


RESEARCH ARTICLE

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Detection of Macrolide Resistant *Mycoplasma pneumoniae* in Children with Lower Respiratory Tract Infection by Sanger Sequencing Targeting Domain V Region of 23S rRNA Gene

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Abstract

Mycoplasma pneumoniae is recognized as the leading cause of community-acquired lower respiratory tract infection in children, accounting for a significant proportion of pediatric mortality. Macrolides are the first-line treatment for *M. pneumoniae* infections. However, the extensive use of macrolides in clinical practice resulted in the emergence of macrolide-resistant *M. pneumoniae* (MRMP), which has a negative impact on treatment outcomes. Hence, in the present study, MRMP was determined in hospitalized children with community-acquired pneumonia (CAP). Real-time PCR detected *M. pneumoniae* in 41 of 348 clinical samples. Sanger sequencing revealed that none of the isolates were associated with the A2063G or A2064G base mutation, which confers macrolide resistance, in domain V of the 23S rRNA gene. Although MRMP was not observed in children with CAP in our study, healthcare practitioners should be vigilant about the potential risk of MRMP infections.

Keywords: Macrolide Resistant *Mycoplasma pneumoniae*, Community-acquired Pneumonia, 23S rRNA Gene Sequencing

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INTRODUCTION

Community-acquired pneumonia (CAP) is the leading cause of hospitalizations, with substantial morbidity and a high mortality rate in children.¹ Bacterial pathogens were considered one of the major causes of CAP, with *Mycoplasma pneumoniae* responsible for 10–40% of CAP infections in children worldwide.^{2,3} The CAP caused by *M. pneumoniae* was underestimated due to its mild symptoms, being overshadowed by other bacterial illnesses, and having limited rapid diagnostic methods. The World Health Organization (WHO) estimates *M. pneumoniae* is responsible for 20% of childhood mortality.⁴ In Southeast Asia, *M. pneumoniae* is responsible for 6.8% and 22% of deaths in neonates and children aged under 5 years, respectively.⁵ Most cases of childhood pneumonia in India are caused by bacteria; nevertheless, *M. pneumoniae* is one of the major respiratory pathogens causing atypical pneumonia in children.⁴ Waning immunity is believed to be a major cause of recurring infections in children, and it is therefore endemic in nature irrespective of risk factors. *M. pneumoniae* infection can be effectively managed with early detection and adequate antibiotic therapy. *M. pneumoniae* infection can be effectively managed with early detection and adequate antibiotic therapy. The beta-lactam group of antibiotics was ineffective against the cell wall, *M. pneumoniae*. Protein synthesis inhibitors, such as macrolides, are recommended as first-line antibiotics for *M. pneumoniae* infection. The overuse of macrolide antibiotics has led to the emergence of macrolide resistant *M. pneumoniae* (MRMP) strains caused by mutations in domain V of 23S rRNA, resulting in varying degrees of macrolide resistance. The prevalence of these MRMP infections has steadily increased during the last decade. It varies between 80 and 95 percent, especially in the Asian subcontinent.⁶ Rates have escalated to more than 90% in China, Japan, and Korea,⁶ more than 30% in Israel, and 12–13% in the United States and Canada.⁷ Despite the fact that the MRMP is increasing in other parts of the world, limited data is available in our region. Regular surveillance of *M. pneumoniae* associated with CAP will provide data on the impact of infection on children. Hence,

in the present study, the prevalence of MRMP infection was determined among the hospitalised children with pneumonia in our region.

MATERIALS AND METHODS

Patient population

The prospective study was conducted from September 2019 to July 2021. A total of 348 hospitalized children, aged between 2 months to 12 years, at a tertiary care centre in Chennai, South India, were included in the study. Children with the symptoms of community-acquired pneumonia as per the WHO criteria were included in the study (WHO, 2014). Children with aspiration pneumonia, hospital-acquired pneumonia, congenital heart diseases, airway anomalies, cystic fibrosis, congenital and acquired immunodeficiency were excluded from the study. The institutional human ethical committee approved our study, and informed consent was obtained from the children's parents before collecting the samples.

Sample collection and DNA isolation

Sputum samples were collected from children diagnosed with CAP and transported immediately to the laboratory at 4°C. Further, DNA was extracted from the sputum sample using the QIAamp DNA mini kit (Qiagen, Germany) as per manufacturer instructions. The extracted DNA samples were stored at -20°C until further processing.

