

Original Article

Comparison of P1 and 16S rRNA genes for detection of *Mycoplasma pneumoniae* by nested PCR in adults in Zhejiang, China

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Abstract

Introduction: *Mycoplasma pneumoniae* (*M. pneumoniae*) is the most common atypical pathogen that causes respiratory infections in humans. Laboratory tests are important in the diagnosis of *M. pneumoniae* because of the atypical features in clinical signs and symptoms. Nowadays, both the P1 adhesin gene and 16S ribosomal (r) RNA (rRNA) gene of *M. pneumoniae* have been widely detected by polymerase chain reaction (PCR). The purpose of the present study was to evaluate the most suitable target in the detection of *M. pneumoniae* via nested PCR.

Methodology: Both the P1 adhesin gene and 16S rRNA gene for nested PCR reaction conditions were optimized through an orthogonal test and single-factor experiment. Then, the sensitivity of the two sets of targets was evaluated. Finally, based on the optimal conditions, 55 clinical samples of throat swabs collected from adult patients in 2013 were examined by established nested PCR.

Result: The results revealed that PCR detection of the 16S rRNA gene was more sensitive than the P1 adhesin gene because the detection limits for both the P1 gene and 16S rRNA gene were at least 100 fg and 10 fg of *M. pneumoniae* DNA, respectively. Furthermore, the positive rate for detection of the 16S rRNA gene (30/55; 54.5%) was higher than that of the P1 adhesin gene (25/55; 45.5%).

Conclusion: Our results indicate that the 16S rRNA gene is more suitable for diagnosis of *M. pneumoniae* infection than the P1 adhesin gene due to its higher sensitivity and positive rate in clinical samples.

Key words: *Mycoplasma pneumoniae*; nested PCR; P1 adhesin gene; 16S rRNA gene; adult patients.

J Infect Dev Ctries 2015; 9(3):244-253. doi:10.3855/jidc.5149

(Received 19 April 2014 – Accepted 17 November 2014)

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Introduction

Mycoplasma pneumoniae is one of the main etiological agents causing atypical pneumonia, especially in children and young adults [1-3]. It accounts for as many as 10% to 30% of all cases of community-acquired pneumonia (CAP) [2,4]. Epidemiological studies in China have demonstrated that *M. pneumoniae* infections account for 20.7% in adult cases, more than *Streptococcus pneumoniae*, and turn out to be the leading pathogen of CAP [2]. Although most of these infections are mild and often self-limiting, severe bronchopneumonia and lung abscesses can occur. Furthermore, *M. pneumoniae* infections may also lead to several extra-pulmonary conditions, such as myocarditis, pericarditis, meningitis, neuritis, and hemolytic anemia, sometimes with fatal outcomes [5-7]. Because treatment of *M.*

pneumoniae infections with β -lactam antibiotics is ineffective and the clinical manifestations of *M. pneumoniae* infections are complicated and nonspecific, a rapid, sensitive, and specific laboratory test is vital for early diagnosis of *M. pneumoniae* infections [8,9].

Conventional tests, including cultivation and serological methods, have their limitations in detecting *M. pneumoniae*. For example, *M. pneumoniae* culture is difficult, time consuming, and lacks sensitivity, and is therefore not recommended for clinical practice [4,10]. Serological methods are currently the most common tool used in the clinical laboratory. However, these methods have practical limitations because of the availability of paired serum samples from both acute and convalescent phases, and provide results of questionable specificity and sensitivity [10-12].

Polymerase chain reaction (PCR), especially nested PCR, has been developed and clinically utilized to detect *M. pneumoniae* due to its rapidity and high sensitivity and specificity [4,13].

Both the P1 adhesin gene and 16S rRNA gene have been utilized widely in PCR techniques as the targets for detection of *M. pneumoniae* [13-15]. The P1 adhesin gene is an intriguing target gene for PCR because of its repetitive nature within the genome [14]. About 8% of the *M. pneumoniae* genome consists of repetitive DNA elements with regions homologous to the P1 adhesin gene, thus allowing an increase in the sensitivity of a PCR assay [16]. The 16S rRNA gene is also an attractive candidate as a target due to its unique organization and the presence of conserved and variable regions on its abundant high-copy number [14,17]. As for which of them is the better choice, there is still no uniform standard.

In this study, we sought to evaluate the more sensitive and specific target (P1 or 16S rRNA) in *M. pneumoniae* detection and to evaluate the use of nested PCR for the diagnosis of *M. pneumoniae* infections from patients in whom a *M. pneumoniae* infection was suspected.

Methodology

Strains and clinical samples

The reference strain *M. pneumoniae* FH (ATCC 15531) was applied to optimize the conditions of nested PCR and to assess its sensitivity. Between January 2013 and January 2014, 55 throat swab samples were routinely obtained from adult patients (range, 18 years to 82 years; mean age, 47.9 years) at three hospitals in Zhejiang (the Second Affiliated Hospital of Wenzhou Medical University, Yueqing Third People's Hospital, and Zhujiaji People's Hospital). This study was approved by the hospitals' ethics committees. The diagnosis was based on clinical signs and symptoms (fever, sore throat, cough, chills, expectoration, dyspnea, chest pain, or abnormal breath sounds) and pulmonary radiography. Passive particle agglutination (Serodia-Myco II; Fujirebio, Japan) assays were also performed; a fourfold rise in antibody titers of paired sera and titers of $\geq 1:40$ were regarded as positive. The specimens were transported to the laboratory (Department of Parasitology, Wenzhou Medical University) rapidly upon collection and stored at -20°C until DNA extract, which should be done within three days.

