Original Article

Evaluation of a New Real-time PCR Assay for Detection of *Mycoplasma Pneumoniae* in Clinical Specimens*

ZHAO Fei[1,3,†], CAO Bin[2,4,†], HE Li Hua[1,3], YIN Yu Dong[2,4], TAO Xiao Xia[1,3], SONG Shu Fan[2,4], MENG Fan Liang[1,3], and ZHANG Jian Zhong[1,3,#]

1. National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; 2. Department of Infectious Diseases and Clinical Microbiology, Beijing Chao-Yang Hospital, Beijing Institute of Respiratory Medicine, Capital Medical University, Beijing 100020，China; 3. State Key Laboratory for Infectious Disease Prevention and Control, Beijing 102206, China; 4. Beijing Key Laboratory of Respiratory and Pulmonary Circulation, Beijing 100020, China

Abstract

Objective To establish and evaluate a real-time PCR assay to detect *Mycoplasma pneumoniae* (*M.pneumoniae*) in clinical specimens.

Methods By analysing the whole p1 gene sequence of 60 *M.pneumoniae* clinical isolates in Beijing of China, an optimized real-time PCR assay (MpP1) using p1 gene conserved region was designed. The specificity and sensitivity of this assay were evaluated and compared with other two reported assays (RepMp1 and Mp181) using 40 positive and 100 negative clinical specimens.

Results The detection limit of the new assay was 8.1 fg (about 1~3CFU) *M.pneumoniae* DNA. The sensitivity of MpP1, RepMp1, and Mp181 assays appeared to be 100%, 100%, and 85%, respectively.

Conclusion MpP1 assay is suitable for the detection of *M.pneumoniae* in Chinese clinical specimens.

Key words: *Mycoplasma pneumoniae*; Real-time PCR; p1 gene

Biomed Environ Sci, 2012; 25(1):77-81 *doi:10.3967/0895-3988.2012.01.011* *ISSN:0895-3988*

www.besjournal.com(full text) *CN: 11-2816/Q*

INTRODUCTION

Mycoplasma pneumoniae (*M.pneumoniae*) is one of the most common pathogens that cause respiratory tract infections. It accounts for approximately 10% to 40% of community-acquired pneumonia (CAP) cases[1-2]. *M.pneumoniae* is distributed globally. Epidemics occur every 4 to 7 years and have been reported in America[3], Europe[4-5], and Asia[6-7]. Conventional tests for detecting *M.pneumoniae* are fraught with limitations[8]. Cultivation is time consuming and insensitive and requires special media. Serological methods, such as the complement fixation test and commercially enzyme-linked immunosorbent assay, are commonly used in clinical laboratories but provide questionable specificity and sensitivity of results. PCR approaches have been found to be useful for the rapid, sensitive and specific detection of *M. pneumoniae*[9].

Recently, different real-time PCR approaches targeting extensive genes of *M.pneumoniae*, such as ATPase operon gene[10], p1 adhesin gene[11], RepMp1 gene[12], and CARDS toxin gene[13], have been developed. All these assays were developed by European or American researchers, verified virtually by local isolates or specimens, and investigated with limited specimens to evaluate their sensitivity and

*This study was supported by the National Key Program for Infectious Diseases of China, No. 2008ZX10004-002.

#Correspondence should be addressed to ZHANG Jian Zhong. Tel: 86-10-58900707. E-mail: zhangjianzhong@icdc.cn

†ZHAO Fei and CAO Bin contributed equally to the work.

Biographical note of the first author: ZHAO Fei, male, born in 1979, majoring in microbiology; CAO Bin, male, born in 1972, associate professor, majoring in clinical medicine.

Received: March 15, 2011; Accepted: June 7, 2011

specificity. No real-time PCR assay designed according to the Chinese *M.pneumoniae* isolates sequence was reported before. The present study aimed to sequence the whole p1 gene of Chinese clinical *M.pneumoniae* isolates, design real-time PCR assay in conserved gene region, and compare the assay with other two reported assays: Mp181[13] and RepMp1[12].

