

Epidemiology and mechanism of drug resistance of *Mycoplasma pneumoniae* in Beijing, China: A multicenter study

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ABSTRACT

Mycoplasma pneumoniae (*M. pneumoniae*) is one of the most common causes of community-acquired respiratory tract infections (RTIs). We aimed to investigate the prevalence of *M. pneumoniae* infection, antibiotic resistance and genetic diversity of *M. pneumoniae* isolates across multiple centers in Beijing, China. P1 protein was detected by Nested PCR to analyze the occurrence of *M. pneumoniae* in pediatric patients with RTI. *M. pneumoniae* isolates were cultured and analyzed by Nested-PCR to determine their genotypes. Broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of antibiotics. Out of 822 children with RTI admitted to 11 hospitals in Beijing, 341 (41.48%) were positive for *M. pneumoniae* by Nested PCR and 236 (69.21%) samples had mutations in 23S rRNA domain V. The highest proportion of *M. pneumoniae* positive samples was observed in school-age children (118/190; 62.11%) and in pediatric patients with pneumonia (220/389; 56.56%). Out of 341 *M. pneumoniae* positive samples, 99 (12.04%) isolates were successfully cultured and the MIC values were determined for 65 *M. pneumoniae* strains. Out of these, 57 (87.69%) strains were resistant to macrolides, and all 65 strains were sensitive to tetracyclines or quinolones. *M. pneumoniae* P1 type I and P1 type II strains were found in 57/65 (87.69%) and 8/65 (12.31%) of cultured isolates, respectively. Overall, we demonstrated a high prevalence of *M. pneumoniae* infection and high macrolide resistance of *M. pneumoniae* strains in Beijing. School-age children were more susceptible to *M. pneumoniae*, particularly the children with pneumonia. Thus, establishment of a systematic surveillance program to fully understand the epidemiology of *M. pneumoniae* is critical for the standardized use of antibiotics in China.

KEY WORDS: *Mycoplasma pneumoniae*; epidemiology; drug resistance; infection

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INTRODUCTION

Mycoplasma pneumoniae (*M. pneumoniae*) is a small, pleomorphic, fastidious, and highly evolved pleomorphic bacteria

lacking the cell wall, which was first isolated from a patient with primary atypical pneumonia [1,2]. *M. pneumoniae* is one of the most common pathogens causing community-acquired respiratory tract infections (RTIs) and has been recognized as a worldwide cause of primary atypical pneumonia [3-5]. It can affect people of all ages, especially the most vulnerable groups children and adolescents [6-8]. Nevertheless, as currently there are no reliable and rapid diagnostic tests for the detection of *M. pneumoniae*, the treatment of community-acquired pneumonia is empirical in most cases [9].

In addition to mild upper RTIs (URTIs; e.g., pharyngitis and sinusitis) and severe lower RTIs (LRTIs; e.g., bronchitis

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and pneumonia), *M. pneumoniae* may also cause damage to extrapulmonary systems [10,11]. *M. pneumoniae* infections mainly occur in preschool children, however, a recent report suggested that the incidence in infants is also high [12]. Although the symptoms are mild to moderate in most cases of *M. pneumoniae* infections, hospitalization is occasionally required in severe cases. In addition, multiple organ system damage may occur [13]. *M. pneumoniae* infection appears as a cyclic epidemic disease with intervals of four to seven years worldwide and persists for one to two years [10,14,15]. This periodicity in *M. pneumoniae* infection may be related to changes in the sequence of P1 adhesin, which is the main method of *M. pneumoniae* typing. According to the differences in the sequence of P1 adhesin gene *MPN141*, *M. pneumoniae* is divided into P1 type I and P1 type II with several subtypes [16,17]. The prevalence of *M. pneumoniae* subtypes differs between countries and years, and the dominance of one subtype is followed by the dominance of another *M. pneumoniae* subtype [18].

Although *M. pneumoniae* is sensitive to macrolide, tetracycline, and quinolone antibiotics, which affect the synthesis of proteins and nucleic acids, macrolides remain the first choice for the treatment of *M. pneumoniae* infection in children [19]. However, the phenomenon of *M. pneumoniae* resistance continues to increase worldwide, especially in China and Japan, where the resistance rate is reported to be over 90% [20,21]. Since 2004, tetracycline and quinolone antibiotics have been approved for use in children over eight years of age with macrolide resistance or refractory *M. pneumoniae* infection [22]. Therefore, with the increasing use of these two drugs in clinical practice, the drug resistance becomes an even more serious problem. Moreover, only a few studies have focused on the *M. pneumoniae* tetracycline or quinolone resistance in China.

