Pediatric clinical features of *Mycoplasma pneumoniae* infection are associated with bacterial P1 genotype

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Abstract. The present study evaluated the association between different *Mycoplasma pneumoniae (M. pneumonia)* genotypes and clinical features of pediatric patients. Subjects were children diagnosed with community-acquired pneumonia at the Children's Hospital of Soochow University (Suzhou, China) from January 2012 to December 2013. Clinical and laboratory tests were conducted and clinical samples positive for *M. pneumoniae* were genotyped by nested-multiplex polymerase chain reaction. Three type I strains and three type II strains were also randomly selected for sequencing. A total of 335 clinical samples positive for *M. pneumoniae* were obtained. The average age of *M. pneumonia*-infected pediatric patients was 4.8±3.3 (years). Genotyping results identified 304 positive samples as group I strains and 30 samples as group II strains, in which 1 sample was type II variant 2a. It was also observed that point mutations were more likely to occur in type I strains compared with type II strains. Although clinical pulmonary infection scores between patients with type I and type II strains did not significantly differ, patients with type I strains had a higher risk of developing severe *M. pneumoniae* pneumonia (SMPP) and extrapulmonary complications, and had significantly higher percentages of peripheral blood neutrophils than patients with type II strains ($P<0.05$). Collectively, these data indicate that the predominant strains of *M. pneumoniae* in Suzhou between 2012 and 2013 were type I, and that pediatric pneumonia patients with type I strains of *M. pneumoniae* were more likely to progress to SMPP.

Introduction

A variety of mycoplasmas have so far been identified and among these, *Mycoplasma pneumoniae* (*M. pneumoniae*) is the best-known human pathogen. *M. pneumoniae* is generally smaller than conventional bacteria in genome size and cellular dimensions, and has the ability to grow and proliferate within cell-free culture media (1). Infection with *M. pneumoniae* occurs worldwide and typically becomes endemic every 5-7 years, each time lasting 6-8 months (2). *M. pneumoniae* adheres to and colonizes host cells through P1 adhesin, encoded by the P1 gene. The P1 protein is also involved in the immunopathogenetic effects of *M. pneumoniae,* suggesting that the P1 gene serves a key role in *M. pneumoniae* infection (3). Previous research has demonstrated that the epidemic cycle of *M. pneumoniae* is associated with P1 gene polymorphisms, and that different subtypes may dominate alternately (4). According to repeat sequences in the *M. pneumoniae* genome (RepMP4, 8 copies and RepMP2/3, 10 copies), clinical strains of *M. pneumoniae* may be classified into two major classes, P1 type I and P1 type II, or as classed as minor variations generated by DNA recombination between repeat sequences and the P1 gene locus (5,6). *M. pneumoniae* is a major respiratory pathogen that may cause various clinical disease ranging from mild upper respiratory tract infection (URTI) to severe, or in some instances, fatal pneumonia. *M. pneumoniae* may account for as many as 40% of community acquired pneumonia cases and 18% of these patients require hospitalization (1). Severe M. pneumoniae pneumonia appears to occur in defined age groups, whereas older children aged 5-15 years are more likely to develop bronchopneumonia, involving one or more lobes (2).

However, it remains unknown whether there is a correlation between different *M. pneumoniae* genotypes and the clinical characteristics of pneumonia. Therefore, the present study genotyped *M. pneumoniae* strains using nested multiplex polymerase chain reaction (PCR) and evaluated whether different bacterial genotypes are associated with the clinical features of pediatric pneumonia patients, in order to identify the association between different genotypes of *M. pneumoniae* and disease severity.

