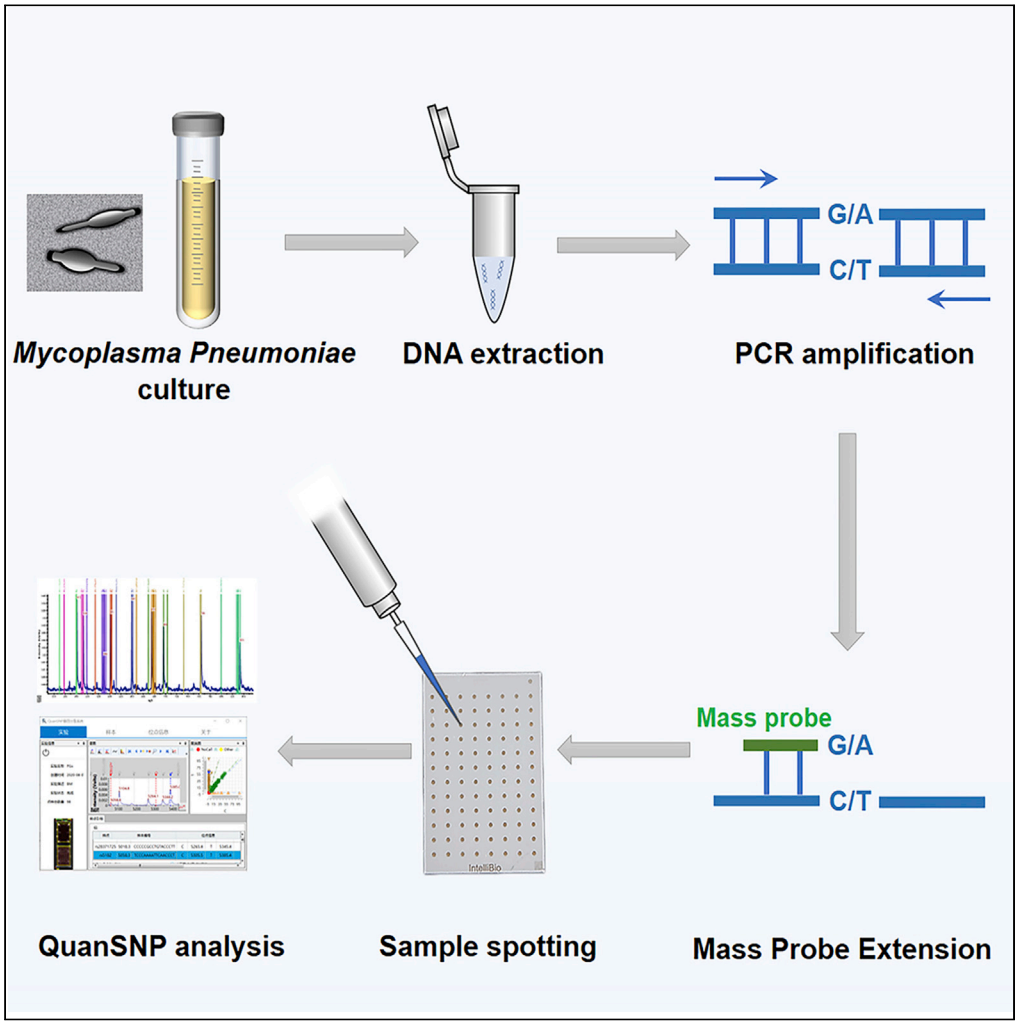
iScience

CellPress
OPEN ACCESS

Article

A multisite SNP genotyping and macrolide susceptibility gene method for *Mycoplasma pneumoniae* based on MALDI-TOF MS



Fei Zhao, Jianzhong Zhang, Xuemei Wang, ..., Lihua He, Fanliang Meng, Di Xiao

xiaodi@icdc.cn

Highlights

An all-in-one genotyping and macrolide resistance testing method for *M. pneumoniae*

Multisite SNP detection technology was used for genotyping and resistance testing

The cost of *M. pneumoniae* genotyping and macrolide resistance detection was reduced

Zhao et al., iScience 24, 102447
May 21, 2021
https://doi.org/10.1016/j.isci.2021.102447

iScience

CellPress
OPEN ACCESS

Article

A multisite SNP genotyping and macrolide susceptibility gene method for *Mycoplasma pneumoniae* based on MALDI-TOF MS

Fei Zhao,[1] Jianzhong Zhang,[1] Xuemei Wang,[2] Liyong Liu,[1] Jie Gong,[1] Zhixiang Zhai,[2] Lihua He,[1] Fanliang Meng,[1] and Di Xiao[1,3,*]

SUMMARY

In this study, a multisite SNP genotyping and macrolide (ML) susceptibility gene test method for *Mycoplasma pneumoniae* (*M. pneumoniae*) was developed based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The detection limit of this method for nucleic acids was 10^2 -10^3 copies/reaction. Six SNP site-based genotyping and 3 ML susceptibility sites could be detected simultaneously based on multiplex PCR and mass probe. Using the method constructed in this study, 141 Chinese clinical isolates were divided into 8 SNP types. All the SNP test results for the ML susceptibility gene were in line with those of the 23S rRNA sequencing results. With this method, the multisite SNP genotyping and ML susceptibility determination of *M. pneumoniae* can be completed simultaneously in one test, which greatly reduces the workload and cost, improves the genotyping ability of *M. pneumoniae* and deserves clinical application.

INTRODUCTION

Mycoplasma pneumoniae (*M. pneumoniae*), an important pathogenic bacterium of respiratory tract infection in children and adults, is responsible for approximately 10%–30% of community-acquired pneumonia (Jacobs et al., 2015; Loens et al., 2010). There has been epidemic spread of *M. pneumoniae* in many European and Asian countries since 2010 (Blystad et al., 2012; Chalker et al., 2011; Gadsby et al., 2012; Lenglet et al., 2012; Linde et al., 2012; Uldum et al., 2012). Generally, the prognosis of *M. pneumoniae* is good, with only a few pediatric cases progressing to refractory *Mycoplasma pneumoniae* pneumonia (RMPP) or severe *Mycoplasma pneumoniae* pneumonia (SMPP), resulting in severe complications and poor prognosis (Liu et al., 2018; Okumura et al., 2019). To date, macrolides (MLs) have been recommended as the first-line agent for treating clinical infection of *M. pneumoniae* infections, especially in children (Waites et al., 2009, 2017).

In the past two decades, the proportion of ML susceptibility in *M. pneumoniae* (MRMP) has shown an increasing trend, especially in Japan and the Republic of Korea (Hong et al., 2013; Kawai et al., 2013). In mainland China, the ML susceptibility ratio of *M. pneumoniae* is still high to some extent (Cao et al., 2010; Liu et al., 2009; Qu et al., 2013; Xin et al., 2009;Zhao et al., 2012).Studies have shown that the ML resistance mechanism of *M. pneumoniae* is very fixed and only closely related to 23S rRNA point mutations. The ML susceptibility of *M. pneumoniae* is closely related to the 23S rRNA mutation, among which the mutations at the 2063 and 2064 positions are correlated with high ML susceptibility (>64 μg/mL for erythromycin) and the mutation at the 2617 position is correlated with low ML susceptibility (Pereyre et al., 2016). Therefore, gene detection based on these mutation sites may serve as an alternative to antimicrobial susceptibility testing of *M. pneumonia in vitro*. To date, the most common methods of detecting ML resistant of *M. pneumoniae* strains include real-time PCR-HRM, PCR-RFLP, and pyrosequencing assays (Liu et al., 2014; Matsuoka et al., 2004; Nummi et al., 2015; Peuchant et al., 2009; Spuesens et al., 2010; Wolff et al., 2008).

Genotyping is an important method for studying the molecular biology and epidemiologic features of *M. pneumoniae*. There may be a close relationship between the transformation of *M. pneumoniae* genotypes in the population and the epidemics of *M. pneumoniae*, and genotyping is also an important technique for tracing the origin of *M. pneumoniae* local outbreaks. The *M. pneumoniae* genome is highly conserved, with a sequence similarity of up to 99% among strains. Based on single nucleotide polymorphisms (SNPs) and indels, two main subgroups of *M. pneumoniae*, genotype I and genotype II, can be

[1]National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, State Key Laboratory of Infectious Disease Prevention and Control, Beijing 102206, China

[2]Intelligene Biosystems (Qingdao) Co., Ltd, Qingdao, China

[3]Lead contact

*Correspondence: xiaodi@icdc.cn

https://doi.org/10.1016/j.isci.2021.102447

clearly differentiated (Lluch-Senar et al., 2015; Xiao et al., 2015). Currently, the most common genotyping techniques for these two genotypes are based mainly on PCR-RFLP genotyping of the *p1* gene (Dorigo-Zetsma et al., 2000), VNTR of the *pl* gene, and real-time PCR of the MPN459/MPNA5864 gene (Zhao et al., 2015). These techniques are easy to perform, but the discrimination ability is not adequate due to the presence of a single target site. Specifically, multiple-locus variable-number tandem-repeat analysis (MLVA) (Degrange et al., 2009) and multilocus sequence typing (MLST) (Brown et al., 2015) contribute to the genotyping of *M. pneumoniae*. However, these genotyping procedures are time-consuming and technically demanding, which hampers their applications. Both genotyping analysis and ML susceptibility nucleic acid testing for *M. pneumoniae* involve complicated procedures, and it is necessary to develop new methods that are rapid and inexpensive.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been utilized for detecting SNPs in recent decades. Multiplex PCR is used to amplify the genes containing the targets of SNPs. Subsequently, an extension mass probe has been utilized for the extension of SNP sites. Finally, MALDI-TOF MS is performed to identify the mass-to-charge ratio (m/z) of extended mass probes. Recently, MALDI-TOF MS has been commonly used in microbiological detection and analysis, and is considered a new gold standard for the identification of many kinds of microorganisms (Jang and Kim, 2018;Kostrzewa, 2018;Rahi et al., 2016). To date, SNP genotyping based on MALDI-TOF MS has been frequently conducted for the screening of hand-foot-and-mouth disease virus, hepatitis B virus (HBV) detection, and the human papillomavirus (HPV) genotyping and serves as an effective alternative to conventional techniques (Peng et al., 2013a, 2013b;Sjoholm et al., 2008). SNP analysis based on MALDI-TOF MS features high throughput, rapid detection, and simultaneous detection of multiple targets. Therefore, it is one of the most promising biomolecular techniques.

