

RESEARCH ARTICLE

Comparison of the molecular characteristics of *Mycoplasma pneumoniae* from children across different regions of China

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Abstract

Previous molecular characterization of *Mycoplasma pneumoniae* in China focused only on one or two cities. In this study, we characterized 835 samples from patients suspected to be infected with *M. pneumoniae*; these samples were collected in 2016 from pediatric patients from different regions of China. Multiple locus variable number tandem repeat analysis (MLVA), P1-restriction fragment length polymorphism (RFLP) analysis, and sequencing of the domain V of 23S rRNA were performed to compare genotype distribution across different locations. Two-hundred-and-thirteen samples tested positive for *M. pneumoniae* by PCR. P1 types were identified in 154 samples: 78.6% were type I and 21.4% were type II. Type I was the most prevalent genotype in five locations, except Nanjing where type II was the most common type ($p < 0.01$). Five distinct MLVA types were identified in the 172 samples. Genotype M4-5-7-2 was predominant at all locations, except Nanjing where type 3-5-6-2 was the most common ($p < 0.01$). Macrolide resistance-associated mutations were identified in 186 (76.3%) samples. The resistance rate differed with the location. This study showed that genotypes and macrolide resistance rate differed across China. The most prevalent genotype in China remains M4-5-7-2/P1-1. The resistance rate decreased, along with changes to the M4-5-7-2 genotype.

Introduction

Mycoplasma pneumoniae is a common cause of respiratory infections, accounting for approximately 10–30% of all cases of community-acquired pneumonia (CAP); the prevalence increased to 50% in recent years [1]. The clinical presentation of *M. pneumoniae* infection widely varies, ranging from self-limiting to severe pneumonia, with extrapulmonary complications in children and adults [2].

Competing interests: The authors have declared that no competing interests exist.

This pathogen often spreads slowly through close contact in families, and the epidemic occurs every 3–7 years. The most recent world epidemic was first reported in Northern Europe, followed by some Asian countries [3–5]. Since the end of 2015, an increase has been noted in the incidence of these infections across Japan, China, and England [6–8], although incidence has remained stable in other countries. This scenario raises a question whether this is an endemic infection restricted to some local countries or a global epidemic. Extensive data collection and detailed studies are necessary to answer this question.

Macrolides are the first line of antibiotics used to treat *M. pneumoniae* infections. However, the resistance rate dramatically increased in the recent years. In Asia, over 90% of the isolates were resistant to macrolide [9]. In the United States, the macrolide-resistance rate was around 10% [10]. The rate varied from 1% to 30% in European countries [11]. Molecular characterization is the best way to understand the mechanisms underlying *M. pneumoniae* macrolide resistance. Certain multiple locus variable number tandem repeat analysis (MLVA) types are known to be associated with macrolide resistance [12], and the genotypes and the extent of macrolide resistance differ across countries as well as within a country [13, 14]. In China, the molecular characterization of *M. pneumoniae* has extensively focused on data from Beijing and Shanghai [15, 16]; data from other areas are scarce.

During the second half of 2015, we observed an increase in the prevalence of *M. pneumoniae* in Beijing [7]. To better understand the molecular characteristics of this endemic, we performed a multicentric study to compare the samples from five different areas (including six cities) in China in 2016.

Materials and methods

Ethics statement

This study was performed in compliance with the Helsinki Declaration (Ethical Principles for Medical Research Involving Human Subjects), and was approved by the Research Board of the Ethics Committee of the Capital Institute of Pediatrics, Beijing, China. All patient information was anonymized, and therefore, informed consent was not required, per the guidelines of the Ethics Committee of the Institute.

Clinical specimens

From January to December 2016, clinical respiratory specimens were collected from 835 pediatric patients presenting with symptoms of respiratory tract infection (RTI), belonging to five different geographical locations in China. Two-hundred-and-forty-three samples were obtained from Beijing, which is located in mid-north China (63 sputum, 45 oropharyngeal swab, 122 bronchoalveolar lavage, 12 pleural fluid, and 1 puncture fluid samples); 100, from Shanghai, which is located in the east (all nasopharyngeal aspirate samples); 275, from Kunming, Yunnan province, which is in the south (121 sputum and 154 BAL samples); 140, from Harbin, Heilongjiang province, which is located in the north (all oropharyngeal swab samples); and 72 from Urumqi, Xinjiang province, which is in the west (62 sputum and 10 BAL samples). Moreover, we obtained 10 *M. pneumoniae*-positive DNA samples (isolated from 10 oropharyngeal swabs) from different pediatric patients at different admission times in Nanjing, which is close to Shanghai (Fig 1). All specimens used in this study were part of routine patient management without any additional collection, sample types were different by site due to distinct study protocols. Most cases were clinically diagnosed as pneumonia, followed by bronchitis, asthma, and respiratory tract infections, associated with central nervous system infections. DNA was extracted from clinical samples using the QIAamp DNA Mini Kit (Qiagen, German), and *M. pneumoniae* was detected by real-time PCR [17].