Several serology-based studies were conducted in Beijing [23-25], however, they were all single-center studies and thus limited by their design. To the best of our knowledge, there are no multicenter studies investigating the changes in the prevalence of *M. pneumoniae* infections in Beijing. Therefore, in the present study, we collected samples from several major children's hospitals in Beijing area to study the prevalence of *M. pneumoniae* infection and the antibiotic resistance of *M. pneumoniae*. In addition, we used molecular assays to identify the genotypes of isolated *M. pneumoniae* and to infer their potential antibiotic (macrolide, tetracycline, and quinolone) resistance mechanism.

MATERIALS AND METHODS

Patients' samples and *M. pneumoniae* testing

This study was conducted at 11 centers in Beijing, China, from January 2014 to December 2014. Participating centers

included Beijing Dongfang Hospital, The First Hospital of Tsinghua University, Beijing Children's Hospital, China Meitan General Hospital, Beijing Chao-Yang Hospital, Civil Aviation General Hospital, Xiyuan Hospital, Beijing Changping Hospital of Integrated Chinese and Western Medicine, New Century Women's and Children's Hospital, New Century International Children's Hospital, and Peking University Third Hospital. Ethics approvals were obtained from local or national institutional review boards, as appropriate. Tracheal swab specimens were collected from 822 pediatric patients with RTI symptoms. The pediatric patients were clinically diagnosed with pneumonia, URTI, or bronchitis. Patient data were collected, including sex, age, disease duration, and clinical diagnosis.

DNA was isolated from tracheal swab specimens using a Universal Genomic DNA Kit (Beijing Kangwei Century Biotech Co., Ltd., China), according to the manufacturer's instructions. Nested polymerase chain reaction (PCR) was performed to test the presence of *M. pneumoniae* DNA in samples, according to the previous research [26]. Each *M. pneumoniae*-positive PCR sample was cultivated to obtain pure *M. pneumoniae* isolates.

Culture of *M. pneumoniae*-positive samples

Culture of *M. pneumoniae* was performed using PPLO (pleuropneumonia-like organisms) basic medium (BD-Difco, USA) containing 15% newborn calf serum, 10% fresh yeast extract (OXOID, UK), 0.4% phenol red indicator (Sigma, USA), 1% glucose, and 50000 U/100 mL penicillin, as described previously [21]. Tracheal swab specimens were inoculated on the *M. pneumoniae* liquid medium and maintained in an incubator at 37°C with a 5% CO₂ atmosphere. The obtained isolates were stored at -80°C until further testing.

Measurement of minimum inhibitory concentration (MIC)

The measurement of MIC of antibiotics was performed using the standard broth microdilution method (standard MIC procedure) [27]. We used six antibiotics from three classes as follows: macrolides (erythromycin, azithromycin, and josamycin), tetracyclines (tetracycline and minocycline), and quinolones (levofloxacin). Antibiotic susceptibility test was conducted to distinguish between sensitive and clinically resistant strains according to the Clinical and Laboratory Standards Institute (CLSI) criteria [28]. The breakpoints of MIC were as follows: for macrolides, MIC ≥ 1.0 µg/ml was considered as resistant, 0.5 < MIC < 1.0 µg/ml as intermediate, and MIC ≤ 0.5 µg/ml as susceptible; for tetracyclines, MIC > 2.0 µg/ml was considered

as resistant and MIC ≤ 2.0 $\mu\text{g/ml}$ as susceptible; and for quinolones, MIC > 1.0 $\mu\text{g/ml}$ was considered as resistant and MIC ≤ 1.0 $\mu\text{g/ml}$ as susceptible. Two *M. pneumoniae* reference strains, M129 (ATCC 29342) and FH (ATCC 15531) were used as a drug-sensitive control.

M. pneumoniae genotyping and detection of resistance-related genes

P1 protein was detected by Nested PCR and classified according to the differences in the sequence of P1 adhesin gene *MPN141* [29]. The reaction conditions were as follows: initial denaturation at 95°C for two minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension step of 72°C for two minutes. PCR primer sequences are shown in Table 1.