Materials and methods

Clinical data. Subjects were pediatric patients who attended the outpatient clinic or were hospitalized at the Children's

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Hospital of Soochow University (Suzhou, China) from January 2012 to December 2013. All patients were diagnosed with community-acquired pneumonia (CAP) on the basis of the following symptoms: A cough, chest pain, wheezing, tachypnea, chest retractions, abnormal auscultatory findings such as crackles or reduced breath sounds, and radiological abnormalities with pulmonary infiltrates and consolidation. Digital radiography examinations of all patients were conducted by a radiologist. From January 2012 to December 2013, a total of 1,982 CAP cases were admitted to hospital; however 48 patients refused to participate in the study. Consequently, a total of 1,934 patients were enrolled. A total of 76 patients who had taken macrolides medication prior to admission and chronic underlying diseases were excluded. A final 335 patients were confirmed to have *M. pneumoniae* infection by PCR and ELISA tests, and were subsequently enrolled for *M. pneumonia* genotyping analysis. The demographic characteristics of patients with *M. pneumoniae* infection were also recorded. During the admission period, severe *M. pneumoniae* pneumonia (SMPP) was diagnosed on the basis of fever (prolonged temperature of >38.5˚C), radiological deterioration or consolidation of >2/3 of the lung lobes, and intra- and extra- pulmonary complications. Intra- and extra-pulmonary complications included pleural effusion, bronchiolitis obliterans, necrotizing pneumonia, neurological disorders (encephalitis, aseptic meningitis and meningoencephalitis), dermatological disorders (erythematous maculopapular and vesicular rashes), hepatic function abnormality and arthralgias. Clinical data and laboratory test results of hospitalized patients were collected and used to calculate clinical pulmonary infection scores (CPIS; Table I) (7). The present study was approved by the Ethics Committee of the Children's Hospital of Soochow University and written informed consent was obtained from all parents.

Sample collection. Nasopharyngeal and serum samples were collected from all patients. Nasopharyngeal samples were obtained by aspiration under negative pressure in a sterile environment within 24 h of patient admission. Briefly, a plastic suction tube was advanced 7-8 cm through the nasal cavity and into the throat, and 1-2 ml secretory fluid was aspirated and transferred to a test tube containing sterile saline. The mixture was then shaken, split into three aliquots and analyzed within 30 min. Nasopharyngeal aspirates (NPAs) were initially examined by smear microscopy, with squamous cells at <10 cells/field and white blood cells at >25 cells/low magnification field deemed as qualified samples. If this requirement was not met, additional samples were collected again until the requirements were met.

For serum collection, a total of 2 ml venous blood was collected from patients during the acute (upon admission) and convalescent (upon discharge, 1-2 weeks after admission) infection phases. Levels of *M. pneumonia*-specific antibodies [Immunoglobulin (IgG)-M and IgG) present in serum samples were subsequently measured by ELISA.

Multi-pathogen detection. Multiple pathogens were screened for ten viruses using NPAs as previously described (8). Respiratory syncytial virus, influenza A and B viruses, parainfluenza viruses 1-3 and adenovirus were detected using direct immunofluorescence assays. Human metapneumovirus, bocavirus and rhinovirus were detected using PCR.

Diagnosis of M. pneumoniae infection by quantitative PCR (qPCR). The procedure was described previously (9). DNA was extracted for diagnosis of *M. pneumoniae* infection. NPA samples were diluted with 2 ml normal saline prior to centrifugation at 500 x g for 10 min at 4˚C. DNA was then extracted from the resulting 400 μl samples using DNA Purification Kit (B518267; Sangon Biotech Co., Ltd., Shanghai, China), according to the manufacturer's instructions. *M. pneumoniae* DNA loads in the NPAs were subsequently determined by fluorescence quantitative PCR, using a Bio-Rad real-time fluorescence PCR amplifier (Bio-Rad laboratories, Inc., Hercules, CA, USA). Primers and fluorescent probes were purchased from Daan Gene Co., Ltd. (Guangzhou, China).