In this study, multiplex PCR coupled with MALDI-TOF MS (PCR-MALDI-TOF MS) was used to genotype and detect the antimicrobial susceptibility of *M. pneumoniae*.

RESULTS

MALDI-TOF MS-based method establishment and optimization

Multiplex PCR coupled with MALDI-TOF MS assay was utilized to automate the detection of the nine SNPs and avoid sequencing the gene fragments. To avoid the formation of a dimer between the probes at the 23S rRNA^{2063} and 23S rRNA^{2064} sites, the quality probe at the 2064 site was designed at the reverse complementary strand. Therefore, the SNP of this site was the basis of the complementary strand (Table 1, Figure 1). MALDI-TOF MS-based amplification and identification were performed on 10 epidemiologically unrelated *M. pneumoniae* strains. Six SNP genotyping sites and three ML susceptibility gene sites were detected, and no MS peak was observed in the blank control. There was complete consistency between the MS SNP results of three ML susceptibility sites and the sequencing results of the strains. Among the 10 strains, there were 6 SNP types (0, 1, 3, 11, 15 and 32). Six odd number SNP types were classified into genotype I, and 4 even number SNP types were classified into genotype II (Table 2) . For example, there were 9 MS peaks for M129 in Figure 1A, representing 6 SNP typing mass probes and 3 ML susceptibility mass probes. The dotted line of the same color represents the mass spectrum peak for various mass probes extending the same base pairs in the SNP sites. The peak value represents the m/z of the MPE after extension. After multiplex PCR amplification and MPE probes extension for M129, the peak of the spectrum is shown in Figure 1B. The red arrow represents the quality value of the nine MPE probes after SNP site extension. The optimized concentration of each extension primer in the primer mixture was 6.9 μM (MPN114^{1461}), 7.1μM (MPN372^{1112}), 7.7μM (23S rRNA^{2063}), 8.0μM (MPN213^{47}), 8.6μM (MPN280^{1641}), 9.2μM (23S rRNA^{2617}), 9.5μM (MPN126^{470}), 10.4μM (MPN262^{192}), and 11.2μM (23S rRNA^{2064}).

Specificity and detection limit of PCR-MALDI-TOF MS

There was no extension signal on the quality probe for the nucleic acids from the 20 non-*M. pneumoniae* pathogens, yielding a specificity of 100%. In addition, the nucleic acids of *M. pneumoniae* M129 strains subjected to 10-fold dilution (10^0-10^5copies/μl) were utilized for the validation of LDL, using 1 μL template. In the presence of an *M. pneumoniae* load of 10^3-10^5copies/reaction, all the nine SNP sites could be accurately identified after two tests. In the presence of a load of 10^2 copies/reaction, 23S rRNA^{2617} site could be accurately examined only once, while there was no MS peak in the other test. For MPN262, there was no MS peak after two tests, and no signals were noticed in the MS peak in the blank control (Figure 2). The detection limit of the nucleic acid derived from the *M. pneumoniae* strains was in the range of 10^2-10^3copies/reaction.

Table 1. Primer sequences for *M. pneumoniae* SNP typing and susceptibility target site amplification and MPE probes for SNP detection

			Multiple-PCR		Mass probe extension									
	Target gene	SNP	Forward primer sequence	Reverse primer sequence	Mass probe sequence	Mass probe mass (Da)	Extension call	Extended mass (Da)	Extension call	Extended mass (Da)	Extension call	Extended mass (Da)	Extension call	Extended mass (Da)
GENO TYPING	MP N114[1461]	C/T	acgttggatgCA CCGAGTGTC TTCGTCCTT	acgttggatgG GTTGACCCC ACTACCCTTT	CCCTTTTGC GCTAACCG	5097.4	C	5410.4	T	5394.4				
	MP N126[470]	C/T	acgttggatgAA ATTCCCCGT AATCCGAGA	acgttggatgGC CTTCTGCTTGT TAGGTAGATG	AGTGATTT AACGTTCC ATTTTC	6690.4	C	6963.4	T	7032.4				
	MP N213[47]	G/T	acgttggatgA TCAGTCGCT GGTACTGTGG	acgttggatgGC TGTGCTTTTG GCAACTTA	TACTACCACC GCAGTTGTT	5738.8	G	6011.8	T	6035.8				
	MP N262[192]	C/T	acgttggatgAC CACTTAGAG CCGGAACAA	acgttggatgTT GCTGAAACTG ACCATCCA	GCCCAATT TAGTCTTAT TTTGGAT	7323.8	C	7636.8	T	7620.8				
	MP N280[1641]	G/T	acgttggatgCTC AATTAAACGCG GTTTGG	acgttggatgC GGTTGAAATC AACGGGTTA	TTCCAAAT GAAGTAAG ACAC	6118.0	G	6391.0	T	6415.0				
	MP N372[1112]	G/T	acgttggatgAGTG TTAGCGCGGTT AATGC	acgttggatgTC GTGTTGTGAA CCACCACT	AATGACAC CGCAAGACA	5181.4	T	5523.4	G	5494.4				
ML DETECTION	23S rRNA[2063]	A/T/ C/G	acgttggatgCCA GGTACGGGTG AAGACAC	acgttggatgA TTCCACCTTT CGCATCAAC	TTAGGCGCA ACGGGACGG	5589.6	G	5902.6	A	5886.6	C	5862.6	T	5931.6
	[a]23S rRNA[2064]	A/T/ C/G	acgttggatgCC AGGTACGGGTG AAGACAC	acgttggatgA TTCCACCTTTC GCATCAAC	AGCTACAGT AAAGCTTCA CGGGGTCT	7995.2	T	8337.2	C	8268.2	G	8308.2	A	8292.2
	23S rRNA[2617]	A/T/ C/G	acgttggatgGC TGTTCGCCGA TTAAAGAG	acgttggatgG CGCTACAACT GGAGCATAA	CCGTCGT GAGACAGG TTGGTC	6478.2	C	6751.2	G	6791.2	A	6775.2	T	6820.2

[a]The MPE extension probe for the SNP of 23S rRNA[2064] was the reverse complementary strand, which detected the complementary bases for the SNP.

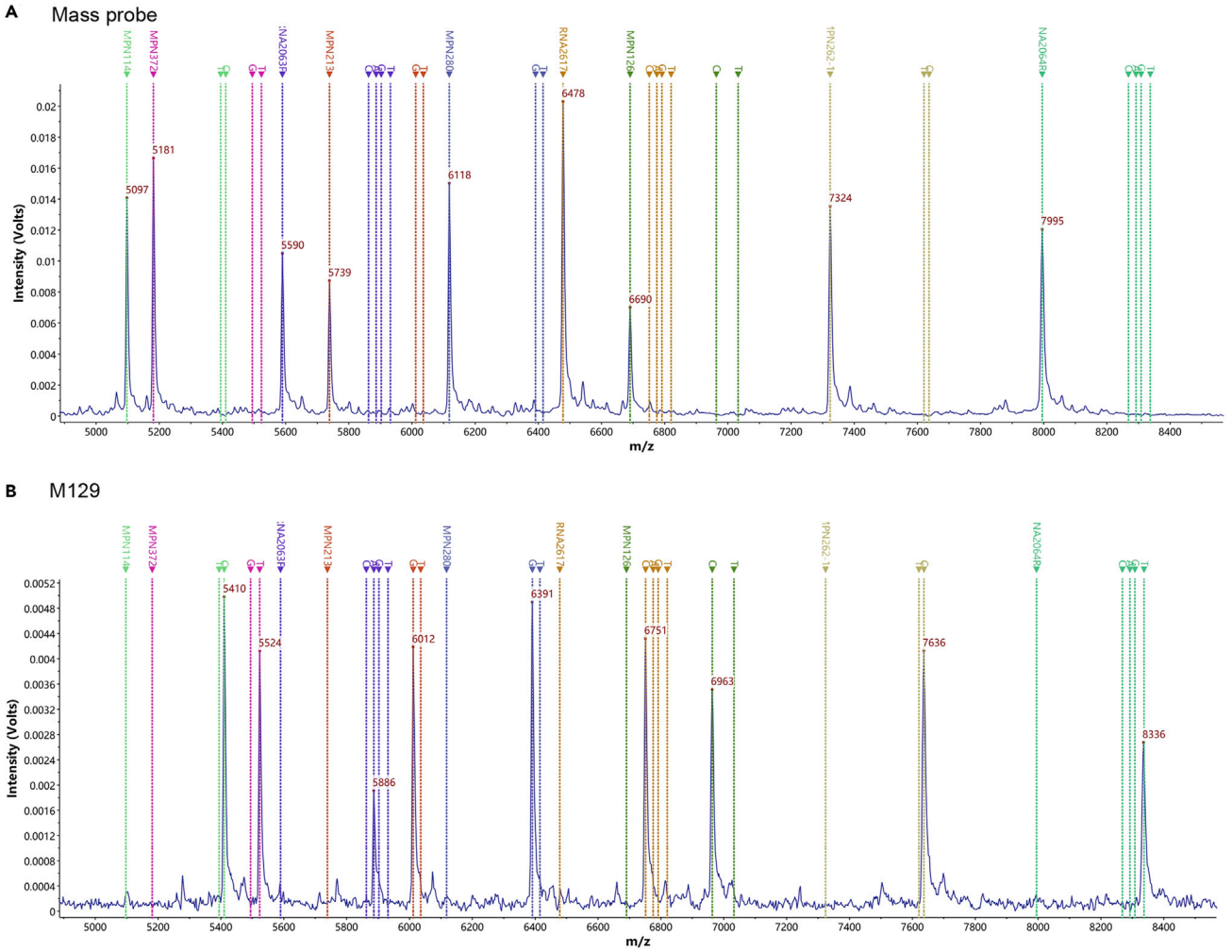


Figure 1. MS peak of the MPE probes

(A) MS peaks of 9 MPE probes without extension;

(B) SNP peak of the 9 MPE probes extended with *M. pneumoniae* M129.