DNA preparation

M. pneumoniae FH strains (ATCC 15531) were cultured in PPLO broth (65 mL PPLO broth supplemented with 20 mL fetal bovine serum, 4 mL 5% TC yeastolate, 6 mL mycoplasma growth supplement, 3 mL 50% yeast extract solution, and 2 mL 0.1% phenol red solution) in plastic tubes at 37°C with 5% CO_2 . The samples were harvested when the phenol red pH indicator turned yellow, which resulted from acid production of glucose utilization by the bacteria. *M. pneumoniae* strains were stored in aliquots at -80°C until use.

DNA from strain culture and clinical samples were extracted with a QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The DNA were measured photometrically for concentration and then were stored at -20°C before PCR was done.

Orthogonal array design

The orthogonal array method was adopted to optimize four common factors affecting the nested PCR: the concentrations of primers and Mg^{2+} , dilution multiple of the first round PCR product, and annealing temperature. The three levels for each of the four factors were arranged into an orthogonal array to find the more appropriate combinations and were chosen on the basis of experiences and references (Tables 1 and 2). Primers targeted to the P1 gene (GenBank accession no. AB691539.1) and 16S rRNA gene (GenBank accession no. NC020076.1) of *M. pneumoniae* are listed in Table 3. The amplification conditions of first round were as follows: 2 μL template (adjusted to 20 ng/ μL), 2.5 μL 10 \times Taq buffer with KCl, 2.5 μL dNTPs, 0.125 μL Taq DNA polymerase, MgCl_2 (all from Thermo, Shanghai, China), outside primers of P1 or 16S rRNA gene, and finally ddH_2O was added to make the total volume 25 μL . The plates were placed in a PCR processor and were processed by initial heating at 94°C for 3 minutes, 25 cycles of denaturation at 94°C for 40 seconds, annealing at indicated temperature for 40 seconds, and extension at 72°C for 40 seconds, followed by a 5-minute extension at 72°C . Three μL of the different dilution products from the first-round PCR were used as templates in the second round of PCR with the same reaction mixture and the inner primer. The amplification conditions were identical to the first round. One negative control was included in each round. PCR products were analyzed by electrophoresis in 2.5% agarose gels stained with 0.5 $\mu\text{L}/\text{mL}$ of ethidium bromide.

Optimization of single-factor conditions

To research the influence of each factor to nested PCR and determine the optimal factor combination of the two gene targets, single-factor complete experiments were done under the results of the orthogonal experiment to optimize the experimental conditions for each factor.

Nested PCR sensitivity test

To compare the relative sensitivities of the primers of the P1 gene and 16S rRNA gene set in nested PCR, the serially diluted *M. pneumoniae* (ATCC 15531) standard DNA templates from 10 ng/μL to 10⁻⁸ ng/μL were used under the optimal PCR conditions. Distilled water served as a negative control and was always included per plate. The experiment was repeated three times.

Detection of clinical samples

A total of 55 clinical specimens were retrieved from storage at -20°C, all of which had previously been collected from adult patients described above. All the specimens were examined for *M. pneumoniae* by nested PCR with the two different primers (the P1 adhesin gene primers and 16S rRNA gene primers) under their respective optimal PCR conditions. For all *M. pneumoniae*-positive samples, the products of nested PCR were sequenced (Sangon Biotech Co., Ltd., Shanghai, China). For all negative samples by nested PCR, human β-actin gene was used as an internal control to evaluate further whether there were inhibitors in samples. All the experiments were repeated three times.

Table 1. Nested PCR factors and their levels for orthogonal array design

Factor	Level		
	1	2	3
Primer (μM)	0.1	0.3	0.5
Mg ²⁺ (mM)	1.5	2.5	4
Annealing temperature (°C)	P1	60	62
	16S rRNA	54	58
Dilution multiple	NO	50	100

Table 2. Orthogonal array design for nested PCR

Reaction	Primer (μM)	Mg ²⁺ (mM)	Annealing temperature(°C)		Dilution multiple
			P1	16S rRNA	
1	0.1	1.5	58	54	NO
2	0.1	2.5	60	56	50
3	0.1	4.0	62	58	100
4	0.3	1.5	60	56	100
5	0.3	2.5	62	58	NO
6	0.3	4.0	58	54	50
7	0.5	1.5	62	58	50
8	0.5	2.5	58	54	100
9	0.5	4.0	60	56	NO