Diagnosis of M. pneumoniae infection: Quantitative ELISA A total of each 2 ml of acute phase (on admission) and convalescent phase (on discharge) patient blood samples were collected and centrifuged at 3,000 r/min for 5 min at 4˚C. A total of 100 μl was harvested and analyzed using a quantitative ELISA kit (ESR127; Institut Virion/Serion GmbH, Würzburg, Germany) to detect *M. pneumonia*-specific IgM and IgG, according to the manufacturer's instructions. Results are presented in U/ml. For acute serum samples, IgM >13 U/ml indicated an acute infection. For convalescent serum samples, a 4-fold increase in the IgG titer of convalescent serum also indicated an acute infection (10). *M. pneumoniae* infection was diagnosed when both PCR and ELISA exhibited positive results.

M. pneumoniae genotyping by nested multiplex PCR. DNA was extracted from clinical samples and reference *M. pneumoniae* strains. Extraction from clinical samples was performed as above. *M. pneumoniae* strains M129 and FH were used as representatives of strain types I and II, respectively, and their genomic DNA was extracted using DNA Purification Kit (B518267; Sangon Biotech Co., Ltd., Shanghai, China), according to the manufacturer's instructions. Primers were designed for the two variable regions of RepMP4 and RepMP2/3 within the P1 gene, according to a previously described method (11) and were synthesized by Sangon Biotech Co., Ltd. (Table II). Nested PCR was then performed, as follows: Primers for ADH2-forward (F)/ADH2-reverse (R), ADH3F/ADH3R, ADH3/MP2/3-R1, and MP2/3-R2/MP2/3-F2 (Table II) were used to amplify the *M. pneumonia* target gene in clinical specimens and reference strains. PCR was performed in a reaction system containing 10X Buffer Concentrate (Shinegene, Shanghai, China; 4 μl), 25 mM $MgCl_2$ (3 μl), 10 mM dNTPs (Shinegene; 3 μl), 10 μM of forward and reverse primers, respectively (1 μl), extracted DNA as a template (5 μl) and AmpliTaq (0.3 μl; Takara Biotechnology, Co., Ltd., Shanghai, China). The reaction system was brought to 25 μl by addition of bi-distilled H_2O (DNAse-free). PCR amplification was performed by an initial denaturation step at 95˚C for 5 min, followed by 30 cycles at 95˚C for 30 sec, 50˚C for 30 sec, 72˚C for 2.5 min and a final extension step at 72˚C for 5 min. Amplification was performed using a Bio-Rad real-time PCR amplifier (Bio-Rad laboratories, Inc.).

Table I. Clinical pulmonary infection scores based on clinical and laboratory test results.

Parameters	Score		
	0	1	2
Body temperature (˚C)	36-38	38-39	<36 or >39
Leukocyte count	4-11	11-17	<4 or >17
Volume and Character of tracheal secretions	Little	Medium	Excessive
Arterial oxygenation (PaO_2/FiO_2, kPa)	>33		<33
Chest X-ray	Normal	Bronchopneumonia	Lobar pneumonia

PaO_2, partial pressure of oxygen; FiO_2, fraction of inspired oxygen; kPa, kilopascal.

Table II. Polymerase chain reaction primer sequences of *Mycoplasma pneumonia* P1 genotypes.

Primer	Sequence (5'→3')
ADH1	CTGCCTTGTCCAAGTCCACT
ADH2	AACCTTGTCGGGAAGAGCTG
ADH3	CGAGTTTGCTGCTAACGAGT
ADH4	CTTGACTGATACCTGTGCGG
ADH2F	GGCAGTGGCAGTCAACAAACCACGTAT
ADH2R	GAACTTAGCGCCAGCAACTGCCAT
ADH3F	GAACCGAAGCGGCTTTGACCGCAT
ADH3R	GTTGACCATGCCTGAGAACAGTAA
ADH4F	GACCGCATCAACCACCTTTGCGTTACG
N1	CCCGGTGGTGGAAGTATTTT
2N2C	TGCCTTGGTCACCGGAGTTG
MP2/3-R1	AGATTGACCTGAGCCTGAAG
MP2/3-F2	CACAAGTGGTTCGCGTTCCT
MP2/3-R2	GGCTGGGTGGAATGGTCTGT
MP2/3-F3	TCGACCAAGCCAACCTCCAG
R3-1	TTGGAATCGGACCCACTTCG
R3-2	CGACGTTGTGTTTGTGCCAC
R3-2V	CGGTATAGCTAATTTGGTAC

