which can be of major importance in heavily pre-treated patients and in NNRTI-experienced candidates about to receive etravirine.

Line probe assay (LiPA) based on the principle of reverse DNA hybridization. It identifies specific point mutations from the reverse transcriptase and protease genes by using short oligonucleotide probes immobilized as parallel bands on nitrocellulose strips. The LiPA offers a simplified platform of mutation identification, an easier interpretation of relevant mutations, and an increased sensitivity to detect mutations present in minor viral population (Stuyver et al., 1997). However, the current LiPA has been developed in the laboratory's company (VERSANT® Bayer HealthCare LLC, Tarrytown, NY, USA) and is available only a limited number of mutations in the reverse transcriptase and protease genes that encompasses only a limited number of drugs.

In this study, the probe-based oligonucleotide ligation assay (OLA) is proposed as an alternative to the sequence-based method of HIV-1 drug resistance detection. The OLA is rapid, specific and sensitive reaction for the detection of known point mutation. The advantages of OLA include relatively inexpensive reagents, require minimal technical skill and ease of interpretation. The machines to perform OLA include thermocycler and spectrophotometer that are usually equipped in most laboratories. The high throughput of assay can be achieved by employing the multi-well streptavidin coated plate, such as the use of 96-well can be genotyped a codon of interest in 43 specimens in less than 5 hours. The cost of OLA reagents is less than 200 baht/codon/specimen.

In our study, we developed an OLA for the detection of Human Immunodeficiency Virus type 1 (HIV-1) M184V variant associated with lamivudine (3TC) and emtricitabine (FTC) resistance. The archival plasma specimens from HIV-1 infected patients who were diagnosed as having treatment failure (N = 40) served as

clinical specimens to determine the performance of M184V detection by OLA. According to nucleotide sequencing information, 97.5 % of the samples (39 of 40) in this study were HIV-1 subtype CRF01_AE with 2.5% subtype B sample (1 of 40). The thermostable DNA ligase enzyme, template and oligonucleotide probes, including wild-type, mutant and common probes were optimized to obtain the condition that the wild-type and mutant variants could be optimally distinguished.

The lower limit of the *pol* PCR amplification in our system is 10 copies (≈80% success rate) to 100 copies (≈100% success rate) according to initial data during setting up the assay (data not shown). The PCR products of such template amount usually contain 52-117 ng/µL of DNA. In the lower limit of detection analysis, the HIV-1 CRF01_AE mutant variants present at 3%-5% in a total of 25ng of DNA (equivalent to the amount of 11.16 log copies/mL to 11.38 log copies/mL) or as low as 1% (10.68 log copies/mL) of some variants in the PCR products could be detected by OLA ($p<0.005$). This 3% to 5% detectable range was consistent with other studies using OLA in the detection of HIV-1 drug resistant subtype B (Edelstein et al., 1998; Vega et al., 2005). The presence of HIV-1 variants in the range of 3%-5% of viral population would likely be undetectable by using most standard sequencing methods (Halvas et al., 2006). The capability to detect mutant variants by OLA when present at the low level is likely due to the use of amplified PCR-based products as the starting materials to generate the ligated products through multiple ligation cycles that are further captured by ELISA involving the sensitive enzymatic reaction.

For the performance evaluation of the HIV-1 M184V OLA, the performance of M184V detection by OLA in a small set of plasmid controls and 40 clinical specimens were assessed. The OLA results by using amplified PCR products of

reference plasmids representing wild-type (HM-12, BaL, CM019) and mutant (CM20, CM28) genotypes at codon 184 in the RT-gene as OLA templates, gave 100% concordance with standard nucleotide sequencing results. The results showed that these clones were successfully detected although the wild-type CM019 had the mutation at codon 181 (Y181I) that confer nevirapine resistance, and the clone CM020 in particular, had both M184V and Y181C mutations (see Table 6). Therefore, the multiple-drug resistant mutation at codon 181 and 184 in RT gene does not affect this OLA system to detect HIV-1 M184V mutation.

The performance evaluation of OLA results among 40 clinical specimens identified by the in-house sequencing genotype method which was the gold standard method, 30 samples (75.0%) were exclusively 184V-mutant genotype, 7 samples (17.5%) were 184M-wild-type, 1 sample (2.5%) was the mix of wild-type and 184V-mutant and 2 samples (5%) were exclusively 184I-mutant. The results of the OLA showed that among 40 clinical specimens, 28 samples were 184V-mutant genotype, 9 samples were 184M-wild-type genotype, 1 sample (2.5%) was the mix of wild-type and 2 samples were indeterminate results. Because our current OLA was not designed for 184I detection, therefore, the 2 samples with 184I were removed from the analysis. The overall results showed the comparison of OLA with in-house sequence analysis gave a concordance of 90.0% for the M184V mutation detection and provided a 93.5% of sensitivity and 100% of specificity for the 184V-mutant detection by using this method.

