Molecular beacon based real-time PCR p1 gene genotyping, macrolide resistance mutation detection and clinical characteristics analysis of *Mycoplasma pneumoniae* infections in children

Lifeng Li1,2, Jiayue Ma1, Pengbo Guo1, Xiaorui Song1, Mingchao Li2, Zengyuan Yu2, Zhidan Yu1, Ping Cheng2, Huiqing Sun2* and Wancun Zhang1*

**Abstract**

**Background:** *Mycoplasma pneumoniae* can be divided into different subtypes on the basis of the sequence differences of adhesive protein P1, but the relationship between different subtypes, macrolide resistance and clinical manifestations are still unclear. In the present study, we established a molecular beacon based real-time polymerase chain reaction (real-time PCR) p1 gene genotyping method, analyzed the macrolide resistance gene mutations and the relationship of clinical characteristics with the genotypes.

**Methods:** A molecular beacon based real-time PCR p1 gene genotyping method was established, the mutation sites of macrolide resistance genes were analyzed by PCR and sequenced, and the relationship of clinical characteristics with the genotypes was analyzed.

**Results:** The detection limit was 1–100 copies/reaction. No cross-reactivity was observed in the two subtypes. In total, samples from 100 patients with positive *M. pneumoniae* detection results in 2019 and 2021 were genotyped using the beacon based real-time PCR method and P1-1 *M. pneumoniae* accounted for 69.0%. All the patients had the A2063G mutation in the macrolide resistance related 23S rRNA gene. Novel mutations were also found, which were C2622T, C2150A, C2202G and C2443A mutations. The relationship between p1 gene genotyping and the clinical characteristics were not statistically related.

**Conclusion:** A rapid and easy clinical application molecular beacon based real-time PCR genotyping method targeting the p1 gene was established. A shift from type 1 to type 2 was found and 100.0% macrolide resistance was detected. Our study provided an efficient method for genotyping *M. pneumoniae*, valuable epidemiological monitoring information and clinical treatment guidance to control high macrolide resistance.

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Background

*Mycoplasma pneumoniae* (M. pneumoniae, MP) is a common pathogen that can cause moderate upper respiratory tract infection, severe lower respiratory tract infection, and extrapulmonary clinical symptoms such as encephalitis, Stevens-Johnson syndrome, myocarditis and hemolytic anemia [1]. The most common infection of *M. pneumoniae* is community-acquired pneumonia (CAP). Statistically, 10–40% of pneumonia pathogens in school-aged children and adolescents consists of *M. pneumoniae*, and 4–8% consists of *M. pneumoniae* in adults, whereas this proportion increased to 20–70% during the epidemic period [2].

The adhesion of respiratory epithelial cells through the attachment organelle of *M. pneumoniae* is a key step for colonization and pathogenesis [4]. P1 is the major component of the adhesion protein complex at the surface of the organelle, which is essential for cytoadherence of *M. pneumoniae* [13]. According to the sequence differences of the *p1* gene, *M. pneumoniae* can be divided into two large subtypes, type 1 and type 2, but the clinical significance of different subtypes is controversial. Although in vitro analysis of immunogenicity of different subtypes showed differences [14], the earlier reports on *M. pneumoniae* P1 typing showed no correlation with susceptibility and severity of clinical symptoms [15, 16]. However, severe pneumonia and additional extrapulmonary clinical manifestations were reported for type 1 *M. pneumoniae* infection manifestations [17]. In another study, type 2 *M. pneumoniae* pneumonia patients were reported with more neurological and cardiovascular symptoms [18]. Simultaneously, the dynamic change in the proportion of two subtypes of P1 may also be related to the periodic outbreak and epidemic of *M. pneumoniae* [5]. Studies on *M. pneumoniae* typing and antibiotic susceptibility analysis showed that different *p1* gene types may be associated with macrolide resistance to a certain degree, and type 2 strains may be more susceptible to macrolides [19, 20]. Hence, it is critical to monitor the molecular epidemiological features of *M. pneumoniae* since the genotypes may be related to macrolide susceptibility, disease severity and the periodic outbreak and epidemic of the pathogen.

The main treatments for *M. pneumoniae* infection are antibiotics. Due to the lack of a cell wall, *M. pneumoniae* is naturally resistant to antibiotics acting on the cell wall, such as β-lactam drugs, glycopeptides and fosfomycin, and it is also resistant to polymixins, sulfonamides, trimethoprim, rifampicin and linezolid. Although aminoglycosides, chloramphenicol and gentamicin have activity against *M. pneumoniae*, they are not recommended for clinical use [2, 3]. Macrolides restrained bacterial growth by binding of the 23S rRNA to inhibit protein synthesis, hence macrolides, tetracycline and fluoroquinolone have better performance for the clinical treatment of *M. pneumoniae* infection. Due to the possible impact on children's development, tetracycline and fluoroquinolone are not recommended for children. Hence, macrolides, such as erythromycin and azithromycin, serve as the primary choice for the clinical treatment of *M. pneumoniae* pneumonia in children. However, macrolide-resistant *M. pneumoniae* is gradually increasing worldwide, especially in Asia, showing a high rate of drug resistance [4–11]. In China, the drug resistance rate of macrolides can be as high as 100%, whereas it is lower than 12% in North America, Europe and Australia, and declined from 90% in 2010–2011 to 11% in 2018–2019 in Japan, which may be explained by a decrease in macrolide use and a shift in the prevalent genotype of *M. pneumoniae* from macrolide-resistant type 1 to the susceptible type 2 [12]. Studies have found that the main macrolide resistance mechanism in *M. pneumoniae* is the mutation in the 23S rRNA V region, in which A2063G and A2064G mutations lead to high level resistance, and mutations at A2067G and C2617G are associated with lower resistance [8]. Thus, it is necessary to perform epidemiological monitoring of *M. pneumoniae* in different regions to monitor local epidemic characteristics.