The primer sites of ADH1, ADH2R, ADH2, ADH3, MP2/3-R1 and ADH4 are located outside the RepMP4 and RepMP2/3 regions of the P1 gene. Primers N1 and R3-1 are specific to the type I P1 gene. Primers 2N2C and R3-2 are specific to the type II and type II variant 2a P1 genes. Primer R3-2V is specific to type II variant 2a. RepMP, repeat Mycoplasma pneumonia sequences; P1, P1 adhesin gene.

Nested PCR products were diluted to a ratio of 1:10,000 using ddH_2O, then multiplex PCR was performed on the diluted PCR products using the primers ADH4F, N1, 2N2C and MP2/3-F3, R3-1, R3-2, and R3-2V (Table II). PCR was performed in a reaction system containing 10X Buffer Concentrate (4 μl), 25 mM $MgCl_2$ (3 μl), 10 mM dNTPs (3 μl), 10 uM of forward and reverse primers (1 μl) repectively, nested PCR products as a DNA template (3 μl) and AmpliTaq (0.3 μl). The reaction mix was brought to 25 μl by addition of ddH_2O. PCR amplification conditions were as stated above. A total of 12 μl PCR products were separated by 2% agarose gel electrophoresis for *M. pneumoniae* genotyping. Experiments were performed in duplicate.

Gene sequencing. Three P1 type I and three P1 type II strains were randomly selected from nested multiplex PCR products. Amplified products were sent to Sangon Biotech Co., Ltd. for P1 gene sequencing. Sequenced genes were then compared with the P1 genes of reference strains M129 and FH, as representatives of strain types I and II, respectively, archived in GenBank (https://www.ncbi.nlm.nih.gov/nuccore/) (Access No. M18639 and FJ215693). Comparisons were made using the Basic Local Alignment Search Tool (BLAST) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis. Statistical analysis was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Measurement data are presented as the mean ± standard deviation. If data were normally distributed, the two groups were compared using Student's t-test. Nonparametric tests were used for data that did not follow a normal distribution. Categorical data were compared using χ^2 or Fisher's exact tests if sample sizes were small or the data was unequally distributed among the cells of the table. For all analyses, $P<0.05$ was considered to indicate statistical significance.

Results

Demographic characteristics of pediatric patients with M. pneumoniae pneumonia (MPP). The average age of the 335 children admitted to hospital with *M. pneumoniae* infection was 4.8±3.3 years. There were 182 males (54.32%) and 153 females (45.67%), with a male to female ratio of 1.19:1. Of the 335 *M. pneumoniae* cases, 35 patients (10.45%) were <1 year old, 79 patients (23.58%) were 1-3 years old, 106 patients (31.64%) were 3-6 years old, 56 patients (16.72%) were 6-9 years old and 59 patients (17.61%) were >9 years old.

M. pneumoniae genotypes. Genotyping results of twice repeated nested multiplex PCR products are depicted in Fig. 1. Regarding genotyping of the RepMP4 region, a 343 bp product from the p1 gene DNA of type I strains and a 560 bp product from that of type II strains were indicated. For genotyping of the RepMP2/3 region, a 394 bp product from the