Nucleic acid detection of *M. pneumoniae* isolates

The genotyping and ML susceptibility of the nucleic acids from 141 *M. pneumoniae* isolates were evaluated using this method. The genotyping and ML susceptibility results are shown in Table 3. For the ML susceptibility sites, 106 were confirmed with the *A2063G* mutation, 3 with the *A2064T* mutation, 1 with the *A2063T* mutation, and 31 with no mutations. The MS findings were consistent with previous sequencing results and drug sensitivity tests. SNP genotyping indicated that there were eight genotypes for the 141 isolates, namely, SNP0, 1, 3, 11, 15, 26, 32, and 27. The 109 strains of genotype I showed an odd number SNP genotype (1, 3, 11, 15, 17), among which SNP 11, 15, and 27 were the major types. Thirty-two genotype II strains showed an even number SNP genotype (0, 26, 32), among which SNP32 was the major type. There were large variations in the number and proportion of the SNP genotypes in the *M. pneumoniae* isolates (Table 4).

Mimic SNP genotyping of international *M. pneumoniae* strains

There were 6 genotypes, namely, SNP 0,1,3,11,15 and 27, among the genomes of 79 international *M. pneumoniae* strains with sequences in the NCBI databases. There were 30 strains with SNP 0 genotypes, including AP012303.1, AP017318.1, AP017319.1, CP002077.1, CP010539.1, CP010540.1, CP010546.1, CP010547.1, CP010548.1, CP010549.1, CP010550.1, CP010551.1, CP017327.1, CP017329.1, CP017334.1,

Table 2. Characteristics of the ten *M. pneumoniae* clinical isolates and strains selected for method establishment

Strain/ Isolate	Year of isolation	Susceptibility to macrolides	Sequence 23S rRNA mutation	P1 gene type	MLVA	Geographical origin	SNP typing	ML SNP 23S rRNA mutation	Reference
M129 (ATCC29342)	1969	S	None	1	4572	USA	SNP1	None	Himmelreich et al. (1996)
FH(ATCC15531)	1954	S	None	2	3562	Boston, USA	SNP0	None	Xiao et al. (2015)
ICDC SY15-11	2012	S	None	1	4572	Beijng, China	SNP11	None	Zhao et al., 2013b
ICDC P028	2008	S	None	2	3562	Beijng, China	SNP32	None	Zhao et al., 2013b
ICDC 21075	2016	S	None	2	3572	Beijng, China	SNP0	None	Zhao et al. (2019a)
ICDC AH-012	2017	R	A2063G	1	4572	Fuyang, China	SNP11	A2063G	Zhao et al. (2019b)
ICDC SD14-08	2017	R	A2063G	2	3562	Jinan, China	SNP0	A2063G	Zhao et al. (2019b)
ICDC SL066	2018	R	A2064G	1	4572	Jilin, China	SNP3	A2064G	Zhao et al. (2019b)
ICDC JSSZ031	2018	R	A2063G	1	4573	Soochow, China	SNP15	A2063G	Zhao et al. (2019b)
ICDC W081	2019	R	A2063T	1	4573	Weihai, China	SNP15	A2063T	This study

CP017335.1, CP017336.1, CP017337.1, CP017338.1, CP017339.1, CP017340.1, CP017341.1, CP017342.1, CP039761.1, CP039772.1, CP039775.1, CP039777.1, CP039781.1, CP039784.1, and LR214945.1. There were 14 SNP 1 genotype including CP003913.2, CP017330.1, CP017343.1, CP020689.1, CP020690.1, CP020691.1, CP020692.1, CP020693.1, CP020710.1, CP020711.1, CP020712.1, CP039787.1, CP039790.1, and U00089.2. There were 17 SNP 3 genotype including CP008895.1, CP010538.1, CP010541.1, CP010542.1, CP010543.1, CP010544.1, CP010545.1, CP017328.1, CP017331.1, CP017332.1, CP017333.1, CP039776.1, CP039779.1, CP039782.1, CP039783.1, CP039785.1, and CP039788.1. There were 4 SNP11 genotypes (i.e. CP013829.1, CP014267.1, CP039773.1, and CP039780.1). There were 7 SNP16 genotypes (CP039762.1, CP039763.1, CP039764.1, CP039765.1, CP039766.1, CP039767.1, and CP039769.1) and 7 SNP27 genotype (CP039768.1, CP039770.1, CP039771.1, CP039774.1, CP039778.1, CP039786.1, and CP039789.1). Among these genotypes, all the odd number SNP genotypes were genotype I, with SNP1 and SNP3 as the major types, and all the even number SNP genotypes were genotype II. Compared with the Chinese strains, the international strains lacked the genotypes SNP26 and SNP32 (Table 4).

Detection results for the nucleic acid positive clinical specimens of *M. pneumoniae*

Our assay was performed directly on clinical specimens using an increase in the template volume (5 μL) and PCR volume (20 μL). Among the 30 *M. pneumoniae* nucleic acid positive clinical specimens, 9 SNP sites were detected in 25 isolates (83.3%). The detection limit of the clinical specimens was 5.2×10^2 copies/reaction. In addition, the genotype and ML susceptibility results in the 25 isolates were consistent with the detection of *M. pneumoniae*. For the remaining 5 specimens, incomplete SNP profiles were obtained. On this basis, complete data interpretation could not be given.

DISCUSSION

With the increased accessibility to markers through high-throughput whole-genome sequencing (WGS), SNP analysis is increasingly useful for evaluating drug susceptibility, evolution, and molecular epidemiology (Kuglik et al., 2014; Lipworth et al., 2019). To date, the single or small proportion of SNP site detection mainly relies on the first-generation sequencing technique or TaqMAN probe-based real-time PCR detection. For the analysis of multiple SNP sites that are extensively distributed, the WGS technique is the preferred option. However, these techniques are not adequate in the detection of more than tens or dozens of SNP sites. The MS technique facilitates these two SNP-based techniques. Multiplex PCR and

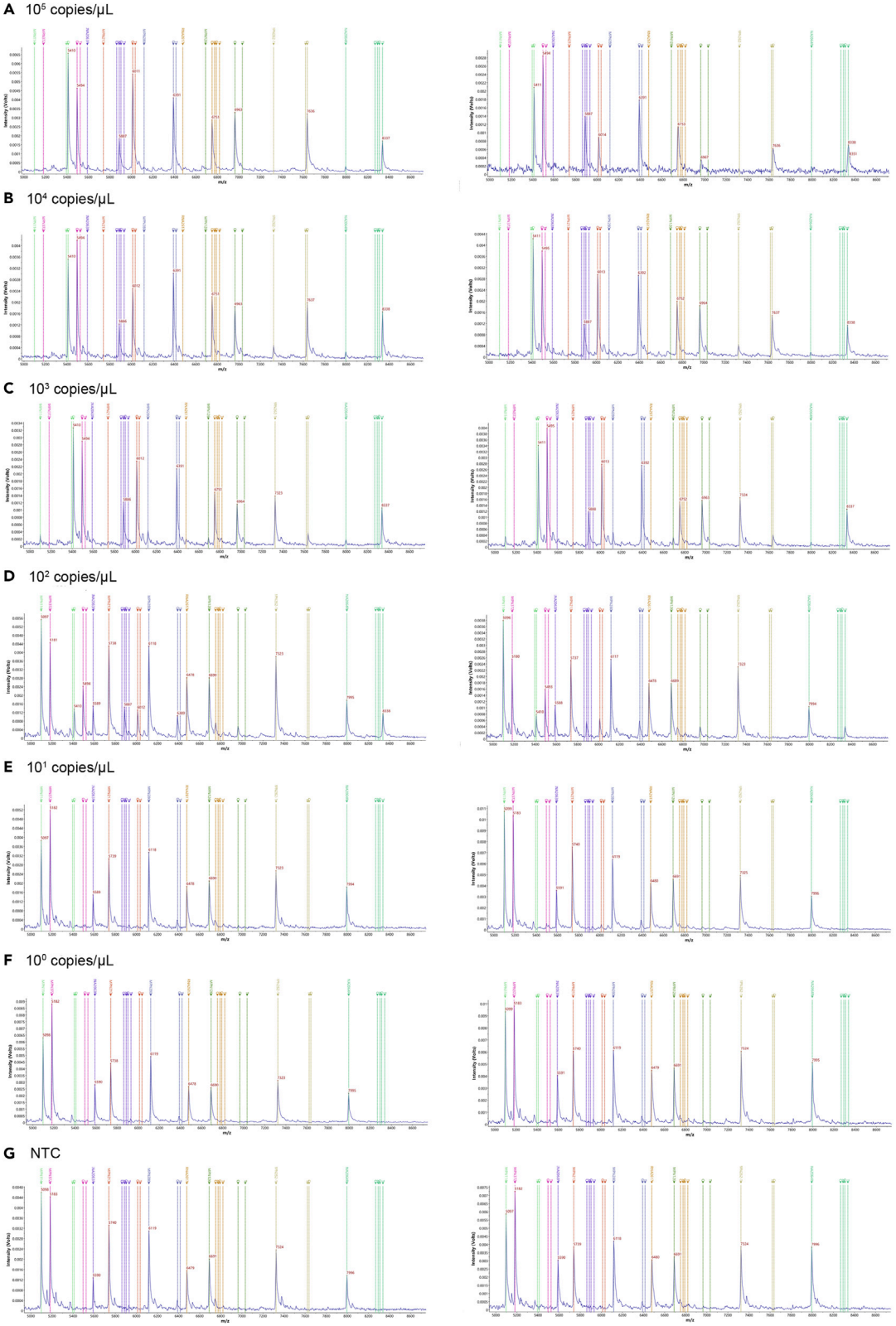


Figure 2. Detection of 9 SNP sites in the presence of different nucleic acid from *M.* pneumoniae based on the PCR-MALDI-TOF MS method

The test was performed twice for each concentration. a-g: of a concentration range of 10^5-10^0copies/μL and the blank control.