Generally, the classification of *M. pneumoniae* is mainly based on the differences between two repeated regions RepMP4 and RepMP2/3 contained in the *p1* gene. Commonly used methods for *p1* genotyping include nested PCR, PCR product restriction fragment length polymorphism (PCR–RFLP), rapid cycle PCR and real-time PCR high-resolution melt (HRM) genotyping assay. [1, 4, 21, 22]. Nested PCR, rapid cycle PCR and PCR–RFLP have high accuracy advantage, but are time-consuming and labor intensive. Compared with traditional PCR, real-time PCR has the advantages of high sensitivity and shorter time consumption by amplifying a small target. The real-time PCR HRM genotyping assay requires amplification of the 1900 bp long region of the *p1* gene and consists of a HRM collection procedure, which may require longer time to obtain genotyping result [22]. Hence, we aimed to establish a molecular beacon based real-time PCR genotyping method targeting the *p1* gene, which can obtain genotype results rapidly and is easy for clinical application. Meanwhile, we investigated the

Keywords: *Mycoplasma pneumoniae*, Genotyping, Molecular beacon, Macrolide resistance, Clinical characteristics
prevalent genotypes in Henan, China using the method established and analyzed the clinical significance of genotyping by analyzing the relationship between genotypes, macrolide resistance and clinical symptoms.

In the present study, we developed a new genotyping method that uses molecular beacon based real-time PCR for *M. pneumoniae* p1 gene genotyping. We examined the prevalent genotypes in Henan, China using the method established and analyzed the mutation sites of drug resistance genes by PCR and sequencing. The relationship of the clinical symptoms with the subtypes and macrolide resistance of *M. pneumoniae* was analyzed.

**Methods**

**Clinical sample collection and nucleic acid extraction**

Samples were collected from children who visited Henan Children's Hospital for *M. pneumoniae* detection in 2019 and 2021. *M. pneumoniae* was detected by amplification of 16S rRNA using SAT-MP kit (Shanghai Rendu Biotechnology Co, Ltd). The samples with positive results were stored in −80 °C for further use. This study was approved by the Ethics Committee of the Henan Children's Hospital. Nucleic acid was extracted from sputum, pharyngeal swab and alveolar lavage fluid of the patients using Shengxiang Biological nucleic acid extraction or purification kit (Changsha, China) following the manufacturer's instructions.

**Plasmid construction and extraction**

The representative strain of p1 subtype 1 (P1-1) was *M. pneumoniae* M129, the coding gene was MPNE_RS00820, and the GenBank accession number was NC_000912.1. The representative strain of p1 subtype 2 (P1-2) was *M. pneumoniae* FH, the coding gene was MPNE_RS00820, and the GenBank accession number was NC_017504.1. The partial sequences of p1 subtype 1 (*M. pneumoniae* M129) and p1 subtype 2 (*M. pneumoniae* FH) genes were inserted into plasmid pUC57 using primer pairs P1-1 F/R and P1-2 F/R (Table 1) to construct the recombinant plasmids, which were transformed into *Escherichia coli* TOP10 to obtain the recombinant strains (Sangon, China). The plasmids were extracted using the plasmid extraction kit (DP103) of Tiangen (Beijing, China). The plasmids were prepared using the gradient dilution method. Positive quality control plasmids were detected by real-time PCR to analyze the detection feasibility and sensitivity. The 20 µL detection reaction was composed of 10 µL qPCR premix (Vazyme, China), M129 F/M129 R/MP FH F/MP FH R 0.4 µL (final concentration was 0.2 µM), MB1/MB2 0.5 µL (final concentration was 0.25 µM), template 1µL, and 5.4 µL nuclease free water. Real-time PCR was used to detect the fluorescence signal of specific reaction, and the reaction conditions were as follows: 95 °C, 5 min; 40 cycles at 95 °C, 10 s, 37 °C, 30 s, 60 °C, 30 s. FAM and HEX signals were collected using the CFX96 thermal cycler (Biorad, USA). Amplification results of the clinical *M. pneumoniae* infection samples were drawn together using Origin 2016 using the amplification data exported [29].

**Molecular beacon based real-time PCR for p1 gene genotyping**

The p1 gene containing plasmids were prepared using the gradient dilution method. Positive quality control plasmids were detected by real-time PCR to analyze the detection feasibility and sensitivity. The 20 µL detection reaction was composed of 10 µL qPCR premix (Vazyme, China), M129 F/M129 R/MP FH F/MP FH R 0.4 µL (final concentration was 0.2 µM), MB1/MB2 0.5 µL (final concentration was 0.25 µM), template 1µL, and 5.4 µL nuclease free water. Real-time PCR was used to detect the fluorescence signal of specific reaction, and the reaction conditions were as follows: 95 °C, 5 min; 40 cycles at 95 °C, 10 s, 37 °C, 30 s, 60 °C, 30 s. FAM and HEX signals were collected using the CFX96 thermal cycler (Biorad, USA). Amplification results of the clinical *M. pneumoniae* infection samples were drawn together using Origin 2016 using the amplification data exported [29].

**Detection of mutations in domain V of Mycoplasma pneumoniae 23S rRNA**

The primers (Table 1) used to amplify the domain V of *M. pneumoniae* 23S rRNA gene were designed using Primer Premier 5 [24] and synthesized by Sangon (Shanghai, China) following the manufacturer's instructions.