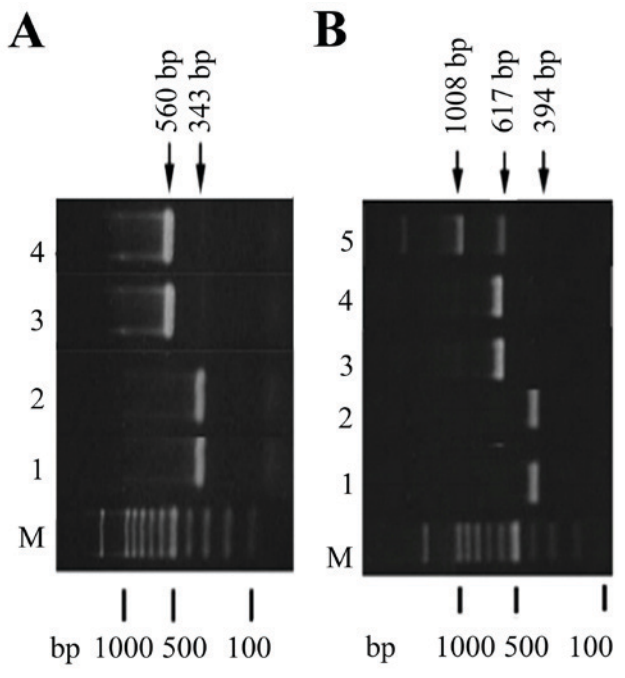

Figure 1. Genotyping of the (A) RepMP4 and (B) RepMP2/3 regions within the *Mycoplasma pneumoniae* P1 gene by nested-multiplex PCR. Representative images of four patient samples are shown of agarose gel (2%) electrophoresis patterns obtained from the final PCR products of genotype strains P1-I and P1-II. M: DNA ladder, lane 1: standard P1-I strain M129, lane 2: P1-I genotype strain, lane 3: standard P1-II strain MP-FH, lane 4: P1-II genotype strain, lane 5: P1-II genotype variant strain. P1, P1 adhesin gene; PCR, polymerase chain reaction; RepMP, repeat Mycoplasma pneumonia sequences.

p1 gene DNA of type I strains, a 617 bp product from that of type II strains and 617 and 1008 bp products from that of type II variant strains were demonstrated. Among the clinical strains obtained from the 335 pediatric patients with MPP, 304 (90.75%) were type I, 30 (8.96%) were type II and 1 (0.3%) was type II variant 2a. Among the 198 clinical strains detected in 2012, 186 (93.94%) were type I and 12 (6.06%) were type II, with no variants identified. Of the 137 clinical strains detected in 2013, 118 (86.13%) were type I, 18 (13.14%) were type II, and 1 (0.73%) was type II variant 2a. The numbers and age distribution of patients with each strain is depicted in Fig. 2.

Nucleotide sequences of type I and type II strains. Type I and II strains (3 of each) obtained from nested multiplex PCR of patient DNA samples were randomly selected for sequencing. Sequenced genes were then compared with the P1 genes of reference strains M129 and FH, as representatives of strain types I and II, respectively. The point mutations C to T and A to C were identified in two type I strains (Fig. 3). All three type II strains had identical sequences to that of the FH strain and contained no mutations (data not shown).

Clinical features of pediatric pneumonia patients with type I and II M. pneumonia. Among the 335 pediatric patients admitted to hospital with MPP, 52 presented with additional viral infections (Table III). Of these co-infection cases, 49 patients had type I *M. pneumoniae* and 3 patients had type II. The remaining 283 patients were infected with *M. pneumoniae* alone; 252 patients with type I and 31 patients with type II. Although the CPIS between patients with type I or II strains did not differ significantly, patients with type I *M. pneumoniae* were at greater risk of developing SMPP and extrapulmonary complications ($P<0.05$), but had significantly lower percentages of peripheral blood neutrophils than patients with type II *M. pneumoniae* ($P<0.05$; Table IV).

Discussion

M. pneumoniae is the primary pathogen responsible for respiratory tract infections in children, causing a worldwide health burden (12-14), and represents an incidence of nearly 20% with respiratory tract infections in Beijing, China (15). The P1 gene of *M. pneumoniae* is highly conserved and linked to the adhesive, colonization and pathogenic properties of the pathogen (16,17). Although rare, severe and/or refractory pneumonia due to *M. pneumoniae* infection can occur. While its exact etiology remains unclear, severe or refractory MPP has been associated with an excessive inflammatory response and/or macrolide resistance (9,18).