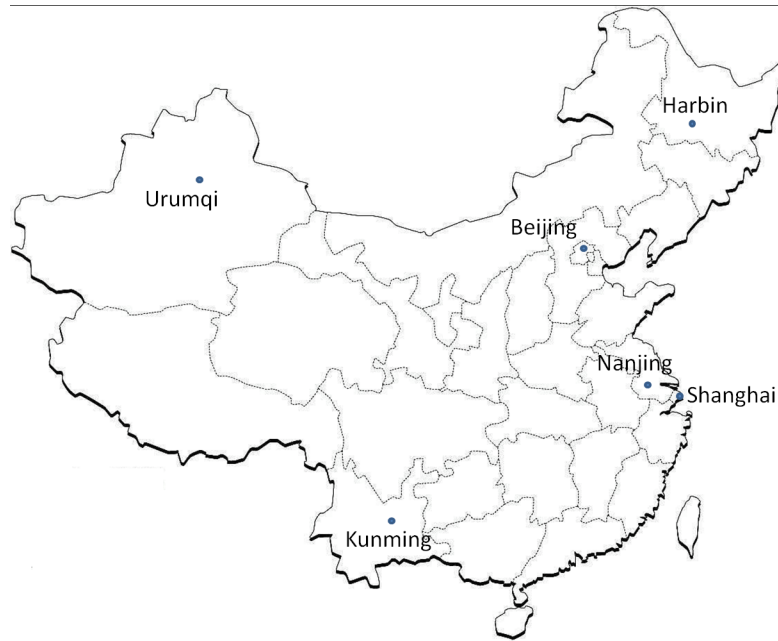


Fig 1. Locality map of different cities included in this study. A total of 835 samples from pediatric patients presenting with the symptoms of respiratory tract infection (RTI) belonging to five different geographical locations in China were collected from January to December 2016. Two-hundred-and-forty-three samples were from Beijing, which is located in mid-north China; 100, from Shanghai, located in the east; 275, from Kunming, Yunnan province, located in the south; 140, from Harbin, Heilongjiang province, located in the north; and 72, from Urumqi, Xinjiang province, located in the west. Ten *M. pneumoniae*-positive DNA samples isolated from 10 clinical specimens were collected from pediatric patients in Nanjing, which is close to Shanghai.

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P1 gene typing

Nested PCR-restriction fragment length polymorphism analysis was used for P1 genotyping, as described previously [15], by directly using the DNA extracted from PCR-positive specimens. In brief, to detect the RepMP4 region of the P1 gene, the ADH1/ADH2 primer pair was used in the first PCR, and the ADH1in/ADH1M and ADH2in/ADH2M primer pairs were designed and used in the second PCR. To detect the RepMp2/3 region, the ADH3/ADH4 primer pair was used for the first PCR, and the ADH3in/ADH3M and ADH4in/ADH4M primer pairs were designed and used for the second PCR (S1 Table). The two nested RepMP4 PCR products were mixed together and digested using the restriction enzyme *Hae*III; the products were electrophoresed on 2% agarose gel (S1 Fig). The subtypes were tested by DNA sequencing and compared with the reference strain.

MLVA typing

Multiplex PCR amplification-linked capillary electrophoresis of four loci (Mpn13, Mpn14, Mpn15, and Mpn16) was used for modified MLVA genotyping, and performed according to a previously described method [18, 19] and international guidelines [20].

Detection of macrolide resistance

Mutations conferring macrolide resistance, including the common point mutations at positions 2063, 2064, 2611, and 2617 (according to *M. pneumoniae* numbering) within 23S rRNA gene, were tested as previously described [9].

Statistical analysis

SPSS 21 package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. For comparison of proportions across different groups, the χ^2 test was used. A *p* value of <0.05 was considered significant. For sites such as Nanjing that offered lesser samples, Fisher’s exact test was used.