Table 3. Primers used in this study

Primer target	Primer name	Sequence	Product size (bp)
P1 adhesin gene (Talkington <i>et al.</i> , 1998)	Mp-F1	5'-ATTCTCATCCTCACCGCCACC-3'	285
	Mp-R1	5'-CGTGGTTTGTGACTGCCACTGCCG-3'	
	Mpn-F1	5'-CAATGCCATCAACCCGCCCTTAACC-3'	107
	Mpn-R1	5'-GTTGTCGCGCACTAAGGCCACG-3'	
16S rRNA gene (Han <i>et al.</i> , 2012)	Mp-F2	5'-AAGGACCTGCAAGGGTTCGT-3'	277
	Mp-R2	5'-CTCTAGCCATTACCTGCTAA-3'	
	Mpn-F2	5'-CTCTAGCCATTACCTGCTAA-3'	141
	Mpn-R2	5'-ACTCCTACGGGAGGCAGCAGTA-3'	
Human β-actin	B-F	5'-GGGACCTGACTGACTACCTC-3'	541
	B-R	5'-TCATACTCCTGCTTGCTGAT-3'	

Results

Orthogonal test

To optimize the P1 gene and 16S rRNA gene amplified by nested PCR, the orthogonal experiments were first designed. The PCR results showed that the eighth reaction system was the most suitable for the P1 gene (Figure 1A) and the sixth reaction system was the most suitable for the 16S rRNA gene (Figure 1B), because the positive bands were clearer and more specific. Consequently, both the eighth and sixth systems were regarded as comparatively suitable reaction systems and used in the following experiments.

Single-factor experiment

To further optimize the reaction system, the single-factor experiment was taken to study the influence of different processing levels (Figures 2 and 3). Through the test on the P1 gene primers and 16S gene primers, excessively low primer concentrations were found to reduce PCR yield, and excessively high primer concentration increased the probability of mispriming. The best performance occurred at the primer concentration of 0.3 μM for amplification of both the P1 and 16S rRNA genes by nested PCR.

The influence of Mg^{2+} concentration was also evaluated. If Mg^{2+} concentration is too low, it reduces the efficiency of the enzyme, and the yield of PCR product could be reduced. On the contrary, non-specific PCR products may appear and the PCR fidelity may be reduced. In the present experiments, for the 16S rRNA gene, the best amplification effect appeared when Mg^{2+} concentration was 3 mM; for the P1 gene, the difference between 2.5 mM and 3 mM was not obvious, so the optimal amount of primer was 2.5 mM.

The specificity of PCR depends on annealing temperature. Figures 2C and 3C show that poor specificity of an amplified band appears at low annealing temperature, weakened amplified bands appear at high annealing temperature, and the optimal annealing temperature of the P1 gene was 60°C, and that of the 16S gene was 56°C.

For second-round PCR, 3 μL of undiluted, 1:50 diluted, or 1:100 diluted first-round PCR products were used as templates, respectively. The results showed that the second amplification based on the undiluted first round of PCR production had many non-specific bands; the dilution multiple of the 1:50 was optimal.

Sensitivity assay

The sensitivity of the nested PCR was tested by using serial dilutions (1:10) of *M. pneumoniae* (ATCC 15531) standard DNA. The results showed that the detection limit of P1 nested PCR was 100 fg of *M. pneumoniae* DNA (Figure 4A) and that of 16S rRNA nested PCR was 10 fg (Figure 4B), respectively.

Analysis of clinical samples

According to the results of clinical tests, the 16S rRNA gene proved clearly to be the best target for this purpose, yielding a positive PCR result in 54.5% (30/55) of cases, while the positive rate was 45.5% (25/55) for the P1 adhesin gene. The coincidence rate of the two gene primers can reach 76.4% (42/55). Nine patients were positive by the 16S rRNA gene primers but negative by the P1 adhesin gene primers, and four patients were positive by the P1 adhesin gene but negative by the 16S rRNA ones.

Figure 1. Electrophoresis analysis of *M. pneumoniae* nested PCR by using the orthogonal array design (Table 2). Primers based on (A) P1 adhesin gene and (B) 16S rRNA gene. M: molecular markers of the DNA ladder; lane 1 to lane 9 correspond to each nested PCR reaction based on the orthogonal array design (reaction column in Table 2), respectively; lane 10: negative control.