In the present study, nested multiplex PCR and gene sequencing techniques were used to determine the genotypes of dominant *M. pneumoniae* strains in pediatric patients in Suzhou, and their association with disease severity. The results demonstrated that type I *M. pneumoniae* was dominant in Suzhou between 2012 and 2013, accounting for 90.75% of all cases (304/335). Similarly, a multilocus variable number tandem repeat analysis conducted in France demonstrated that 81.6% of *M. pneumoniae* strains were adhesin P1 type I (19). However, a recent study in Germany documented a higher incidence of P1 type II variants (39.08%, 34/87) between 2011 and 2012 (20) than that identified in the present study (0.3%, 1/335). The current study also determined that MPP patients with type I strains were at greater risk of developing SMPP. In addition, the higher rate of point mutations identified in type I strains suggests that type I *M. pneumoniae* is more susceptible to point mutations, although this was confirmed in only 2 out of 3 of the randomly selected cases. These data indicate that mutations in the P1 gene may be partly associated with SMPP; however, the corresponding mechanisms remain unknown.

Different genotypes have been identified in prevalent *M. pneumoniae* strains during different time periods. In Denmark, the majority of strains isolated between 1962 and 1986, and between 1991 and 1993 were type I, while the majority of those isolated between 1987 and 1988 were type II (21). This type shift has also been documented in France (22) and in Japan, where a type shift is considered to occur every eight to ten years (23). It has also been documented in Japan that the type shift process itself may take two to three years, though after dominance is established, a strain type remains dominant for approximately seven years (23). In addition, it has been observed that outbreaks of *M. pneumoniae* coincide with a type shift in *M. pneumoniae* strains, suggesting that type shifts may be involved in *M. pneumonia* outbreaks (11). The mechanism underlying the type shift process remains poorly characterized and may be related to the immune status of the population involved (11). More recently, the genotype of predominant *M. pneumoniae* strains in Beijing, China between 2008 and 2010 was type I (24,25), which is consistent with the

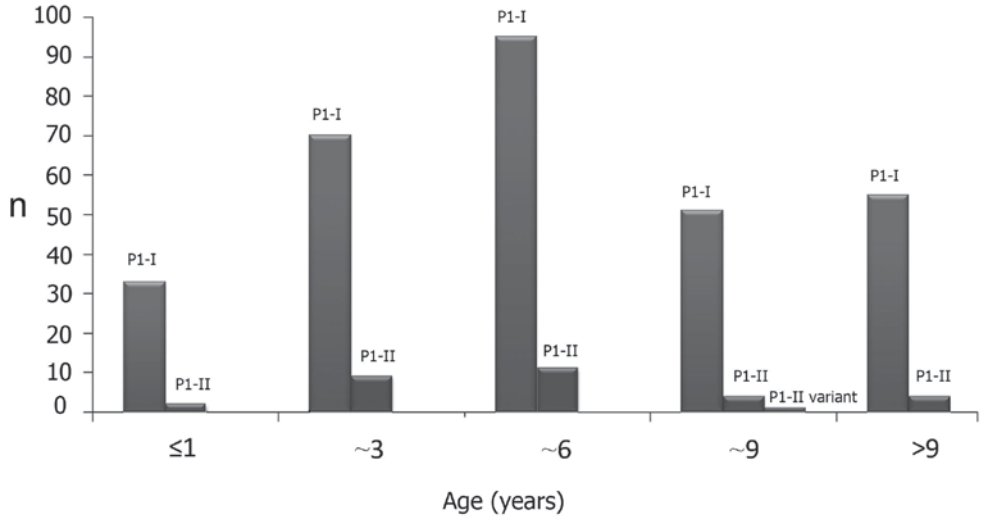

Figure 2. Age distribution of pediatric patients in Suzhou infected with genotype strains P1-I, P1-II and P1-II variant 2a. P1, P1 adhesin gene.

