Antimicrobial Susceptibility Profiles and Genetic Characteristics of *Mycoplasma pneumoniae* in Shanghai, China, from 2017 to 19

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**Objective:** The current study investigated the recent genetic characteristics and antimicrobial susceptibility profiles of *Mycoplasma pneumoniae* (*M. pneumoniae*) in Shanghai, becoming a clinical reference for treating *M. pneumoniae* infection in Shanghai.

**Methods:** Clinical strains were isolated from nasopharyngeal aspirates of the pediatric patients in Shanghai from 2017 to 2019. Nine antimicrobial agents of three antimicrobial classes macrolides, fluoroquinolones and tetracyclines, against *M. pneumoniae* isolates were investigated using the broth microdilution method. The mechanism of macrolide resistance was analyzed by evaluating the sequences of the 23S rRNA gene and the ribosomal protein genes L4 and L22. Molecular genotyping was undergone to classify the P1 subtypes and the multi-locus variable-number tandem-repeat analysis (MLVA) types.

**Results:** A total of 72 isolates were resistant to macrolides (MICs > 64 mg/L for erythromycin) based on the A2063G mutation in the 23S rRNA gene. These strains were susceptible to tetracyclines and fluoroquinolones. P1 type 1 (166/182, 91.2%) and MLVA type 4-5-7-2 (165/182, 90.7%) were the dominant subtypes. MLVA type was associated with the P1 subtypes. The distribution of the P1 subtypes and MLVA types did not change over time. The macrolide-resistant rate in P1 type 2 and MLVA type 3-5-6-2 strains were increased during the three-year study. The 5-loci MLVA typing scheme revealed the clonal expansion of MLVA type 3-4-5-7-2 strains which are macrolide-resistant in 2019.

**Conclusion:** Macrolide resistance in *M. pneumoniae* in Shanghai is very high and is evolving among certain subtypes. Cautions should be taken for the possible clonal spreading of macrolide-resistant genotypes within this populated region.

**Keywords:** *Mycoplasma pneumoniae*, molecular genotyping, macrolide resistance, resistant mechanism

**Introduction**

*Mycoplasmas* are small self-replicating organisms without a cell wall. More than 200 *Mycoplasma* species are found in plants, animals, arthropods and humans.1 Several of the *Mycoplasmas* are related to human infections, in which *M. pneumoniae* is one of the most investigated species. *M. pneumoniae* causes upper and lower respiratory tract infections among adults and children.2,3 It is responsible for about 10–50% of pediatric community-acquired pneumonia (CAP) with a prevalence of approximately 70% in closed populations.1,2 *M. pneumoniae* also causes many extrapulmonary diseases, such as encephalitis, dermatological disorders, and septic arthritis. In addition, some researchers also observed that *M. pneumoniae* could present asymptotically within the upper respiratory tract of children.4,5 Therefore, the positive result of serology or quantitative polymerase chain reaction (PCR) or culture could not differentiate the asymptomatic carriage from any infection.5
Molecular characteristics help in monitoring the epidemiology of *M. pneumoniae* infections. In this regard, several genotyping methods have been developed. Among them, P1 typing is one of the most common methods. The MLVA typing has a higher discriminability than the P1 subtyping method. This method was amended to a 4-loci scheme and standardized using multiple laboratories. The 4-loci system has been used extensively in general epidemiological research worldwide. In contrast, the 5-loci scheme contains the highly variable loci Mpn1, which is useful in studies involving strain tracking.

Macrolides are the first-line antimicrobials for treating *M. pneumoniae* infections. However, macrolide resistance has emerged since the early 1990s and is increasing globally. The highest resistance rate was over 90% in some Asian regions, including our reports Shanghai, China, 10 years ago. Recently, based on the information from China, Japan and South Korea, the macrolide resistance rate decreased, corresponding to the genotype shift within this area. However, only sparse data are available for the recent *M. pneumoniae* status in Shanghai. As bacteriostatic agents, macrolides block the protein synthesis of *M. pneumoniae* by binding to specific nucleotides in domains II and/or V of 23S rRNA within the 50S bacterial ribosomal subunit. Research demonstrated that point mutations in the peptidyl transferase loop of 23S rRNA of *M. pneumoniae*, including C2617G, A2063G/C/T, and A2064G/C, could naturally cause macrolide resistance. Other studies also reported that insertions or deletions within the ribosomal proteins L4 and L22 were associated with macrolide resistance in *M. pneumoniae*.

In this study, the antimicrobial susceptibility profiles of 182 *M. pneumoniae* clinical isolates were evaluated between 2017 and 2019 in Shanghai, and their resistance mechanisms were also identified. Finally, we analyzed their molecular typing depending on P1 subtyping and MLVA genotyping.

