CHAPTER V
DISCUSSION

Ten primers were selected and modified from previous studies [5, 27, 160] for the detection and identification of mycobacteria by Multiplex PCR. These primers were calculated their product sizes by the FastPCR computerized program. The results showed the clearly differences in size within 6 species mycobacteria. Additional, each pair of primers were determined chance of annealing by using the Oligonucleotide Analyzer Program. The results revealed that dG value of each primer was lower than 0 kcal/mol. This indicated the high affinity of primers pairing. Only MACF vs MACF and INT vs KAN1 showed higher dG values than 0 but less than 1kcal/mol. Therefore, the opportunity of annealing was lower than others but they still be appropriated for this study.

The efficiency and specificity of selected primers were tested by single PCR assay with standard condition for PCR reaction [27, 158] against 6 referent stains of mycobacteria. The first pair, ITS-F1 vs MYCOM-2 could amplify all of mycobacteria DNA at 16S-23S rDNA sequences with 300-400 bp products. The sizes of genus specific products depend on the species. The second pair, FORF vs ITS-F1 could amplify the target specific of *M. fortuitum* DNA with the product size of 210 bp. In order to amplify completely all types of *M. kansassii* type 1, 2, 3 the reaction required 3 forward primers KAN1, KAN2 and mKAN pairing to only one reverse primer ITS-F1 resulting of 170 bp amplified product. The mKAN primer was modified for this experiment by changing AAA AGC ACC CCA ATA GGT GG to AAA AGT GCC CCA ATT GGT GG from KAN2 [27]. The fourth pair, ITS-F1 vs SCOR could amplify *M. scrofulaceum* resulting product size of 340 bp. The fifth pair, TBR vs MYCOM-2 could amplify *M. tuberculosis* DNA resulting product size of 260 bp. The sixth and the seventh pairs, MACF vs MYCOM-2 and INT vs MYCOM-2 could amplify the target sequences of both *M. avium* and *M. intracellulare*; both pairs amplified the product size of 95 and 75 bp, respectively. This may cause by the similarity of target DNA which is the characteristic of the

complex group of mycobacteria. *M. avium* and *M. intracellulare* are grouping in *M. avium- intracellulare* complex (MAC), so this reaction could be understanded.

There are many factors that have to be optimized for Multiplex PCR, such as primer sequence, reaction buffer, annealing temperature, concentration of primer, DNA template and deoxynucleotide concentration [161]. Since there are many primers composed in the reaction of this study, so, the condition of Multiplex PCR should be optimized. Firstly, the optimization of Tm was found out between 55-66 ºC. The highest Tm which provided the clearest band of amplified products observed within 6 strains of mycobacteria, was selected (**Figure 9-14**). The higher Tm required higher binding specificity of primer and target and decreased the nonspecific annealing of the primer. Finally, the results showed the optimized Tm was 63.5 ºC following by the optimization of $MgCl_2$ concentration. High concentration of $MgCl_2$ provides the stabilized double-stranded DNA and protects the products in the next cycles by prevent completely denaturation of double strand DNA. Excess concentration of $MgCl_2$ can generate non-specific binding of primer to target. This is lead to a lot of false positive products. On the other hand, low $MgCl_2$ concentration impairs the extension reaction as Mg^{2+} is a required co-factor for enzymatic activity of most DNA polymerases. The optimal concentration of $MgCl_2$ selected in this study was 1.5 mM due to the clearest band of amplified products without nonspecific band (**Figure 15-20**). Last of all, the amount of dNTPs was optimized. Too small amount of dNTPs is lead to an unwanted size of products. However, the excess amount of dNTPs may cause misincorporation error. Therefore, the lowest amount of dNTPs, which yielded the clearest band of amplified product, was 200 µM (**Figure 21-23**).

The Single-tube Multiplex PCR in this study could detect the lowest limited concentration of *M. avium* at 500 pg, *M. fortuitum* at 5 pg, *M. intracellulare* at 5 pg, *M. kansasii* at 50 pg, *M. scrofulaceum* at 50 pg and *M. tuberculosis* at 50 pg. Therefore, a sufficient amount of genomic DNA necessary for this technique is $\geq$500 pg (**Figure 24-29**). This sensitivity indicated at least 10^4 of the mycobacteria cells enable to detected by Multiplex PCR, if one cell haboring 5 fg of genome DNA [162].

Once the condition of Multiplex PCR assay was well optimized, the 15 referenct strains of mycobacteria and 3 reference strains of nonmycobacteria were tested by this method. The results showed the accurate product sizes specific to their species (**Figure 30-31**). Only *M. gordonae* TB-D 071-05 could be amplified resulting 150 and 330 bp products. But *M. gordonae* ATCC 14470 was amplified only 330 bp product, which is the genus specific band. When, 16S-23S rDNA spacer sequence of *M. gordonae* complementary to mKAN primer, the result showed 80% binding. This revealed the nucleotide sequences of *M. gordonae* and *M. kansasii* are close to each other. However, the product size of *M. gordonae* is totally different from others (6 species of mycobacteria). So, if the 150 and 330 bp products are found, it may be assumed as *M. gordonae* or repeat with another technique such as PCR-REA, INNOLiPA and Biochemical test.

The efficiency of multiplex PCR assay was studied by comparing the identication results of Multiplex PCR and PCR-REA. The 64 clinical isolates of *Mycobacterium* species were tested and both assays showed almost the same results. Exception one strains, which the multiplex PCR assay could identify *M. intracellulare* whereas PCR-REA could only identify as MAC[158]. The reason might be due to DNA sequences of MAC close to *M. intracellure* and the both of them are was in same group. So a concordance was 98.4%.This reflects the better ability of Multiplex PCR than PCR-REA to distinguish MAC group into each species. The results of Multiplex PCR were also compared with INNOLiPA for another 3 species and both assays could show no disagreement result. However, the concordance value was not calculated since the number of sample was undersized. Additional, the results of Multiplex PCR assay was also compared with of PNB screening test and the results showed 100% agreement for MTB complex identification. There was one out of 8 NTM isolates, identified as MTB complex by Multiplex PCR. The biochemical test using for screening the MTB complex is PNB inhibition test. This could inhibit 95.9% of MTB complex [163], so, there is about 4.1% of tolerant strains that could grow on PNB medium. Therefore, it may be the advantage of the Multiplex PCR in identifying the PNB resistant strains of MTB complex.

In the present, the detection and identification based on molecular technique can be done as Real time PCR [164] which is comfortable, rapid, high through put and

safe from contamination of cary over, but require the complicated and expensive instrutment, so it is more expensive cost. PCR- reverse hybridization (such as INNOLiPA) [161] is another test which is easy and high accuracy but the cost is very high too. PCR –REA [165] are low cost, simple assay but there are many steps of experiment and the results sometimes are not clear cut.DNA sequencing [166] is very sensitive, specific technique but expensive and require the well trained technician, so, it is not suitable for routine. The Single- tube Multiplex PCR could perform as single PCR. Single-tube Multiplex PCR is the well established technique, can be done successfully oneday, the test is simple, and does not high expense. In conclusion, Single-tube Multiplex PCR will be one choice assay due to comfortable, rapid and reliable assay.