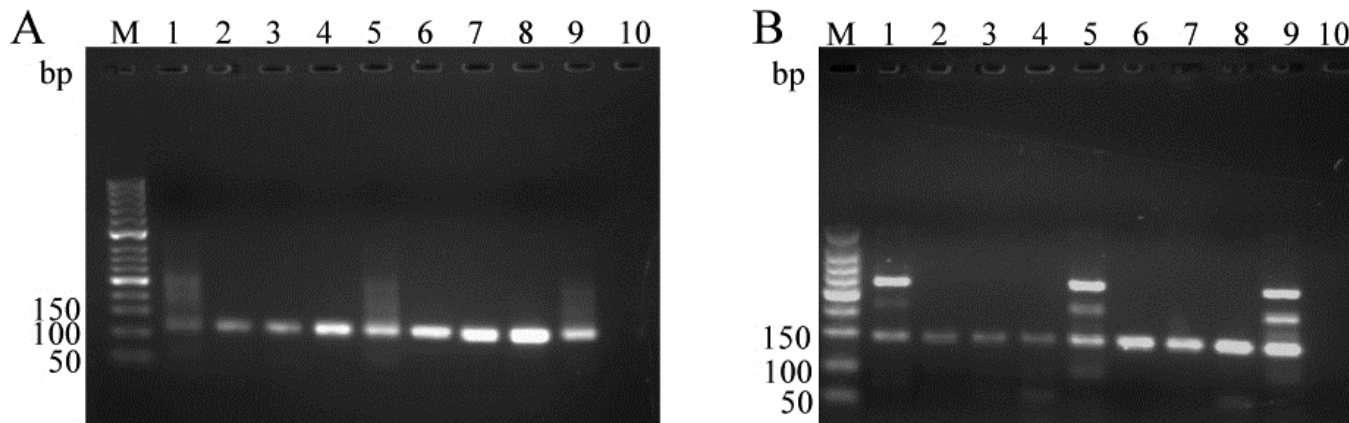


Figure 2. Electrophoresis analysis of P1 nested PCR based on a single-factor experiment. (A) Effect of different concentration of the P1 adhesin gene primers. M: molecular markers of the DNA ladder; lane 1: 0.1 μ M; lane 2: 0.2 μ M; lane 3: 0.3 μ M; lane 4: 0.4 μ M; lane 5: 0.5 μ M; lane 6: negative control. (B) Effect of different concentration of the Mg^{2+} . M, molecular markers of the DNA ladder; lane 1: 1.5 mM; lane 2: 2 mM; lane 3: 2.5 mM; lane 4: 3 mM; lane 5: 4 mM; lane 6: negative control. (C) Effect of different annealing temperature. M: molecular markers of the DNA ladder; lane 1: 56°C; lane 2: 58°C; lane 3: 59°C; lane 4: 60°C; lane 5: 62°C; lane 6: negative control. (D) Effect of the dilution of the first round of PCR product. M: molecular markers of the DNA ladder; lane 1: undiluted; lane 2: 50-fold dilution; lane 3: 100-fold dilution; lane 4: negative control.