**Materials and Methods**

**Clinical *M. pneumoniae* Strains**

*M. pneumoniae* clinical strains from November 2017 to August 2019 were secured from the strain bank of the Institute of Antibiotics, Huashan Hospital, Shanghai. All the samples were previously isolated during routine clinical testing previously, and then stored in the strain bank. The study was authorized and approved by the ethics committee of Huashan Hospital, and written informed consent was not necessary. This study did not harm the rights, benefits and health of the subjects. Moreover, the privacy and personal identity information of the subjects remained protected.

The culture of *M. pneumoniae* was carried out as described previously. *M. pneumoniae* strain M129 (ATCC 29342) became the quality control for identification. All the isolates were identified through colony morphologies and ascertained by sequencing the P1 gene. PCR was performed through the primers (5' - GCCACCCTCGGGGGCAGTCAG −3' and 5'- GAGTCGGGATTCCCCGCGGAGG −3') amplifying a 209bp fragment of the P1 gene. Sequencing was undergone by Shanghai MAP Biotech CO., Ltd and analyzed using the Basic local alignment search tool (BLAST) by comparing with the reference strain M129.

**Antimicrobial Susceptibility Test (AST)**

The minimum inhibitory concentration (MIC) broth microdilution method determined the susceptibility of the isolates to the antimicrobials through the standard procedures of CLSI. Three classes antimicrobials were included in the test. The macrolides were erythromycin, roxithromycin, azithromycin, and josamycin. The tetracyclines included tetracycline, minocycline, and doxycycline. In addition, the fluoroquinolones included levofloxacin and moxifloxacin. *M. pneumoniae* reference strain M129 (ATCC 29342) become the control. Isolates with erythromycin MIC ≥ 1 mg/L were regarded as macrolide resistant.

**Sequencing of 23S rRNA Gene and L4 and L22 Ribosomal Protein Genes**

The 23S rRNA gene point mutations were detected by nested PCR using a previous method. The L4 and L22 ribosomal protein genes were amplified through primer pairs MPL4 - 1/ MPL4 - 2 (5 ' - GAACCATGAAAATGCCC- 3 ' and 5 ' - TTTGTCAAGAGCTTGCCAC - 3 ') and MPL22 – 1/MPL22 - 2 (5 ' – CCGTGGAATCTCCACCC – 3 ' and 5 ' – CTGCTTTTGACGTGCCATC – 3 '). All the amplicons were sequenced and analyzed.
P1 Genotyping (P1 Sequencing as an Alternative Method)
P1 subtyping was undergone through the PCR restriction fragment length polymorphism (PCR-RFLP) method and P1 sequencing was the alternative method. Briefly, the P1 gene was amplified in two fragments using two primer sets: ADH1/ADH2 and ADH3/ADH4. They were subjected to restriction endonuclease digestion with HaeIII (Takara Bio Inc., Kyoto, Japan). Isolates were classified into two P1 subtypes compared with P1-1 reference strain M129 and P1-2 reference strain FH (ATCC 15531).

MLVA Genotyping
MLVA typing was performed through primers amplifying five VNTR loci (Mpn1, Mpn13, Mpn14, Mpn15, and Mpn16). The PCR products were sequenced and analyzed. The MLVA types were assigned to each strain in both the 5-loci scheme and the 4-loci scheme. MLVA typing data were uploaded within the BioNumerics software 7.6 (Applied Maths, Austin, TX) and clustered through an unweighted pair group method with arithmetic mean (UPGMA) algorithm. A cutoff value of 80% similarity defined the MLVA clusters. Minimum spanning trees (MST) were generated through the standard MST with single and double loci variants on priority.

Statistical Analysis
SPSS 26 (IBM Corp., Armonk, NY) was used to perform the Chi-square or Fisher’s exact test to analyze the correlation between the P1 subtype and the MLVA type and their relationships with collection year and the macrolide susceptibility status. A p-value of <0.05 was considered statistically significant, except for the Bonferroni adjustment.

Results
M. pneumoniae Culture and Antimicrobial Susceptibility Test
One hundred eighty-two clinical M. pneumoniae isolates were successfully obtained from November 2017 to August 2019. Among them, seven were isolated in 2017, 108 in 2018, and 67 in 2019. The morphological features of all clinical isolates showed typical spherical colonies under the stereomicroscope. PCR and sequencing confirmed that all isolates were M. pneumoniae.