The resistance-associated mutation in the 23S rRNA gene of *M. pneumoniae* was detected by Nested PCR. The reaction conditions were as follows: initial denaturation at 95°C for two minutes, followed by 35 cycles of 95°C for one minute, 55°C for one minute, and 72°C for 100 seconds, with a final extension step of 72°C for five minutes. The identification of nucleotide sequences was performed using BLAST against the NCBI GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences of the resistance-associated genes and PCR primers are shown in Table 1.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, NY, USA). Quantitative data were presented as mean \pm standard deviation (SD). Qualitative data were described as numbers or percentages. The normality of data distribution was tested by the Kolmogorov–Smirnov test. Qualitative variables were analyzed using Chi-squared test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Detection of *M. pneumoniae* in pediatric patients with RTI

The mean age of children with RTI was 4.68 ± 3.23 years (range, one month to 17 years). Out of 822 patients with RTI admitted to 11 hospitals in Beijing, 341 (41.48%) had a positive PCR for *M. pneumoniae*. Ninety-nine (12.04%, 99/822) *M. pneumoniae*-positive samples were successfully cultured. The sex of 779 patients was available. There were 417 males and 362 females. There was no statistically significant difference with regard to the *M. pneumoniae* positivity rates between male and female samples (38.85% [162/417] vs. 45.58% [165/362], $p = 0.06$; Figure 1A).

The age of 776 children was collected. School-age children (7–17 years old) had the highest *M. pneumoniae* positivity rate (62.11%, 118/190), followed by preschool children [3–6 years old] (40.30%, 160/397) and infants [< 2 years old] (24.87%, 47/189). There was a significant difference in the *M. pneumoniae* positivity rate between the three groups ($p < 0.001$).

The diagnosis of 775 children was available. The majority of *M. pneumoniae*-positive samples were found in patients with pneumonia (56.56%, 220/389), followed by those with URTI (28.71%, 60/209) and bronchitis (26.55%, 47/177). The *M. pneumoniae* detection rate in pneumonia samples was significantly higher than in URTI and bronchitis samples ($p < 0.001$), whereas there was no significant difference between URTI and bronchitis samples ($p = 0.67$).

Detection of mutations in domain V of the 23S rRNA gene

The 341 *M. pneumoniae*-positive PCR samples were analyzed by Nested PCR. Mutations in domain V of the 23S rRNA gene were found in 236 (69.21%) samples. The predominant mutation was A2063G (199/341, 58.36%), followed by A2064G (25/341, 7.33%). Twelve (3.52%) samples had the co-mutation of A2063G and A2064G. The mutation detection rate of

TABLE 1. Primers used for Nested PCR analysis

Gene	Primer sequence (Forward, 5'-3')	Primer sequence (Reverse, 5'-3')	Product length (bp)
<i>23II</i>	AGTACCGTGAGGGAAAGGTG	TCCCAAGCGTTACTCATGCC	816
<i>l4</i>	AAAAGCAGCACCAGTTGTAG	GGTTAGAAGTGGTTTTAGCA	722
<i>l22</i>	GTACATAACGGCAAGACCTT	GCAAGCCGTTGGAGTTTACT	627
<i>23V-1</i>	GCACTGAAGAAGAACGAGGGG	CACACTTAGATGCTTTCAGCG	1012
<i>23V-2</i>	TAAGTATAACGGTCTTAAGG	CGTACAACCTGGAGCATAAGA	793
<i>528a-1</i>	TACGACTAGGAGATCATCCAG	TTAAGGTGTTTGGATTGGTGT	232
<i>528a-2</i>	ATCTACCGATTCAACCAACTGCT	GCTAACTGCGCTAGAGCAAAT	166
<i>gyrA</i>	TGGAGTATGCGATGTCGGT	ACAGTCTCTAAGCGTTCCT	2335
<i>gyrB</i>	CATTCCTGTTGACATCCATCCT	GGAAGTTCATCGCCATT	1653
<i>parC</i>	GCGTTATTACTCCAGGCCAT	GTTATAGACACCACCATCACTG	2136
<i>Tet</i>	GAACGTATCCTAATGTGTG	GATACTCTAACCGAATCTCG	377
<i>16S</i>	CTGAGAGTTTGATCCTGGCT	CCGGTGCTATCCTTGACAT	1450

PCR: Polymerase chain reaction; bp: Base pairs

23S rRNA domain V in different groups is shown in Figure 1B. The detection rate of the mutant *M. pneumoniae* strains was 69.14% (112/162) in male patients and 70.30% (116/165) in female patients, with no significant difference between the sexes ($p = 0.82$). School-age children (7–17 years old) had the highest detection rate of *M. pneumoniae* mutant strains (77.78%, 26/91), followed by preschool children [3–6 years old] (71.07%, 113/159) and infants [<2 years old] (48.94%, 23/47). The detection rate of mutant *M. pneumoniae* strains was significantly higher in school-age and preschool children than in infants ($p < 0.05$). The majority of mutant strains were

found in pneumonia samples (77.27%, 170/220), followed by URTI samples (59.57%, 28/47) and bronchitis samples (50.00%, 30/60). The percentage of mutant *M. pneumoniae* strains was significantly higher in pneumonia and bronchitis samples than in URTI samples (pneumonia vs. URTI, $p < 0.001$ and bronchitis vs. URTI, $p = 0.012$).