Results

Detection of *M. pneumoniae* from clinical specimens

Among 835 clinical samples, 213 (25.5%) tested positive for *M. pneumoniae* by real-time PCR including 72 (29.6%, 72/243) from Beijing; 28 (28%, 28/100) from Shanghai; 77 (28%, 77/ 275) from Kunming, Yunnan province; 20 (27.8%, 20/72) from Urumqi, Xinjiang province; and 16 (11.4%, 16/140) from Harbin, Heilongjiang province. The prevalence rate across these sites was similar, except in Harbin, where it was lower than other sites (*p* < 0.05).

P1 genotyping

Due to the low levels of DNA in some samples, P1 genotyping was successful obtained from 154 *M. pneumoniae*-positive DNA samples. Type I (P1-1) was found in 78.6% (121/154), and type II (P1-2 and its variants P1-2a, P1-2c) in 21.4% (33/154) in these samples (Table 1). The proportion of type I and type II differs across cities: 89.1% and 10.9% in Beijing, 80% and 20% in Shanghai, 78% and 22% in Kunming, 83.3% and 16.7% in Harbin, 71.4% and 28.6% in Urumqi, and 20% and 80% in Nanjing. The distribution of the two types is similar in these cities, except in Nanjing, wherein type II is dominant (*p* < 0.01, Table 1).

Table 1. Molecular characteristics of *Mycoplasma pneumoniae* from different areas.

	Total	Beijing	Shanghai	Kunming, Yunnan	Urumqi, Xinjiang	Harbin, Heilongjiang	Nanjing
Specimens collected	N = 835	N = 243	N = 100	N = 275	N = 72	N = 140	-
<i>M. pneumoniae</i> -positive	213 (25.5%)	72 (29.6%)	28 (28%)	77 (28%)	20 (27.8%)	16 (11.4%)	10
P1 genotypes	N = 154	N = 55	N = 20	N = 50	N = 12	N = 7	N = 10
Type I (P1-1)	121 (78.6%)	49 (89.1%)	16 (80%)	39 (78%)	10 (83.3%)	5 (71.4%)	2 (20%)
Type II (P1-2)	6 (3.9%)	0	0	2 (4%)	1 (8.35%)	0	3 (30%)
Type II (P1-2a) ^b	1(0.65%)	0	0	1(2%)	0	0	0
Type II (P1-2c) ^b	26(16.9%)	6 (10.9%)	4 (20%)	8(16%)	1 (8.35%)	2 (28.6%)	5 (50%)
MLVA genotypes	N = 172	N = 59	N = 22	N = 56	N = 16	N = 9	N = 10
M4-5-7-2	124 (72.1%)	49 (83.1%)	17 (77.3%)	38 (67.9%)	12 (75%)	6 (66.7%)	2 (20%)
M3-5-6-2	38 (22.1%)	7 (11.9%)	5 (22.7%)	13(23.2%)	2 (12.5%)	3 (33.3%)	8 (80%)
M4-5-7-3	7 (4.1%)	2 (3.3%)	0	5 (8.9%) ^a	0	0	0
M4-5-5-2	2 (1.2%)	1 (1.7%)	0	0	1 (6.25%)	0	0
M3-6-6-2	1 (0.6%)	0	0	0	1 (6.25%)	0	0
Macrolide	N = 186	N = 60	N = 22	N = 70	N = 15	N = 9	N = 10
Resistance	142 (76.3%)	52 (86.7%)	18(81.8%)	52(74.3%)	12 (80%)	6 (66.7%)	2 (20%)
Sensitive	44 (23.7%)	8 (13.3%)	4 (18.2%)	18(25.7%)	3 (20%)	3 (33.3%)	8 (80%)

^aSpecial repeat number of Locus Mpn15

^bType II variants were examined by DNA sequencing.

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MLVA genotyping

Due to the low levels of DNA, some specimens were failure to amplify all targets in MLVA. Full MLVA profile were obtained from 172 *M. pneumoniae* PCR-positive specimens and five distinct MLVA types were identified. The most common type was 4-5-7-2 (72.1%, 124/172), followed by type 3-5-6-2 (22.1%, 38/172), M4-5-7-3 (4.1%, 7/172), M4-5-5-2 (1.2%, 2/172), and M3-6-6-2 (0.6%, 1/172). Type M4-5-7-2 was predominant at all sites during the entire study duration, except in Nanjing where type 3-5-6-2 was most common ($p < 0.01$, Fig 2). Comparing the MLVA types with P1 genotype showed that the type M4-5-7-2 correlated with P1 Type I, and type M3-5-6-2 correlated with P1 Type II ($p < 0.01$, Table 1).