The antimicrobial susceptibility of 110 isolates obtained between 2017 and 2018 was reported in our previous study. For the remaining 72 isolates, 100% (72/72) were resistant to erythromycin (MIC ≥ 64mg/L). The MIC summary (including MIC50 values, MIC90 values, MIC ranges, percentages of resistance and susceptibility) and the cumulative bacteriostatic rates of the agents for these isolates are depicted in Tables 1S and 2S. The summary data of all the isolates are shown in Tables 1 and 2. Of all strains, 97.3% (177/ 182) were resistant to erythromycin (MIC ≥ 64mg/L). Only five (2.7%) isolates were susceptible to macrolides with the MIC ≤ 0.125 mg/L for all the macrolides. Although not

| Antimicrobials | MIC (mg/L) | Interpretive Criteria* |
|----------------|------------|------------------------|
|                | MIC Range  | MIC50 | MIC90 | S, % | R, % | S | R |
| Moxifloxacin   | 0.015–0.25 | 0.125 | 0.25  | 100  | 0   | ≤0.25 | – |
| Levofloxacin   | 0.03–1     | 0.5   | 1     | 100  | 0   | ≤1   | – |
| Tetracycline   | 0.06–2     | 0.5   | 1     | 100  | 0   | ≤2   | – |
| Minocycline    | 0.03–4     | 0.5   | 2     | –    | –   | –    | – |
| Doxycycline    | 0.015–1    | 0.25  | 0.5   | 100  | 0   | ≤2   | – |
| Erythromycin   | ≤0.06–>128 | >128  | >128  | 0    | 100 | ≤0.5 | ≥1 |
| Roxithromycin  | ≤0.06–>128 | 128   | >128  | -    | -   | -    | - |
| Azithromycin   | ≤0.06–64   | 16    | 32    | 0    | 100 | ≤0.5 | ≥1 |
| Josamycin      | ≤0.06–32   | 4     | 4     | -    | -   | -    | - |

Notes: *Interpretive criteria from the CLSI M43-A. Abbreviations: S, susceptible; R, resistant.
statistically significant ($p = 0.055$), the macrolide resistance rate has been increasing in the recent three years, from 85.7% (6/7) in 2017 to 100% (67/67) in 2019 (Figure 1). All the 182 clinical isolates were susceptible to tetracycline (MIC range: 0.06–2 mg/L) and doxycycline (MIC range: 0.015–1 mg/L), and fluoroquinolones (MIC range: ≤ 0.06–1 mg/L). The MIC range of minocycline was 0.03–4 mg/L, slightly higher than tetracycline and doxycycline. In addition, moxifloxacin (MIC range: 0.015–0.25 mg/L; MIC50, 0.125mg/L; MIC90, 0.25mg/L) was more active than levofloxacin (MIC range, 0.03–1 mg/L; MIC50, 0.5mg/L; MIC90, 1mg/L).

Mutations Associated with Macrolide Resistance in *M. pneumoniae* Isolates

Mutation A2063G (*E. coli* numbering 2058) in the domain V of the 23S rRNA gene was detected within all the 72 macrolide resistance isolates of 2019. No mutation was identified in the ribosomal protein genes L4 and L22.

**P1 Genotypes**

A total of 91.2% (166/182) of all the isolates were classified as P1-1, and the other 8.8% (16/182) were P1-2 (Table 3). The macrolide resistance rate was 99.4% (165/166) in P1-1 isolates, which was significantly higher than in the P1-2 isolates (75.0%, 12/16; $p < 0.001$). Over the three years, the P1 subtype distribution was without significant changes ($p = 0.624$, Figure 1). Macrolide resistance rate in P1-1 isolates was stable ($p = 0.699$). In contrast, in P1-2 isolates, the rate rapidly

| Antimicrobials   | MIC value (mg/L) | ≤0.06 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | >128 |
|------------------|------------------|-------|-------|------|-----|---|---|---|---|----|----|----|-----|------|
| Moxifloxacin     |                  | 2.2   | 84.2  | 100.0| 29.7| 89.6| 99.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0|
| Levofloxacin     |                  | 0.6   | 6.0   | 57.4 | 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0|
| Tetracycline     |                  | 1.7   | 6.6   | 29.7 | 89.6 | 99.5| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0|
| Minocycline      |                  | 2.2   | 16.5  | 45.6 | 81.3 | 89.0| 97.3| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0|
| Doxycycline      |                  | 7.7   | 36.8  | 69.8 | 95.1 | 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0|
| Erythromycin     |                  | 2.7   | 3.3   | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  |
| Roxithromycin    |                  | 2.7   | 3.3   | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  |
| Azithromycin     |                  | 2.7   | 3.3   | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  | 3.3  |
| Josamycin        |                  | 2.2   | 2.7   | 2.7  | 2.7  | 2.7  | 2.7  | 2.7  | 2.7  | 2.7  | 2.7  | 2.7  | 2.7  | 2.7  |

Figure 1 Comparison of *M. pneumoniae* P1 subtypes in Shanghai between 2017 and 2019. (A) Distribution of the P1-1 and P1-2 subtypes ($p = 0.693$). (B) Macrolide resistance rate in P1-1 ($p = 0.347$) and P1-2 ($p = 0.054$).
increased from 0% (0/1) in 2017 to 72.7% (8/11) in 2018, and 100% (4/4) in 2019, without any statistical significance ($p = 0.113$) (Figure 1).