**Table 1: Primers used in the present study**

| Primer name | Sequence (5'-3') |
|-------------|-----------------|
| P1-1 F      | GCTCTAGAGGTGTTCAGTGAGGCTGAC |
| P1-1 R      | GGGGTACCGAGCAGAAACATCGCCGCCC |
| P1-2 F      | ACGCGTCGACCTTGTGTGGGCTGAC |
| P1-2 R      | CGGGATCCATGTCGATGACCTCAACCCGTC |
| M129 F      | CGAACCGAGGAGTGCTCAAAAT |
| M129 R      | CGAAGCTGAAAGGCGATCG |
| MB1         | FAM-CCCCCATCGGAGAGTGCTCGAGTCAGTTCAGGG-BHQ1 |
| MB2         | HEX-CGCGAACAATCCGGTGACCAAGGCACTTCGCGG-BHQ1 |
| MP FH F     | AGACAGCACTAACAAAAACAGGC |
| MP FH R     | CCGAACTGGAAGGGCGAG |
| M129C       | GGACTGACCGACCTCTCGCT |
| FHC         | GTGCCCTGTGTCACCGGAGTTG |
| MP 23S 5em  | GTCTCCGCTATAGACTCCTGGT |
| MP 23S Ant  | GCTCAACTGGGACGATAAGAG |

**Plasmid construction and extraction**

The representative strain of p1 subtype 1 (P1-1) was *M. pneumoniae* M129, the coding gene was MPNE_RS00820, and the GenBank accession number was NC_000912.1. The representative strain of p1 subtype 2 (P1-2) was *M. pneumoniae* FH, the coding gene was MPNE_RS00820, and the GenBank accession number was NC_017504.1. The partial sequences of p1 subtype 1 (*M. pneumoniae* M129) and p1 subtype 2 (*M. pneumoniae* FH) genes were inserted into plasmid pUC57 using primer pairs P1-1 F/R and P1-2 F/R (Table 1) to construct the recombinant plasmids, which were transformed into *Escherichia coli* TOP10 to obtain the recombinant strains (Sangon, China). The plasmids were extracted using the plasmid extraction kit (DP103) of Tiangen (Beijing, China). The plasmids were prepared using the gradient dilution method. Positive quality control plasmids were detected by real-time PCR to analyze the detection feasibility and sensitivity. The 20 µL detection reaction was composed of 10 µL qPCR premix (Vazyme, China), M129 F/M129 R/MP FH F/MP FH R 0.4 µL (final concentration was 0.2 µM), MB1/MB2 0.5 µL (final concentration was 0.25 µM), template 1µL, and 5.4 µL nuclease free water. Real-time PCR was used to detect the fluorescence signal of specific reaction, and the reaction conditions were as follows: 95 °C, 5 min; 40 cycles at 95 °C, 10 s, 37 °C, 30 s, 60 °C, 30 s. FAM and HEX signals were collected using the CFX96 thermal cycler (Biorad, USA). Amplification results of the clinical *M. pneumoniae* infection samples were drawn together using Origin 2016 using the amplification data exported [29].
China). PCR was performed to amplify the fragments of the gene using the \textit{M. pneumoniae} positive samples as templates with the enzyme (Vazyme, Nanjing, China). The PCR products were sequenced in Sangon (Shanghai, China) and compared with the reference 23S rRNA gene using the Bioedit software [23].

**Statistical analysis**

The statistical analyses were performed using IBM SPSS statistics 25.0 (IBM Corp., Armonk, NY, USA). Normality of data was detected by Kolmogorov–Smirnov test. The statistical significance was determined at the two-tailed 0.1 level. The two groups were compared using a two-sample independent \(t\)-test or the Mann–Whitney \(U\) test. Means \(\pm\) standard deviations (SDs) and median values (interquartile ranges: Q25–Q75) were used to present normally distributed data and skewed distribution data, respectively. The Chi-squared test was used for categorical variables. \(P < 0.05\) was regarded as statistically significant.

**Results**

**Molecular beacon based real-time-PCR for \(p1\) gene genotyping assay**

To establish the beacon based genotyping method, the primer sets M129 F/M129 R and MP FH F/MP FH R were designed to amplify the two \(p1\) gene RepMP4 regions, and MB1/MB2 were designed to have typical secondary structures with FAM/HEX fluorescence labels (Table 1, Fig. 1 and Additional file 1: Fig. S1). The melting temperature (\(T_m\)) was the temperature at which half of the hairpin structure was unfolded into single-stranded DNA and the fluorescence signals reached half of the peak value. \(T_m\) values for two beacon probes

![Fig. 1](image-url)  
**Fig. 1** Composition of the \(p1\) gene and sequence alignments of primer binding sites. A The illustration of the \(p1\) gene indicating the RepMP4 and RepMP2/3 regions; B Sequence alignments of type 1 and 2 primer and molecular beacon binding sites. Sequence alignment picture was drawn using the tool reported [38]. Blue solid lines were used to indicate binding sequences of type 1 primer and molecular beacon. Green dotted lines were used to indicate binding sequences of type 2 primer and molecular beacon.
were determined, which was 59 °C for MB1 and 58 °C for MB2 (Additional file 1: Fig. S1). To determine the optimal reaction temperature of the molecular beacons, the fluorescence signal changes of MB1 and MB2 in the presence and absence of the targets were detected by PCR by increasing temperature from 10 °C to 92 °C. The temperature corresponding to the maximum difference of fluorescence signal intensity in the presence and absence of the targets was the optimal reaction temperature, which was 30 °C for MB1 and 40 °C for MB2, hence 37 °C was used as the annealing temperature of the real-time PCR reaction.