M. pneumoniae detection rate in different months

We analyzed the distribution of *M. pneumoniae* infection during all 12 months in 2014 (Figure 2) and found that the sample size varied from month to month, ranging from

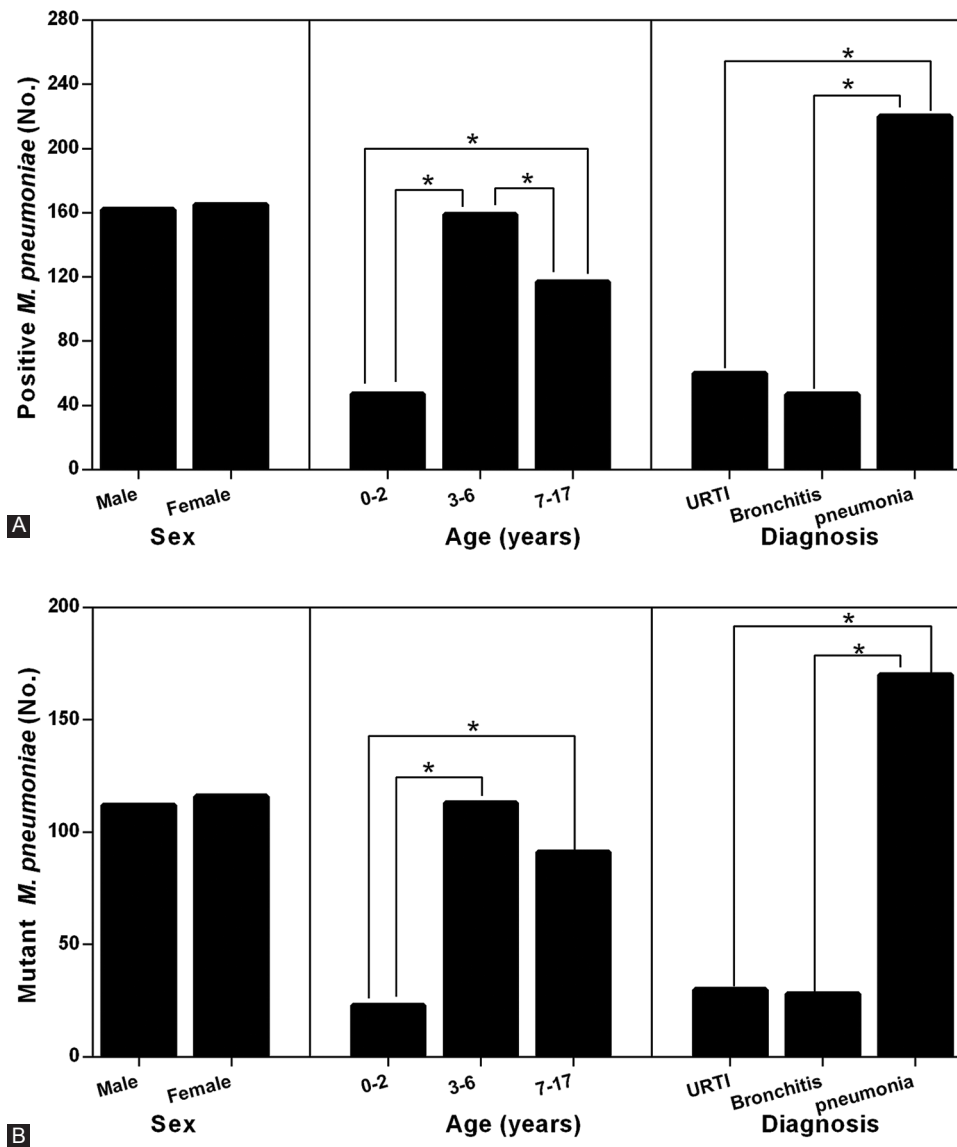


FIGURE 1. Distribution of *Mycoplasma pneumoniae* (*M. pneumoniae*) in children with RTIs. A) Distribution of *M. pneumoniae*-positive samples in children. There was no statistically significant difference in the *M. pneumoniae* positivity rates between boys and girls ($p = 0.06$). School-age children had the highest *M. pneumoniae* positivity rate, followed by preschool children and infants, with a significant difference ($p < 0.001$). Patients with pneumonia had the highest *M. pneumoniae* positivity rate, followed by those with URTI and bronchitis, with a significant difference ($p < 0.001$). B) Distribution of *M. pneumoniae* samples with mutations in domain V of 23S rRNA genes in children. No significant difference was found in the detection rate of mutant *M. pneumoniae* strains between boys and girls ($p = 0.82$). School-age children (7–17 years old) had the highest detection rate of *M. pneumoniae* mutant strains, followed by preschool children (3–6 years old) and infants (<2 years old), with a significant difference ($p < 0.001$). The majority of mutant strains were found in pneumonia samples, followed by URTI samples and bronchitis samples, with a significant difference ($p < 0.001$). * $p < 0.05$. RTI: Respiratory tract infection; URTI: Upper RTI.

2 to 181 positive samples. However, it should be noted that a smaller number of samples were collected during February and March, due to the Chinese Spring Festival. During the 12-month period, the *M. pneumoniae* positivity rate ranged from 22% (31/141) to 100% (2/2) and the resistance rate ranged from 38% (5/13) to 100% (3/3).

