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The Clinical Significance of Pathogen Loads and Macrolide Resistance Levels for Macrolide-Resistant *Mycoplasma pneumoniae* Pneumonia in Children

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Objective: *Mycoplasma pneumoniae* (*M. pneumoniae*) pneumonia presses a serious threat on children's health. This study was aimed to investigate the clinical significance of pathogen loads and macrolide resistance levels for macrolide-resistant *M. pneumoniae* (MRMP)-induced pneumonia in children.

Methods: Serum levels of inflammatory markers including lactic dehydrogenase (LDH), D-dimer, C-reactive protein (CRP) were tested. RT–PCR was used for the detection of *M. pneumoniae infection* and the macrolide resistance levels. The patients were classified into high pathogen load and low pathogen load groups based on the Ct values of the *p1* gene, and high macrolide resistance level and low macrolide resistance level groups based on the relative levels of macrolide resistance associated mutations to that of the *p1* gene. The rates of alternative antibiotic use and hospitalization days were recorded, and the leukocyte counts were tested.

Results: The rates of elevated inflammatory markers from high to low were LDH, CRP and D-dimer. The Ct values of the *p1* gene ranged from 19 to 35, and patients with higher pathogen loads had greater rates of alternative antibiotic use; higher levels of LDH, D-dimer, CRP and neutrophil counts (NEUT); and longer hospitalization durations. The range of the macrolide resistance levels was 0.31–2.11, and the rates of alternative antibiotic use, NEUT, CRP and D-dimer levels were higher in patients with higher macrolide resistance levels.

Conclusion: LDH was a more frequently elevated serum inflammatory marker than D-dimer and CRP, and the pathogen load and macrolide resistance levels possessed important clinical significance for MRMP-induced pneumonia in children.

Keywords: *Mycoplasma pneumoniae* pneumonia, children, inflammation, macrolide resistance levels, pathogen load

Introduction

Mycoplasma pneumoniae (*M. pneumoniae*) is an important pathogen that can cause pneumonia, accounting for 10–40% of community-acquired pneumonia cases.[1] Due to the self-limited nature of *M. pneumoniae* infection, the symptoms are often mild, but it can cause severe and refractory pneumonia in children, which places a heavy burden on children's health and even endangers lives.[2,3] Macrolide antibiotics are regarded as the first-line treatment for *M. pneumoniae* infections in children.[4] But due to the widespread and inappropriate use of macrolides, macrolide resistance has developed rapidly among *M. pneumoniae* strains, which has increased the morbidity of refractory pneumonia in children.[5–9] However, effective quinolones and tetracyclines for *M. pneumoniae* infection may have side effects on children. As a result, alternative antibiotics will be selected for the treatment of macrolide-resistant *M. pneumoniae* (MRMP)-induced pneumonia patients by pediatric clinicians after full considerations of the disease severity, benefits of alternative antibiotics and possible side effects.[10,11]

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Real-time PCR (RT–PCR) for the detection of DNA from *M. pneumoniae* strains has been used for the detection of infections, which has advantages including being fast, accurate and reflecting the pathogen load in the body.[4] The protein encoded by the *p1* gene was identified as the major determinant for *M. pneumoniae* adhesion.[12] RT–PCR targeting the *p1* gene can be used for the detection of *M. pneumoniae* infection. The macrolide resistance of *M. pneumoniae* is attributed to single-point mutations in region V of the 23S rRNA gene, including mutations at sites 2063, 2064, 2611, 2061, 2617 and 2067, among which A2063G and A2064G are the dominant mutations and other mutations rarely exist.[13–15] RT–PCR targeting the A2063G and A2064G mutations can be applied for the detection of the macrolide resistance of the infected *M. pneumoniae*. RT-PCR test is a kind of quantitative method, and the Ct values of the test results are negatively correlated with the amount of the initial templates. The Ct values of RT-PCR tests of *p1* gene from *M. pneumoniae* strains can be used to evaluate the amount of pathogen loads. On the other hand, the Ct values of RT-PCR tests of A2063G and A2064G can be used to evaluate the amount of genes with macrolide resistance associated mutations from *M. pneumoniae* strains. Due to the stability of *p1* gene, the relative levels of macrolide resistance of *M. pneumoniae* strains can be evaluated by the difference of the Ct values of RT-PCR tests of A2063G and A2064G with that of *p1* gene. Therefore, RT-PCR tests can be applied to evaluate the pathogen loads and macrolide resistance levels of *M. pneumoniae* strains.