Two samples from Kunming showed a variation in MLVA types. Both samples were M4-5-7-3, with a size variation at the locus Mpn15. Sequencing showed that one of the repeats (repeat 3) in this locus was only 14-bp-long, compared to the other 21-bp-long samples (Fig 3). We then tested the presence of the highly variable locus Mpn1 in these two samples and found that both samples had the same copy number of 3. The two samples were collected from two different patients at different admission times. They might represent a new variation of MLVA types.

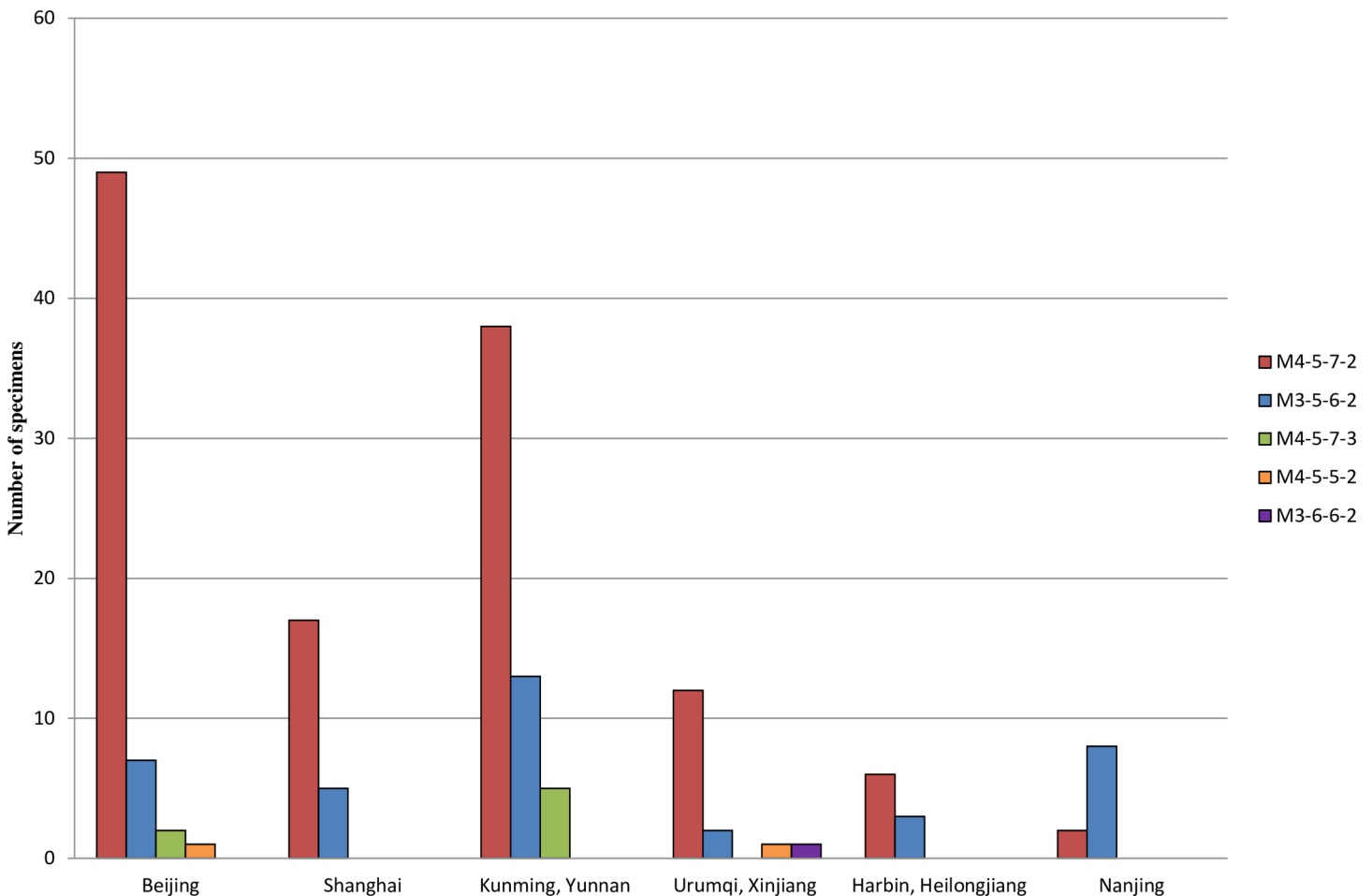


Fig 2. Genotype distribution across different sites. Different colors are used to represent the genotypes observed at different sites.