Table 3. Genotyping and macrolide susceptibility results of *M. pneumoniae* strains used in this study

					Nucleotide of SNP									23S rRNA SNP		
					SNP1	SNP2	SNP3	SNP4	SNP5	SNP6				2063	2064	2617
Isolates name	City	year of isolation	MLVA type	Geno type	[a]MP N114[1461]	MP N126[470]	MP N213[47]	MP N262[192]	MP N280[1641]	MP N372[1112]	SNP1-6 to digital string	SNP type	SNP (odd/even)	23S rRNA[2063]	23S rRNA[2064]	23S rRNA[2617]
ICDC SD14-02	Jinan	2017	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC SD14-07	Jinan	2017	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC SD14-09	Jinan	2017	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC SD14-26	Jinan	2017	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC SD14-50	Jinan	2018	3662	II	C	C	G	C	G	G	000000	0	even	G	A	C
ICDC SD14-58	Jinan	2018	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC SD14-61	Jinan	2018	3562	II	C	C	G	C	G	G	000000	0	even	G	A	C
ICDC SD14-69	Jinan	2018	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC SD14-77	Jinan	2018	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC SD14-13	Jinan	2017	3562	II	C	T	T	C	T	G	011010	26	even	A	A	C
ICDC SD14-91	Jinan	2018	3562	II	C	T	T	C	T	G	011010	26	even	A	A	C
ICDC SD14-03	Jinan	2017	4572	I	C	C	G	C	T	T	000011	3	odd	A	G	C
ICDC SD14-01	Jinan	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC SD14-06	Jinan	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC SD14-22	Jinan	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC SD14-23	Jinan	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C

(*Continued on next page*)

Table 3. *Continued*

Isolates name	City	year of isolation	MLVA type	Geno type	Nucleotide of SNP						SNP1-6 to digital string	SNP type	SNP (odd/even)	23S rRNA SNP		
					SNP1	SNP2	SNP3	SNP4	SNP5	SNP6				2063	2064	2617
					[a]MP N114[1461]	MP N126[470]	MP N213[47]	MP N262[192]	MP N280[1641]	MP N372[1112]				23S rRNA[2063]	23S rRNA[2064]	23S rRNA[2617]
ICDC SD14-37	Jinan	2018	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC SD14-88	Jinan	2018	4572	I	C	C	T	C	T	T	001011	11	odd	A	A	C
ICDC SD14-45	Jinan	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC SD14-14	Jinan	2017	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SD14-25	Jinan	2017	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL002	Jilin	2017	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL008	Jilin	2017	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL011	Jilin	2017	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL019	Jilin	2017	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL033	Jilin	2018	4572	I	C	C	G	C	T	T	000011	3	odd	G	A	C
ICDC SL037	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL044	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL061	Jilin	2018	4573	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL066	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL069	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL075	Jilin	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC SL083	Jilin	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC SL087	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL091	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL093	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL121	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL134	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL144	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL145	Jilin	2018	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC SL181	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL182	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C

(*Continued on next page*)

Table 3. *Continued*

Isolates name	City	year of isolation	MLVA type	Geno type	Nucleotide of SNP						SNP1-6 to digital string	SNP type	SNP (odd/even)	23S rRNA SNP		
					SNP1	SNP2	SNP3	SNP4	SNP5	SNP6				2063	2064	2617
					[a]MP N114[1461]	MP N126[470]	MP N213[47]	MP N262[192]	MP N280[1641]	MP N372[1112]				23S rRNA[2063]	23S rRNA[2064]	23S rRNA[2617]
ICDC SL199	Jilin	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC SL200	Jilin	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL251	Jilin	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC SL266	Jilin	2018	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC SL277	Jilin	2018	4472	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC SL281	Jilin	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC SL288	Jilin	2018	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC SL309	Jilin	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC SL331	Jilin	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH001	Beijing	2017	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC BCH077	Beijing	2017	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC BCH336	Beijing	2018	3562	II	C	C	G	C	G	G	000000	0	even	G	A	C
ICDC BCH423	Beijing	2018	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC BCH437	Beijing	2018	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC BCH443	Beijing	2018	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC BCH118	Beijing	2017	3562	II	C	T	T	C	T	G	011010	26	even	A	A	C
ICDC BCH023	Beijing	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC BCH031	Beijing	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC BCH039	Beijing	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC BCH145	Beijing	2018	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C

(*Continued on next page*)

Table 3. *Continued*

					Nucleotide of SNP									23S rRNA SNP		
					SNP1	SNP2	SNP3	SNP4	SNP5	SNP6				2063	2064	2617
Isolates name	City	year of isolation	MLVA type	Geno type	[a]MP N114[1461]	MP N126[470]	MP N213[47]	MP N262[192]	MP N280[1641]	MP N372[1112]	SNP1-6 to digital string	SNP type	SNP (odd/ even)	23S rRNA[2063]	23S rRNA[2064]	23S rRNA[2617]
ICDC BCH331	Beijing	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH012	Beijing	2017	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH057	Beijing	2017	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH112	Beijing	2017	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH120	Beijing	2017	4573	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH133	Beijing	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH178	Beijing	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH215	Beijing	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH281	Beijing	2018	4472	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH399	Beijing	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH451	Beijing	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC BCH008	Beijing	2017	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC BCH016	Beijing	2017	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC BCH019	Beijing	2017	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC BCH022	Beijing	2017	4572	I	C	T	T	C	T	T	011011	27	odd	A	A	C
ICDC BCH090	Beijing	2017	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C

(*Continued on next page*)

Table 3. *Continued*

Isolates name	City	year of isolation	MLVA type	Geno type	Nucleotide of SNP						SNP1-6 to digital string	SNP type	SNP (odd/even)	23S rRNA SNP		
					SNP1	SNP2	SNP3	SNP4	SNP5	SNP6				2063	2064	2617
					[a]MP N114[1461]	MP N126[470]	MP N213[47]	MP N262[192]	MP N280[1641]	MP N372[1112]				23S rRNA[2063]	23S rRNA[2064]	23S rRNA[2617]
ICDC BCH264	Beijing	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC BCH271	Beijing	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC BCH412	Beijing	2018	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC U016	Beijing	2008	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC U098	Beijing	2008	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC 12013	Beijing	2012	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC 21055	Beijing	2012	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC 21077	Beijing	2012	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC 21109	Beijing	2013	3562	II	C	C	G	C	G	G	000000	0	even	A	G	C
ICDC B117	Beijing	2013	3562	II	C	C	G	C	G	G	000000	0	even	G	A	C
ICDC P0118	Beijing	2008	3562	II	T	C	G	C	G	G	100000	32	even	A	A	C
ICDC P033	Beijing	2008	3562	II	T	C	G	C	G	G	100000	32	even	A	A	C
ICDC P054	Beijing	2008	3562	II	T	C	G	C	G	G	100000	32	even	A	A	C
ICDC 21010	Beijing	2012	4572	I	C	C	G	C	G	T	000001	1	odd	G	A	C
ICDC SR15-22	Beijing	2015	4472	I	C	C	G	C	G	T	000001	1	odd	G	A	C
ICDC U014	Beijing	2008	4572	I	C	C	G	C	T	T	000011	3	odd	G	A	C
ICDC P396	Beijing	2009	4572	I	C	C	G	C	T	T	000011	3	odd	G	A	C
ICDC 21001	Beijing	2012	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC 12066	Beijing	2013	4572	I	C	C	T	C	T	T	001011	11	odd	T	A	C
ICDC B271	Beijing	2013	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC SY15-77	Beijing	2015	4572	I	C	C	T	T	T	T	001111	15	odd	A	G	C
ICDC P005	Beijing	2008	4572	I	C	C	T	T	T	T	001111	15	odd	A	A	C
ICDC 12075	Beijing	2013	4572	I	C	C	T	T	T	T	001111	15	odd	A	A	C
ICDC B023	Beijing	2013	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC B370	Beijing	2013	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC B385	Beijing	2014	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC P135	Beijing	2008	4573	I	C	T	T	C	T	T	011011	27	odd	G	A	C

(*Continued on next page*)

Table 3. *Continued*

Isolates name	City	year of isolation	MLVA type	Geno type	Nucleotide of SNP						SNP1-6 to digital string	SNP type	SNP (odd/even)	23S rRNA SNP		
					SNP1	SNP2	SNP3	SNP4	SNP5	SNP6				2063	2064	2617
					[a]MP N114[1461]	MP N126[470]	MP N213[47]	MP N262[192]	MP N280[1641]	MP N372[1112]				23S rRNA[2063]	23S rRNA[2064]	23S rRNA[2617]
ICDC 10577	Beijing	2012	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC 12038	Beijing	2012	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC 21000	Beijing	2012	4573	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC 21114	Beijing	2013	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC B429	Beijing	2014	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC B434	Beijing	2014	4572	I	C	T	T	C	T	T	011011	27	odd	G	A	C
ICDC JSSZ002	Soochow	2017	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC JSSZ012	Soochow	2017	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC JSSZ023	Soochow	2017	4572	I	C	C	G	C	T	T	000011	3	odd	G	A	C
ICDC JSSZ038	Soochow	2017	4572	I	C	C	G	C	T	T	000011	3	odd	G	A	C
ICDC JSSZ007	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ010	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ024	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ028	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ037	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ041	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ046	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ047	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ048	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C

(*Continued on next page*)

Table 3. *Continued*

Isolates name	City	year of isolation	MLVA type	Geno type	Nucleotide of SNP						SNP1-6 to digital string	SNP type	SNP (odd/even)	23S rRNA SNP		
					SNP1	SNP2	SNP3	SNP4	SNP5	SNP6				2063	2064	2617
					[a]MP N114[1461]	MP N126[470]	MP N213[47]	MP N262[192]	MP N280[1641]	MP N372[1112]				23S rRNA[2063]	23S rRNA[2064]	23S rRNA[2617]
ICDC JSSZ051	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ055	Soochow	2017	4573	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ064	Soochow	2017	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ072	Soochow	2018	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ078	Soochow	2018	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC JSSZ033	Soochow	2017	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC JSSZ067	Soochow	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC JSSZ071	Soochow	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC JSSZ073	Soochow	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC JSSZ077	Soochow	2018	4572	I	C	C	T	T	T	T	001111	15	odd	G	A	C
ICDC AH-001	Fuyang	2018	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC AH-014	Fuyang	2018	3562	II	C	C	G	C	G	G	000000	0	even	A	A	C
ICDC AH-007	Fuyang	2018	4572	I	C	C	G	C	T	T	000011	3	odd	G	A	C
ICDC AH-004	Fuyang	2018	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC AH-006	Fuyang	2018	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC AH-008	Fuyang	2018	4472	I	C	C	T	C	T	T	001011	11	odd	G	A	C
ICDC AH-010	Fuyang	2018	4572	I	C	C	T	C	T	T	001011	11	odd	G	A	C

The result information for columns 1–5 in the table comes from reference Zhao et al. (2013a), 2013b, Zhao et al. (2019b).