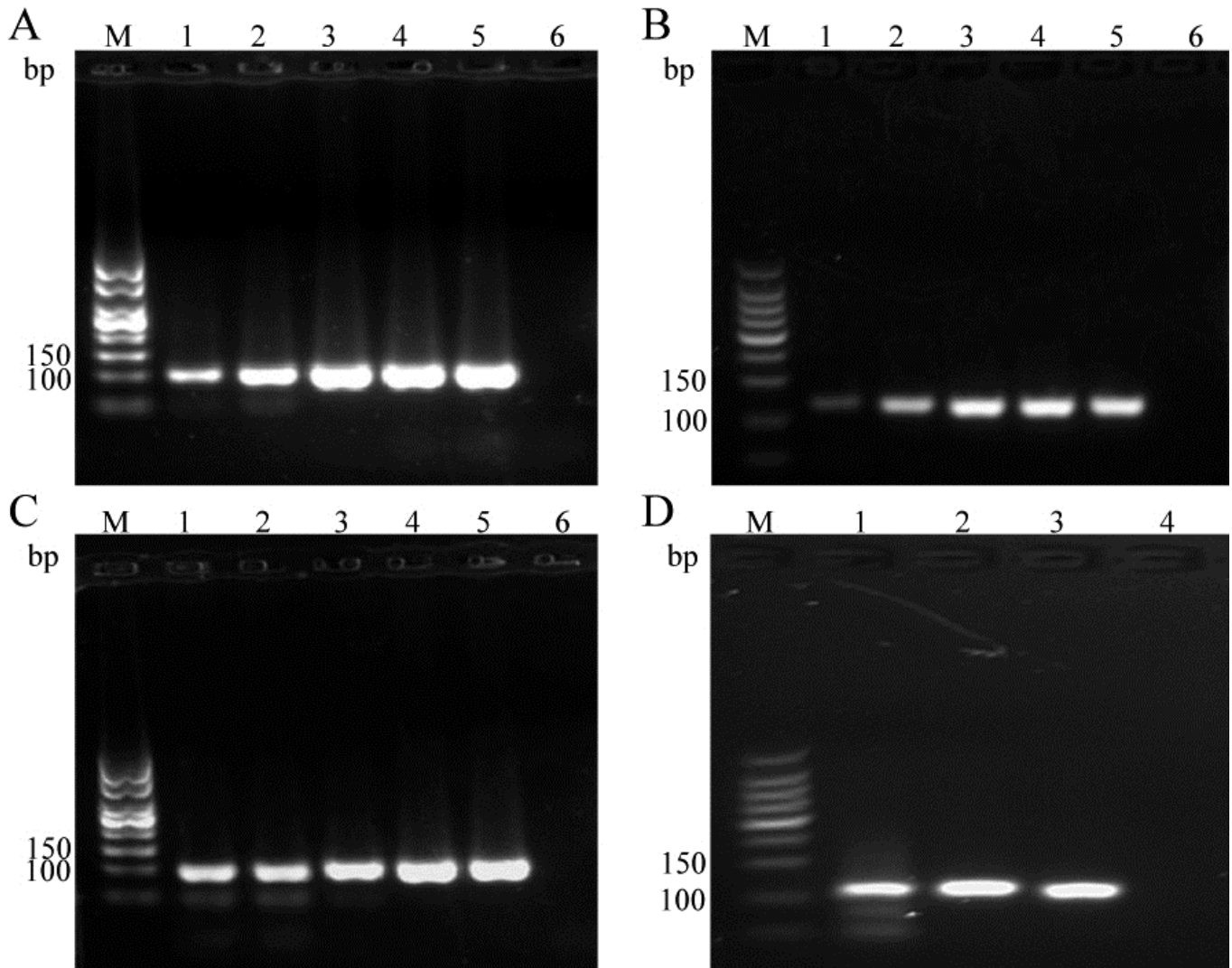


Figure 3. Electrophoresis analysis of 16S rRNA nested PCR based on a single-factor experiment. (A) Effect of different concentration of the 16S rRNA gene primers. M: molecular markers of the DNA ladder; lane 1: 0.1 μ M; lane 2: 0.2 μ M; lane 3: 0.3 μ M; lane 4: 0.4 μ M; lane 5: 0.5 μ M; lane 6: negative control. (B) Effect of different concentration of the Mg^{2+} . M: molecular markers of the DNA ladder; lane 1: 1.5 mM; lane 2: 2 mM; lane 3: 2.5 mM; lane 4: 3 mM; lane 5: 4 mM; lane 6: negative control. (C) Effect of different annealing temperature. M: molecular markers of the DNA ladder; lane 1: 52°C; lane 2: 54°C; lane 3: 55°C; lane 4: 56°C; lane 5: 58°C; lane 6: negative control. (D) Effect of the dilution of the first round of PCR product. M: molecular markers of the DNA ladder; lane 1: undiluted; lane 2: 50-fold dilution; lane 3: 100-fold dilution; lane 4: negative control.