**MLVA Genotyping of *M. pneumoniae***

Five MLVA types were identified through the 4-loci typing scheme (Table 3). The dominant type was 4-5-7-2 (90.7%, 165/182), followed by 3-5-6-2 (7.7%, 14/182). The other three MLVA types were singletons: 3-6-6-2, 4-4-7-2 and 4-5-6-2. The 5-loci typing scheme was adopted to differentiate the isolates to investigate whether the clonal spreading of MLVA type 4-5-7-2 existed. Thirteen MLVA types, represented by 43 to 1 specimens, were identified within the 182 isolates through the 5-loci typing scheme (Table 3). No dominant types were observed. However, there was a relative even distribution of the several major types: 5-4-5-7-2 (23.6%, 43/182), followed by type 3-4-5-7-2 (19.8%, 36/182), 2-4-5-7-2 (17.0%, 31/182), 4-4-5-7-2 (15.9%, 29/182), and 6-4-5-7-2 (12.1%, 22/182). The rest of the types were lower than 10%. The 182 isolates were clustered into two major lineages based on the 5-loci scheme (Figure 2A). Lineage 1 contained types X-4-5-7-2 and 5-4-5-7-2, while lineage 2 included X-3-5-6-2, 4-3-6-6-2 and 4-4-5-6-2. Two MLVA clusters (MC1 and MC2) containing 165 and 14 isolates and three singletons were identified based on an arbitrary cutoff value of 80% genetic similarity (Figure S1). MC1 corresponded to MLVA types X-4-5-7-2, and MC2 corresponded to MLVA types X-3-5-6-2. The three singletons were types, 4-3-6-6-2, 4-4-5-6-2 and 5-4-5-7-2. The distribution of the 4-loci scheme MLVA types was stable over the three years ($p = 0.940$), while the 5-loci scheme types altered significantly ($p = 0.035$, Figure 2A). When each locus was analyzed separately, the change was associated with the variation in locus.

### Table 3 Summary of the Genotypes of 182 *M. pneumoniae* Isolates in Shanghai from 2017 to 2019

| Genotypes          | Year, n (%) | Macrolide Susceptibility, n (%) | Total, n (%) |
|--------------------|-------------|---------------------------------|--------------|
|                    | 2017        | 2018                           | 2019         |
|                    | Susceptible | Resistant                      |              |
| P1 subtype         |             |                                 |              |
| P1-1               | 6 (85.7)    | 97 (89.8)                      | 63 (94.0)    |
| P1-2               | 1 (14.3)    | 11 (10.2)                      | 4 (6.0)      |
| MLVA Type          |             |                                 |              |
| 3-5-6-2 (n=14)     | 1 (14.3)    | 1 (0.9)                        | 2 (40.0)     |
| 4-3-5-6-2          | 0 (0)       | 5 (4.6)                        | 3 (4.5)      |
| 5-3-5-6-2          | 0 (0)       | 2 (1.9)                        | 1 (1.5)      |
| 6-3-5-6-2          | 0 (0)       | 1 (0.9)                        | 0 (0)        |
| 3-6-6-2 (n=1)      | 0 (0)       | 1 (0.9)                        | 1 (20.0)     |
| 4-5-6-2            | 0 (0)       | 1 (0.9)                        | 0 (0)        |
| 4-4-7-2 (n=1)      | 0 (0)       | 1 (0.9)                        | 0 (0)        |
| 4-5-7-2 (n=167)    | 1 (14.3)    | 19 (17.6)                      | 11 (16.4)    |
| 3-4-5-7-2          | 2 (28.6)    | 12 (11.1)                      | 22 (32.8)    |
| 4-4-5-7-2          | 0 (0)       | 24 (22.2)                      | 5 (7.5)      |
| 5-4-5-7-2          | 1 (14.3)    | 27 (25.0)                      | 15 (22.4)    |
| 6-4-5-7-2          | 2 (28.6)    | 10 (9.3)                       | 10 (14.9)    |
| 7-4-5-7-2          | 0 (0)       | 4 (3.7)                        | 0 (0)        |
| Macrolide          |             |                                 |              |
| susceptibility      |             |                                 |              |
| Susceptible        | 1 (14.3)    | 4 (3.7)                        | 0 (0)        |
| Resistant          | 6 (85.7)    | 104 (96.3)                     | 67 (100)     |
| Total, n (%)       | 7 (3.8)     | 108 (59.3)                     | 67 (36.8)    |

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Mpn1 ($p = 0.01$) but not the others. MLVA type 3-4-5-7-2 was significantly elevated in 2019 (32.8%, 22/67), indicating a possible clonal expansion of the isolate types in 2019. There was a significant association between macrolide resistance and MLVA types of both the schemes ($p < 0.001$). The most common MLVA type among the macrolide-susceptible M. pneumoniae isolates was 3-5-6-2 (60%, 3/5), whereas the 4-5-7-2 was most common among macrolide-resistant isolates (92.7%, 164/177).