Mycoplasma pneumoniae detection by RT-PCR

RT-PCR assays, which include primers (5'-GTGAACGTATCGTAACACGAGCTTT-3' and 5'-TGGTTTGTGACTGCCACTGC-3') and probes [5'FAMTTGTCGCGACTAAGGCCACG-BHQ-1-3'] that were specific to a 125-bp fragment of the P1-cytadhesin gene, were used to confirm the presence of *M. pneumoniae* in the clinical samples. The reaction was performed on a QuantStudio3 Flex real-time PCR machine (Thermo Fisher Scientific, Inc.) using a total reaction volume of 10 µL containing probe fast universal master mix (KAPA. REF: KK4702), 10 picomoles each of forward and reverse primers, and a 10ng DNA template. For RT-PCR, the following thermocycling conditions were used: 3 minutes at 95°C, 3 seconds

at 95°C followed by 20 seconds at 60°C and 30 seconds at 60°C for 40 cycles.

Screening of macrolide resistant *Mycoplasma pneumoniae*

MRMP was determined by amplifying and sequencing the domain V region of the 23S rRNA gene using the primers and PCR conditions described in previous literature.⁸ The Big Dye Terminator cycle sequencing kit (version 3.1; catalogue number 4337454; Applied Biosystems Inc.) was used to perform the sequencing reaction on the sense strand of the amplicon under the following thermal conditions: 1 cycle at 96°C for 1 min, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. The PCR products were purified by ethanol precipitation, and the ABI 3130XL instrument (Applied Biosystems Inc.) was used to sequence them under conditions appropriate for a 50-cm capillary. The raw sequencing data was analysed using the Bioedit programme to identify the gene mutations. Appropriate positive and negative controls were included in the study.

Statistical Analysis

Descriptive statistics were employed to analyse and summarize the important features of the study data.

RESULTS

Detection of *M. pneumoniae*

M. pneumoniae was observed in 11.7% (41/348) children presented with CAP using real-

time PCR. Figure 1 shows the prevalence of *M. pneumoniae*.

Clinical manifestations of children infected with *M. pneumoniae*

The majority of the *M. pneumoniae* positive samples (53.66%) were observed among the male children. Children aged one to five years have a higher prevalence of children with *M. pneumoniae* infection (63.41%) compared to other age groups were represented in the Table 1. Simultaneously, Table 2 shows the clinical symptoms such as cold, cough, breathlessness, chest in drawing and fever were observed among the children tested positive for *M. pneumoniae* infection. The results of X-rays showed that 58.6% of children had lobar pneumonia and bronchopneumonia, and that 17 out of 41 children (41.4%) who had a negative chest X-ray for pneumonia had tested positive for *M. pneumoniae* in a real-time PCR experiment. This emphasises the superiority of molecular detection over other traditional methods.

Mycoplasma pneumoniae 23S rRNA domain V gene sequence analysis by Sanger sequencing

PCR amplification and sequencing of the domain V region of the 23S rRNA gene were analyzed for A2063G and A2064G mutations. Figure 2 represents a multiple sequence alignment

Table 1. Table representing the proportions of age and sex of the *M. pneumoniae* positive and negative samples

Prevalence of <i>Mycoplasma pneumoniae</i>			
Mycoplasma infection			
Age & sex		Positive (%) N=41	Negative (%) N=307
Gender	Male	22 (53.66)	169 (55.05)
	Female	19 (46.34)	138 (44.95)
Age	< 1 year	2 (4.87)	32 (10.43)
	1-5 years	26 (63.41)	165 (53.74)
	> 5 years (5-12)	13(31.71)	110 (35.83)

Table 2. Table representing the proportions of clinical parameters of the *M. pneumoniae* positive and negative samples

Mycoplasma infection Clinical Parameter	Positive (%) N=41	Negative (%) N=307
	Cough & Cold	
Present	35 (85.37)	231 (75.24)
Nil (Absent)	6 (14.63)	76 (24.76)
Fever duration		
Present	39 (95.12)	296 (96.42)
Nil (Absent)	2 (4.88)	11 (3.58)
Breathlessness		
Present	30 (73.17)	193 (62.87)
Nil (Absent)	11 (26.83)	114 (37.13)
Chest indrawing		
Present	10 (24.39)	97 (31.6)
Nil (Absent)	31 (75.61)	210 (68.4)

file of the domain V region of the *M. pneumoniae* 23S rRNA gene. In our investigation, there are no variations and all strains are of the wild type.

