ORIGINAL RESEARCH

Antimicrobial Susceptibility Profiles and Genetic Characteristics of *Mycoplasma pneumoniae* in Shanghai, China, from 2017 to 2019

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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.[2] In addition, some researchers also observed that *M. pneumoniae* could present asymptomatically 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]

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Molecular characteristics help in monitoring the epidemiology of *M. pneumoniae* infections. In this regard, several genotyping methods have been developed.[6] Among them, P1 typing is one of the most common methods.[7,8] 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.[9,10] 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.[2] The highest resistance rate was over 90% in some Asian regions, including our reports Shanghai, China, 10 years ago.[11–14] Recently, based on the information from China, Japan and South Korea, the macrolide resistance rate decreased, corresponding to the genotype shift within this area.[15–19] However, only sparse data are available for the recent *M. pneumoniae* status in Shanghai.[15,17,20] 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.[2] 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.[2,21] Other studies also reported that insertions or deletions within the ribosomal proteins L4 and L22 were associated with macrolide resistance in *M. pneumoniae*.[22,23]

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.[3,24] *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.[25] PCR was performed through the primers (5' - GCCACCCTCGGGGGGCAGTCAG −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.[26] 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 $\geq$ 1 mg/L were regarded as macrolide resistant.[26]

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.[9] The *L4* and *L22* ribosomal protein genes were amplified through primer pairs MPL4 - 1/ MPL4 - 2 (5 ' - GAACCAGTGAAACTAAGCCC- 3 ' and 5 ' - TTTGTCCAAGAGCTTGGCAC - 3 ') and MPL22 – 1/MPL22 - 2 (5 ' – CCGTGTGAGAATCTCACCCC – 3 ' and 5 ' – CTGCTTTTTGACGTGCCATC – 3 ').[23] 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.[7] 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).[27] 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.[9,27] 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.[28] For the remaining 72 isolates, 100% (72/72) were resistant to erythromycin (MIC ≥ 64mg/L). The MIC summary (including MIC_{50} values, MIC_{90} 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

Table 1 Summary of the Minimum Inhibitory Concentrations of Nine Antimicrobials Against 182 *M. pneumoniae* Isolates

Antimicrobials	MIC (mg/L)					Interpretive Criteria*	
	MIC Range	MIC_{50}	MIC_{90}	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.

Table 2 Cumulative Bacteriostatic Rates (%) of the Nine Antimicrobials Against 182 *M. pneumoniae* Isolates

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	–	–	–	–	–	–	–	–	–	–
Levofloxacin	0.6	–	6.0	57.4	100.0	–	–	–	–	–	–	–	–
Tetracycline	1.7	6.6	29.7	89.6	99.5	100.0	–	–	–	–	–	–	–
Minocycline	2.2	16.5	45.6	81.3	89.0	97.3	100.0	–	–	–	–	–	–
Doxycycline	7.7	36.8	69.8	95.1	100.0	–	–	–	–	–	–	–	–
Erythromycin	2.7	–	–	–	–	–	–	–	–	–	3.8	23.6	100.0
Roxithromycin	2.7	–	–	–	–	–	–	–	–	6.6	20.9	63.2	100.0
Azithromycin	2.7	–	–	–	–	3.3	9.9	27.5	76.9	96.7	100.0	–	–
Josamycin	2.2	2.7	–	–	7.7	28.6	94.0	99.5	–	100.0	–	–	–