**Correlation Between MLVA Types and P1 Subtypes**
MLVA types of both the schemes were significantly correlated with P1 subtypes ($p < 0.001$) (Table 4). P1-1 had two MLVA types, MLVA 4-5-7-2 and 4-4-7-2, while P1-2 had three: MLVA types 3-5-6-2, 3-6-6-2, and 4-5-6-2. The VNTR loci Mpn1, Mpn13, and Mpn15 were significantly related to the P1 subtype differentiation ($p < 0.01$). When looking at the MLVA lineages, all the isolates in the MLVA lineage 1 were P1-1 and MLVA lineage 2 isolates were P1-2 (Figure 2B and Figure S1).

**Discussion**
The current study reported the latest epidemiological status of M. pneumoniae in Shanghai, China. The data indicated that the macrolide resistance rate was increasing in Shanghai. P1-1 was still predominant, and the primary MLVA types was 4-5-7-2. Macrolide resistance was evolving among specific subtypes.

The study revealed that the average macrolide resistance rate within the recent three years in Shanghai was 97.3%. Shanghai is still the city with the highest resistance rate than other regions in China and other countries that show decreasing macrolide resistance. This difficult resistant situation in Shanghai could be related to the antimicrobial selection pressure. The selection was correlated with antimicrobial usage as observed in the US. No data indicated whether the macrolides were more intensively used in Shanghai than in other regions in China. On the other hand, a significantly increased proportion of MLVA type 3-4-5-7-2 was identified in 2019 and all strains with this subtype were
Macrolide-resistant. Thus, the clonal spread of macrolide-resistant strains could contribute to the high macrolide-resistant rate in Shanghai.

Macrolide resistance in *M. pneumoniae* is strongly associated with mutations in the 23S rRNA gene and mutations in L4 and L22 ribosomal proteins.\(^2\) The mutations in the 23S rRNA gene include A2063G/C/T, A2064G/C, and C2617G, (corresponding to 2058, 2059, and 2611 in the *E. coli* numbering system).\(^2\) In this study, only A2063G mutation was identified within the macrolide-resistant isolates. No mutations were found in the L4 and L22 ribosomal protein genes. This is a consistent trend based on our previous findings that A2063G was the predominant mutation associated with macrolide resistance 10 years ago in Shanghai,\(^13,14\) and agrees with observations from other regions worldwide.\(^2\)

We found that fluoroquinolones and tetracyclines showed significant activities against *M. pneumoniae*. Interestingly, five isolates had a MIC of 4 mg/L for minocycline. CLSI does not have a standard for minocycline yet. Since minocycline is an older drug with less potency, and these isolates could be classified as susceptible to tetracycline. Based on the high macrolide resistance and teeth damaged by tetracyclines, new guidelines recommending respiratory fluoroquinolone/ tosufloxacin as a second-line drug for *M. pneumoniae* infections were documented in Japan, which has helped reducing the prevalence of macrolide-resistant strains.\(^36\) A similar treatment strategy could help control the current severe situation of macrolide-resistant *M. pneumoniae* in Shanghai be helpful. In addition, a continued comprehensive surveillance program involving more local hospitals and with more diverse patient populations is necessary to monitor *M. pneumoniae* macrolide resistance in Shanghai.

This study showed that P1-1 was predominant, the same as we observed 10 years ago.\(^14\) Several studies reported that there was a regional difference in P1 subtype distribution in China and a transition trend from P1-1 to P1-2 was observed recently in some regions.\(^15,17,37\) However, data from previous studies and other reports suggested that P1-1 strains were constantly dominant in Shanghai, lacking a trend of subtype shift.\(^15\) Further investigation needs of whether this stable strain dominance is due to the stable herd immunity in Shanghai. We also noticed a sharp increase in macrolide-resistant

### Table 4 Correlations Between the P1 Subtypes and the MLVA Types

| P1 Subtype | MLVA Type, 4-Loci | Number of Isolates, n (%) | MLVA Type, 5-Loci | Number of Isolates, n (%) |
|------------|------------------|--------------------------|------------------|--------------------------|
| P1-1 (n=171) | 3-5-6-2 | 3 (1.8%) | 2-3-5-6-2 | 1 (0.6%) |
| | 4-3-5-6-2 | 2 (1.2%) |
| | 3-6-6-2 | 1 (0.6%) | 4-3-6-6-2 | 1 (0.6%) |
| | 4-4-7-2 | 1 (0.6%) | 5-4-4-7-2 | 1 (0.6%) |
| | 4-5-7-2 | 166 (97.1\%)* | 2-4-5-7-2 | 31 (18.1%) |
| | 3-4-5-7-2 | 36 (21.1%) |
| | 4-4-5-7-2 | 29 (17.0%) |
| | 5-4-5-7-2 | 43 (25.1%) |
| | 6-4-5-7-2 | 23 (13.5%) |
| | 7-4-5-7-2 | 4 (2.3%) |
| P1-2 (n=12) | 3-5-6-2 | 11 (91.7\%)* | 2-3-5-6-2 | 1 (8.3%) |
| | 4-3-5-6-2 | 6 (50.0\%)* |
| | 5-3-5-6-2 | 3 (25.0\%)* |
| | 6-3-5-6-2 | 1 (8.3\%)* |
| | 4-5-7-2 | 1 (8.3%) |
| | 4-4-5-7-2 | 1 (8.3%) |