For the M184I mutation, is associated with 1,000-fold phenotypic resistance to 3TC, similar to the M184V mutation, additionally, it was reported that M184I appears earlier (Diallo et al., 2003) and is then outgrown by the 184V mutant (Larder et al.,

1995), which has superior RT polymerase function (Boyer et al., 1993; Beck et al., 2002). There was a notion that the M184I mutations were transitional mutations with a much lower fitness. Their presence indicates the near emergence of the M184V mutations. It was then, interesting to follow such patients for the length of transitional stage from 184I to 184V emergence, which may help to predict the optimal time to switch from 3TC to other retroviral agents.

Five percents (2 of 40) of the samples were 184I variants, which were not the target of our designed probes, and thus were not detected by OLA. However, the OLA was able to recognize the presence of wild-type strains in these samples which were missed by sequencing, this finding supports the greater potential of OLA to detect minor genotypes presence in quasispecies of virus circulating in patients. Consensus sequencing does not consistently detect genotypes present at concentrations <20% of the viral population (Schuurman et al., 2002). In contrast, the OLA can detect mutant genotypes present at concentrations as low as 5% among wild-type virus (Edenstein et al., 1998; Beck et al., 2002). The negative detection results of these 2 samples by OLA, therefore, further emphasize the high specificity of this assay.

Specificity of OLA is chiefly due to the nature of the thermostable-type of the DNA ligase and not the DNA annealing conditions (Landegren, 1988; Frenkel et al., 1995). Detection of single-base mutation based on the properties of DNA annealing generally require fine-tuning of the reaction conditions to achieve a high degree of specificity, whereas, the conditions used in OLA were not stringent in respect to DNA annealing. Additionally, 3 other factors could have played a role; specificity of hybridization of the probes to their complementary sequences on the template,

hybridization of the probes in the 5′-to-3′ orientation, and perfect base pairing at the target junctions (ligation site) of the probes. These conditions allow nonstringent annealing conditions without compromising specificity (Tobe et al., 1996).

Like other assays that based on the point mutation detection, the major limitation of these assays including OLA is that only selected nucleic acid is examined in which the new patterns of mutation cannot be revealed. Sequencing, on the other hand, give comprehensive information on all possible mutations within the region probed. Sequencing may be preferred for genotyping of specimens from highly drug-experienced individuals, with the caveat that mutation at low levels may not be detected. In contrast, the OLA, with a higher sensitivity for detection of low-level mutation, may be of great utility for the surveillance of drug resistance in settings where the antiretroviral mutational pattern is predictable, such as antiretroviral roll-out programs adopted by developing countries recently offering antiretroviral treatment. However, one disadvantage of the OLA compared with consensus sequencing is that new oligonucleotide probes must be developed as new drug resistant mutation is identified. Otherwise, when genetic polymorphisms occur near the target codon, hybridization or probe annealing can be failed.

In this study, the 5.0% (2/40) rate of indeterminate results at codon 184 of HIV-1 subtype CRF01_AE was observed. This rate, was lower than 31% of overall non-B subtypes and 6.6% of subtype E of HIV-1 that had been reported (Beck et al., 2008). From the inspection of nucleotide sequences data, the polymorphisms within the probes binding regions were abundant, as expected, in these HIV-1 genomes. The presence of several polymorphisms located in the sample's sequence, would inevitably resulting in decreased complementary to the probe. The probes used to

identify the genotypes; wild-type versus mutant variants, in the OLA are in the region of 40-50 bases long and due to the error-prone nature of HIV replication, the chance of mismatches existing in the probe region is high (Wallis et al., 2005). For the cost of polymorphisms that affect the OLA efficiency, not only the number but the site of polymorphisms appears to play a major role. Beck et al. (2002) and Edenstein et al. (1998) both reported that their indeterminate probe binding is attributable to the mismatch of the base located 3 base-upsteam of the ligation site. Nucleotide match at this site seems to be the paramount requirement for the ligation.

In conclusion, the HIV-1 DR detection using OLA was successfully developed for M184V variants emergence in the Thai population. The performance of the assay in plasmid controls with known genotype was reliable, sensitive and specific. However, further study needs to be carried out in order to improve the current OLA system and to increase the sensitivity when apply to clinical specimen testing. Such study include information of patients regarding the duration of HIV-1 infection, the treatment regimens, the duration of HIV-1 treatment, the drug compliance, the duration of treatment failure, as well as the CD_4 status the viral load, would be helpful to a better understanding the incidence of patients infected with HIV-1 DR among those who are receiving HIV-1 anti-retroviral therapy in Thailand.