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
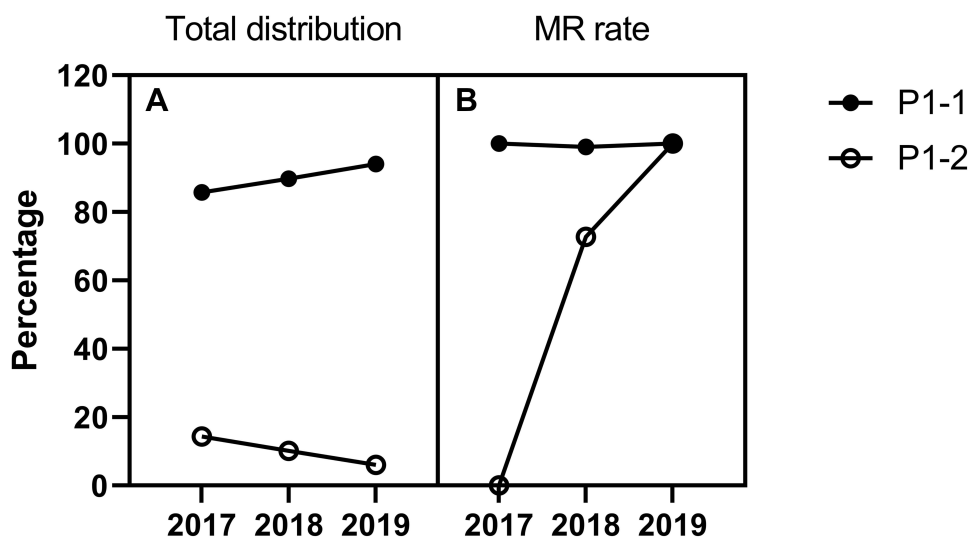

Figure 1 Comparison of *M. pneumoniae* P1 subtypes in Shanghai between 2017 and 2019. (**A**) Distribution of the P1-1 and P1-2 subtypes ($p = 0.693$). (**B**) Macrolide resistance rate in P1-1 ($p = 0.347$) and P1-2 ($p = 0.054$).

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)	1 (0.6)	165 (99.4)	166 (91.2)
		P1-2	1 (14.3)	11 (10.2)	4 (6.0)	4 (25.0)	12 (75.0)	16 (8.8)
MLVA Type	3-5-6-2 (n=14)	2-3-5-6-2	1 (14.3)	1 (0.9)	0 (0)	2 (40.0)	0 (0)	2 (1.1)
		4-3-5-6-2	0 (0)	5 (4.6)	3 (4.5)	0 (0)	8 (4.5)	8 (4.4)
		5-3-5-6-2	0 (0)	2 (1.9)	1 (1.5)	1 (20.0)	2 (1.1)	3 (1.6)
		6-3-5-6-2	0 (0)	1 (0.9)	0 (0)	0 (0)	1 (0.6)	1 (0.5)
	3-6-6-2 (n=1)	4-3-6-6-2	0 (0)	1 (0.9)	0 (0)	1 (20.0)	0 (0)	1 (0.5)
	4-5-6-2	4-4-5-6-2	0 (0)	1 (0.9)	0 (0)	0 (0)	1 (0.6)	1 (0.5)
	4-4-7-2 (n=1)	5-4-4-7-2	0 (0)	1 (0.9)	0 (0)	0 (0)	1 (0.6)	1 (0.5)
	4-5-7-2 (n=167)	2-4-5-7-2	1 (14.3)	19 (17.6)	11 (16.4)	0 (0)	31 (17.4)	31 (17.0)
		3-4-5-7-2	2 (28.6)	12 (11.1)	22 (32.8)	0 (0)	36 (20.2)	36 (19.8)
		4-4-5-7-2	0 (0)	24 (22.2)	5 (7.5)	1 (20.0)	28 (16.3)	30 (16.5)
		5-4-5-7-2	1 (14.3)	27 (25.0)	15 (22.4)	0 (0)	43 (24.2)	43 (23.6)
		6-4-5-7-2	2 (28.6)	10 (9.3)	10 (14.9)	0 (0)	22 (12.9)	23 (12.6)
		7-4-5-7-2	0 (0)	4 (3.7)	0 (0)	0 (0)	4 (2.2)	4 (2.2)
Macrolide susceptibility		Susceptible	1 (14.3)	4 (3.7)	0 (0)	-	-	5 (2.7)
		Resistant	6 (85.7)	104 (96.3)	67 (100)	-	-	178 (97.3)
Total, n (%)			7 (3.8)	108 (59.3)	67 (36.8)	5 (2.7)	177 (97.3)	182 (100)