**Notes:** Chi-Square tests for the P1 subtypes vs the MLVA type 4-loci and 5 loci, both overall p < 0.001. *Significant after Bonferroni correction.
rate in P1-2 isolates during the 3-year study period under a stable distribution of the P1 subtypes. This observation indicated that P1-2 strains were adapted to the antimicrobial exposure and developed resistance. Clonal expansion of the resistant P1-2 strains was unlikely to happen based on the 5-loci scheme MLVA typing data.

In this study, 13 distinct 5-loci scheme MLVA types were identified. This number was slightly lower than that of our previous study (17 types) from 2005 to 2009.38 We observed that the distribution of the 4-loci MLVA types was stable in Shanghai between 2017 and 2019, continuing the trend from 2016.15 There were only five MLVA types by this scheme. Three of them were singletons, and a suspicion of possible clonal expansion in Shanghai was raised. When the highly variable Mpn1 locus was included in the analysis, the two major types, 4-5-7-2 and 3-5-6-2, were divided into several subtypes, and a pattern of no evident dominance was observed. We then noticed an association of the 5-loci MLVA types with collection year, and type 3-4-5-7-2 was significantly more in 2019. This finding suggests that clonal expansion of this type of strains was possible in 2019 in Shanghai. Unfortunately, we did not obtain demographic information, which can provide references for this conclusion in this study.

We found that MLVA types in 4-loci and 5-loci schemes were correlated with the P1 subtypes. The 5-loci MLVA types were clustered within two lineages, corresponding to the two P1 subtypes. P1-1 was associated with MLVA 4-5-7-2, while P1-2 was related to MLVA 3-5-6-2. These findings were similar to reports from other regions worldwide.9,19,20,39,40 There was one MLVA 3-6-6-2 isolate classified as P1-1, which was also reported by other studies.17,41–44

Macrolide resistance in the strains having different MLVA types was different and evolving in this study. In the MLVA type 4-5-7-2 strains, 99.4% (164/165) were resistant, in concordance with some previous studies. In Beijing and other cities in China, the rate of resistance occurring in the type 4-5-7-2 was also over 90%.17 Among the 14 MLVA type 3-5-6-2 strains, macrolides resistance was rapidly increasing from 0% (0/1) in 2017 to 77.8% (7/9) in 2018, and to 100% (4/4) in 2019, contributing to a similar trend in P1-2 strains. Although there is an increasing trend of macrolide resistance in the MLVA type 3-5-6-2 strains in other regions of China, to our knowledge, such a rapid pace has not been observed.17 Therefore, continued monitoring of the development of macrolide resistance in different MLVA types should be conducted. Close attention should also be paid to the possible clonal spread of specific subtypes, especially the macrolide-resistant strains, within a local region having a high population density, such as Shanghai.

There are some limitations in this study. First, the age distributions and the clinical characteristics of the patients were not enrolled. Second, any differences among the strains collected from different patients were unknown. Third, the strains numbers in 2017 were small affecting the statistical outcomes.

Conclusion
The macrolide resistance rate of M. pneumoniae in Shanghai is still very high and has the mutation A2063G within domain V of the 23S rRNA in the recent three years, with an alarming increase in P1-2 strains. We also identified a significantly increased proportion of MLVA type 3-4-5-7-2 in 2019 and all the strains with this subtype were macrolide-resistant. MLVA types were significantly associated with P1 subtypes. Continued surveillance and updated treatment guidelines are urgently needed to reduce the high resistance. Moreover, new guidelines recommending other respiratory drugs as a second-line drugs for M. pneumoniae infections must be documented in Shanghai, which could help reduce the prevalence of macrolide-resistant strains.

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Disclosure
Na Wang, Hong Zhang and Yihua Yin share first authorship. The authors report no conflicts of interest in this work.

References
1. Atkinson TP, Balish MF, Waites KB. Epidemiology, clinical manifestations, pathogenesis and laboratory detection of Mycoplasma pneumoniae infections. *FEMS Microbiol Rev*. 2008;32(6):956–973. doi:10.1111/j.1574-6976.2008.00129.x
2. Waites KB, Xiao L, Liu Y, Balish MF, Atkinson TP. Mycoplasma pneumoniae from the respiratory tract and beyond. Clin Microbiol Rev. 2017;30(3):747–809. doi:10.1128/CMR.00114-16

3. Waites KB, Talkington DF. Mycoplasma pneumoniae and its role as a human pathogen. Clin Microbiol Rev. 2004;17(4):697–728. doi:10.1128/CMR.17.4.697-728.2004