Positive control plasmids carrying the p1 gene with a gradient concentration were used to assess the sensitivity of the beacon based real-time PCR method (Fig. 2). The detection limit was $1 \times 10^2$ copies for P1-1, and when the copy number of standard DNA ranged from $1 \times 10^3$ copies/μL to $1 \times 10^9$ copies/μL, there was a good linear relationship. The detection limit was $1 \times 10^2$ copies for P1-2, and when the copy number of standard DNA ranged from $1 \times 10^3$ copies/μL to $1 \times 10^9$ copies/μL, there was a good linear relationship. No amplification was found when the two pairs of primers and probes were cross-examined. Eight samples were genotyped using the method in the present study and the reported nest-PCR method [4], the two assays were in perfect agreement with five P1-1 genotypes and three P1-2 genotypes. This indicated that the method could be used to the following genotyping of *M. pneumoniae* infection.

### Genotyping of clinical *M. pneumoniae* infection samples

In total, the samples were obtained from 100 patients with positive *M. pneumoniae* detection results in 2019 and 2021 including 59 boys and 41 girls (Table 2). There were 69 patients infected with P1-1 type *M. pneumoniae*, which included 40 boys and 29 girls; 31

|  | P1-1 | P1-II | Total | $\chi^2$ | p       |
|---|------|-------|-------|---------|---------|
| 2019 | 55 (76.4%) | 17 (23.4%) | 72 | 6.653 | 0.010  |
| 2021 | 14 (50.0%)  | 14 (50.0%)  | 28 |         |        |
| Total | 69 (69.0%) | 31 (31.0%) | 100 |         |        |

**Table 2** Genotyping results of *M. pneumoniae* positive clinical samples

![Amplification results of gradient positive quality control DNA.](image)

**Fig. 2** Amplification results of gradient positive quality control DNA. **A** The results of P1-1 amplification; **B** The standard curve of P1-1 amplification; **C** The results of P1-2 amplification; **D** The standard curve of P1-2 amplification. The numbers 1–10 represent $1 \times 10^3$ copies/μL, $1 \times 10^4$ copies/μL, $1 \times 10^5$ copies/μL, $1 \times 10^6$ copies/μL, $1 \times 10^7$ copies/μL, $1 \times 10^8$ copies/μL, $1 \times 10^9$ copies/μL, and 1 copies/μL, respectively. NC was the negative control.
patients with P1-2 M. pneumoniae, which included 19 boys and 12 girls. Moreover, the median age of patients were 4.90 years, 4.65 and 5.47 years in P1-1 and P1-2 groups respectively. The amplification results of 100 samples were shown in Fig. 3. To further verify the accuracy of our method, we verified the genotyping results by our method by analyzing half of the samples (50 of 100) using nest-PCR method, and the results were consistent with the method established in this study. No significant difference was observed in gender and age composition between the two groups. There was a significant difference between the M. pneumoniae infection group in 2019 and 2021 ($P = 0.010$) where the infection rate of P1-1 was higher than P1-2.

**Macrolide resistance mutation detection in the 23S rRNA gene**

To analyze the macrolide resistance, the samples with genotyping results were further used to amplify the 23S rRNA gene and analyze the mutations in the domain V by sequencing. Among the 100 M. pneumoniae samples, all were found to have A2063G mutation compared with the sequence of the reference M129 strain. Two M. pneumoniae strains contained another C2622T mutation (2.0%). One M. pneumoniae strain had C2150A and C2202G mutation, which was subtype 2 genotype. One M. pneumoniae strain had C2443A mutation. The other three with new mutations belonged to the genotype P1-1.

**Clinical characteristics of pediatric M. pneumoniae infection**

To analyze the relationship of p1 gene genotyping and the clinical characteristics of M. pneumoniae infection, the demographics, clinical manifestations and laboratory examination results were analyzed and shown in Table 3. Aside from a higher lymphocyte count in the P1-1 group compared to the P1-2 group (2.83 [2.08–3.6] × 10⁹ cells/L vs 2.54 [1.31–2.99] × 10⁹ cells/L; Z = -2.132; $P = 0.033$), no significant difference was observed in the clinical symptoms analyzed between the two groups.

**Discussion**

In the present study, we described the development of a molecular beacon based M. pneumoniae genotyping method based on real-time PCR targeting p1 gene, in which one reaction can detect two genotypes P1-1 and P1-2. First, the accuracy of this method was evaluated by comparing the results with the ones generated by nest-PCR [4]. Further, 100 M. pneumoniae infection samples were genotyped using this molecular beacon based method, the mutations in the domain V of 23S rRNA gene were analyzed by PCR and sequencing, and the clinical significances of genotyping were analyzed.