METHODS

Clinical Specimens

Three hundred and two throat swabs were collected from CAP patients treated at Beijing Chao-Yang Hospital from August 2008 to February 2010. Each swab was aliquot to 200 μL. The DNAs of specimens were extracted with the QIAamp DNA Mini Kit (QIAGEN 51 306) and *M.pneumoniae* was cultured in selective broth liquid media (Oxoid) at 37 °C for 40 days. All 302 swabs DNA extractions were detected with a RepMp1 real-time PCR method[12]. Swabs with culture positive were defined as *M.pneumoniae* positive respiratory specimens, and swabs with both culture and RepMp1 assay real-time PCR negative were defined as negative specimens.

P1 Gene Sequence and Real-time PCR Assay Design

The p1 gene of 60 isolates cultured from 302 throat swabs were amplified using the primer pairs SeqP1-F (5'-ATGCACCAAACCAAAAAAACTGCCT-3') and SeqP1-R (5'-CTAAGCGGGTTTTTTAGGT GGTTGC-3'). The reaction conditions were 30 cycles of 98 °C for 15 sec, 56 °C for 15 sec, and 72 °C for 3 min. The product of about 4 900 bp size was amplified by this reaction. PCR reactions were performed using the PrimeSTAR Kit (TaKaRa DR010A). The amplicons were sequenced in two directions and spliced by Beijing Genomics Institute. The 60 sequenced whole p1 genes of clinical isolates and other reported p1 genes in NCBI were compared, and a novel TaqMan assay targeting the conserved region of p1 gene was designed (Table 1).

Table 1. Probs and Primers for Real-time PCR of *M.pneumoniae* Targets

Prob/Primer	Sequence (5'→3')	Target Gene	Assay Efficiency (%)	Assay Lowest Detectable Limit (CFU)	Reference
MpP1-F	CAATAACCGCTGGTTTGAATATGT				
MpP1-R	AACGAGTTCCCTACCAACGAAC	p1 gene	99.6	1～3	This study
MpP1-P	(FAM)-CCACGGATGGCAGTTGCTGG-(BHQ1)				
RepMp1-F	TCTTTACGCGTTACGTATTC				
RepMp1-R	AGTGTGGAATTCTCTGGCA	RepMp1	99.6	about 0.3	[12]
RepMp1-P	(FAM)-TTCACTGGTATAACCGGTTTGTTAAG-(BHQ1)				
Mp181-F	TTTGGTAGCTGGTTACGGGAAT				
Mp181-R	GGTCGGCACGAATTTCATATAAG	CARDS gene	99.7	1～3	[13]
Mp181-P	(FAM)-TGTACCAGAGCACCCCAGAAGGGCT-(BHQ1)				

Real–time PCR Amplification

The real-time PCR mixture was prepared in a total volume of 25 μL. Each PCR mixture contained the following per reaction: 12.5 μL of Platinum quantitative PCR SuperMix-UDG (Invitrogen C11730-025), 2.0 μL of 50 mmol/L $MgCl_2$, 0.5 μmol/L final concentrations of each primer, a 0.2 μmol/L final concentration of the probe, 1.25 U of Platinum Taq DNA polymerase (Invitrogen C10966-034), 1 μL of 10 mmol/L PCR nucleotide mix (Promega C1141), 5 μL of extracted nucleic acid from each specimen, and nuclease-free water (Promega P1193) to 25 μL final volume. Real-time PCR for each target was performed using a C1000 Thermal Cycler (BIO RAD) under the following conditions: initial activation of 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s and 59 °C for 30 s.

Specificity, Sensitivity, and Lowest Detectable Limit of Three Assays

DNA extracted from several Mycoplasma species and bacteria, as well as human DNA were amplified (Table 2) with MpP1, Mp181, and RepMp1 assays. A standardized dilution series of quantified DNA (from 8.1 ng to 0.81 fg)of *M.pneumoniae* (ATCC2934) were also tested with each assay.

