CHAPTER VI

DISCUSSION

PCR in general offers the potential for a rapid and sensitive assay for the diagnosis of infections caused by organisms that grow slowly in vitro or organisms which growth conditions are suboptimal, leading to a low sensitivity of the culture technique. The low sensitivity of the culture of *M. pneumoniae* (23%-64%) has been well documented by comparison with serologic results (29,35). However, for diagnostic purposes serology offers no alternative, because significant rises in IgG titers appear late, and detection of IgM is often unreliable due to the cross-reactivity of rheumatoid disease (22). The purposes of this study were to evaluate a nested PCR for detection of *M. pneumoniae* infection by the use of two sets of primers, P1 gene and 16S rRNA gene.

In this study, *M. pneumoniae* from throat swab samples of patients could not be isolated by culture on modified Hayflick medium. According to its fastidious nature, the process of culture of *M. pneumoniae* is very difficult to perform (75). However, a few laboratories could successfully set up this method. The sensitivity of this method is quite low (30-60%) in comparison with serology method (101). In addition, Skakni and coworkers suggested that culture positivity may be partly related to the presence of a large number of

organisms (>10^4 CCU/ml) in the sample with the culture system (37). Our negative culture result may be from the low number of bacteria (<10^4 CCU/ml) in sample. It may be possible to collect higher number of bacteria by using two swabs together for specimen collection. One swab is then used for culture and the other one for PCR. Other reasons were probably due to undefined problem of the transport technique, i.e. specimens were not immediately sent to laboratory for culture, so that the number of organisms may be reduced to the undetectable level.

For PCR to be applicable in the diagnostic microbiology laboratory, sample preparation and amplicon detection should be as simple as possible. Preparation of samples was simplified by replacing the time-consuming phenol-chloroform extraction by treatment with proteinase K (41,99). This proved to be a fast method which did not result in loss of DNA (99). Two primer sets were used for the amplification. The MP-primer set was 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 (46). The 16S rDNA primer set was also selected. The 16S rRNA sequences have revealed the existence of regions with highly conserved sequences. This results showed that the 466-bp segment of the P1 gene and 277-bp of 16S rRNA gene were successfully amplified from pure culture of *M. pneumoniae* Mac strain. The product of MP-PCR could be obtained from a minimum of 10 fg of *M. pneumoniae* DNA. This result was agreed with that from Buck et al. (38). With similar conditions excepted the selected primers, they were able to detect between 1 to 10 organisms.

However, the product of 16S rDNA PCR could be detected from a minimum of 10 fg of *M. pneumoniae* DNA, while earlier study from Jeroen et al. (99), with the similar conditions and primers, 1.5 CFU of organisms could be detected. The reason for the difference in results is not entirely clear but is probably related to the error of CFU counts.

In order to increase the sensitivity of detection, two-step PCR (nested PCR) was used instead of a time-consuming hybridization protocol. By this method, the detection limit is increased for both primers sets to 1 and 0.1 fg of *M. pneumoniae* DNA by MP nested PCR and 16S rDNA PCR, respectively. However, increase in sensitivity was accompanied by an enhanced risk of contamination of PCR product (amplicon) (101). To prevent the amplicon carryover, strict following the guidelines for the general handling of the PCR procedure (105), and the use of enzymatic degradation procedure were selected. Using incorporation of dUTP instead of dTTP and addition of UNG in PCR reaction (48), UNG cleave uracil residues from single stranded and double stranded DNA. Further heat treatment destroyed the phosphodiester bond at the apyrimidinic site. Thus, the amplicon was destroyed and played no role as target for amplification.

In normal volunteers, it was found that PCR and culture were negative in all subjects. The results of the serological test (MAG assay) showed that the titers ranging from ≤ 1:40 to 1:80 were observed (Table 2). The titer of ≥ 1: 40 was interpreted as positive according to manufacturer's recommendation. It is possible that volunteers with titer of ≥ 1: 40 had persistence of antibody from previous infection. False

positive by serological method could be excluded despite of the low possibility. Several authors have reported carriage of *M. pneumoniae* after symptomatic infection (4) or after treatment (106). However in this study, indications for carriership were not found. All controls were PCR and culture negative.

The PCR for the detection of *M. pneumoniae* was evaluated on clinical samples by comparing the results obtained by the *M. pneumoniae* PCR with those obtained by culture and serology. The MAG assay and *M. pneumoniae* PCR yielded concordant results for samples from 92 % of the patients (Table 6). Although a good correlation was found between serology and PCR results, samples from eight patients gave discordant results, as shown in Table 7.

In children patients (Group I), the best correlation between serology and PCR results was observed. This result was agreed with previous reports (12,107). The MAG assay and *M. pneumoniae* PCR yielded concordant results for samples from 21 patients (Table 4 and Table 5). Discrepant results were obtained for samples from 2 patients (sample no.8 and no.9), which were serologically positive (titers of 1:80 and 1:160) as shown in Table 5. This is probably due to several reasons: first, the persistence of antibodies from past infection such as sample no.8, the patient had a stable titer in paired sera; second, false-positive serological test; third, false negative PCR because of the amount of DNA is too low to be detected. However, these cases were interpreted as negative results by laboratory diagnosis because results of culture and PCR (two targets) were negative.

In adults patients (Group II), 4 of 77 samples were interpreted as positive by laboratory diagnostic criteria (Table 4). The interpretation of positive results was, with exception of one case, more difficult because serological result needs paired sera to convince the positive result in this patients. In the one exception case, the patient had a positive PCR results (MP-nested PCR and 16S rDNA nested PCR), and a high antibody titer was detected in the first serum by the MAG assay. The other three patients with a positive nested PCR gave negative results by serological (MAG) test at first serum (titer ≤ 1:40) which could be explained that serological samples were probably taken too early in the disease. The possibility of false-positive PCR results seems very unlikely, because PCR was positive in two primer sets, and it had the negative control of amplification. In addition, the second serum of three samples were tested to confirm and showed four-fold rising or greater in titers. It suggests that paired sera should be taken for serological test for the accurate of results. For the one patient, whose second serum was missed, it should be regarded as positive case followed by laboratory criteria. In the adult patients group, there were 5 patients with the titers of >1:40, but negative PCR (Table 5). These samples were considered positive by MAG test. It is possible that these patients were infected by *M. pneumoniae* in the past time with the long-term persistence of antibodies (25,71). On the other hand, it may be false positive of serology or false negative of PCR. In 68 samples that PCR negative were also negative by serological test. From the serological test by MAG assay in this study, it is suggested that the single serum with low positive titer were less diagnostic value. It required paired sera which is the problem in clinical practice. Other reports suggested that a single titer of

1:80 was regarded as indeterminate and titer ≥ 1:160 as positive (37,101,107). In this respect, PCR is a major improvement on existing methods for the diagnostic of *M. pneumoniae* infection.

At present, there is no single gold standard for diagnosis of *M. pneumoniae* infection. The combinations of the above three methods are probable the most optimal choice for higher confidence, this choice can detect both early phases of infection and after onset of immune response. For rapid diagnosis, 16S rDNA nested PCR has significant potential alternative with sensitive, specific for detection of *M. pneumoniae* and it leaves undetected some very early infections. It was more sensitive than culture and serological commercial kits. The method of pretreat specimens is a simple, that could be used in any laboratory. Therefore, this test could easily be used as a rapid diagnostic test for *M. pneumoniae* infections.