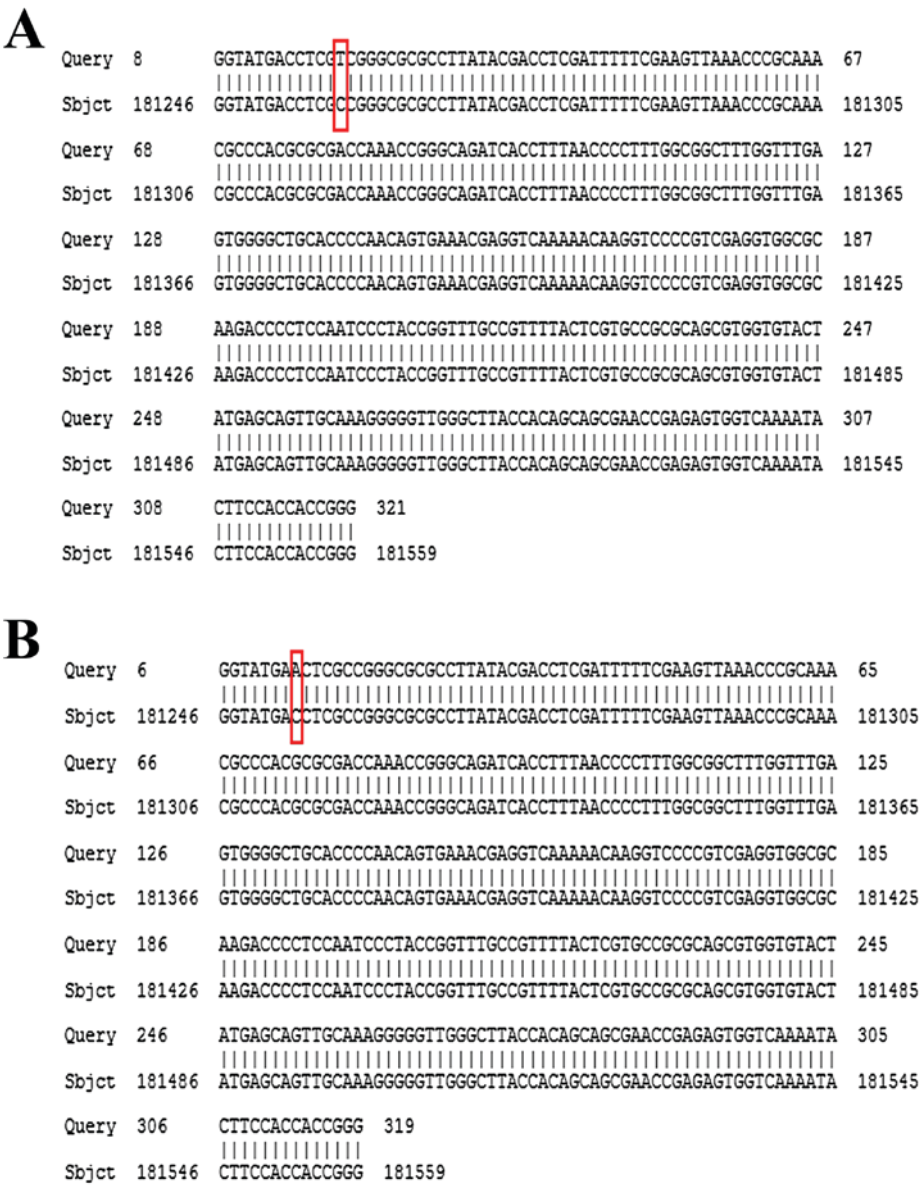

Figure 3. Sequencing of randomly selected patient samples positive for genotype strain P1-I. Point mutations of (A) C to T and (B) C to A compared with the standard P1-I strain M129 are highlighted (red boxes). P1, P1 adhesin gene; sbjct, M129; query, selected sample.