The pathogen load of *M. pneumoniae* strains has been studied in previous studies, which can be correlated with disease conditions and drug resistance.[16–18] However, the studies have not focused on the clinical significance of the pathogen loads of only MRMP strains. The clinical characteristics of pneumonia caused by MRMP infection and macrolide-sensitive *M. pneumoniae* (MSMP) infection has been reported in several studies,[19–22] but the clinical significance of macrolide resistance levels among pneumonia patients caused by only MRMP infection deserves more attention.

Therefore, in this study, the clinical value of pathogen loads and macrolide resistance levels for the pneumonia caused by MRMP in children were investigated, including the rate of alternative antibiotic use, the duration of hospitalization, lactic dehydrogenase (LDH), D-dimer and C-reactive protein (CRP) levels and leukocyte counts. The results of the study may provide some new insights into the pneumonia caused by MRMP strains in children.

Methods

Study Subjects

The study subjects were recruited from patients who were hospitalized at the Children's Hospital Affiliated to Zhengzhou University due to the diagnosis of *M. pneumoniae* pneumonia (MPP), and the study was performed from July 2023 to May 2024. The inclusion criteria for cases included: 1. positive of *M. pneumoniae* infection; 2. the infected *M. pneumoniae* strains were macrolide resistant; 3. pneumonia identified by CT scans. The exclusion criteria for cases were: 1. patients with coinfections of other pathogens, such as *Haemophilus influenzae, streptococcus pneumoniae*, influenza virus, respiratory adenovirus and parainfluenza virus in addition to *M. pneumoniae*; 2. patients with basic diseases, such as heart disease. The sex and age of all the patients were recorded. The patients were pretreated with macrolides before attempting alternative antibiotics. The use of quinolones or tetracyclines during treatment and hospitalization durations were also recorded.

Co-Infection Pathogen Detection Method

For the detection of *Haemophilus influenzae* and *streptococcus pneumoniae* co-infections, sputum was collected from the patients and cultured on Columbia blood agar and chocolate agar plates for 2 days, then the bacterial colony were identified by mass spectrometer. To detect the co-infections of influenza virus and respiratory adenovirus, oropharyngeal swab specimens were collected from the patients and dissolved in the lysis buffer for minutes, then the buffer was added in the immunochromatography test strips to test the virus antigens. For the RT-PCR test of parainfluenza virus, oropharyngeal swab specimens were collected from the patients and dissolved in preserving fluid, RNA was extracted and then tested on the PCR test instruments with commercialized RT–PCR detection kits.

Inflammatory Marker and Leukocyte Count Tests

Peripheral blood was collected from all patients within one day of admission for inflammatory marker and leukocyte count analyses. Serum levels of LDH and plasma levels of D-dimer were tested with an AU5800 biochemical analyzer (Beckman, America) and CS5100 coagulation analyzer (Sysmex, Japan). The CRP level and leukocyte counts in the whole blood were analyzed by a BC7500 routine blood analyzer (Mindray, China). Then, the levels of inflammatory markers and leukocyte counts were compared between patients in different groups classified by pathogen loads and macrolide resistance levels of infected *M. pneumoniae*.

RT–PCR Tests for MRMP

Oropharyngeal swab specimens were taken from all the patients for RT–PCR analysis of *M. pneumoniae* infection and the macrolide resistance within one day after the onset of symptoms. The specimens were first put into tubes with 3mL preserving fluid (DAAN GENE, Guangzhou, China), and then DNA was extracted from the fluid after mixing the specimen with the preserving fluid adequately by oscillation. Commercialized DNA extraction kits (Zybio, China) were used for DNA extraction.