In terms of the quarterly distribution, the *M. pneumoniae* positivity rate was relatively higher in the first (63.64%) and second quarter (65.31%) of the year, followed by the third (56.33%) and fourth quarter (30.12%). Additionally, the resistance rate was the highest in the first quarter (85.71%), followed by the fourth quarter (72.79%).

Antibiotic resistance of *M. pneumoniae* isolates and related gene mutations

Out of 99 *M. pneumoniae* cultured isolates, MIC measurement was successfully performed in 65 *M. pneumoniae* isolates. As shown in Table 2, among these 65 *M. pneumoniae* isolates, 57 (87.69%) strains with A2063G mutation in domain V of the 23S rRNA gene were resistant to macrolides, and the MIC₉₀ values were 1024 µg/ml (erythromycin), 128 µg/ml (azithromycin), and 8 µg/ml (josamycin). The other eight wild-type *M. pneumoniae* isolates were macrolide-sensitive strains

with MIC ≤ 0.5 µg/ml. With regard to tetracyclines, all *M. pneumoniae* isolates were sensitive with MIC ≤ 2.0 µg/ml. Besides, all *M. pneumoniae* isolates were sensitive to levofloxacin with MIC 0.25 to 1.0 µg/ml.

One macrolide-resistant strain harbored a missense mutation (K27N) in L4 ribosomal protein. Eight macrolide-sensitive strains harbored the mutation M144V in L4 ribosomal protein and S170P in L22, which was consistent with the reference strain FH (Table 3). No mutation was found in the tetracycline-resistance genes (*tet* and 16S rRNA). The mutations in the quinolone-resistance genes are summarized in Table 4. For all 65 *M. pneumoniae*-cultured isolates, no mutation was found in the *gyrB* gene. Two strains had D149G, A533S, and C705 mutations in the *gyrA* gene. One strain had R454C and G652R mutations in the *parC* gene. Another six strains had a co-mutation in the *gyrA* and *parC* genes.

M. pneumoniae genotyping and susceptibility

M. pneumoniae P1 type I and P1 type II strains were found in 87.69% (57/65) and 12.31% (8/65) of cultured isolates, respectively. One of the P1 type I strains was sensitive to macrolides and the remaining 56 (98.25%) strains were resistant to macrolides. One P1 type II strain was resistant to macrolides and