Table2. DNA Template and Related Source Used in the MpP1 Real-time PCR Assay Specificity Test

Source of DNA	Reference Strain Number or Sample Name
Mycoplasma pneuminiae	ATCC39505[a]
Mycoplasma.pneuminiae	ATCC15531[a]
Mycoplasma.pneuminiae	ATCC29085[a]
Mycoplasma.pneuminiae	ATCC29342[a]
Mycoplasma.pneuminiae	ATCC29343[a]
Mycoplasma.pneuminiae	ATCC15537[a]
Mycoplasma.pneuminiae	ATCC15492[a]
Mycoplasma.pneuminiae	ATCC49894[a]
Mycoplasma.pneuminiae	ATCC15293[a]
Mycoplasma.salivarium	ATCC23064[a]
Mycoplasma.orale	ATCC23714[a]
Mycoplasma.faucium	ATCC25293[a]
Mycoplasma.hominis	ATCC23114[a]
Mycoplasma.penetrans	ATCC55252[a]
Mycoplasma.fermentans	ATCC19989[a]
Mycoplasma.hyorhinis	ATCC17981[a]
Ureaplasma.urealyticum	ATCC27618[a]
Mycoplasma.genitalium	ATCC33530[a]
Chlamydophila.pneumoniae	Clinical isolate[b]
Staphylococcus.aureus	Clinical isolate[b]
Staphylococcus.epidermidis	Clinical isolate[b]
Streptococcus.pneumoniae	Clinical isolate[b]
Escherichia.coli	Clinical isolate[b]
Legionella.pneumophila	Clinical isolate[b]
Haemophilus.influenzae	Clinical isolate[b]
Mycobacterium.tuberculosis	Clinical isolate[b]
Neisseria.meningitides	Clinical isolate[b]
Pseudomonas.aeruginosa	Clinical isolate[b]
Human DNA	Clinical isolate[b]

Note. [a]ATCC, American Type Culture Collection. [b]Isolates or sample stored by National Institute for Communicable Disease Control and Prevention(ICDC).

Evaluation of Three Real-time PCR Assays with Clinical Specimens

MpP1 assay and other two reported assay, RepMp1 and Mp181, were evaluated using 40 *M.pneumoniae* positive respiratory specimens and 100 *M.pneumoniae* negative respiratory specimens from clinical patients.

RESULTS

Clinical Specimens Detection

Among 302 throat swabs 85 swabs DNA extractions were detected positive with real-time PCR[12], and the other 217 swabs were negative. Sixty *M.pneumoniae* were isolated from 302 throat swabs. Each positive culture specimen was also detected positive with real-time PCR method, and each negative real-time PCR specimen was also negative in culture. These 60 swabs were defined as *M.pneumoniae* positive respiratory specimens, and 217 swabs were defined as negative respiratory specimens.

Sequence Analysis of p1 Gene and Real-time PCR Assay Design

DNAs of all 60 *M.pneumoniae* isolates were extracted with the QIAamp DNA Mini Kit (QIAGEN). The 60 complete p1 gene sequences obtained in this study with those of strains M129 (gi:150166), P11428 (gi:12382267), Mp1842 (gi:15213523), Mp3896 (gi:157383331), and Mp4817 (gi:15213519) in the GenBank were compared by using the Vector NTI suite 6 software and the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The conserved sequence in each p1 gene was selected to design the probs and primers (Table 1). MpP1 real-time PCR production was located from nt1689 to nt1762 (corresponding in the p1 gene of strain M129) in conserved p1 sequence, only one copy in the *M.pneumoniae* genome.

Specificity, Sensitivity, and Lowest Detectable Limit of Three Assays

RepMP1, MpP1, and Mp181 assays proved to be highly specific, reacting only with DNA extracted from *M.pneumoniae* but with none of the other species tested (Table 2). The standardized dilution series (8.1 ng to 8.1 fg)of quantified DNA of *M.pneumoniae* (ATCC2934) were detected with MpP1 and Mp181 assays. The detection limit of these two assays was 3 CFU (8.1 fg). RepMP1 assay could detect 0.81 fg DNA (0.3 CFU). The efficiency of MpP1, Mp181, and RepMP1 assays was demonstrated 99.6%, 99.7%, and 99.6%, repsectively (Table 1).

Evaluation of Three Real-time PCR Assays with Clinical Specimens

According to the results of 40 positive and 100 negative respiratory tract specimens detection, the sensitivity of MpP1, RepMp1, and Mp181 assays was 100%, 100%, and 85%, respectively, and the respective specificity of RepMP1, MpP1, and Mp181 assays was 100%, 98%, and 99% (Table 3). Six positive samples in low DNA concentration (Crossing threshold values later than 36.5 with MpP1 assay)

could not have been detected with Mp181 assay (Table 4).