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Fig 3. Sequence comparison of the locus Mpn 15. M129, *M. pneumoniae* reference strain; KM-Mpn15, the special sequence of Mpn15 from Kunming; BJ-Mpn15, the normal sequence of Mpn15 from Beijing; all repeat sequences in Mpn15 were 21-bp-long, except repeat 3, which was a 14-bp-long truncated sequence.

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Macrolide resistance gene mutations

Among the 223 *M. pneumoniae*-positive samples from six cities, 186 specimens were successfully tested for mutations in the 23S rRNA gene due to the low levels of DNA in some specimens. Of them, 142 (76.3%) had mutations associated with macrolide resistance, and 44 (23.7%) were wild type (Table 1). The resistance rates in different areas were as follows: Beijing, 86.7% (52/60); Shanghai, 81.8% (18/22); Kunming, 74.3% (52/70); Harbin, 66.7% (6/9); Urumqi, 80% (12/15); and Nanjing, 20% (2/10). The resistance rate in Nanjing was significantly lower compared to that at the other five sites ($p < 0.01$, Table 1). Sequencing analysis revealed the presence of the A2063G mutation in 137 (74.1%) specimens, and both A2063G and A2065C mutations were detected in a single specimen from Beijing. Three specimens had a mixture of A2063G and wild type, and one specimen had a mixture of A2064G and wild type. Of the 142 resistant specimens, 131 were genotyped successfully. The most prevalent MLVA type among resistant specimens was M4-5-7-2 (92.4%, 121/131), followed by M3-5-6-2 (3.8%, 5/131), M4-5-7-3 (2.3%, 3/131), and M4-5-5-2 (1.5%, 2/131) (Fig 4).

Discussion

To better understand the recent endemic outbreaks as well as the molecular characteristics of *M. pneumoniae* strains found in China, we collected samples from six cities across the country in 2016. In China, the total detection rate was 25.51% in 2016, which is still higher compared to that in the previous non-epidemic years [21]. This indicated a new epidemic in some areas of China, which is consistent with our previous finding that the infection rate has been increasing from late 2015 [7]. Such a trend was observed in Japan and England in 2015 [6, 8] and in Central Slovenia in 2014 [22]. It is necessary to acquire more data from other sites to survey this trend. In this study, the detection rate at different locations was similar (>27%), except in the Heilongjiang province in northeast China (11.43%). The reasons underlying the low detection rate in this area are unclear. Sample types and transport methods might be two of the reasons, because all samples collected from Heilongjiang province were swabs, and the general consensus of several studies is that sputum is the best specimen for *M. pneumoniae*, the other specimen types varied in different studies [23–25]. Also, as distinct study protocols used by site, the transport medium and methods were different. It can also affect the detection rate. Besides, colder climate can be another reason. In fact, a previous study showed that *M. pneumoniae* infection positively correlated with temperature, and that the infection rate of *M. pneumoniae* gradually increased with the increase in the minimum temperature [26].

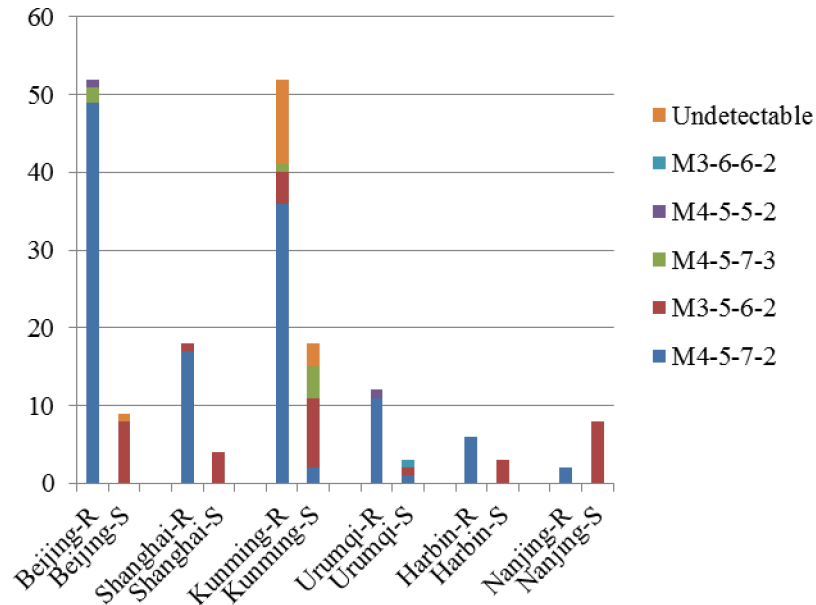


Fig 4. Different genotypes observed in macrolide-resistant and macrolide-sensitive specimens obtained from different sites in China. R, resistant; S, sensitive; Undetectable, the repeat number of the four loci could not be fully determined because of low DNA concentration.