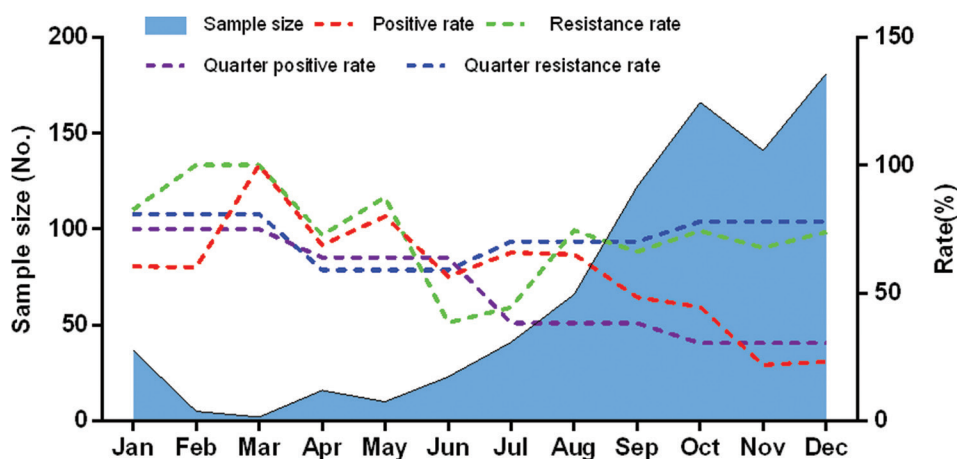


FIGURE 2. Distribution of *Mycoplasma pneumoniae* infections in children with respiratory tract infections (RTIs) over time, from January 2014 to December 2014. The sample size varied from month to month, ranging from 2 to 181 positive samples. During the 12-month period, the *M. pneumoniae* positivity rate ranged from 22% (31/141) to 100% (2/2) and the resistance rate ranged from 38% (5/13) to 100% (3/3). In terms of the quarterly distribution, the *M. pneumoniae* positivity rate was relatively higher in the first (63.64%) and second quarter (65.31%) of the year, followed by the third (56.33%) and fourth quarter (30.12%). Resistance rate was the highest in the first quarter (85.71%), followed by the fourth quarter (72.79%).

TABLE 2. Antimicrobial susceptibility of *Mycoplasma pneumoniae* strains to antimicrobial agents (MIC range/MIC₉₀, µg/ml)

Antibiotic	Wild-type strains (n=8)		A2063G mutated strains (n=57)		M129/FH *MIC (µg/ml)
	MIC range (µg/ml)	MIC ₉₀ (µg/ml)	MIC range (µg/ml)	MIC ₉₀ (µg/ml)	
Erythromycin	0.008–0.032	0.016	128–>1024	1024	0.032
Azithromycin	≤0.002–0.004	≤0.002	16–256	128	0.008
Josamycin	0.008–0.125	0.063	2–16	8	0.063
Tetracycline	0.063–0.25	0.25	0.063–0.25	0.25	0.125
Minocycline	0.125–0.5	0.25	0.032–0.25	0.25	0.125
Levofloxacin	0.25–1	0.5	0.25–1	0.5	0.5

*Reference strain. MIC: Minimum inhibitory concentration

TABLE 3. Mutations in macrolide-resistance genes and susceptibility of *M. pneumoniae* isolates to macrolide antibiotics

Sample number	L4		L22	MIC (µg/ml)		
	27	144	170	Erythromycin	Azithromycin	Josamycin
M129	K	M	S	0.032	0.008	0.063
FH	-	M-V	S-P	0.032	0.008	0.063
MHM041	K-N	-	-	0.032	≤0.002	0.016
MHM117	-	M-V	S-P	0.008	≤0.002	0.008
MHM144	-	M-V	S-P	0.016	≤0.002	0.008
DFB118	-	M-V	S-P	0.016	≤0.002	0.008
XY15	-	M-V	S-P	0.008	≤0.002	0.063
XY20	-	M-V	S-P	0.008	≤0.002	0.032
XY23	-	M-V	S-P	0.008	≤0.002	0.032
MHM032	-	M-V	S-P	0.016	0.004	0.125
MHM049	-	M-V	S-P	256	16	4

MIC: Minimum inhibitory concentration; K: Lysine; M: Methionine; S: Serine; V: Valine; P: Proline; N: Asparagine

TABLE 4. Mutations in quinolone-resistance genes and susceptibility of *M. pneumoniae* isolates to quinolone antibiotics

Sample number	gyrA			parC		MIC (µg/ml)
	149	533	705	454	652	Levofloxacin
M129	D	A	C	R	G	0.5
FH	-	-	-	-	-	0.5
MHM117	D-G	A-S	C-G	-	-	0.5
MHM144	D-G	A-S	C-G	-	-	0.5
DFB118	D-G	A-S	C-G	R-C	G-R	0.5
XY15	D-G	A-S	C-G	R-C	G-R	0.5
XY20	D-G	A-S	C-G	R-C	G-R	0.5
XY23	D-G	A-S	C-G	R-C	G-R	0.25
MHM032	D-G	A-S	-	R-C	G-R	0.5
MHM049	D-G	A-S	C-G	R-C	G-R	0.5
MHM170	-	-	-	R-C	G-R	0.5

MIC: Minimum inhibitory concentration; D: Aspartic acid; A: Alanine; C: Cysteine; R: Arginine; G: Glycine; S: Serine

the remaining seven were sensitive to macrolides. The MIC values of various antibiotics for different *M. pneumoniae* P1 type strains are shown in Table 5. The results indicated that the MIC of macrolides for P1 type I strains was significantly higher than for P1 type II strains ($p < 0.001$) and no significant difference was detected in the MIC of tetracyclines (tetracycline, $p = 0.06$; minocycline, $p = 0.43$) and levofloxacin ($p = 0.11$) between the two subtypes.