Commercialized RT–PCR detection kits from Mole (Jiangsu, China) were used for the detection of *M. pneumoniae* infection and the macrolide resistance-associated mutations at the same time. The *p1* gene in the genome of *M. pneumoniae* was the target of RT–PCR for the infection tests, and the A2063G and A2064G mutations in region V of the 23SrRNA gene were tested for macrolide resistance. The detection limit of *M. pneumoniae* and the macrolide resistance associated mutations was 500 copies/mL, and the reaction volume was 25 μL, so 13 copies could be detected per test. RT–PCR was performed under the following conditions: 1 cycle (50°C, 2 min); 1 cycle (95°C, 2 min); and 40 cycles (91°C, 15 sec and 64°C, 1 min). Internal positive and negative controls were included in each run, and two replicates were performed for each sample. All the tests were run at the same PCR instrument to exclude the influence of different instruments on the results. Positive results of *M. pneumoniae* infection and macrolide resistance genes were determined when Ct values were lower than 35 for the *p1* gene and macrolide resistance-associated mutations. However, the detection method was not able to distinguish between mutations at positions 2063/2064 of region V of the 23S rRNA gene. The precise Ct values of the *p1* gene and macrolide resistance-associated mutations of the infected MRMP strains were recorded for all the patients. The pathogen load of the patients was analyzed based on Ct values of the *p1* gene, and the patients were classified into two groups: high pathogen load (Ct<28) and low pathogen load (Ct≥28). Macrolide resistance levels of MRMP strains were calculated by the difference of Ct values of macrolide resistance levels and those of the *p1* gene with the formula: $2^{-\Delta Ct}$. Then, the macrolide resistance levels were standardized to the mean value of macrolide resistance levels (the mean value was regarded as 1), and the patients were grouped based on the relative macrolide resistance levels as: high macrolide resistance level (>1) and low macrolide resistance level (≤1). The rates of alternative antibiotic use, hospitalization days, the levels of serum inflammatory markers and leukocyte counts were compared between patients in different groups classified by pathogen loads and macrolide resistance levels of infected *M. pneumoniae*.

Statistical Methods

IBM SPSS 21.0 was used for the statistical analysis in the present study. For normally distributed quantitative data, the arithmetic mean ± standard deviation (SD) was used to express the mean and variation; the median and interquartile range were used for the mean and variation when the data were not normally distributed. Student's *t* test or nonparametric test was used for the statistical analysis of the quantitative data. The chi-square test was used for statistical analysis of qualitative data. $P<0.05$ indicated statistical significance.

Results

Population Characteristics of the Study Subjects

A total of 258 study subjects, including 144 male and 114 female children, were included in the study. The median age of all the children was 5.50 years, with an interquartile range of 3.35 years, and the oldest and youngest ages were 14.00 years and 0.39 years, respectively.

Ratios of Elevated Inflammatory Markers

The normal reference intervals of LDH, D-dimer and CRP applied were 0–10mg/l, 120–246U/L and 0–0.5μg/mL respectively. Among all the patients with MRMP-induced pneumonia, 76.36% (197/258), 27.91% (72/258) and 43.41% (112/258) had elevated LDH, D-dimer and CRP in contrast to their respective normal reference intervals. The elevated ratio of LDH was significantly greater than that of D-dimer (P=0.000) and CRP (P=0.000), and the elevated ratio of CRP was significantly greater than that of D-dimer (P=0.001) (see Figure 1).

The Clinical Significance of Pathogen Loads

In this study, 110 patients with high pathogen loads (Ct<28) and 148 patients with low pathogen loads (Ct≥28) were identified. The Ct values of the *p1* gene ranged from 19 to 35. The population characteristics (sex and age), laboratory test results (NEUT, CRP, LDH and D-dimer), rate of antibiotic use and hospitalization days between patients with high pathogen loads and those with low pathogen loads are shown in Table 1. No statistically significant differences were observed with regard to sex and age between the two groups. However, the neutrophil count (NEUT), CRP, LDH and D-dimer levels, rate of alternative antibiotic use and duration of hospitalization days were significantly greater in patients with high pathogen loads than those in patients with low pathogen loads.

The Clinical Significance of Macrolide Resistance Levels of Infected *M. Pneumoniae*

After the calculation and standardization of macrolide resistance levels of all the infected MRMP strains, 120 patients were classified as having high macrolide resistance levels of MRMP infection (median:1.110; interquartile range:0.168), and 138 patients were classified as having low macrolide resistance levels of MRMP infection (median:0.850; interquartile range:0.180). The range of macrolide resistance levels was 0.31–2.11. The population characteristics (sex and age), laboratory