4. Spuesens EB, Frauij PL, Visser EG, et al. Carriage of Mycoplasma pneumoniae in the upper respiratory tract of symptomatic and asymptomatic children: an observational study. PLoS Med. 2013;10(5):e1001444. doi:10.1371/journal.pmed.1001444

5. Meyer Sauteur PM, Unger WW, Nadal D, Berger C, Vianc C, van Rossum AM. Infection with and carriage of Mycoplasma pneumoniae in children. Front Microbiol. 2016;7:329. doi:10.3389/fmicb.2016.00329

6. Dumke R. Molecular tools for typing Mycoplasma pneumoniae and Mycoplasma genitalium. Front Microbiol. 2022;13:904944.

7. Cousin-Allery A, Charron A, de Barbycay B, et al. Molecular typing of Mycoplasma pneumoniae strains by PCR-based methods and pulsed-field gel electrophoresis. Application to French and Danish isolates. Epidemiol Infect. 2000;124(1):103–111. doi:10.1017/S0950268899003313

8. 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(Pt 4):469–475. doi:10.1099/jmm.0.47644-0

9. Sun H, Xue G, Yan C, et al. Multiple-locus variable-number tandem-repeat analysis of Mycoplasma pneumoniae clinical specimens and proposal for amendment of MLVA nomenclature. PLoS One. 2013;8(5):e64607. doi:10.1371/journal.pone.0064607

10. Chalker VJ, Pereyre S, Dumke R, et al. International Mycoplasma pneumoniae typing study: interpretation of M. pneumoniae multilocus variable-number tandem-repeat analysis. New Microbes New Infect. 2015;7:37–40. doi:10.1016/j.micratio.2015.05.005

11. Xin D, Mi Z, Han X, et al. Molecular mechanisms of macrolide resistance in clinical isolates of Mycoplasma pneumoniae from China. Antimicrob Agents Chemother. 2009;53(5):2158–2159. doi:10.1128/AAC.01563-08

12. Komatsu H, Tsunoda T, Inui A, Sogo T, Fujisawa T. Characteristics of hospitalized children infected with macrolide-resistant Mycoplasma pneumoniae. Braz J Infect Dis. 2014;18(3):294–299. doi:10.1016/j.bjid.2013.09.004

13. Liu Y, Ye X, Zhang H, et al. Anti-macrolide susceptibility of Mycoplasma pneumoniae isolates and molecular analysis of macrolide-resistant strains from Shanghai, China. Antimicrob Agents Chemother. 2009;53(5):2160–2162. doi:10.1128/AAC.01684-08

14. Liu Y, Ye X, Zhang H, et al. Characterization of macrolide resistance in Mycoplasma pneumoniae isolated from children in Shanghai, China. Diag Microbiol Infect Dis. 2010;67(4):335–358. doi:10.1016/j.diagmicrobio.2010.03.004

15. Xue G, Li M, Wang N, et al. Comparison of the molecular characteristics of Mycoplasma pneumoniae from children across different regions of China. PLoS One. 2018;13(8):e0198557. doi:10.1371/journal.pone.0198557

16. Katsukawa C, Kenri T, Shibayama K, Takahashi K. Genetic characterization of Mycoplasma pneumoniae isolates in Osaka between 2011 and 2017: decreased detection rate of macrolide-resistance and increase of p1 gene type 2 lineage strains. PLoS One. 2019;14(1):e0209938. doi:10.1371/journal.pone.0209938

17. Zhao F, Li J, Liu J, et al. Anti-macrolide susceptibility and molecular characteristics of Mycoplasma pneumoniae isolates across different regions of China. Antimicrob Resist Infect Control. 2019;8:143. doi:10.1186/s13756-019-0576-5

18. Lee JK, Lee JH, Lee H, et al. Clonal expansion of macrolide-resistant sequence Type 3 Mycoplasma pneumoniae, South Korea. Emerg Infect Dis. 2018;24(8):1465–1471. doi:10.3201/eid2408.180081

19. Suzuki S, Konno T, Shibata C, Saito H. Low Incidence of Macrolide-Resistant Mycoplasma pneumoniae between April 2016 and March 2017 in Akita Prefecture, Japan. Jpn J Infect Dis. 2018;71(6):477–478. doi:10.7883/yoken.JJID.2018.170

20. Zhao F, Liu J, Shi W, et al. Anti-macrolide susceptibility and genotyping of Mycoplasma pneumoniae isolates in Beijing, China, from 2014 to 2016. Antimicrob Resist Infect Control. 2019;8:18. doi:10.1186/s13756-019-0469-7

21. Lucier TS, Heitzman K, Liu SK, Hu PC. Transition mutations in the 23S rRNA of erythromycin-resistant isolates of Mycoplasma pneumoniae. Antimicrob Agents Chemother. 1995;39(12):2770–2773. doi:10.1128/AAC.39.12.2770