DISCUSSION

M. pneumoniae causes upper and lower RTIs in children and adults. Although the symptoms are mild and self-limited in the majority of patients, or the infections are asymptomatic, approximately 25% of patients will be hospitalized due to extrapulmonary complications or severe pneumonia [30]. It is generally known that the incidence and prevalence of *M. pneumoniae* vary in different periods and regions. Thus, the surveillance of *M. pneumoniae* infection is particularly important

in the monitoring and prevention of acquired pneumonia. Here, we conducted for the first time a multicenter study of *M. pneumoniae* infection in Beijing, China. Overall, 822 tracheal swab specimens were collected from pediatric patients with RTI symptoms in 11 hospitals in Beijing. Out of 822 samples, 341 (41.48%) were positive for *M. pneumoniae* by PCR. Previous study showed that the positivity rate of *M. pneumoniae* ranged from 19.13% to 29.07% in Beijing [5,25,31]. Thus, we may conclude that *M. pneumoniae* infection in Beijing has a gradual upward trend. This may be due to the rise of drug-resistant strains caused by the overuse of antibiotics in recent years.

Excessive or inappropriate use of antibiotics may lead to a selective pressure for the development of antibiotic resistance [32]. This greatly increases the number of resistant strains and, consequently, the severity of clinical symptoms, making the treatment more difficult. The prevalence of *M. pneumoniae* infections has obvious regional differences. Ishiguro et al. reported that the macrolide resistance in different cities in Hokkaido significantly varied, from 0.0% to 100% [33]. Therefore, enhanced surveillance of drug-resistant bacteria across a country is essential for the prevention of *M. pneumoniae* infection. In *M. pneumoniae* infections, macrolide resistance has become a potential threat worldwide, especially in China. According to a national survey on antibiotic usage, the rate of macrolide use in China has been around 70% [34] and the rate of macrolide sales reached 7.9 billion in 2014. In such circumstances, the high prevalence of *M. pneumoniae* macrolide-resistant strains observed in our study is not unexpected. Similarly, other studies showed that more than 90% of *M. pneumoniae* infections in China were caused by resistant strains [35-37]. Based on these data, we assume that the macrolide-resistant strains have developed in the southern or northern China. On the other hand, in Western countries, the proportion of resistant bacteria has been relatively low, ranging from 1% to 10% [10,16,32,38]. This phenomenon is mainly owing to the careful use of macrolides in these areas.

TABLE 5. *Mycoplasma pneumoniae* P1 type strains and resistance phenotype analysis (MIC range/MIC90, µg/ml)

Antibiotic	P1 type I (n=57)		P1 type II (n=8)		p	M129/FH*
	MIC range (µg/ml)	MIC90 (µg/ml)	MIC range (µg/ml)	MIC90 (µg/ml)		
Erythromycin	0.032->1024	1024	0.008->256	0.016	<0.001	0.032
Azithromycin	≤0.002-256	128	≤0.002-16	0.004	<0.001	0.008
Josamycin	0.016-16	8	0.008-4	0.125	<0.001	0.063
Tetracycline	0.063-0.25	0.25	0.063-0.25	0.25	0.06	0.125
Minocycline	0.063-0.5	0.25	0.125-0.25	0.25	0.43	0.125
Levofloxacin	0.25-1	0.5	0.25-0.5	0.5	0.11	0.5

*Reference strain. MIC: Minimum inhibitory concentration

For that reason, a systematic and strict surveillance program is necessary to control the use of macrolides in our country and to manage the threat of emerging resistance of *M. pneumoniae* to the first-line antibiotic therapy.

