CHAPTER V

DISCUSSION

The methods presently used to diagnose *M. pneumoniae* infections are inadequate because they lack speed, sensitivity and specificity. Since PCR technology can overcome these problems, it is a promising candidate to replace current diagnostic methods. The goal of this study was to develop PCR method that was suitable and reliable for detection of *M. pneumoniae* in routine diagnostic laboratory and evaluate the use of developed PCR in simulated clinical samples.

The primers, MP-1 & MP-2, were selected for the reason that the target for these primers is the P1 cytadhesin, a virulence factor of *M. pneumoniae*. P1 cytadhesin is a protein that allows this organism to attach to respiratory epithelium cells in human and animal (36). This result showed that the 466-bp segment of the P1 gene was successfully amplified from pure cultures of all five ATCC strains of *M. pneumoniae*. No false positive was detected after using other mycoplasmas, other bacteria and human leukocytes DNAs as templates.

With this method, the 466-bp product specific for *M. pneumoniae* could be obtained from a minimum of 10 fg of *M. pneumoniae* DNA. This result was agreed with that from Buck et al. (31). With similar conditions excepted the selected primers, they

were able to detect between 1 and 10 organisms. Comparing with the study from Bernet et al. (28), which amplified specific clone DNA as target, this study and Buck's provided more sensitivity results. The reason for the difference in results is not entirely clear but is probably related to the target sequence selected for amplification. From this study indicated that only 10 fg corresponding to 10 genome copies (35) of *M. pneumoniae* DNA could be detected. It will be very useful to apply to routine diagnostic test.

At this point, amplification of the 466-bp segment provided an exquisitely sensitive and specific. However, it should be noted that these results were obtained with purified DNA as the template in PCR. Therefore, the same achieved sensitivity and specificity might not obtained with clinical specimens.

In order to set up as a routine procedure, the confirmation of PCR amplification products needs to be simplified with regard to saving time and avoiding the use of radioactively labelled compounds. The nested amplification and the fluorescein-labelled dot blot hybridization were performed to compare the efficiency of *M. pneumoniae* detection. The same concentration of PCR product from 1 fg template was able to be detected. However, the amplicon carryover which will give the false-positive for the PCR should be awared. It sounded that the dot blot hybridization provide the more reliable result than nested PCR. To solved the amplicon carryover in nested PCR, the enzymatic degradation procedure was selected. From the principle that contamination was due to the presence of amplicons,

not *M. pneumoniae* bacteria or DNA (88). Using incorporation of dUTP instead of dTTP and addition of UNG in the PCR reaction (89). The UNG cleave uracil residues from single stranded and double stranded DNA. Increasing the temperature to 94°C for 10 min, the UNG was inactivated and phosphodiaster bond in abasic site of DNA was hydrolysed result in the break down of amplicons into small fragments. As dNTP appear to quantitatively bind to Mg^{2+}, the amount of dNTPs present in a reaction will determine the amount of free magnesium available. When the dUTP was replaced dTTP, the optimal dUTP concentration is approximately increase 10 times of dTTP, a compensatory change in $MgCl_2$ was, therefore, necessary. The optimal concentration of $MgCl_2$ was changed from 1.5 to be 3.0 mM. After setting the dUTP condition, sensitivity of dU-PCR was tested, the minimum of *M. pneumoniae* DNA can be detected also 10 fg as same as that of dT-PCR.

To evaluate the ability of UNG. PCR performed with 10 ul of a 10^6-times diluted 15 ng/ul 466-bp amplicon solution and 0.1 U of UNG, which is about 150 fg. still gives a clearly visible band in an agarose gel. The use of 0.1 U of UNG was sufficient to break down 15 pg of 466-bp amplicon. As far as the literatures were reviewed the report concerning the enzymatic degradation of the amplicon, in *M. pneumoniae* was not found.

An alternative to the pre-PCR enzymatic amplicon degradation method is the post-PCR photochemical method. Many investigators prefer the former since high concentration of

isopsoralen that are needed in the post-PCR photochemical method for inactivation of small and GC-rich amplicons can reduce the activity of *Taq* polymerase (90); moreover, isopsoralen is mutagenic.

For the clinical diagnostic, the ten simulated clinical specimens were employed. The results showed that 10 pg spiked DNA in each sample was able to detected with the differnt intensity (Fig. 18). This might be due to certain unknown inhibitors containing in the specimens. To recheck the sensitivity of the PCR, the serial dilution of spiked DNA, 10 pg to 1 fg were added to the pooled specimens. It was found that the amplified product was able to be detected at 100 fg DNA template. Comparing the sensitivity from the purified DNA without throat swab experiment was 10 times decreased. This results was corresponded to the unpooled samples results.

All the results suggest that PCR has significant potential as a rapid, sensitive and specific for detecting *M. pneumoniae*. It was 100- to 1,000-fold more sensitive than culture or commercial gene probe. The method used to treat specimens to remove DNA prior to amplification is a simple, rapid procedure that could be used in any laboratory. With the addition of more simplified procedure for detecting the amplified DNA, this test could easily be used as a rapid diagnostic test for *M. pneumoniae* infections. The establish protocol is under way to further evaluate the performance of this procedure by a prospective study with patient specimens.