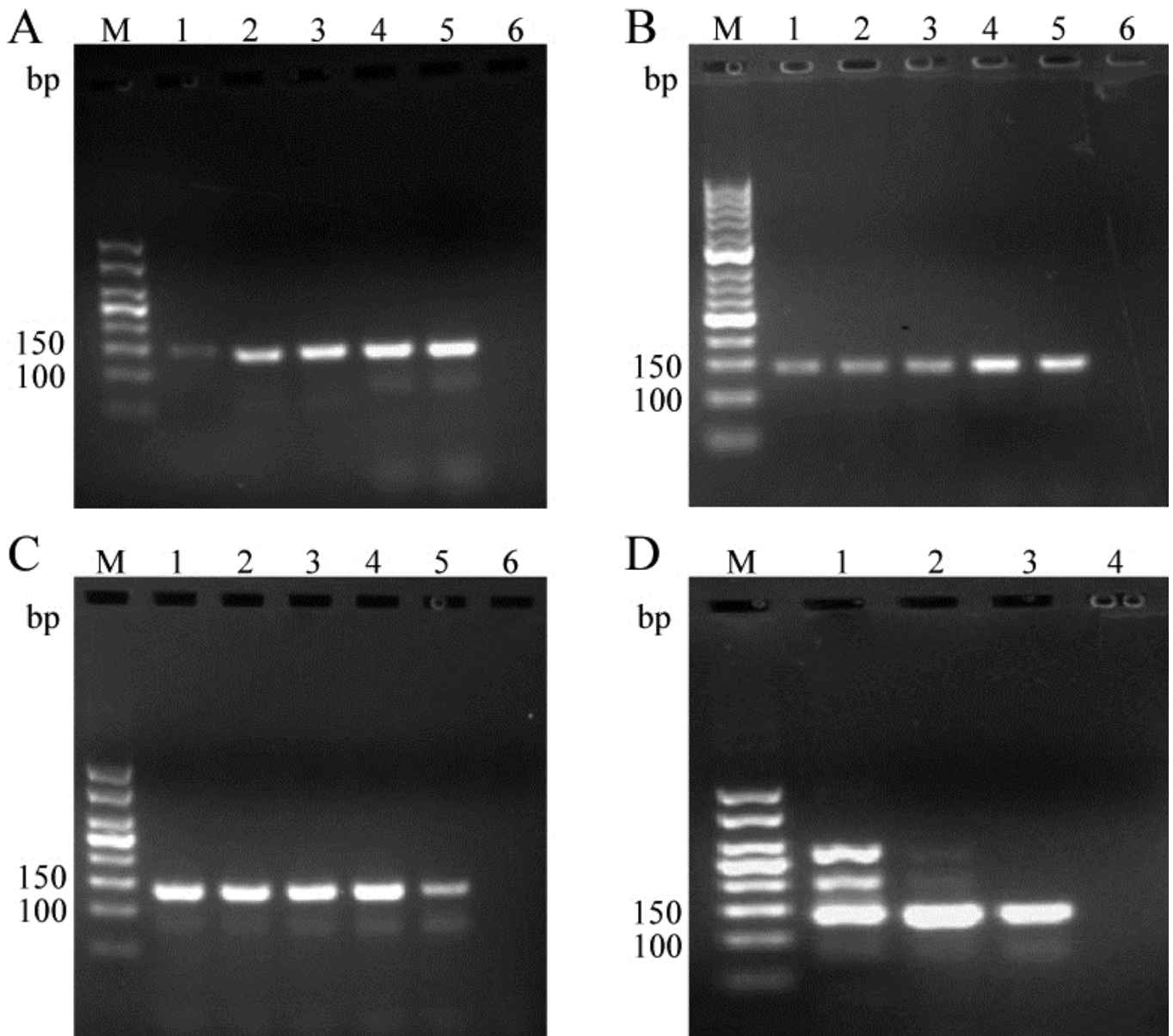


Figure 4. Sensitivity of the optimized nested PCR based on (A) P1 adhesin gene and (B) 16S rRNA gene. M: molecular markers of the DNA ladder; lane 1: 10 ng of *M. pneumoniae* DNA; lane 2: 1 ng of *M. pneumoniae* DNA; lane 3: 10⁻² ng of *M. pneumoniae* DNA; lane 4: 10⁻³ ng of *M. pneumoniae* DNA; lane 5: 10⁻⁴ ng of *M. pneumoniae* DNA; lane 6: 10⁻⁵ ng of *M. pneumoniae* DNA; lane 7: 10⁻⁶ ng of *M. pneumoniae* DNA; lane 8: 10⁻⁷ ng of *M. pneumoniae* DNA; lane 9: 10⁻⁸ ng of *M. pneumoniae* DNA; lane 10: negative control.

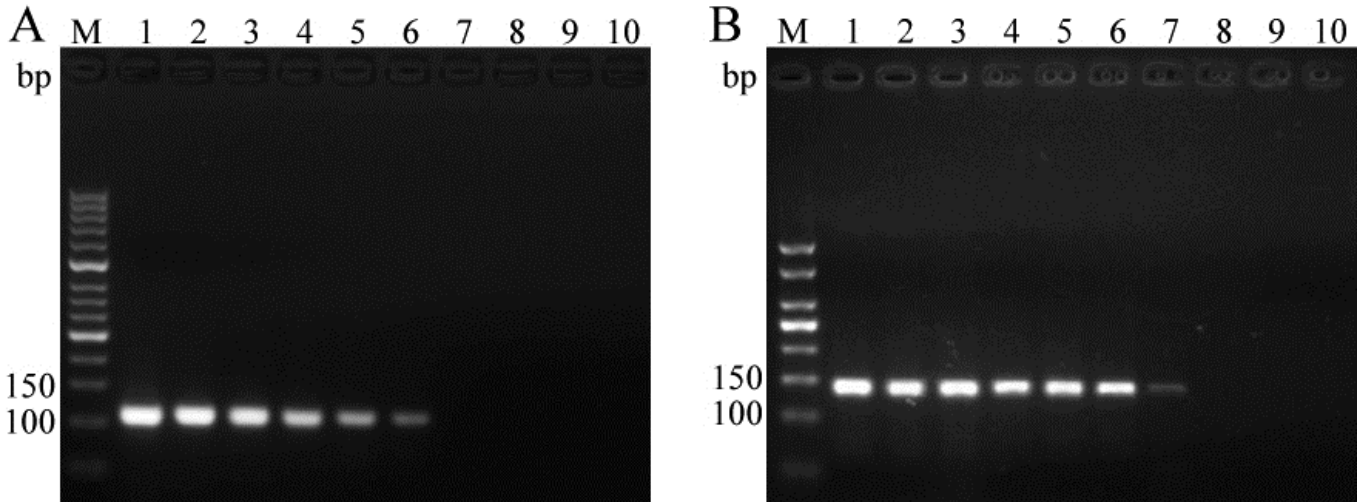
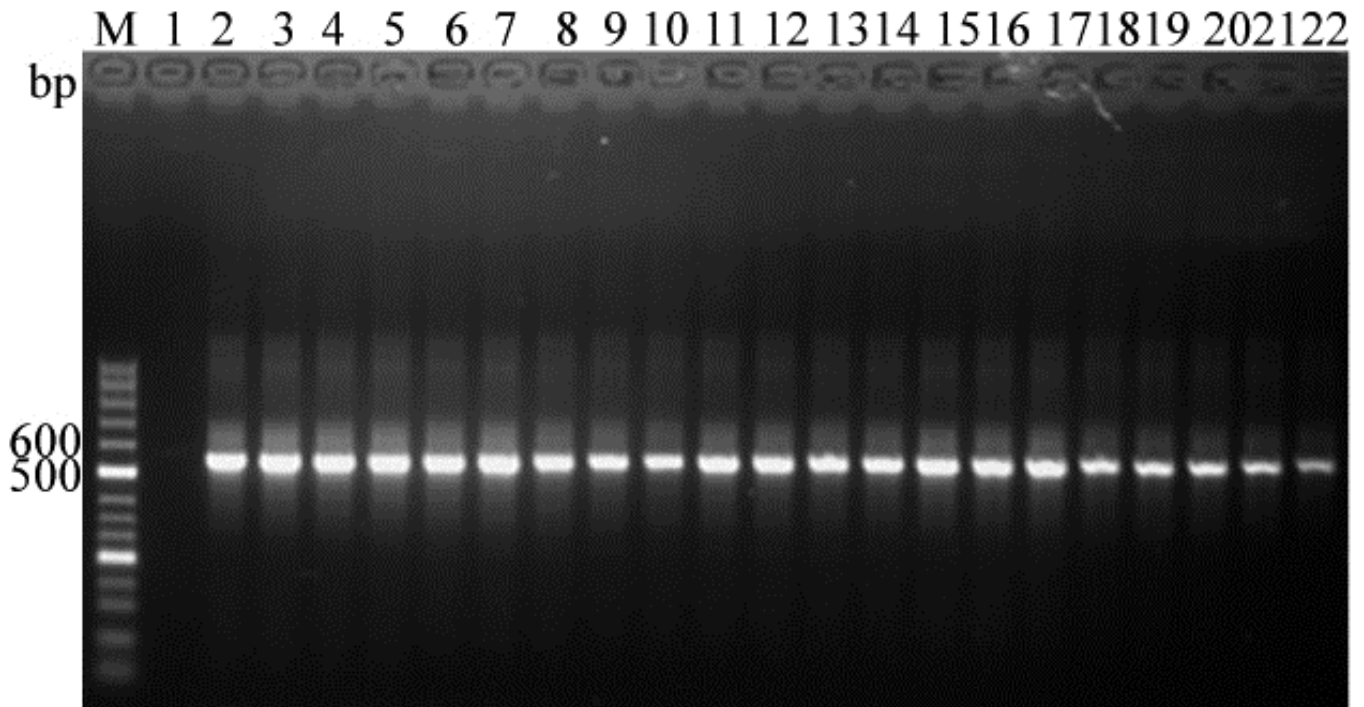