The reasons why M. pneumoniae infection can lead to different manifestations are not clear. Genotypes are associated with the clinical outcomes were reported [17, 18]. Hence, we conducted the genotyping of M. pneumoniae infections to further analyze its relationship with clinical symptoms in the present study. According to the sequence differences of repetitive elements RepMP2/3 and RepMP4 in the P1 protein gene, M. pneumoniae...
can be classified into subtype 1 and subtype 2 two major genotypes [30]. HRM analysis based real-time PCR was reported to distinguish two subtypes of P1 protein gene by amplifying the 1.9 kb fragment [22]. Compared to the HRM analysis based real-time PCR method, our method had a shorter amplification fragment and hence needed a shorter time (about 1.5 h vs 2.5 h) to obtain the genotyping result. Additionally, our method can quantify the sample according to the standard curve. PCR–RFLP method was also used in the P1 gene genotyping, which could detect the subtypes by PCR amplification and agarose gel electrophoresis [4]. In this study, we developed

| Table 3 Clinical characteristics of M. pneumoniae infection children |
|--------------------------|------------------|------------------|-----------------|------------------|
| Variable | Total (n = 100) | P I-I (n = 69) | P I-II (n = 31) | t/Z/χ² | p |
| Male/Female | 100 | 40/29 | 19/12 | -0.311 | 0.756 |
| Age (years) | 4.90 | 4.65±2.16 | 5.47±2.80 | -1.606 | 0.112<sup>a</sup> |
| Leukocyte count (x 10<sup>9</sup> cells/L) | 9.69 | 10.14 (7.23–10.80) | 8.7 (5.94–10.14) | -1.170 | 0.242 |
| Neutrophil count (x 10<sup>9</sup> cells/L) | 6.18 | 6.53 (4.03–7.14) | 5.38 (2.97–6.97) | -0.332 | 0.740 |
| Lymphocyte count (x 10<sup>9</sup> cells/L) | 2.74 | 2.83 (2.08–3.60) | 2.54 (1.31–2.99) | -2.132 | 0.033 |
| CRP (mg/L) | 25.11 | 25.43 (7.28–35.75) | 24.45 (3.83–26.42) | -1.108 | 0.268 |
| PCT | 0.242 | 0.200 (0.060–0.197) | 0.229 (0.085–0.210) | -0.565 | 0.572 |
| Length of hospitalization (days) | 11.40 | 11.35 (9–13) | 11.52 (8–15) | -0.193 | 0.847 |
| Duration of fever (days) | 6.79 | 7 (5.00–8.25) | 6.29 (4.00–8.75) | -0.429 | 0.668 |
| Thermal spike (°C) | 39.43 | 39.42 (39.00–39.90) | 39.45 (39.00–40.00) | -0.634 | 0.526 |
| Pulmonary manifestations | | | | |
| Positive (n, %) | 52 | 35 (50.8%) | 17 (54.8%) | 0.145 | 0.703<sup>b</sup> |
| Negative (n, %) | 48 | 34 (49.3%) | 14 (45.2%) | 0.740 | 0.459 |
| A: Pleural effusion | 32 | 20 | 12 | -0.959 | 0.337 |
| B: Pulmonary atelectasis | 2 | 1 | 1 | -0.584 | 0.559 |
| C: Pleuritis | 1 | 1 | 0 | 0.670 | 0.503 |
| D: Respiratory insufficiency; Respiratory failure | 10 | 7 | 3 | 0.072 | 0.943 |
| E: Pulmonary consolidation | 27 | 17 | 10 | 0.790 | 0.430 |
| F: Pneumothorax | 3 | 2 | 1 | 0.088 | 0.930 |
| G: Severe pneumonia | 5 | 4 | 1 | 0.543 | 0.587 |
| H: Pertussis syndrome | 3 | 2 | 1 | 0.088 | 0.930 |
| Extrapulmonary manifestations | | | | |
| Positive (n, %) | 49 | 33 (47.8%) | 16 (51.6%) | 0.123 | 0.726<sup>b</sup> |
| Negative (n, %) | 51 | 36 (52.4%) | 15 (48.4%) | 0.740 | 0.459 |
| A: Digestive system | | | | |
| A1: Peritoneal effusion | 14 | 10 | 4 | -0.211 | 0.833 |
| A2: Others<sup>a</sup> | 19 | 13 | 6 | -0.600 | 0.523 |
| B: Cardiovascular system | 2 | 2 | 0 | 0.953 | 0.341 |
| C: Nervous system | 3 | 2 | 1 | -0.088 | 0.930 |
| D: Blood-Lymphatic system | | | | |
| D1: Sepsis | 3 | 1 | 2 | 1.349 | 0.177 |
| D2: Lymphadenectasis | 20 | 12 | 8 | 0.968 | 0.333 |
| D3: Anemia | 2 | 2 | 0 | 0.953 | 0.341 |
| E: Rash | 4 | 3 | 1 | 0.263 | 0.792 |
| F: Electrolyte disturbance | 3 | 1 | 2 | 1.349 | 0.177 |
| G: Immune system (allergy, immunodeficiency) | 6 | 6 | 0 | 1.685 | 0.092 |
| H: Urinary system | 2 | 2 | 0 | 0.953 | 0.341 |
| Two systems | 4 | 3 | 1 | 0.263 | 0.792 |
| Three systems | 1 | 1 | 0 | 0.670 | 0.503 |

<sup>a</sup>Hepatomegaly, hepatic injury, abnormal liver function, Enteritis and gastrointestinal dysfunction

<sup>b</sup>Two-sample independent t-test

<sup>b</sup>Chi-squared test
a molecular beacon probes based real-time PCR method that can identify two subtypes by detection of different fluorescence signals in the amplification process. The genotyping results by our method were consistent with the data generated by the nest-PCR method. Hence, we used the method established to further analyze the genotype of *M. pneumoniae* infection samples in this study.