22. Li BB, Shen JZ, Cao XY, et al. Mutations in 23S rRNA gene associated with decreased susceptibility to tiamulin and valnemulin in Mycoplasma gallisepticum. FEMS Microbiol Lett. 2016;308(2):144–149. doi:10.1111/1574-6968.2010.02003.x

23. Pereyre S, Guyot C, Renaudin H, Charron A, Bébér C, Bébér CM. In vitro selection and characterization of resistance to macrolides and related antibiotics in Mycoplasma pneumoniae. Antimicrob Agents Chemother. 2004;48(4):460–465. doi:10.1128/AAC.48.2.460-465.2004

24. Dorigo-Zetsma JW, Zaat SA, Wertheim-van Dillen PM, et al. Comparison of PCR, culture, and serological tests for diagnosis of Mycoplasma pneumoniae respiratory tract infection in children. J Clin Microbiol. 1999;37(1):14–17. doi:10.1128/JCM.37.1.14-17.1999

25. De Grager S, Cazanave C, Charron A, Renaudin H, Charron A, Renaudin H, Bebear C, Bebear CM. Development of multiple locus variable-number tandem repeat analysis for molecular typing of Mycoplasma pneumoniae. J Clin Microbiol. 2004;4(4):914–923. doi:10.1128/JCM.01935-08

26. Wang N, Zhou Y, Zhang H, Liu Y. In vitro activities of acetylmidecamycin and other antimicrobials against human macrolide-resistant Mycoplasma pneumoniae isolates. J Antimicrob Chemother. 2020;75(6):1513–1517. doi:10.1093/jac/dkaa027

27. Hu PC, Liu SK, Heitzman K, Helin H, et al. Transition mutations in the 23S rRNA of erythromycin-resistant isolates of Mycoplasma pneumoniae. Antimicrob Agents Chemother. 1995;39:2770–2773. doi:10.1128/AAC.39.12.2770

28. Wang et al. J Clin Microbiol Infect Dis. 2019;16(1):23–34. doi:10.1080/14787210.2018.141599
35. Waite KB, Ratliff A, Crabb DM, et al. Macrolide-resistant Mycoplasma pneumoniae in the United States as determined from a national surveillance program. J Clin Microbiol. 2019;57(11):e00968–00919. doi:10.1128/JCM.00968-19
36. Tanaka T, Oishi T, Miyata I, et al. Macrolide-Resistant Mycoplasma pneumoniae Infection, Japan, 2008-2015. Emerg Infect Dis. 2017;23(10):1703–1706. doi:10.3201/eid2310.170106
37. Guo Z, Liu L, Gong J, et al. Molecular features and antimicrobial susceptibility of Mycoplasma pneumoniae isolates from paediatric inpatients in Weihai, China: characteristics of M. pneumoniae In Weihai. J Glob Antimicrob Resist. 2020;28:180–184. doi:10.1016/j.jgar.2020.01.002
38. Liu Y, Ye X, Zhang H, Xu X, Wang M. Multiclonal origin of macrolide-resistant Mycoplasma pneumoniae isolates as determined by multilocus variable-number tandem-repeat analysis. J Clin Microbiol. 2012;50(8):2793–2795. doi:10.1128/JCM.00678-12
39. Kogoj R, Praprotnik M, Mrvic T, Korva M, Kese D. Genetic diversity and macrolide resistance of Mycoplasma pneumoniae isolates from two consecutive epidemics in Slovenia. Eur J Clin Microbiol Infect Dis. 2018;37(1):99–107. doi:10.1007/s10096-017-3106-5
40. Gullsby K, Olsen B, Bondeson K. Molecular typing of mycoplasma pneumoniae strains in Sweden from 1996 to 2017 and the emergence of a new P1 cytadhesin gene, variant 2c. J Clin Microbiol. 2019;57(6):e00049–00019. doi:10.1128/JCM.00049-19
41. Yan C, Yang H, Sun H, et al. Diversity in genotype distribution of mycoplasma pneumoniae obtained from children and adults. Jpn J Infect Dis. 2020;73(1):14–18. doi:10.7883/yoken.JJID.2019.037
42. Diaz MH, Benitez AJ, Cross KE, et al. Molecular detection and characterization of mycoplasma pneumoniae among patients hospitalized with community-acquired Pneumonia in the United States. Open Forum Infect Dis. 2015;2(3):ofv106. doi:10.1093/ofid/ofv106
43. Qu J, Chen S, Bao F, Gu L, Cao B. Molecular characterization and analysis of Mycoplasma pneumoniae among patients of all ages with community-acquired pneumonia during an epidemic in China. Int J Infect Dis. 2019;83:26–31. doi:10.1016/j.ijid.2019.03.028
44. Whistler T, Sawatwong P, Diaz MH, et al. Molecular characterization of Mycoplasma pneumoniae infections in two rural populations of Thailand from 2009 to 2012. J Clin Microbiol. 2017;55(7):2222–2233. doi:10.1128/JCM.00350-17