Figure 5. Agarose gel electrophoresis analysis of human β -actin PCR products from clinical nested PCR-negative samples. M: molecular markers of the DNA ladder; lane 1: negative control; lanes 2–22: β -actin PCR products from clinical nested PCR-negative samples.



The 107-bp and 141-bp products from positive clinical samples by *M. pneumoniae* nested PCR were sequenced, and all of the sequencing results were correct. All of the negative clinical samples produced a band after the human β -actin PCR (Figure 5), suggesting that there were no significant PCR inhibitory factors.

Discussion

In the present study, we joined orthogonal experiment and single-factor tests to optimize several crucial factors in nested PCR assay based on both the 16S rRNA gene and P1 adhesin gene designed for *M. pneumoniae* detection. The results revealed that the 16S rRNA gene is more sensitive than the P1 adhesin gene under the optimum reaction conditions. Afterwards, 55 serology-positive specimens from adults with CAP in Zhejiang in China were detected by optimized nested PCR on the basis of two target genes. We observed that 54.5% (30/55) of the specimens were positive with the 16S rRNA gene nested PCR and 45.4% (25/55) of the specimens were positive with the P1 adhesin gene nested PCR.

Cultivation is rarely used for diagnosis of *M. pneumoniae* infection in most clinical laboratories because the fastidious growth requirements and length of time necessary to culture *M. pneumoniae* (three to six weeks) make growing the organism impractical for patient management [4,10]. Currently, serological assay is the most widely used means for laboratory confirmation of mycoplasmal respiratory infections [4]. However, there are concerns about the use of single qualitative tests to identify acute *M. pneumoniae* infections in adults, since many persons may not mount an IgM response, presumably because of re-infection, and when it is produced, IgM may persist for long periods [18-20]. Furthermore, the percentage of individuals with acute infection who demonstrated a positive IgG response in the acute phase was less than 50% in a recent study. It has been suggested that cross-reactivity with antigen preparations used in some of the commercial enzyme immunoassays (EIAs) result in over-diagnosis of *M. pneumoniae* infections [21]. Therefore, even if serology is a useful epidemiologic tool in areas where the infection rate of *M. pneumoniae* is high, it is less suited for assessment of individual patients in clinical laboratories [11].

Compared with serology and culture, a direct detection of pathogens in clinical specimens has been done more regularly using molecular biology techniques. PCR approaches have been the most

valuable method for rapid, sensitive, and specific diagnosis of *M. pneumoniae* infection [4]. However, the application of molecular methods of enhanced sensitivity may be necessary since the pathogens are probably present in small quantities. Among PCR methods, nested PCR assays have significant advantages over traditional PCR, including superior sensitivity and specificity, because they involve the reamplification of a PCR product with a second primer set. As demonstrated by other researchers, nested PCR enables the detection of 1–100 fg of DNA, and single-step PCR assays can only detect 10–100 pg of DNA [4,22]. Consistent with these results, under optimal reaction conditions, the nested PCR established in this study was able to detect 10 fg of the 16S rDNA gene and 100 fg of the P1 gene. In addition, the results of sequencing indicated that both nested PCRs had high specificity.

Gene targets used widely in various types of PCR assays for *M. pneumoniae* include the P1 adhesin gene and 16S rRNA gene [13]. The P1 adhesin gene is an attractive target for PCR because it repeats up to 10 times within the *M. pneumoniae* genome, which increases the sensitivity of PCR assay [16]. Another important target is 16S rRNA, or rather rDNA. The advantage of using rDNA sequences is the high degree of conservation of the target and the presence of the highly variable regions [14]. Therefore, it would be greatly valuable for guiding treatment decisions and follow-ups, particularly in countries with a high frequency of strains resistant to antibiotics, because the confirmation of 16S rDNA could be applied in discriminating between bacterial and viral causes of pneumonia. To our knowledge, however, it is still controversial which target is more effective. For instance, Loens *et al.* suggested that the P1 adhesin gene may be more sensitive than the 16S rRNA one [13]. Two independent researchers, nevertheless, showed that the amplification of the 16S rRNA gene was more sensitive for the detection of *M. pneumoniae* because more positive samples were found by 16S rDNA PCR than by a PCR with the P1 gene [14,15]. The main reason for the ambivalent conclusions is that the researchers detected the DNA directly from clinical samples, rather than a standard strain DNA of *M. pneumoniae*, to compare the sensitivity of 16S rDNA PCR with P1 gene PCR. In the present study, the sensitivity of both nested PCR assays was compared using tenfold serial dilutions of a standard strain DNA of *M. pneumoniae*. These results confirmed that the 16S rRNA gene primers are more sensitive than the P1 adhesin gene primers, as the 16S