Different and changing ratios of the P1-1 and P1-2 subtype *M. pneumoniae* infections were reported worldwide [4, 22], and the incidence of P1-1 infections were usually more than P1-2 (Table 4). Genotyping is crucial for molecular epidemiological studies and the development of an effective vaccine [31]. In total, 69 (69.0%) of 100 children analyzed in this study were infected with P1-1 *M. pneumoniae* whereas the rate of P1-1 in 2019 was 76.4% and 50.0% in 2021. In 2015, 92.0% type 1 strain was reported on the basis of P1 gene PCR–RFLP analysis among 71 adults in Zhejiang province [32]. Zhao et al. performed a multicenter study analyzing molecular characteristics of *M. pneumoniae* by genotyping 154 isolates from 5 cities in mainland China in 2017–2018 and found that type 1 accounted for 76.6%, 23.4% for type 2 strains, and a large variance was found ranging from 100% type 1 in Jilin to 45.5% in Jinan [33]. Jia et al. reported 57.1% type 1 *M. pneumoniae* infection by nested PCR from children with pneumonia in Qingdao, China, in 2019 [34]. Guo et al. analyzed the molecular features of *M. pneumoniae* isolates in paediatric inpatients in Weihai, China in 2019 and found that genotype 2 was identified in 42 isolates of 82 culture-positive samples [35]. Whistler et al. reported type 1 genotype *M. pneumoniae* accounted for a ratio of 61.8% (97/157) in the rural populations of Thailand from 2009 to 2012, and no macrolide resistance mutations were detected [36]. Kenri et al. found that the genotypes changed periodically in Japan where type 1 *M. pneumoniae* strains reduced from 100% of the strains isolated in 2012 to 8.3% in 2018 [37]. Hence, *p1* subtype 1 was the prevalent genotypes in several regions analyzed, and different epidemiological genotypes were distributed in different regions in China and other countries, whereas the prevalent genotypes of Japan indicated a substantial periodical change in the epidemiological features of *M. pneumoniae*.

As an important causative pathogen in CAP, *M. pneumoniae* increased in macrolide resistance. The high resistance rate was reported in East Asia, which was 81.6% in Japan, 87.2% in Korea, and 90% to 100% in China [10]. In our study, the macrolide resistance rate was 100.0% and all had the high resistance related A2063G mutation, which was consistent with a study in Qingdao, China in 2021 [34]. An earlier study in Zhejiang, China also reported A2063G mutation in all of the 71 *M. pneumoniae* strains isolated from adults with CAP in 2015 [32]. It is noteworthy that high resistance rate was also found in the type 2 strains in our study, while type 2 strains were related with the lower macrolide resistance rate in other studies reported [19, 20, 37]. Meanwhile, new mutations were found in the samples, which were C2622T, C2150A, C2202G and C2443A. Among four new mutations found, three was in P1-1 group and one was P1-2 group. The strains were needed to analyze the concrete impact of the new mutations on the macrolide resistance levels. Therefore, the high resistance rate requires special attention and macrolide antibiotic use in the clinical practice should be adjusted accordingly to reduce selection stress for the pathogen. Acute reduction in macrolide-resistant *M. pneumoniae* infections among Japanese children was reported, which may indicate the importance of changing antibiotic usage and the impact of *p1* genotype distribution [20].

Despite advances in genotyping methods to characterize different *M. pneumoniae* strains, the relationship between different genotypes and specific clinical outcomes it is still unclear. Hence, we analyzed the relationship between P1-1 genotypes and clinical characteristics. Although the majority clinical outcomes of infections caused by P1-1 and P1-2 subtype *M. pneumoniae* isolates are not significantly different, patients infected with P1-1 isolates had a higher lymphocyte count (2.83 × 10^9 cells/L). Fan et al. analyzed 304 cases of type

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**Table 4** Distribution of *p1* gene subtypes of *M. pneumoniae* infection in different regions

| Region      | Year     | Genotyping results                                      | References |
|-------------|----------|---------------------------------------------------------|------------|
| Henan, China| 2019/2021| P1 subtype 1: 76.4% in 2019 and 50.0% in 2021           | This study |
| Zhejiang, China| 2015    | P1 subtype 1: 92.0%                                     | [32]       |
| Multicenter, China| 2017–2018 | Overall P1 subtype 1: 76.6%; P1 subtype 1: 100.0% in Jilin and 45.5% in Jinan | [33]       |
| Qingdao, China  | 2019    | P1 subtype 1: 57.1%                                     | [34]       |
| Weihai, China   | 2019    | P1 subtype 2: 51.2%                                     | [35]       |
| Thailand      | 2009–2012| P1 subtype 1: 61.8%                                     | [36]       |
| Japan         | 2012–2018| P1 subtype 1: 100.0% in 2012 and 8.3% in 2018          | [37]       |
1 M. pneumoniae and 30 cases of type 2 M. pneumoniae infection (type 1 91.0%) in children with pneumonia, and found that children infected with type 1 M. pneumoniae strain had a higher risk of developing severe pneumonia and with more extrapulmonary clinical manifestations [17]. Berlot et al. analyzed 356 cases of type 1 M. pneumoniae and 126 cases of type 2 M. pneumoniae pneumonia in children, which found that different types of M. pneumoniae infections in patients showed different clinical features. Type 2 M. pneumoniae pneumonia patients were with more neurological and cardiovascular symptoms, but patients infected with type 1 M. pneumoniae had other clinical manifestations, which indicated that different types of M. pneumoniae may have different pathogenicity [18].

Our study has limitations. First, the samples collected were geographically confined to Zhengzhou, Henan, China. Second, the number of analyzed M. pneumoniae strains were still relatively small, especially the samples in 2021, and it is not enough to analyze the impact of COVID-19 to epidemiological features of M. pneumoniae. Third, although the current design does not affect the results of this study, but variant 2d may be missed by our method, which should be noted in future uses.