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P1-RFLP genotyping method has been applied for 20 years since its development [27, 28]. This method helps classify the organism into two major genotypes according to the variations in the adhesin P1 gene type I and type II. In this study, the most prevalent genotype was type I (P1-1), which was in line with the findings in other countries [13, 4]. Although the proportion of P1-1 isolates varied from 71.4% to 89% in the five cities, except Nanjing, it was the dominant subtype. The samples from Nanjing were different in that type II (P1-2 and P1-2c) was the dominant subtype in those samples. Our previous studies in Beijing indicated that the P1 genotype started to switch from type I to type II as early as 2013 [29]. Since no historical data are available for Nanjing and because fewer samples were obtained from this site, even though these samples collected from different patients at different admission times, it is unclear whether this type II-dominant phenomenon is stable or dynamic in nature. Extensive data collection and detailed studies are necessary to answer this question.

Previous studies have established that the P1 type correlates with certain MLVA types [30]. In this study, MLVA 4-5-7-2 was found to be related to P1-1, and M3-5-6-2 to P1-2 and its variants. MLVA type M4-5-7-2 was the most prevalent type in our study, consistent with a previous study [4]. Unlike the other five cities, the most prevalent MLVA genotype in Nanjing was M3-5-6-2, corresponding to the predominant P1-2 and P1-2c subtypes. Differing geographical distribution of MLVA genotypes has also been observed in the USA [13]. Interestingly, we found two samples from Kunming, which showed a repeat length variation in the Mpn15 locus, where a 7-bp-long deletion (CTATTTT) was noticed in the third repeat sequence. This indicates that specific genotypes exist in certain regions. The biological significance of this variation is unknown.

The macrolide resistance rate is very high in China, reaching >90% annually [31, 9]. Interestingly, in this study, the average resistance rate was 76.3%. Considering single cities, the rate was decreased in Beijing and Shanghai [29, 32]. However, the specimens being compared belonged to different studies, and therefore, other factors such as the number of specimens tested and the method used among laboratories might affect this comparison. More studies are

needed to verify the observed trends. Of all single cities, differences were observed in Nanjing compared to other sites, including a lower proportion of macrolide resistance (20%) and predominant genotype of M3-5-6-2. It was consistent with the study that the macrolide non-resistant rate of *M. pneumoniae* isolates with Mpn13-14-15-16 profile of 3-5-6-2 was significantly higher than that of other types. [33]. Genotype M4-5-7-2, which correlates with macrolide resistance, is predominant in other cities, and can be considered as the reason for the higher rate of resistance. These observations are consistent with those of the reports from China and the USA [34, 29]. The differences in the rate of macrolide resistance in Japan varied from 0% to 100% across four different cities [14]. However, no further studies have been undertaken to study the correlation with MLVA genotypes.

This study has several limitations. First, the number of samples obtained from some areas is quite low, with the least being 10 from Nanjing. This limits the power of statistical analysis as well as further examination of the specific characteristics of the samples obtained from Nanjing. Second, all specimens used in this study were part of routine patient management without any additional collection. As distinct study protocols used, sample types were different by site. The detection rate could be affected due to lack of systematic studies comparing this variable. Third, limited regions were included in this study. China is geographically diverse, and therefore, the microbiological characteristics of *M. pneumoniae* isolates obtained in this study might not completely represent the true status of all regions of China.

To summarize, we investigated the molecular characteristics of six cities across five different areas of China in 2016. The proportion of *M. pneumoniae* positive respiratory specimens was around 25.5%, and the most prevalent genotype was M4-5-7-2/P1-1 at all sites, except Nanjing. The macrolide resistant rate decreased, accompanied with decrease in the M4-5-7-2 genotype.

Supporting information

S1 Table. Primers used in P1-RFLP gene typing.
(DOCX)

S1 Fig. The electrophoresis results of P1-RFLP typing. M: molecular weight marker, lane 1 and 4: type II. lane 2, 3 and 5–8: type I.
(TIF)

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