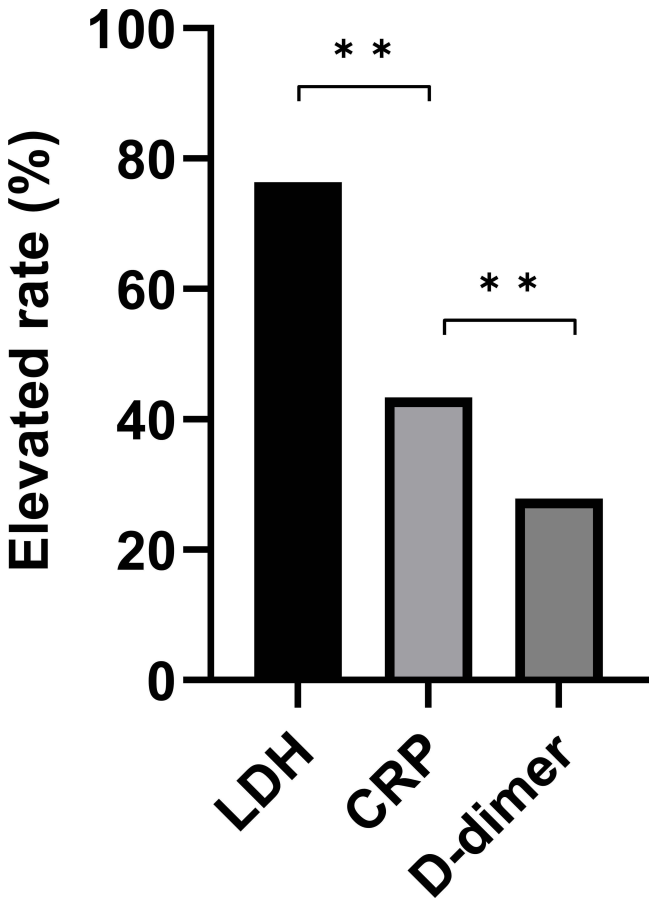

Figure 1 Ratios of elevated inflammatory markers. The ratios were calculated by the elevated numbers of cases to the total number of studied patients. **P<0.05.

Table 1 Laboratory Test Results, Rate of Alternative Antibiotic Use and Hospitalization Days Between Patients with Different Pathogen Loads

Parameters	High Pathogen Loads (Ct<28)	Low Pathogen Loads (Ct≥28)	*P*
Sex (male/female)	63/47	81/67	0.684
Age (median, interquartile range)	5.42, 3.69	5.50, 3.00	0.535
NEUT (10^9/L)	5.27±1.63	4.83±1.39	0.023
CRP (mg/L)	16.75±5.02	13.64±4.18	0.000
LDH (U/L)	309.36±58.92	293.40±49.29	0.019
D-dimer (μg/mL)	0.57±0.18	0.43±0.14	0.000
Rate of alternative antibiotic use	81 (110)	84 (148)	0.005
Hospitalized days (mean±SD)	7.14±2.08	6.35±1.52	0.001

Table 2 Laboratory Test Results, Rate of Alternative Antibiotic Use and Hospitalization Days Between Patients with Different Macrolide Resistance Levels of *M. Pneumoniae* Infection

Parameters	High Macrolide Resistance Levels (>1)	Low Macrolide Resistance Levels (<1)	*P*
Sex (male/female)	69/51	75/63	0.611
Age (median, interquartile range)	5.92, 3.29	4.92, 3.44	0.347
NEUT (10^9/L)	5.36±1.60	4.72±1.42	0.000
CRP (mg/L)	15.86±5.05	14.19±4.45	0.005
LDH (U/L)	298.86±54.16	301.37±54.18	0.710
D-dimer (μg/mL)	0.47±0.15	0.51±0.14	0.028
Rate of alternative antibiotic use	86 (120)	79 (138)	0.019
Hospitalized days (mean±SD)	6.81±1.98	6.58±1.66	0.315

test results (NEUT, CRP, LDH and D-dimer), rate of alternative antibiotic use and hospitalization days between patients with high and low macrolide resistance levels of *M. pneumoniae* infection are shown in Table 2. The rate of alternative antibiotic use, NEUT, CRP and D-dimer levels in patients with high macrolide resistance levels of *M. pneumoniae* infection was significantly greater than those in patients with low macrolide resistance levels of *M. pneumoniae* infection. However, there was no significant difference in the remaining parameters between the two groups.

Discussion

In the study of inflammatory markers, we found that LDH demonstrated a higher elevated rate than CRP and D-dimer in the pneumonia patients caused by MRMP. We detected the pathogen loads and macrolide resistance levels of *M. pneumoniae*-infected children via RT–PCR and investigated their clinical significance for MRMP-induced pneumonia in children. The study showed that high macrolide resistance levels in the infected *M. pneumoniae* strains could lead to increased use of alternative antibiotics and higher NEUT, CRP and D-dimer levels; on the other hand, high pathogen loads could result in increased levels of NEUT, CRP, LDH and D-dimer, greater rate of alternative antibiotic use and longer hospitalization duration.