Conclusions

M. pneumoniae infection is common amongst pneumonia infections in the pediatric community with endemic prevalence worldwide. The prevalent genotypes vary over time and geographic location, which may or may not determine specific clinical outcomes. In summary, the molecular beacon based real-time PCR p1 gene genotyping method was established, which had a detection limit of 1–100 copies depending on the subtypes. 100 M. pneumoniae infection samples from children with pneumonia in Zhengzhou were genotyped using this method. Type 1 M. pneumoniae was the main genotype. A shift from type 1 to type 2 was found and 100% macrolide resistance was detected. The macrolide resistance mutation A2063G could be detected in all the samples analyzed. Simultaneous, new mutations of C2622T, C2150A, C2202G and C2443A were found. Our study is an important addition to the molecular epidemiological features of M. pneumoniae in children in Henan, China combining the genotyping, macrolide resistance profile and analysis of clinical symptoms. Due to the high macrolide resistance rate found in our study and other reports in China, routine M. pneumoniae resistance profile detection would be critical for the control of M. pneumoniae macrolide resistance and guiding clinical treatments. In the future, national surveillance, long-term longitudinal and multi-center studies are needed to examine the molecular epidemiology information such as the periodic genotype shifts and the changes of antibiotic resistance to provide basis for the prevention and treatment of related diseases.

Abbreviations

Real-time PCR: Real-time polymerase chain reaction; MP: Mycoplasma pneumoniae, M. pneumoniae; CAP: Community-acquired pneumonia; PCR–RFLP: PCR product restriction fragment length polymorphism; HRM: High-resolution melt; NCBI: National Center for Biotechnology Information; SDs: Standard deviations; Tm value: Melting temperature values.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-022-07715-6.

Additional file 1: Fig. S1. Secondary structures and Tm values of two molecular beacons. A Secondary structures of MB1; B Secondary structures of MB2; C Tm values of MB1; D Tm values of MB2.

Acknowledgements

Not applicable.

Author contributions

LL: Conceptualization, Methodology, Writing-original draft, Funding acquisition; JM: Investigation, Resources, Data analysis; PG: Methodology, Formal analysis, XS: Methodology, ML: Formal analysis; ZY: Data curation; ZY: Supervision, Ping Cheng: Data analysis; HS: Supervision, Writing-review & editing; WZ: Supervision, Conceptualization, Writing-review & editing. All the authors have revised the manuscript critically and approved the submission. All authors read and approved the final manuscript.

Funding

This work was supported by grants from the Scientific and Technological Projects of Henan Province (212102310897 and 202102310395), the Medical Science and Technology Projects of Henan Province (LHGJ20190955), and the National Natural Science Foundation of China (31900116).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Henan Children’s Hospital (2022-K-041). All the steps were performed in accordance with the relevant guidelines and regulations. The informed consent was waived by the Ethics Committee of Henan Children’s Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interests.

Received: 20 May 2022 Accepted: 29 August 2022
Published online: 06 September 2022

References

1. Gultsbys K, Olsen B, Bondeson K. Molecular typing of mycoplasma pneumoniae strains in Sweden from 1996 to 2017 and the emergence of a new p1 cytadhesin gene, variant 2e. J Clin Microbiol 2019. https://doi.org/10.1128/JCM.00049-19.
2. Bajantri B, Venkatram S, Diaz-Fuentes G. Mycoplasma pneumoniae: a potentially severe infection. J Clin Med Res. 2018;10(7):S35–44.
3. Pereyre S, Goret J, Bébéar C. *Mycoplasma pneumoniae*: current knowledge on macrolide resistance and treatment. *Front Microbiol.* 2016;7:974.

4. Keren T, Okazaki N, Yamazaki T, et al. Genotyping analysis of *Mycoplasma pneumoniae*: clinical strains in Japan between 1995 and 2005: type shift phenomenon of M. pneumoniae clinical strains. *J Med Microbiol.* 2008;57(Pt 4):469–75.

5. Kogoj R, Mrvic T, Prapornik M, et al. Prevalence, genotyping and macrolide resistance of *Mycoplasma pneumoniae* among isolates of patients with respiratory tract infections, Central Slovenia, 2006 to 2014. *Euro Surveill.* 2015. doi:10.2861/j.2079-5171.2015.20.37.30018.

6. Li X, Atkinson TP, Hagood J, et al. Emerging macrolide resistance in *Mycoplasma pneumoniae* in children: detection and characterization of resistant isolates. *Pediatr Infect Dis J.* 2009;28(8):693–6.

7. Wolff BJ, Thacker WL, Schwartz SB, et al. Detection of macrolide resistance in *Mycoplasma pneumoniae* by real-time PCR and high-resolution melt analysis. *Antimicrob Agents Chemother.* 2008;52(10):3542–9.

8. Ye Y, Li S, et al. *Mycoplasma pneumoniae* 23S rRNA Gene mutations and mechanisms of macrolide resistance. *Lab Med.* 2015;44(1):63–8.

9. Zhao F, Liu G, Wu J, et al. Surveillance of macrolide-resistant *Mycoplasma pneumoniae* in Beijing, China, from 2008 to 2012. *Antimicrob Agents Chemother.* 2013;57(3):1521–3.

10. Xu C, Deng H, Zhang J, et al. Mutations in domain V of *Mycoplasma pneumoniae* 23S rRNA and clinical characteristics of pediatric *M. pneumoniae* pneumonia in Nanjing China. *J Int Med Res.* 2021. doi:10.1177/0300060521106376.

11. Wang Y, Xu B, Wu X, et al. Increased macrolide resistance rate of M3562 *Mycoplasma pneumoniae* correlated with macrolide usage and genotype shifting. *Front Cell Infect Microbiol.* 2021;11:675466.