DISCUSSION

The current study developed an internally regulated real-time PCR test that targets the P1 adhesion gene to diagnose *M. pneumoniae* infections. Fluorescent probes improve PCR specificity without requiring post-PCR processing and significantly reduce laboratory result turn around time, which improves patient management. In the present study, *M. pneumoniae* infection was observed in 11.7% of the study population, which is consistent with the previous study.⁹ Macrolides are widely used first-line medications for the treatment of *M. pneumoniae*, which is responsible for 40% of CAP worldwide and 30% of CAP in India.¹⁰⁻¹²

Our investigation revealed that *M. pneumoniae* infection primarily occurs in children aged between 1-5 years, which was consistent with the previous reports.¹³⁻¹⁵ Previous studies showed a significant difference in gender-based approaches, were girls being more susceptible to *M. pneumoniae* infection than boys.¹⁶ On the contrary, we found that male children had a higher incidence than female children, which could be attributed to differences in ethnicity and lifestyle.

In addition to molecular detection, the study examined the clinical subjects' chest X-rays

and found that among the *M. pneumoniae* positive cases, 43.9% of children had lobar pneumonia compared to other pneumonia, which is consistent with the findings of previous study that found a positive correlation between the aetiology of lobar pneumonia and *M. pneumoniae* infection.¹⁷

The study also compared the association of clinical symptoms with *M. pneumoniae* infection, but we did not find any notable change due to the similarity in signs and symptoms displayed by other typical and atypical pathogens. This misinterpretation will lead to antibiotic mismanagement. Thus, advancements in diagnostic techniques are required for specific detection of *M. pneumoniae*, which will undoubtedly aid in antibiotic selection.^{18,19}

Macrolides are widely used as first-line medications for *M. pneumoniae* infection which accounts for 40% of CAP worldwide and 30% of CAP in India.¹⁰⁻¹² Although tetracyclines, fluoroquinolones and erythromycin are effective in treating *M. pneumoniae* infection, they are not recommended for use in children due to their toxicity. Until the rise of the drug resistance in 2000, macrolides were the potential antibiotic in treating pneumonia caused by *M. pneumoniae*.^{2,20} The first MRMP strain was observed in Japan, and since then, the use of macrolides has begun to be reduced as a result of increased resistance, which has become a global burden.^{2,20,21} The majority of MRMP strains have 23S rRNA gene mutations (2063G and A2064G), which have been identified