Table III. Pathogen co-infection with P1-I and P1-II genotype strains of *Mycoplasma pneumoniae*.

Pathogen	Cases (n)	Percentage (%)	P1-I genotype	P1-II genotype
RSV	14	26.92	14	0
HRV	8	15.38	7	1
HBoV	8	15.38	7	1
hMPV	7	13.46	7	0
PIV-3	5	9.62	5	0
IV-A	3	5.77	3	0
ADV	1	1.92	1	0
Two or more viruses	6	11.54	5	1
Total	52	100	49	3

RSV, Respiratory syncytial virus; HRV, human rhinovirus; HBoV, human bocavirus; hMPV, human metapneumovirus; PIV-3, parainfluenza virus 3; IV-A, influenza virus A; ADV, adenovirus. P1, P1 adhesin gene.

Table IV. Comparison of clinical and laboratory profiles of hospitalized children infected by the P1-I and P1-II genotype strains of *Mycoplasma pneumonia*.

Parameters	P1-I genotype (n=252)	P1-II genotype (n=31)	P-value
Average age (months)	54	62	0.692
SMPP (n)	87	4	0.014[a]
Extrapulmonary complications (n)	58	2	0.035[a]
Duration of fever (mean ± SD, day)	4.6±4.25	4.6±3.63	0.856
Fever peak (mean ± SD, ˚C)	38.91±4.12	39.85±0.42	0.373
Hospital days (mean ± SD, day)	11.52±2.99	11.33±3.62	0.827
White blood cell (mean ± SD, $x10^9$/l)	8.17±4.40	5.51±2.72	0.283
Neutrophils percentage (mean ± SD, %)	40.35±20.36	57.67±17.04	0.032[a]
CRP (mean ± SD, mg/l)	41.50±9.57	21.99±3.28	0.194
CPIS (mean ± SD)	25.90±5.97	22.00±3.22	0.135

[a]P<0.05; SMPP, severe *Mycoplasma pneumoniae* pneumonia; CRP, C-reactive protein; CPIS, clinical pulmonary infection score; SD, standard deviation.

prevalent strains identified in Suzhou between 2012 and 2013 in the current study. Therefore, type I strains may have been dominant in China between 2008 and 2013. As the present study was conducted over a short period, no type shifts were observed. Future studies involving long-term surveillance may identify how the two genotypes shift in Suzhou.

Interestingly, although the CPIS of patients infected with type I or II strains did not differ significantly, the present study observed that patients with type I strains were more likely to progress to SMPP, partly due to the high frequency of coinfections in patients with type I strains. However, a previous study in Sweden observed that disease severity was not correlated with genotypes of the P1 gene, but with the *M. pneumoniae* load collected on throat swabs (26). This discrepancy may be due to greater susceptibility of the P1 type I genotype to point mutations, which in turn, may be associated with disease severity. In China, the *M. pneumoniae* resistance-related 23S ribosomal RNA gene is also prone to mutations and results in macrolide resistance (18,27). However, an association between point mutations of the P1 gene and macrolide resistance has not been established (25). Alternatively, mutations in the P1 adhesin protein may alter its structure and function, thus enabling *M. pneumonia* to evade identification and removal by the host and enhance its pathogenicity. A previous study examined *M. pneumoniae* genotypes using multiple-locus variable number tandem repeat analysis (MLVA), demonstrating that patients infected with types U and J had significantly higher CPIS (P<0.001) and longer total cough durations (P=0.011) compared with patients with other MLVA types (28). This appears to confirm that there is an association between *M. pneumoniae* genotype and disease severity.

In conclusion, the predominant *M. pneumoniae* strains in pediatric patients in Suzhou between 2012 and 2013 were type I and MPP patients infected with type I strains exhibited a higher risk of developing SMPP. Thus, P1 genotyping may be a valuable method of identifying the dominant genotype of *M. pneumoniae* strains within various populations.

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