12. Pereyre S, Tardy F. Integrating the human and animal sides of mycoplasmas into medical and public health. *Res Microbiol.* 2000;151(4):259–65.

13. Vizarraga D, Kawamoto A, Matsumoto U, et al. Immunodominant proteins P1 and P40/P90 from human pathogen *Mycoplasma pneumoniae*. *Nat Commun.* 2020;11(1):5188.

14. Dumke R, Schurwanz N, Jacobs E. Characterisation of subtype- and variant-specific antigen regions of the P1 adhesin of *Mycoplasma pneumoniae*: Int J Med Microbiol. 2008;298(5–6):83–91.

15. Su CJ, Dallo SF, Baseman JB. Molecular distinctions among clinical isolates of *Mycoplasma pneumoniae*. *J Clin Microbiol.* 1990;28(7):1538–40.

16. Jacobs E, Vonski M, Oberle K, et al. Are outbreaks and sporadic respiratory infections by *Mycoplasma pneumoniae* due to two distinct subtypes? *Eur J Clin Microbiol Infect Dis.* 1996;15(1):38–44.

17. Fan L, Li D, Zhang L, et al. Pediatric clinical features of *Mycoplasma pneumoniae* infection are associated with bacterial P1 genotype. Exp Ther Med. 2017;14(3):1892–8.

18. Rodman Berlot J, Krivec U, Praprotnik M, et al. Clinical characteristics of infections caused by *Mycoplasma pneumoniae* P1 genotypes in children. *Eur J Clin Microbiol Infect Dis.* 2018;37(7):1265–72.

19. Zhao F, Liu J, Shi W, et al. Antimicrobial susceptibility and genotyping of *Mycoplasma pneumoniae* isolates in Beijing, China, from 2014 to 2016. *Antimicrob Resist Infect Control.* 2019;8:18.

20. Nakamura Y, Oishi T, Kaneko K, et al. Recent acute reduction in macrolide-resistant *Mycoplasma pneumoniae* infections among Japanese children. *J Infect Chemother.* 2021;27(2):271–6.

21. Kong F, Gordon S, Gilbert GL. Rapid-cycle PCR for detection and typing of *Mycoplasma pneumoniae* in clinical specimens. *J Clin Microbiol.* 2000;38(11):4256–9.

22. Schwartz SB, Thurman KA, Mitchell SL, et al. Genotyping of *Mycoplasma pneumoniae* isolates using real-time PCR and high-resolution melt analysis. *Clin Microbiol Infect.* 2009;15(8):756–62.

23. Hall TA. BioEdit: a user-friendly biological sequence alignment program for Windows 95/98/NT. *Nucleic Acids Symp Ser.* 1999;41(41):95–8.

24. Singh VK, Mangalam AK, Dwivedi S, et al. Primer premier program for design of degenerate primers from a protein sequence. *Biotechniques.* 1998;24(2):318–9.

25. Thornton B, Basiu C. Real-time PCR (qPCR) primer design using free online software. Biochem Mol Biol Educ. 2011;39(2):145–54.

26. Reuter JS, Mathews DH. RNAi: a tool for RNA secondary structure prediction and analysis. BMC Bioinformatics. 2010;11:129.

27. Feeney M, Murphy K, Lopilato J. Designing PCR primers painlessly. *J Microbiol Biol Educ.* 2014;15(1):28–9.

28. Zucker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 2003;31(13):3406–15.

29. Deschenes LA. Origin of 60: scientific data analysis and graphing software origin lab corporation (formerly Microcal Software, Inc.). *J Am Chem Soc.* 2000;122(39):9567–8.

30. Spuesens EBM, Oudber M, Hoogenboezem T, et al. Sequence variations in RepMP2/3 and RepMP4 elements reveal intragenomic homologous DNA recombination events in *Mycoplasma pneumoniae*. *Microbiology* (Reading). 2009;155(7):2182–96.

31. Jiang Z, Li S, Zhu C, et al. *Mycoplasma pneumoniae* infections: pathogenesis and vaccine development. *Pathogens.* 2021. doi:10.3390/pathogens10020119.

32. Zhou Z, Li X, Chen X, et al. Macrolide-resistant *Mycoplasma pneumoniae* in adults in Zhejiang, China. *Antimicrob Agents Chemother.* 2015;59(2):1048–51.

33. Zhao F, Li J, Liu J, et al. Antimicrobial susceptibility and molecular characteristics of *Mycoplasma pneumoniae* isolates across different regions of China. *Antimicrob Resist Infect Control.* 2019;8:143.

34. Jiang FC, Wang RF, Chen P, et al. Genotype and mutation patterns of macrolide resistance genes of *Mycoplasma pneumoniae* from children with pneumonia in Qingdao, China, in 2019. *J Glob Antimicrob Resist.* 2021;7:273–8.

35. Guo Z, Liu L, Gong J, et al. Molecular features and antimicrobial susceptibility of *Mycoplasma pneumoniae* isolates from paediatric inpatients in Weihai, China: characteristics of *M. pneumoniae* in Weihai. *J Glob Antimicrob Resist.* 2022;28:180–4.

36. Whister T, Sawatwong P, Diaz MH, et al. Molecular characterization of *Mycoplasma pneumoniae* infections in two rural populations of Thailand from 2009 to 2012. *J Clin Microbiol.* 2017;55(7):2222–33.

37. Keren T, Suzuki M, Sekizuka T, et al. Periodic genotype shifts in clinically prevalent *Mycoplasma pneumoniae* strains in Japan. Front Cell Infect Microbiol. 2020;10:385.

38. Madeira F, Park YM, Lee J, et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res.* 2019;47(W1):W636–w641.

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