[a]ATCC29342 (GenBank: U00089.2) was used as reference. The number after MPN is the gene number, and the superscript number indicates the position of SNP in the gene.

Table 4. SNP genotyping of 141 Chinese *M. pneumoniae* isolates, macrolide susceptibility test results, and mimic genotyping of 79 international *M. pneumoniae*

			Genotype 1					Genotype 2			23S rRNA			
	Year	No	SNP1	SNP3	SNP11	SNP15	SNP27	SNP0	SNP26	SNP32	A2063G	A3064G	A2063T	None
Beijing, China	2008–2012	30	2	2	3	6	7	7	0	3	17	2	1	10
Beijing, China	2017–2018	30	0	0	4	11	8	6	1	0	23	0	0	7
Soochow, China	2017–2018	23	0	2	14	5	0	2	0	0	21	0	0	2
Jinan, China	2017–2018	21	0	1	6	1	2	9	2	0	10	1	0	10
Jilin, China	2017–2018	30	0	1	3	7	19	0	0	0	30	0	0	0
Fuyang, China	2017–2018	7	0	1	4	0	0	2	0	0	5	0	0	2
Total	2008–2018	141	2	7	34	30	36	26	3	3	106	3	1	31
International strains	–	79	14	17	4	7	7	30	0	0	–	–	–	–

Detailed information on each *M. pneumoniae* strain is shown inTable 3.

the single base pair extension technique have proven to be superior in detecting more than ten SNP sites and are extensively utilized in the genotyping and identification of multiple microorganisms(Jang and Kim, 2018; Kostrzewa, 2018; Peng et al., 2013a, 2013b;Rahi et al., 2016;Sjoholm et al., 2008).

To date, there is still a lack of efficiency regarding genotyping techniques for *M. pneumoniae* compared with other pathogens. Currently, the most commonly utilized methods are based on the genotyping technique for the two genotypes. These methods are easy to perform, but their application in tracing pathogenic bacteria is hampered due to insufficient discriminatory capacity. In 2009, Degrange et al. established an MLVA genotyping technique using 5 VNTR sites, which divided *M. pneumoniae* into dozens of MLVA genotypes. However, the classification ability of this method is still limited in practical applications (Benitez et al., 2012;Degrange et al., 2009;Dumke and Jacobs, 2011;Kubota et al., 2015;Pereyre et al., 2013;Qu et al., 2013; Waller et al., 2014;Xue et al., 2014;Yan et al., 2014;Zhao et al., 2013a, 2013b). In 2015, Brown et al. (Brown et al., 2015) established a genotyping technique of MLST. MLST improves the typing ability to a certain extent, but such a technique is not extensively utilized, as it is technically demanding and expensive. In addition, there is a necessity for data uploading. In the same year, Touati et al. developed SNaPshot minisequencing technology based on the single base extension of a specially designed minisequencing primer, which promoted the discrimination of *M. pneumoniae*. However, the cost was still high, even though it was less than that presented in a previous study (4.2 Euro) (Touati et al., 2015). In this study, 6 SNP genotyping sites, which were derived from 1434 SNPs (Table S1) screened from 20 *M. pneumoniae* clinical isolates, were utilized as the targets. In addition, all three sites associated with ML susceptibility were included, and then MS-based amplification was established that could simultaneously accomplish *M. pneumoniae* genotyping of multiple sites and ML susceptibility analysis, which could test 96 samples simultaneously within 6 hr based on PCR amplification and extension (4.5 hr) and MS analysis (0.5 hr). The cost for MS was only 1.0 RMB (approximately 0.1 Euro), which was far less than that reported by Touati (Touati et al., 2015) Meanwhile, our method could provide more data on the ML susceptibility gene based on genotyping. To the best of our knowledge, this method provides the highest throughput for the *M. pneumoniae* genotyping and ML susceptibility gene testing and involves less time and expenditure than the current methods.

The selected SNP site, MPN372^{1112} (*G1112T*), was closely related to the two genotypes of *M. pneumoniae*, with T and G for genotype I and genotype II, respectively. According to the data interpretation standard, stains with an even number SNP genotype were classified into genotype II, while those with an odd number SNP genotype were classified into genotype I (Table S2). The SNP genotyping of the 141 Chinese isolates showed complete consistency, among which 109 genotype I strains showed an odd number SNP genotype and the 32 genotype II strains showed an even number SNP genotype. Moreover, for the 79 international strains, genome sequencing was consistent with the above rules (Table 3). This indicated that our method could improve the discriminatory capacity and present the genotyping traits of the two conventional genotypes of *M. pneumoniae*. For the detection of ML susceptibility sites of 23S rRNA^{2063}, 23S rRNA^{2064} and 23S rRNA^{20617}, 106 isolates showed *A2063G*, 3 showed *A2064G*, 1 shown*A2063T*, and 31 showed no mutations at the 2063/2064 sites. SNP ML susceptibility findings were consistent with the strain sequencing and ML sensitivity test findings (Table 3). The SNP genotyping results indicated that there were 4–7 SNP genotypes in the five selected cities (Table 4). There were

differences in the SNP genotypes in different regions between 2017 and 2018. Specifically, SNP27 was the predominant type in Jilin City in northern China, while SNP14 was found in Suzhou in southern China, SNP15 in Beijing in central China, and SNP0 in Jinan in the mid-eastern part of China. The differences in SNP types for the same region in different years were relatively small. For instance, the genotype I *M. pneumoniae* strains between 2008 and 2012 as well as 2017 and 2018 in Beijing were predominantly of the SNP15 and SNP27 types, while for genotype II, SNP0 was the predominant type. For the 79 international strains, there were 6 SNP genotypes, among which 49 genotype I *M. pneumoniae* strains showed an odd number SNP type and 30 genotype II *M. pneumoniae* strains showed an even number SNP type. The international strains with an odd number SNP type were predominantly SNP1 and SNP3, while those of the Chinese strains were SNP11, SNP15, and SNP27, yielding a large variance. This further confirmed the discriminatory capacity of our method.

In addition to genotyping and the ML susceptibility test with pure culture *M. pneumoniae* nucleic acids, our method could be utilized for the analysis of nucleic acids derived from clinical specimens. The detection limit of the clinical specimens was 5.2×10^2 copies/reaction, which was relatively consistent with that of nucleic acids isolated from the purified *M. pneumoniae* strains (10^2-10^3copies/reaction). The detection limit was on the same order of magnitude (10^2 copies/reaction), which was similar to that of the multiplex PCR. The genotyping and ML susceptibility positive rate of the 30 clinical isolates with nucleic acid load after real-time PCR was 83.3%. This implied that our method contributed to the genotyping of the clinical isolates and the ML susceptibility test, which was appropriate for the rapid genotyping of *M. pneumoniae* with a slow growth and rapid detection of ML susceptibility.

In this study, we developed a multiplex PCR coupled with the MALDI-TOF MS method for the multisite genotyping and ML susceptibility gene testing of *M. pneumoniae*. The method was easy to perform with a high specificity and a low cost as well as a high throughput. Multisite genotyping and ML susceptibility gene testing could be accomplished simultaneously, with a strong discriminatory capacity for strains of genotype I and genotype II. The analysis of the ML susceptibility site was comprehensive and accurate and deserves clinical application. Meanwhile, the PCR-MALDI-TOF MS technique could meet the demands of various regimens, which may contribute to the microbial identification, genotyping, and drug susceptibility testing.

Limitations of the study

The sensitivity of the method constructed in this study is limited by the detection limit of multiplex PCR technology.

Resource availability

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Prof. Di Xiao, xiaodi@icdc.cn.

Material availability

This study did not generate nor use any new or unique reagents.

Data and code availability

The raw data of MALDI-TOF MS of all the samples used in this study are available on the project homepage at https://dx.doi.org/10.17632/b8pnzp2y6c.1.

METHODS

All methods can be found in the accompanying Transparent methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102447.

ACKNOWLEDGMENTS

This study was funded by the National Natural Science Foundation of China (Grant No. 81772154), and The Major Infectious Diseases Such as AIDS and Viral Hepatitis Prevention and Control Technology Major Projects (Grant No. 2018ZX10712001&2018ZX10733402).

AUTHOR CONTRIBUTIONS

Z.F. and X.D. conceived and managed the project, analyzed the data, and wrote the manuscript. Z.J.Z searched the literature. W.X.M. acquired the MS data. L.L.Y. and G.J. operated the experiments. Z.Z.X. helped design the primers. H.L.H. and M.F.L. cultured the bacteria.

DECLARATION OF INTERESTS

The authors declare no conflicts of interests.

Received: January 14, 2021
Revised: March 20, 2021
Accepted: April 14, 2021
Published: May 21, 2021

REFERENCES

Benitez, A.J., Diaz, M.H., Wolff, B.J., Pimentel, G., Njenga, M.K., Estevez, A., and Winchell, J.M. (2012). Multilocus variable-number tandem-repeat analysis of Mycoplasma pneumoniae clinical isolates from 1962 to the present: a retrospective study. J. Clin. Microbiol. *50*, 3620–3626.

Blystad, H., Anestad, G., Vestrheim, D.F., Madsen, S., and Ronning, K. (2012). Increased incidence of Mycoplasma pneumoniae infection in Norway 2011. Euro Surveill. *17*, 20074.

Brown, R.J., Holden, M.T., Spiller, O.B., and Chalker, V.J. (2015). Development of a multilocus sequence typing scheme for molecular typing of mycoplasma pneumoniae. J. Clin. Microbiol. *53*, 3195–3203.

Cao, B., Zhao, C.J., Yin, Y.D., Zhao, F., Song, S.F., Bai, L., Zhang, J.Z., Liu, Y.M., Zhang, Y.Y., Wang, H., and Wang, C. (2010). High prevalence of macrolide resistance in Mycoplasma pneumoniae isolates from adult and adolescent patients with respiratory tract infection in China. Clin.Infect. Dis. *51*, 189–194.