rRNA gene primers can detect up to 10 fg of *M. pneumoniae* DNA and the P1 gene primers can detect 100 fg of *M. pneumoniae* DNA at most. This was mainly because the presence of approximately 10^3 copies of 16S rRNA per mycoplasma cell and the high degree of conservation of the rRNA genes allowed a high fixation of primers on the target and lead to a higher PCR yield [4,14]. Importantly, because RNA is destroyed more rapidly than DNA after the death of the mycoplasma cell, detection of the 16S rRNA gene provides further evidence of viable mycoplasmas in the specimen [4].

Although nested PCR is a rapid and sensitive method for early diagnosis of *M. pneumoniae* infection, the factors that impact on PCR reaction are numerous and it is time consuming to determine the optimum conditions. Thus, at first, we adopted the orthogonal test design to optimize several crucial factors affecting the nested PCR using a standard strain of *M. pneumoniae* because this design can greatly shorten the test number and can quickly arrive at a more appropriate reaction condition. The factors optimized in this study were the concentration of primers, concentration of Mg^{2+} , dilution ratio of the first-round PCR product, and annealing temperature. Based on the results of the orthogonal design, a completely single-factor test design was utilized to confirm the ultimate optimized conditions. Finally, the final optimal reaction conditions of nested PCR were determined by integrated the results of the methods above. For this reason, this study comparing of performance of nested PCR methods with P1 adhesin gene and the 16S rRNA gene primers under this optimal condition could be more objective and valuable for the diagnosis of *M. pneumoniae* infection.

According to recent research, *M. pneumoniae* has been the most prevalent pathogen in adults from China, and the routine treatment with β -lactam antibiotics is often ineffective against this pathogen [2]. Until now, however, the precise incidence of *M. pneumoniae* in acute respiratory tract infections in Zhejiang for adults was unknown because surveillance is not done and laboratory confirmation is usually not obtained. To evaluate further the nested PCR method for the diagnosis of respiratory *M. pneumoniae* infections, to define the procedure best suited for the clinical diagnostic laboratory, and to define the incidence of *M. pneumoniae* in acute respiratory tract infections in Zhejiang for adults, we performed nested PCR analysis for *M. pneumoniae* DNA on 55 throat swab samples from adults who were positive by serological assay. In accordance with results reported

in other publications, the 16S rRNA gene proved clearly to be the best target for this purpose, yielding a positive PCR result in 54.5% (30/55) of cases, while the positive rate was 45.4% (25/55) for the P1 adhesin gene; however, there was no significant difference between the positive rate for detection of the 16S rRNA gene and P1 adhesin gene ($p > 0.05$). That was mainly because the sample quantity was too small. Meanwhile, there was an excellent correspondence of positive subjects detected by both P1 nested PCR and 16S rRNA nested PCR, and the coincidence rate of nested PCR of both genes reached 76.4% (42/55). Combining the results of serology, nested PCR, and sequencing, we determined that these positive patients were indeed infected with *M. pneumoniae*. The major difficulties for the interpretation of the PCR data were the discordant results. First, nine 16S rRNA nested PCR-positive patients were negative by the P1 nested PCR. There is a possible explanation that the nested PCR based on the 16S rRNA gene was more sensitive than the P1 adhesin gene shown in the results of a comparative study. In contrast, there were four positive patients by the P1 adhesin gene but negative by the 16S rRNA gene. The reason for the difference in results is not entirely clear but is probably because the cytoadhesin protein encoded by the P1 gene appears to be a virulence factor and presents in any pathogenic strain of this organism. Finally, the negative PCR results in serologically proven infections may possibly not be true negatives for three possible reasons. First, the inhibitors may be present in specimen. To exclude the impact of inhibitors on PCR assays, we used the human β -actin gene as an internal control to determine if there are inhibitors. The result revealed that the human β -actin gene could be detected in all negative PCR samples, suggesting that there were no inhibitors in the specimens or that the amount of inhibitors was too low to impact on the nested PCR. Second, the number of pathogens in negative specimens may be too low to be detected with the present nested PCR. Third, *M. pneumoniae* may already have been eradicated by antibiotics before the specimens were taken.

Conclusions

Nested PCRs performed with both the 16S rRNA gene and P1 adhesin gene proved to be reliable methods for detecting *M. pneumoniae* infection rapidly and specifically in clinical specimens. For routine clinical practice, we recommend that nested PCR based on the 16S rRNA gene is more suitable for

early diagnosing *M. pneumoniae* infection, especially in adults.

Acknowledgements

This work was supported by Social Development Program of Science and Technology Department of Zhejiang Province of China (No. 2011C13040). We declare that the experiments performed and presented in this report comply with the current laws of the People's Republic of China.

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Conflict of interests: No conflict of interests is declared.