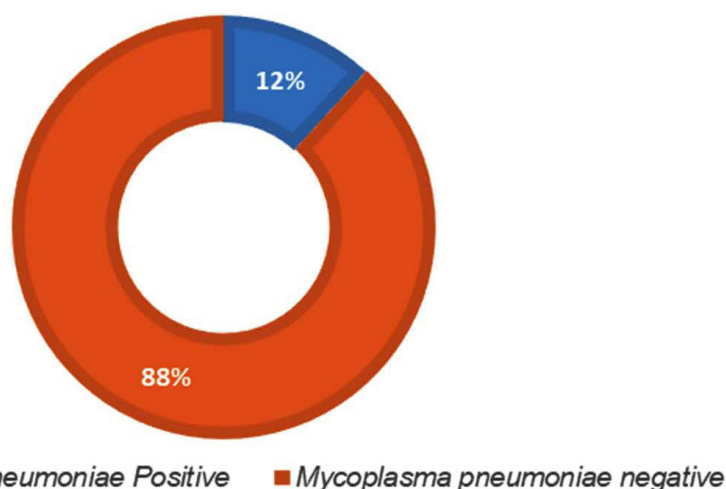


Figure 1. Prevalence of *M. pneumoniae* in the present study

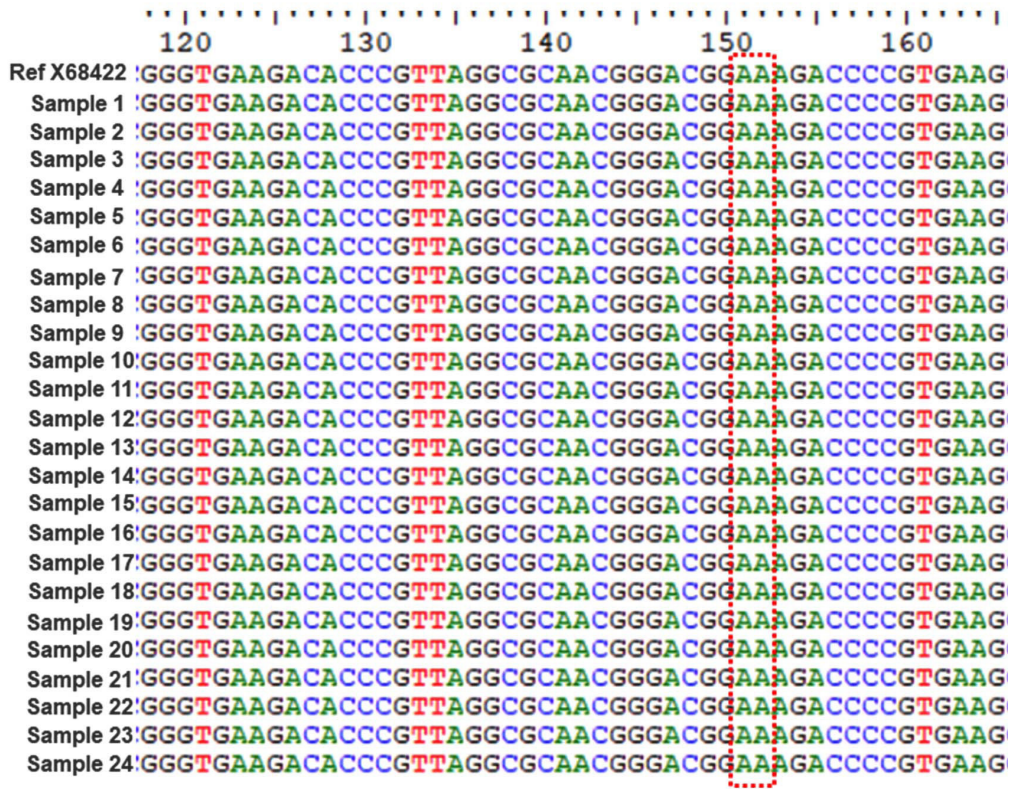


Figure 2. Multiple Sequence Alignment of 23S rRNA sequence of *M. pneumoniae* from 24 clinical samples along with reference sequence showing the A2063G or A2064G base mutations highlighted in the red dotted box, representing no mutations

as one of the major mechanisms by which *M. pneumoniae* acquires macrolide resistance.^{22,23} In the present study, no mutations in domain V of 23S rRNA gene were observed in *M. pneumoniae*, indicating that MRMP is not present in our study population. This suggests that we are in the early stages of macrolide resistance, which may worsen over the next decade. Regardless of the situation in India, the rapid spread of MRMP strains throughout Asia is a major concern. To reduce antibiotic misuse, continuous epidemiological monitoring of macrolide resistance must be prioritised.²⁴ As far as we know, this is the first study in our region to perform direct screening for MRMP in sputum of children with CAP. In the current study, induced sputum from children was found to be more representative of the lower respiratory tract than nasopharyngeal aspirates, which may later indicate the carrier state.

CONCLUSION

Real-time PCR is a sensitive, specific and rapid method for diagnosing *M. pneumoniae* infection in children. Although macrolide resistance was not observed in our study, given the unscrupulous use of macrolides in LRTIs, it may be the beginning of the development of macrolide resistance, which will likely increase over the next decade. Rapid detection of resistant-associated mutations would aid in prescription of an alternate antibiotic regimen, particularly in cases of chronic or recurring *M. pneumoniae* infection. Furthermore, macrolide stewardship is required to limit the use of these antibiotics and avoid excessive antibiotic prescribing, particularly in countries where macrolide resistance rates are still low. Even though the mutations A2063G and A2064G were not observed in our study, other

mutations in the 23S rRNA are still needed to confirm the absence of MRMP in our region.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Human Ethics Committee, Dr. ALM PG IBMS, University of Madras, Taramani Campus, Chennai, India, with approval number UM/IHEC/FRM/2021-X.

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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