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 5-4-4-7-2, 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 5-4-4-7-2. 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
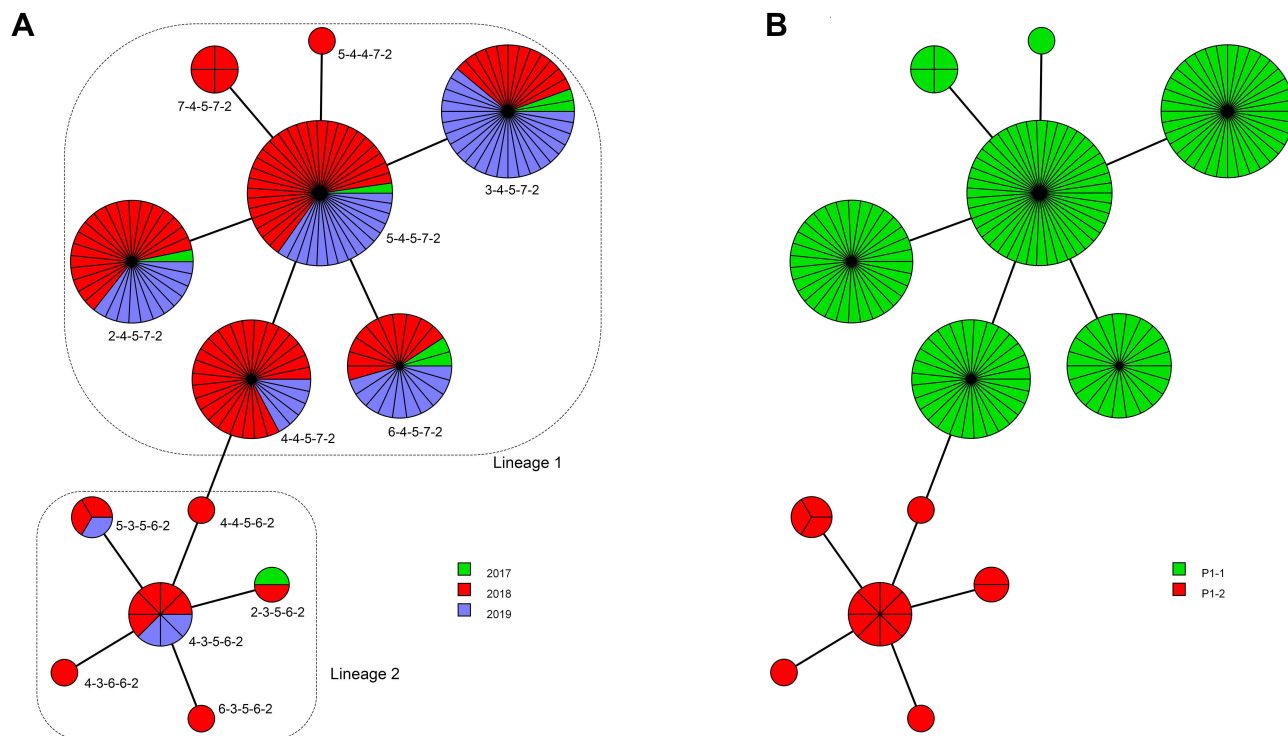
Figure 2 The minimum spanning tree (MST) of the 182 *M. pneumoniae* strains depends on the 5-loci scheme MLVA types. Clustering of the MLVA profiles was based on the categorical coefficient. Each circle represents one MLVA type and the size of the circle is directly proportional to the number of isolates. The distance of the line between the MLVA types corresponds to the total number of allelic changes. (**A**) MST with colors based on the collection years. Circles with dashed lines delineate the MLVA lineages. (**B**). MST with colors depends on the P1 subtypes.

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.[20,29–32] This difficult resistant situation in Shanghai could be related to the antimicrobial selection pressure.[33,34] The selection was correlated with antimicrobial usage as observed in the US.[35] 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

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.

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.[26] 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

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%.[17,41] 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

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