Table 3. Sensitivity and Specificity of Three Assays Tested by Clinical Specimens.

Clinical Samples (The number of specimens)	Ratio of Assays			*P* Value (chi-square test)
	RepMp1	MpP1	Mp181	
Positive Clinical Specimens (40)	100%	100%	85%	$0.01<P<0.05$[a]
Negative Clinical Specimens (100)	100%	98%	99%	$P>0.05$[b]

Note. [a]compared to Mp181 assay. [b]Pairwise comparison.

DISCUSSION

In this study, we designed a novel real-time PCR assay-MpP1 according to the characteristics of the p1 sequence of 60 Chinese *M.pneumoniae* clinical isolates and other reported targets sequence. This is the first report about a real-time PCR assay designed for Chinese *M.pneumoniae* strains detection. This assay, as well as other real-time PCR assays: Mp181 (targeting CARDS toxin gene) and RepMp1 (targeting RepMP1 sequence) were evaluated with 40 *M.pneumoniae* positive respiratory specimens and 100 *M.pneumoniae* negative respiratory specimens. The sensitivity of MpP1, RepMp1, and Mp181 assays appear to be 100%, 100%, and 85%, respectively. The sensitivity of the RepMP1 and MpP1 based assays was slightly higher than that of the Mp181 based assay ($0.01<P<0.05$, chi-square test) and was confirmed, especially in low copy *M.pneumoniae* clinical specimens. There was no significant difference in regard to the specificities of these assays ($P>0.05$, chi-square test).

Although MpP1 and Mp181 assays have nearly similar capability for lowest detectable limit with pure culture extraction DNA, the sensitive MpP1-based real-time PCR approach seems to be an alternative method particularly for Chinese clinical specimens in low concentrations of *M.pneumoniae* DNA in comparison with Mp181 assay alone. Compared to the single-copy of Mp181 and MpP1 assays, the RepMp1 assay amplifies about 89 bp product within the 14 repetitive elements of RepMp1 scattered all over the genome of *M.pneumoniae*[12,14]. Multiple copies RepMP1 based assay have also generated 100% sensitivity and lower crossing threshold values in this study, but the uncertain amplification number of repetitive sequences made it difficult to be used in the quantitative *M.pneumoniae* determination of clinical specimens. This novel MpP1 assay might be used as a superior method for *M.pneumoniae* quantification, especially in the Chinese population.

Table 4. Average Crossing-threshold Values for MpP1 and Mp181 in 40 Positive Clinical Specimens

Specimen No.	Crossing threshold±SD		
	RepmMp1	MpP1	Mp181
1	27.18±0.16	30.59±0.15	30.66±0.01
2	30.28±0.23	33.08±0.04	33.67±0.02
3	28.81±0.01	31.96±0.13	32.16±0.13
4	26.66±0.11	29.79±0.01	30.32±0.25
5	27.69±0.05	30.9±0.06	31.30±0.11
6	31.98±0.21	35.07±0.18	35.61±0.25
7	27.66±0.11	30.33±0.03	30.78±0.08
8	31.68±0.25	34.96±0.18	35.08±0.18
9	30.16±0.13	33.29±0.11	33.56±0.22
10	31.44±0.1	34.29±0.09	34.57±0.10
11	32.46±0.3	35.05±0.08	35.63±0.02
12	29.25±0.06	32.05±0.01	32.38±0.19
13	31.67±0.46	35.1±0.33	35.05±0.09
14	32.06±0.21	36.15±0.15	35.84±0.10
15	28.67±0.21	31.42±0.02	32.09±0.14
16	31.66±0.06	35.06±0.05	35.31±0.06
17	30.12±0.16	33.21±0.12	33.50±0.04
18	30.85±0.06	33.32±0.19	34.17±0.10
19	30.29±0.15	33.91±0.07	33.89±0.25
20	29.1±0.03	32.22±0.02	32.63±0.28
21	29.77±0.24	32.88±0.11	33.00±0.08
22	29.31±0.02	31.97±0.04	32.06±0.06
23	27.75±0.06	30.46±0.07	30.80±0.11
24	27.45±0.03	30.27±0.01	30.58±0.29
25	30.03±0.01	32.82±0.02	33.29±0.30
26	33.03±0.21	35.48±0.41	35.73±0.23
27	31.17±0.44	33.64±0.05	33.90±0.32
28	25.65±0.08	29.17±0.07	29.28±0.06
29	34.44±0.25	38±0.28	38.22±0.40
30	31.67±0.21	34.36±0.08	34.48±0.06
31	34.51±0.07	37.41±0.06	37.60±1.39
32	34.04±0.31	36.26±0.02	36.78±0.67
33	34.09±0.1	37.53±0.95	36.46±0.21
34	35.65±0.65	38.17±0.37	39.01±0.25
35	34.71±0.16	37.34±0.13	N
36	35.65±0.39	38.64±0.42	N
37	34.65±0.17	36.66±0.01	N
38	35.38±0.44	37.63±0.38	N
39	35.83±0.33	37.21±0.36	N
40	36.07±0.31	38.4±0.65	N