Chalker, V., Stocki, T., Mentasti, M., Fleming, D., and Harrison, T. (2011). Increased incidence of Mycoplasma pneumoniae infection in England and Wales in 2010: multiocus variable number tandem repeat analysis typing and macrolide susceptibility. Euro Surveill. *16*, 19865.

Degrange, S., Cazanave, C., Charron, A., Renaudin, H., Bebear, C., and Bebear, C.M. (2009). Development of multiple-locus variable-number tandem-repeat analysis for molecular typing of Mycoplasma pneumoniae. J. Clin. Microbiol. *47*, 914–923.

Dorigo-Zetsma, J.W., Dankert, J., and Zaat, S.A. (2000). Genotyping of Mycoplasma pneumoniae clinical isolates reveals eight P1 subtypes within two genomic groups. J. Clin. Microbiol. *38*, 965–970.

Dumke, R., and Jacobs, E. (2011). Culture-independent multi-locus variable-number tandem-repeat analysis (MLVA) of Mycoplasma pneumoniae. J. Microbiol.Methods *86*, 393–396.

Gadsby, N.J., Reynolds, A.J., McMenamin, J., Gunson, R.N., McDonagh, S., Molyneaux, P.J., Yirrell, D.L., and Templeton, K.E. (2012). Increased reports of Mycoplasma pneumoniae from laboratories in Scotland in 2010 and 2011 - impact of the epidemic in infants. Euro Surveill. *17*, 20110.

Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B.C., and Herrmann, R. (1996). Complete sequence analysis of the genome of the bacterium Mycoplasma pneumoniae. Nucleic Acids Res. *24*, 4420–4449.

Hong, K.B., Choi, E.H., Lee, H.J., Lee, S.Y., Cho, E.Y., Choi, J.H., Kang, H.M., Lee, J., Ahn, Y.M., Kang, Y.H., and Lee, J.H. (2013). Macrolide resistance of Mycoplasma pneumoniae, South Korea, 2000-2011. Emerg.Infect. Dis. *19*, 1281–1284.

Jacobs, E., Ehrhardt, I., and Dumke, R. (2015). New insights in the outbreak pattern of Mycoplasma pneumoniae. Int. J. Med. Microbiol. *305*, 705–708.

Jang, K.S., and Kim, Y.H. (2018). Rapid and robust MALDI-TOF MS techniques for microbial identification: a brief overview of their diverse applications. J. Microbiol. *56*, 209–216.

Kawai, Y., Miyashita, N., Kubo, M., Akaike, H., Kato, A., Nishizawa, Y., Saito, A., Kondo, E., Teranishi, H., Wakabayashi, T., et al. (2013). Nationwide surveillance of macrolide-resistant Mycoplasma pneumoniae infection in pediatric patients. Antimicrob. Agents Chemother. *57*, 4046–4049.

Kostrzewa, M. (2018). Application of the MALDI Biotyper to clinical microbiology: progress and potential. Expert Rev. Proteomics *15*, 193–202.

Kubota, H., Okuno, R., Hatakeyama, K., Uchitani, Y., Sadamasu, K., Hirai, A., and Kai, A. (2015). Molecular typing of Mycoplasma pneumoniae isolated from pediatric patients in Tokyo, Japan. Jpn. J.Infect. Dis. *68*, 76–78.

Kuglik, P., Smetana, J., Vallova, V., Moukova, L., Kasikova, K., Cvanova, M., and Brozova, L. (2014). Genome-wide screening of DNA copy number alterations in cervical carcinoma patients with CGH+SNP microarrays and HPV-FISH. Int. J. Clin. Exp. Pathol. *7*, 5071–5082.

Lenglet, A., Herrador, Z., Magiorakos, A.P., Leitmeyer, K., Coulombier, D., and European Working Group on Mycoplasma pneumoniae, S. (2012). Surveillance status and recent data for mycoplasma pneumoniae infections in the European union and European economic area, January 2012. Euro Surveill. *17*, 20075.

Linde, A., Ternhag, A., Torner, A., and Claesson, B. (2012). Antibiotic prescriptions and laboratory-confirmed cases of Mycoplasma pneumoniae during the epidemic in Sweden in 2011. Euro Surveill. *17*, 20082.

Lipworth, S., Jajou, R., de Neeling, A., Bradley, P., van der Hoek, W., Maphalala, G., Bonnet, M., Sanchez-Padilla, E., Diel, R., Niemann, S., et al. (2019). SNP-IT tool for identifying subspecies and associated lineages of Mycobacterium tuberculosis complex. Emerg.Infect. Dis. *25*, 482–488.

Liu, Y., Ye, X., Zhang, H., Xu, X., Li, W., Zhu, D., and Wang, M. (2009). Antimicrobial susceptibility of Mycoplasma pneumoniae isolates and molecular analysis of macrolide-resistant strains from Shanghai, China. Antimicrob. Agents Chemother. *53*, 2160–2162.

Liu, Y., Ye, X., Zhang, H., Wu, Z., and Xu, X. (2014). Rapid detection of Mycoplasma pneumoniae and its macrolide-resistance mutation by Cycleave PCR. Diagn. Microbiol. Infect. Dis. *78*, 333–337.

Liu, T.Y., Lee, W.J., Tsai, C.M., Kuo, K.C., Lee, C.H., Hsieh, K.S., Chang, C.H., Su, Y.T., Niu, C.K., and Yu, H.R. (2018). Serum lactate dehydrogenase isoenzymes 4 plus 5 is a better biomarker than total lactate dehydrogenase for refractory Mycoplasma pneumoniae pneumonia in children. Pediatr.Neonatol. *59*, 501–506.

Lluch-Senar, M., Cozzuto, L., Cano, J., Delgado, J., Llorens-Rico, V., Pereyre, S., Bebear, C., and Serrano, L. (2015). Comparative "-omics" in mycoplasma pneumoniae clinical isolates reveals key virulence factors. PLoS One *10*, e0137354.

Loens, K., Goossens, H., and Ieven, M. (2010). Acute respiratory infection due to Mycoplasma pneumoniae: current status of diagnostic methods. Eur. J. Clin. Microbiol. Infect. Dis. *29*, 1055–1069.

Matsuoka, M., Narita, M., Okazaki, N., Ohya, H., Yamazaki, T., Ouchi, K., Suzuki, I., Andoh, T., Kenri, T., Sasaki, Y., et al. (2004). Characterization and molecular analysis of macrolide-resistant Mycoplasma pneumoniae clinical isolates obtained in Japan. Antimicrob. Agents Chemother. *48*, 4624–4630.

Nummi, M., Mannonen, L., and Puolakkainen, M. (2015). Development of a multiplex real-time PCR assay for detection of Mycoplasma pneumoniae, Chlamydia pneumoniae and mutations associated with macrolide resistance in Mycoplasma pneumoniae from respiratory clinical specimens. Springerplus *4*, 684.

Okumura, T., Kawada, J.I., Tanaka, M., Narita, K., Ishiguro, T., Hirayama, Y., Narahara, S., Tsuji, G., Sugiyama, Y., Suzuki, M., et al. (2019). Comparison of high-dose and low-dose corticosteroid therapy for refractory Mycoplasma pneumoniae pneumonia in children. J.Infect. Chemother. *25*, 346–350.

Peng, J., Gao, L., Guo, J., Wang, T., Wang, L., Yao, Q., Zhu, H., and Jin, Q. (2013a). Type-specific detection of 30 oncogenic human papillomaviruses by genotyping both E6 and L1 genes. J. Clin. Microbiol. *51*, 402–408.

Peng, J., Yang, F., Xiong, Z., Guo, J., Du, J., Hu, Y., and Jin, Q. (2013b). Sensitive and rapid detection of viruses associated with hand foot and mouth disease using multiplexed MALDI-TOF analysis. J. Clin. Virol. *56*, 170–174.

Pereyre, S., Touati, A., Petitjean-Lecherbonnier, J., Charron, A., Vabret, A., and Bebear, C. (2013). The increased incidence of Mycoplasma pneumoniae in France in 2011 was polyclonal, mainly involving M. pneumoniae type 1 strains. Clin. Microbiol. Infect. *19*, E212–E217.

Pereyre, S., Goret, J., and Bebear, C. (2016). Mycoplasma pneumoniae: current knowledge on macrolide resistance and treatment. Front. Microbiol. *7*, 974.

Peuchant, O., Menard, A., Renaudin, H., Morozumi, M., Ubukata, K., Bebear, C.M., and Pereyre, S. (2009). Increased macrolide resistance of Mycoplasma pneumoniae in France directly detected in clinical specimens by real-time PCR and melting curve analysis. J. Antimicrob. Chemother. *64*, 52–58.

Qu, J., Yu, X., Liu, Y., Yin, Y., Gu, L., Cao, B., and Wang, C. (2013). Specific multilocus variable-number tandem-repeat analysis genotypes of Mycoplasma pneumoniae are associated with diseases severity and macrolide susceptibility. PLoS One *8*, e82174.

Rahi, P., Prakash, O., and Shouche, Y.S. (2016). Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) based microbial identifications: challenges and scopes for microbial ecologists. Front. Microbiol. *7*, 1359.

Sjoholm, M.I., Dillner, J., and Carlson, J. (2008). Multiplex detection of human herpesviruses from archival specimens by using matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. *46*, 540–545.

Spuesens, E.B., Hoogenboezem, T., Sluijter, M., Hartwig, N.G., van Rossum, A.M., and Vink, C. (2010). Macrolide resistance determination and molecular typing of Mycoplasma pneumoniae by pyrosequencing. J. Microbiol.Methods *82*, 214–222.

Touati, A., Blouin, Y., Sirand-Pugnet, P., Renaudin, H., Oishi, T., Vergnaud, G., Bebear, C., and Pereyre, S. (2015). Molecular epidemiology of mycoplasma pneumoniae: genotyping using single nucleotide polymorphisms and SNaPshot technology. J. Clin. Microbiol. *53*, 3182–3194.