LDH, D-dimer and CRP are frequently studied inflammatory markers in pneumonia.[23–25] LDH is a glycolytic enzyme that is present in the cytoplasm of all tissue cells in the body and can be elevated in hepatitis, myocarditis and MPP.[23] D-dimer can be elevated in the serum of patients with COVID-19 and MPP.[23,26] CRP can be elevated during inflammation, and patients with plastic bronchitis caused by *M. pneumoniae* exhibit increased levels of CRP.[24] In the present study, we evaluated the rates of elevated LDH, D-dimer and CRP in patients with pneumonia caused by MRMP to assess the sensitivity of theses inflammatory markers. LDH was the most frequently elevated inflammatory marker in pneumonia patients caused by MRMP with an elevated rate of 76.36%, followed by CRP and D-dimer.

As reported in previous studies, the pathogen load of *M. pneumoniae* can be evaluated via PCR methods.[16,17,27] Although the pathogen load of *M. pneumoniae* in bronchoalveolar lavage fluid (BALF) can better represent the actual infection situation than that in oropharyngeal swabs, the pathogen load from the two kinds of specimens can be consistent. On the other hand, it's more convenient for the collection of oropharyngeal swab specimens than to collect lavage fluid. Therefore, we evaluated the pathogen load of *M. pneumoniae* in oropharyngeal swab specimens with Ct values of the *p1* gene detected by RT-PCR in our study. In the study by Sun H et al,[16] the pathogen load of *M. pneumoniae* in BALF was detected by RT–PCR, and the study revealed a positive correlation between DNA copy number and CRP levels, which was consistent with the higher CRP levels in patients with higher pathogen loads found in our study. Deng F et al reported that patients with a high pathogen load of *M. pneumoniae* in the BALF exhibited increased levels of interleukin (IL)-6, IL-10, and tumor necrosis factor-α (TNF-α), and the load of *M. pneumoniae* was positively correlated with the CRP, LDH and D-dimer levels.[28] In our study, the levels of NEUT, CRP, LDH and D-dimer were greater in patients with higher pathogen loads, suggesting the more severe inflammatory conditions in those patients. In addition, the higher pathogen load of *M. pneumoniae* in patients could also cause a greater rate of alternative antibiotic use and longer hospitalization days. A study by Wang W et al revealed that the *M. pneumoniae* load was positively correlated with the length of hospitalization,[29] which was similar to our results. Therefore, it can be concluded that higher pathogen load of *M. pneumoniae* possessed significant clinical significance for MPP caused by MRMP.

Based on the Ct values of the *p1* gene and macrolide resistance-associated mutations of the infected MRMP strains, the macrolide resistance levels were calculated, and the patients were classified as having high macrolide resistance or low macrolide resistance levels of MRMP infection. In a study by Guo D et al, 61.59% of MRMP and MSMP coinfections were detected in infected children via allele-specific RT–PCR, suggesting that MRMP and MSMP can frequently coexist in one patient.[30] Another study revealed that coinfections of MRMP and MSMP developed from MSMP infection alone after azithromycin treatment.[31] Therefore, the different macrolide resistance levels of *M. pneumoniae* could be attributed from the different proportions of MRMP and MSMP coinfections. According to previous studies, MRMP infection can prolong febrile periods, increase the use of second-line treatment and decrease the efficacy of macrolides.[19–22] In our study, a greater rate of alternative antibiotic use, and higher levels of NEUT, CRP and D-dimer were observed in patients with *M. pneumoniae* infection with higher macrolide resistance levels, suggesting the effect of macrolide resistance levels. On the other hand, it can be speculated that higher macrolide resistance levels might lead to higher minimum inhibitory concentration of macrolide antibiotics for the higher frequency of alternative antibiotic use in patients. Therefore, macrolide resistance levels possessed some clinical significance among pneumonia patients caused by MRMP, but more limited than pathogen loads.

Conclusion

Inflammation is an important pathological characteristic in MPP, and LDH was a more frequently elevated serum inflammatory marker than D-dimer and CRP in MRMP-induced pneumonia patients. The pathogen load and macrolide resistance levels of MRMP determined by RT–PCR possessed great clinical significance for the MRMP-induced pneumonia in children.

Data Sharing Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Ethical Approval

Our study complied with the Declaration of Helsinki and was approved by the Ethics Committee of Henan Children's Hospital (IRB number: 2024-K-041).

Informed Consent

Written informed consent was obtained from all the patients or their guardians.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have no conflicts of interest to declare for this work.

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