Note. N, Detected negative.

REFERENCES

1. Klement E, Talkington DF, Wasserzug O, et al. Identification of risk factors for infection in an outbreak of Mycoplasma pneumoniae respiratory tract disease. Clin Infect Dis, 2006; 43, 1239-45.
2. Waring AL, Halse TA, Csiza CK, et al. Development of a genomics-based PCR assay for detection of Mycoplasma pneumoniae in a large outbreak in New York State. J Clin Microbiol, 2001; 39, 1385-90.
3. Foy HM, Kenny GE, Cooney MK, et al. Long-term epidemiology of infections with Mycoplasma pneumoniae. J Infect Dis, 1979, 139, 681-7.
4. Hauksdottir GS, Jonsson T, Sigurdardottir V, et al. Seroepidemiology of Mycoplasma pneumoniae infections in Iceland 1987-96. Scand J Infect Dis, 1998; 30, 177-80.
5. Rastawicki W, Kaluzewski S, Jagielski M. Occurrence of serologically verified Mycoplasma pneumoniae infections in Poland in 1970-1995. Eur J Epidemiol, 1998; 14, 37-40.
6. Kenri T, Okazaki N, Yamazaki T, et al. Genotyping analysis of Mycoplasma pneumoniae clinical strains in Japan between 1995 and 2005: type shift phenomenon of M. pneumoniae clinical strains. J Med Microbiol, 2008; 57, 469-75.
7. Sasaki T, Kenri T, Okazaki N, et al. Epidemiological study of Mycoplasma pneumoniae infections in Japan based on PCR-restriction fragment length polymorphism of the P1 cytadhesin gene. J Clin Microbiol, 1996; 34, 447-9.
8. Daxboeck F, Krause R, Wenisch C. Laboratory diagnosis of Mycoplasma pneumoniae infection. Clin Microbiol Infect, 2003; 9, 263-73.
9. Loens K, Ursi D, Goossens H, et al. Molecular diagnosis of Mycoplasma pneumoniae respiratory tract infections. J Clin Microbiol, 2003; 41, 4915-23.
10. Daxboeck F, Khanakah G, Bauer C, et al. Detection of Mycoplasma pneumoniae in serum specimens from patients with mycoplasma pneumonia by PCR. Int J Med Microbiol, 2005; 295, 279-85.
11. Ursi D, Dirven K, Loens K, et al. Detection of Mycoplasma pneumoniae in respiratory samples by real-time PCR using an inhibition control. J Microbiol Methods, 2003; 55, 149-53.
12. Dumke R, Schurwanz N, Lenz M, et al. Sensitive detection of Mycoplasma pneumoniae in human respiratory tract samples by optimized real-time PCR approach. J Clin Microbiol, 2007; 45, 2726-30.
13. Winchell JM, Thurman KA, Mitchel SL, et al. Evaluation of three real-time PCR assays for detection of Mycoplasma pneumoniae in an outbreak investigation. J Clin Microbiol, 2008; 46, 3116-8.
14. Himmelreich R, Hilbert H, Plagens H, et al. Complete sequence analysis of the genome of the bacterium Mycoplasma pneumoniae. Nucleic Acids Res, 1996; 24, 4420-49.