Uldum, S.A., Bangsborg, J.M., Gahrn-Hansen, B., Ljung, R., Molvadgaard, M., Fons Petersen, R., and Wiid Svarrer, C. (2012). Epidemic of Mycoplasma pneumoniae infection in Denmark, 2010 and 2011. Euro Surveill. *17*, 20073.

Waites, K.B., Crabb, D.M., and Duffy, L.B. (2009). Comparative in vitro susceptibilities of human mycoplasmas and ureaplasmas to a new investigational ketolide, CEM-101. Antimicrob. Agents Chemother. *53*, 2139–2141.

Waites, K.B., Xiao, L., Liu, Y., Balish, M.F., and Atkinson, T.P. (2017). Mycoplasma pneumoniae from the respiratory tract and beyond. Clin.Microbiol. Rev. *30*, 747–809.

Waller, J.L., Diaz, M.H., Petrone, B.L., Benitez, A.J., Wolff, B.J., Edison, L., Tobin-D'Angelo, M., Moore, A., Martyn, A., Dishman, H., et al. (2014). Detection and characterization of Mycoplasma pneumoniae during an outbreak of respiratory illness at a university. J. Clin. Microbiol. *52*, 849–853.

Wolff, B.J., Thacker, W.L., Schwartz, S.B., and Winchell, J.M. (2008). Detection of macrolide resistance in Mycoplasma pneumoniae by real-time PCR and high-resolution melt analysis. Antimicrob. Agents Chemother. *52*, 3542–3549.

Xiao, L., Ptacek, T., Osborne, J.D., Crabb, D.M., Simmons, W.L., Lefkowitz, E.J., Waites, K.B., Atkinson, T.P., and Dybvig, K. (2015). Comparative genome analysis of Mycoplasma pneumoniae. BMC Genomics *16*, 610.

Xin, D., Mi, Z., Han, X., Qin, L., Li, J., Wei, T., Chen, X., Ma, S., Hou, A., Li, G., and Shi, D. (2009). Molecular mechanisms of macrolide resistance in clinical isolates of Mycoplasma pneumoniae from China. Antimicrob. Agents Chemother. *53*, 2158–2159.

Xue, G., Wang, Q., Yan, C., Jeoffreys, N., Wang, L., Li, S., Gilbert, G.L., and Sun, H. (2014). Molecular characterizations of PCR-positive Mycoplasma pneumoniae specimens collected from Australia and China. J. Clin. Microbiol. *52*, 1478–1482.

Yan, C., Sun, H., Xue, G., Zhao, H., Wang, L., Feng, Y., and Li, S. (2014). A single-tube multiple-locus variable-number tandem-repeat analysis of Mycoplasma pneumoniae clinical specimens by use of multiplex PCR-capillary electrophoresis. J. Clin. Microbiol. *52*, 4168–4171.

Zhao, F., Lv, M., Tao, X., Huang, H., Zhang, B., Zhang, Z., and Zhang, J. (2012). Antibiotic sensitivity of 40 Mycoplasma pneumoniae isolates and molecular analysis of macrolide-resistant isolates from Beijing, China. Antimicrob. Agents Chemother. *56*, 1108–1109.

Zhao, F., Liu, G., Cao, B., Wu, J., Gu, Y., He, L., Meng, F., Zhu, L., Yin, Y., Lv, M., and Zhang, J. (2013a). Multiple-locus variable-number tandem-repeat analysis of 201 Mycoplasma pneumoniae isolates from Beijing, China, from 2008 to 2011. J. Clin. Microbiol. *51*, 636–639.

Zhao, F., Liu, G., Wu, J., Cao, B., Tao, X., He, L., Meng, F., Zhu, L., Lv, M., Yin, Y., and Zhang, J. (2013b). Surveillance of macrolide-resistant mycoplasma pneumoniae in Beijing, China, from 2008 to 2012. Antimicrob. Agents Chemother. *57*, 1521–1523.

Zhao, F., Liu, L., Tao, X., He, L., Meng, F., and Zhang, J. (2015). Culture-Independent detection and genotyping of mycoplasma pneumoniae in clinical specimens from Beijing, China. PLoS One *10*, e0141702.

Zhao, F., Liu, J., Shi, W., Huang, F., Liu, L., Zhao, S., and Zhang, J. (2019a). Antimicrobial susceptibility and genotyping of Mycoplasma pneumoniae isolates in Beijing, China, from 2014 to 2016. Antimicrob. Resist. Infect. Control *8*, 18.

Zhao, F., Li, J., Liu, J., Guan, X., Gong, J., Liu, L., He, L., Meng, F., and Zhang, J. (2019b). Antimicrobial susceptibility and molecular characteristics of Mycoplasma pneumoniae isolates across different regions of China. Antimicrob. Resist. Infect. Control *8*, 143.

iScience, Volume *24*

Supplemental information

A multisite SNP genotyping and macrolide susceptibility gene method for *Mycoplasma pneumoniae* based on MALDI-TOF MS

Fei Zhao, Jianzhong Zhang, Xuemei Wang, Liyong Liu, Jie Gong, Zhixiang Zhai, Lihua He, Fanliang Meng, and Di Xiao

Supplemental information

Table S2. Detailed list of 64 SNP types binary-decimal conversion.

ATCC29342 (GenBank: U00089.2) was used as reference. The number after MPN is the gene number, and the superscript number indicate the position of SNP in the gene.

Transparent Methods

Table S2. Detailed list of 64 SNP types binary-decimal conversion, related to table 2

Six SNPs	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP results	Aligned result of SNPs	SNP type (binary-decimal)
SNP site[a]	MPN114[1461]	MPN126[470]	MPN213[47]	MPN262[192]	MPN280[1641]	MPN372[1112]			
Type of SNP	C/T	C/T	G/T	C/T	G/T	G/T			
SNP 0	C	C	G	C	G	G	CCGCGG	000000	0
SNP 1	C	C	G	C	G	T	CCGCGT	000001	1
SNP 2	C	C	G	C	T	G	CCGCTG	000010	2
SNP 3	C	C	G	C	T	T	CCGCTT	000011	3
SNP 4	C	C	G	T	G	G	CCGTGG	000100	4
SNP 5	C	C	G	T	G	T	CCGTGT	000101	5
SNP 6	C	C	G	T	T	G	CCGTTG	000110	6
SNP 7	C	C	G	T	T	T	CCGTTT	000111	7
SNP 8	C	C	T	C	G	G	CCTCGG	001000	8
SNP 9	C	C	T	C	G	T	CCTCGT	001001	9
SNP 10	C	C	T	C	T	G	CCTCTG	001010	10
SNP 11	C	C	T	C	T	T	CCTCTT	001011	11
SNP 12	C	C	T	T	G	G	CCTTGG	001100	12
SNP 13	C	C	T	T	G	T	CCTTGT	001101	13
SNP 14	C	C	T	T	T	G	CCTTTG	001110	14
SNP 15	C	C	T	T	T	T	CCTTTT	001111	15
SNP 16	C	T	G	C	G	G	CTGCGG	010000	16
SNP 17	C	T	G	C	G	T	CTGCGT	010001	17
SNP 18	C	T	G	C	T	G	CTGCTG	010010	18
SNP 19	C	T	G	C	T	T	CTGCTT	010011	19
SNP 20	C	T	G	T	G	G	CTGTGG	010100	20
SNP 21	C	T	G	T	G	T	CTGTGT	010101	21
SNP 22	C	T	G	T	T	G	CTGTTG	010110	22
SNP 23	C	T	G	T	T	T	CTGTTT	010111	23
SNP 24	C	T	T	C	G	G	CTTCGG	011000	24
SNP 25	C	T	T	C	G	T	CTTCGT	011001	25
SNP 26	C	T	T	C	T	G	CTTCTG	011010	26
SNP 27	C	T	T	C	T	T	CTTCTT	011011	27
SNP 28	C	T	T	T	G	G	CTTTGG	011100	28
SNP 29	C	T	T	T	G	T	CTTTGT	011101	29
SNP 30	C	T	T	T	T	G	CTTTTG	011110	30
SNP 31	C	T	T	T	T	T	CTTTTT	011111	31
SNP 32	T	C	G	C	G	G	TCGCGG	100000	32
SNP 33	T	C	G	C	G	T	TCGCGT	100001	33

SNP 34	T	C	G	C	T	G	TCGCTG	100010	34
SNP 35	T	C	G	C	T	T	TCGCTT	100011	35
SNP 36	T	C	G	T	G	G	TCGTGG	100100	36
SNP 37	T	C	G	T	G	T	TCGTGT	100101	37
SNP 38	T	C	G	T	T	G	TCGTTG	100110	38
SNP 39	T	C	G	T	T	T	TCGTTT	100111	39
SNP 40	T	C	T	C	G	G	TCTCGG	101000	40
SNP 41	T	C	T	C	G	T	TCTCGT	101001	41
SNP 42	T	C	T	C	T	G	TCTCTG	101010	42
SNP 43	T	C	T	C	T	T	TCTCTT	101011	43
SNP 44	T	C	T	T	G	G	TCTTGG	101100	44
SNP 45	T	C	T	T	G	T	TCTTGT	101101	45
SNP 46	T	C	T	T	T	G	TCTTTG	101110	46
SNP 47	T	C	T	T	T	T	TCTTTT	101111	47
SNP 48	T	T	G	C	G	G	TTGCGG	110000	48
SNP 49	T	T	G	C	G	T	TTGCGT	110001	49
SNP 50	T	T	G	C	T	G	TTGCTG	110010	50
SNP 51	T	T	G	C	T	T	TTGCTT	110011	51
SNP 52	T	T	G	T	G	G	TTGTGG	110100	52
SNP 53	T	T	G	T	G	T	TTGTGT	110101	53
SNP 54	T	T	G	T	T	G	TTGTTG	110110	54
SNP 55	T	T	G	T	T	T	TTGTTT	110111	55
SNP 56	T	T	T	C	G	G	TTTCGG	111000	56
SNP 57	T	T	T	C	G	T	TTTCGT	111001	57
SNP 58	T	T	T	C	T	G	TTTCTG	111010	58
SNP 59	T	T	T	C	T	T	TTTCTT	111011	59
SNP 60	T	T	T	T	G	G	TTTTGG	111100	60
SNP 61	T	T	T	T	G	T	TTTTGT	111101	61
SNP 62	T	T	T	T	T	G	TTTTTG	111110	62
SNP 63	T	T	T	T	T	T	TTTTTT	111111	63

[a] ATCC29342 (GenBank: U00089.2) was used as reference. The number after MPN is the gene number, and the superscript number indicate the position of SNP in the gene.