Fortunately, tetracyclines and quinolones remained effective against clinical *M. pneumoniae* isolates. However, although we did not find tetracycline- or quinolone-resistant strains in the current study, an increasing number of *Mycoplasma genitalium*, *Mycoplasma urealyticum*, and *Mycoplasma hominis* strains resistant to these antibiotics has been isolated [39]. The drug resistance is primarily acquired and induced by the external environment. Therefore, with the increasing use of tetracycline and quinolone substitution therapy, the risk of development of clinically resistant strains should not be ignored. Additionally, it is worth noting that all cultured positive *M. pneumoniae* samples in our study had A2063G mutation in domain V of the 23S rRNA gene, which has been recognized as the most prevalent and highly associated with macrolide resistance. Although these *M. pneumoniae* positive strains harbored the same type of mutation, the correlation with resistance is not yet clear and further investigation is warranted to explain this phenomenon. In *M. pneumoniae*, resistance caused by 23S rRNA gene mutation is the most common resistance mechanism, because *M. pneumoniae* harbors only one copy of rRNA operon [40]. According to previous studies, mutations in the 23S rRNA gene, such as A-to-G transition or A-to-C transversion at position 2063 or 2064, respectively, predominantly cause the macrolide resistance in *M. pneumoniae* [41]. Furthermore, mutations at position 2617 in domain V of the 23S rRNA gene are associated with lower resistance to macrolides than the mutations at position 2063 or 2064 [41].

The positivity rate of *M. pneumoniae* infection and detection rate of 23S rRNA domain V gene mutations differed between the quarters of 2014 year and between different age groups of patients in our study. The highest *M. pneumoniae* infection rate was found in the second quarter and among school-age children. Additionally, we found no significant gender-related differences in the *M. pneumoniae* positivity rate. These results are consistent with previous epidemiological research on *M. pneumoniae* in China [5,42]. According to the

age-based distribution, we speculate that the epidemiology of *M. pneumoniae* in Beijing conforms to the traditional epidemiological model of infectious disease diffusion. In this model, the patients are linked by the characteristics of the bacterial strain, indicating a common exposure or person-to-person transmission [32]. Usually, school-age children live in a relatively closed environment and are in close contact with a large number of people, leading to a rapid transmission and outbreak of pathogens.

In our study, 57 (87.69%) clinical isolates were classified as P1 type I strains and 8 (12.31%) as P1 type II strains. The vast majority of P1 type I strains were macrolide-resistant, while most of the P1 type II isolates were macrolide-sensitive strains. The regular distribution of P1 subtypes among macrolide-resistant and macrolide-sensitive *M. pneumoniae* strains suggests an association between P1 subtype and the macrolide susceptibility. This potential correlation should be further investigated in studies with larger sample sizes. In addition, a more thorough analysis of the *M. pneumoniae* genome with whole-genome sequencing might provide more evidence for the resistance mechanism.

We found A2063G mutation in domain V of the 23S rRNA gene in 57 (87.69%) *M. pneumoniae* isolates, which was associated with high MIC90 of macrolides. Mutations in domain V of the 23S rRNA gene are the main cause of macrolides resistance, especially those at positions 2063 and 2064. Many studies have revealed that macrolides could inhibit protein synthesis by binding to large ribosomal subunits, so mutations in 23S rRNA may lead to decreased affinity of the ribosome for drug [21]. While many reports have investigated the role of the mutation in domain V of 23S rRNA gene, no study has analyzed mutations in the ribosomal proteins L4 and L22 [36]. Although we found several *M. pneumoniae* isolates with mutations in L4 and L22, the relationship between L4 and L22 mutations and macrolide resistance is yet unclear. Additionally, no mutation was detected in the tetracycline and quinolone resistance-associated genes, suggesting that tetracyclines and quinolones remain effective against clinical *M. pneumoniae* isolates.

There are several limitations in this study. First, this study only collected data for one year. Although we compared

these data to the data from previous studies in Beijing, this may not be sufficient to observe changes in the prevalence of *M. pneumoniae* circulating types and epidemic trends over time. Second, due to the observational, non-randomized design of the study, we collected data in a retrospective manner, meaning that the information of some pediatric patients was unavailable and the quality of the data was dependent on the accuracy of the medical record.

CONCLUSION

We investigated *M. pneumoniae* infections in 11 hospitals in Beijing and found that school-age children were more susceptible to this disease, particularly pediatric patients with pneumonia. We also found that *M. pneumoniae* P1 type I may be the main cause of the epidemic in Beijing. The rates of macrolide resistance observed in this study were more than 85%. Fortunately, tetracyclines and quinolones remain effective against *M. pneumoniae* isolates in Beijing. In order to fully understand the biology and epidemiology of *M. pneumoniae*, the establishment of a systematic surveillance program is critical.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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