Transparent Methods

M. pneumoniae strains and clinical samples

Ten epidemiologically unrelated *M. pneumoniae* isolates (derived from different geographical locations and isolated in different years) were selected to construct the genotyping and ML susceptibility detection methods The genotyping and ML susceptibility are shown in Table 1. Twenty other pathogens, including *M. genitalium* (ATCC 33530), *M. salivarium* (ATCC 23064), *M. orale* (ATCC 23714), *M. faucium* (ATCC 25293), *Ureaplasma urealyticum* (ATCC 27618), *U. parvum* (ATCC 27813), *M. fermentans* (ATCC 19989), *M. hominis* (ATCC 23114), *M. penetrans* (ATCC 55252), *M. hyorhinis* (ATCC17981), *M. pirum* (ATCC25960), *Escherichia coli* (ATCC 11229), *Streptococcus pneumoniae* (ATCC 49619), *S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 35984), *Legionella pneumophila* (clinical isolate), *M. tuberculosis* (clinical isolate), *Pseudomonas aeruginosa* (clinical isolate), *Haemophilus influenzae* (clinical isolate) and *Neisseria meningitides* (clinical isolate) were selected for specific validation.

A total of 141 *M. pneumoniae* clinical isolates obtained from 5 cities (Beijing, 60 strains; Jinlin, 30 strains; Jinan, 21 strains; Suzhou, 24 strains; and Fuyang, 7 strains) between 2008 and 2018 were used for the evaluation **(**Table 2). M129 served as the reference strain. Then 30 oropharyngeal swabs and BALF samples that were positive for *M. pneumoniae* nucleic acid were used for the validation of the clinical samples. All DNA extraction from the strains and clinical swab specimens was conducted using the QIAamp DNA Mini Kit (No.51306) according to the manufacturer's instructions.

Target gene selection for genotyping and ML susceptibility and design of primers and extension the mass probe

Six SNP target genes were chosen for genotyping based on our previous work, namely, MPN114^{1461} (C1461T), MPN126^{470} (C470T), MPN213^{47} (G47T), MPN262^{192} (C192T), MPN280^{1641} (G1641T), and MPN372^{1112} (G1112T). Three SNP sites from 23S rRNA, namely, 23S rRNA^{2063}, 23S rRNA ^{2064} and 23S rRNA ^{2617}, served as the target sites for ML susceptibility. Initially, BLAST software was used for gene alignment, and the conserved segment was analyzed using BatchPrimer3 (version 1.0) software (https://wheat.pw.usda.gov/demos/BatchPrimer3/) for the design of primers for eight-plex PCR. Among them, the same pair of primers was designed for the amplification of the 23S rRNA^{2063} and 23S rRNA ^{2064} sites. To obtain a molecular weight of >9 kDa for the primers, a 10 nt sequence (acgttggatg) was added to the 5' end of each primer, which extended the scale for the quality extension of the mass probe for the SNP. Subsequently, IntelliBio genetic locus analysis software (V2.0, IntelliBio, China) was utilized for the extension of the mass probe (Table 4). The sequences of the probes met the following conditions: (i) no dimer formation, including hairpin structures and dimmers, between the primers; (ii) no dimer formation at the 3' termini with a length of 5 bp or more; (iii) an annealing temperature in the range of 45-65 ℃; (iv) a molecular weight in the range of 4k-9kDa and a primer length of 17-28 bp; (v) a GC content in the range of 30%-70%; and (vi) a minimal difference between the probe molecular weights of 16Da.

PCR-MALDI-TOF MS

Nucleic acids from 10 *M. pneumoniae* strains (12-83 ng/μL, Qubit 3.0) were utilized (Table 1). Nucleic acid-free water served as the blank control. For multiplex PCR amplification, the total volume was 5 μL containing 1 μL DNA template, 2.0 μL PCR buffer (Intelligene Biosystems, Qingdao, China), and 1.0 μL PCR primer mixture. The amplification conditions were as follows: 95 ℃ for 15 min, followed by 30 cycles

of 95 ℃ for 15 sec, 59 ℃ for 30 sec, and 72 ℃ for 30 sec. The PCR products were digested using shrimp alkaline phosphatase (SAP) to eliminate the free dNTPs. Afterwards, each PCR tube was added to 2 μL SAP (Intelligene Biosystems, Qingdao, China). Then, the mixture was subjected to PCR amplification under the following conditions: 37 ℃ for 40 min, followed by 85 ℃ for 5 min and 4 ℃ for the maintenance.

For the mass probe extension (MPE), a buffer solution of 4 μL was prepared, including 1 μL E-ddNTP, 1.4 μL MPE buffer, 0.6 μL MPE enzyme, and 1.0 μL primer. In each tube containing SAP-treated products, a 4 μL MPE mixture was added followed by vortexing. The PCR conditions were as follows: 95 ℃ for 30 min; followed by 95 ℃ for 5 sec, and 5 cycles of 52 ℃ for 5 sec and 80 ℃ for 5 sec, 40 cycles of 95 ℃ for 5 sec and 5 cycles; and a final extension at 72 ℃ for 3 min. Then, single base pair extension was performed.

For salt purification, 14 μL ultrapure water and resin were added to each well, followed by vortexing for 30 min. Then the supernatant was collected for testing after centrifugation.

For SNP identification and data analysis by MALDI-TOF MS, 3-hydroxypyridine-2-carboxylic acid (3-HPA, 0.9 μL) was dropped at the center of the sample target. After drying, the matrix was covered with 0.3 μL purified supernatant. Then, the samples were subjected to testing after crystallization. The data were acquired from the QuanTOF I system (Intelligene Biosystems, Qingdao, China). The parameters were as follows: data acquisition mode was positive ion, with a mass range of 4000-9000 Da. The focus mass was 4000 Da, and the pulse frequency was 2000 Hz. The detector voltage was -0.53 kV, and the extraction voltage was -3.45 kV. The Laser pulse energy was 25 μJ. A QuanSNP system (V1.0, IntelliBio, China) was used for the analysis of the extension results of the samples.

Determination of the detection limit and specificity

The lowest detection limit (LDL) was confirmed based on the diluted nucleic acid of *M. pneumoniae* (M129) strains (10^0-10^5copies/μl). For each concentration, the test was performed twice. The specificity was validated by quantitative analysis of nucleic acids with a concentration range of 6.3 ng/μl to 221.0 ng/μl derived from 20 non-*M. pneumoniae* pathogen.

Data analysis

The SNP sites of 6 genotypes were aligned based on the MPN gene (i.e., MPN114, 126, 213, 262, 280, 372). For the SNPs, A/T was assigned a value of 1, and C/G was assigned a value of 0. On this basis, each SNP genotyped isolate was represented by six numbers consisting of "0" or "1" from 000000 to 111111. This SNP number was transformed into a decimal scale of 0-63 according to the binary system, yielding 64 genotypes in total. For the M129 strain containing 6 SNP sites of C/C/G/C/G/T, the number was 000001, which resulted in a score of 1 after transformation into a binary digit. Therefore, the M129 strain was defined as the SNP1 type. See Table S2 for specific SNP typing principles.

Nucleic acid validation of *M. pneumoniae* clinical isolates and specimens and mimic genotyping of the international *M. pneumoniae* genome

Genotyping and ML susceptibility analysis were performed for the nucleic acids of 141 clinical isolates to test the accuracy of the method. The nucleic acids were stored in the strain bank of the National Institute for Communicable Disease Control and Prevention Chinese Center for Disease Control and Prevention. The nucleic acid concentration of the *M. pneumoniae* isolates was 13-68 ng/μl (Qubit 3.0). The basic characteristics, genotype and drug susceptibility data of the strains are provided in Table 2. In addition, 30 *M. pneumoniae*-positive clinical specimens were selected for genotyping and drug susceptibility analysis. Multiplex PCR amplification for specimens was performed in a total volume of 20 μl containing 5 μl DNA

template. The nucleic acid load of *M. pneumoniae* clinical isolates was evaluated using real-time PCR (Zhao et al., 2012). Seventy-nine international *M. pneumoniae* stains with full sequences in the NCBI database were selected, and the database serial number was as follows: AP012303.1, AP017318.1, AP017319.1, CP002077.1, CP003913.2, CP008895.1, CP010538.1, CP010539.1, CP010540.1, CP010541.1, CP010542.1, CP010543.1, CP010544.1, CP010545.1, CP010546.1, CP010547.1, CP010548.1, CP010549.1, CP010550.1, CP010551.1, CP013829.1, CP014267.1, CP017327.1, CP017328.1, CP017329.1, CP017330.1, CP017331.1, CP017332.1, CP017333.1, CP017334.1, CP017335.1, CP017336.1, CP017337.1, CP017338.1, CP017339.1, CP017340.1, CP017341.1, CP017342.1, CP017343.1, CP020689.1, CP020690.1, CP020691.1, CP020692.1, CP020693.1, CP020710.1, CP020711.1, CP020712.1, CP039761.1, CP039762.1, CP039763.1, CP039764.1, CP039765.1, CP039766.1, CP039767.1, CP039768.1, CP039769.1, CP039770.1, CP039771.1, CP039772.1, CP039773.1, CP039774.1, CP039775.1, CP039776.1, CP039777.1, CP039778.1, CP039779.1, CP039780.1, CP039781.1, CP039782.1, CP039783.1, CP039784.1, CP039785.1, CP039786.1, CP039787.1, CP039788.1, CP039789.1, CP039790.1, LR214945.1 and U00089.2. Mimic genotyping was performed on the international strains for the 6 SNP sites to evaluate the discriminatory capacity of such genotyping methods for international strains.

REFERENCES

Zhao, F., Cao, B., He, L. H., Yin, Y. D., Tao, X. X., Song, S. F., Meng, F. L., and Zhang, J. Z. (2012). Evaluation of a new real-time PCR assay for detection of Mycoplasma pneumoniae in clinical specimens. Biomed Environ Sci. 25, 77-81.