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Original Article

Paediatrics Section

Prevalence of Genomic Resistance to Macrolide in Mycoplasma Isolates among Children with Community Acquired Pneumonia

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ABSTRACT

Introduction: Mycoplasma pneumonia is traditionally susceptible to macrolides, tetracycline and fluroquinolones. Since, tetracycline and fluoroquinolone are used cautiously in children, macrolides remain the antibiotic of choice for treating Mycoplasma pneumonia. But, resistance to macrolides has been reported in mycoplasma since the 2000s especially from Asia. Currently, there is no evidence on macrolide resistance of *mycoplasma pneumoniae* from India.

Aim: To identify the prevalence of genomic resistance to macrolides in mycoplasma isolates among children hospitalised with community acquired pneumonia.

Materials and Methods: This descriptive cross-sectional study was conducted in Department of Paediatrics at Institute of Child Health and Hospital for Children, Chennai, Tamil Nadu, India (tertiary care centre for children in South India), from September 2019 to August 2020. Children between 2 months to 12 years of age, who were hospitalised with community acquired pneumonia were included in the study. The sampling procedure used was induced sputum or mini bronchoalveolar lavage (in intubated children). The samples were processed for culture in Pleuropneumonia Like Organisms (PPLO) agar. The culture isolates showing the typical fried egg colonies were subjected to polymerase chain reaction to detect the presence of resistance conferring mutation in the P1 adhesin gene *MPN141*. Chi-square test was used to test statistical significance.

Results: Among the 268 children included in the study, mycoplasma pneumonia was positive in 33 (12.3%) cases. *Mycoplasma pneumoniae* was most common in children aged 1-5 years old (51.5%), followed by infants (36.4%) and children aged 5-12 (12.1%). There was no significant difference in distribution among males (39.4%) and females (60.6%) (p-value=0.08). None of the mycoplasma isolates in the study showed mutation for resistance conferring genes.

Conclusion: Macrolide resistance conferring genes were not identified in the study population, which may indicate that the mycoplasma strains from this part of India are still susceptible to macrolides.

Keywords: Antibiotic, Bronchoalveolar lavage, *Mycoplasma pneumoniae*, Mutation, Susceptible

INTRODUCTION

Mycoplasma pneumoniae infections are primarily treated with macrolides. Macrolide-resistant *M. pneumoniae* (MRMp) was first reported in Japan in the early 2000s. The resistance rates increased to more than 90% within 10 years, and was then reported through Asia and finally in Europe and North America [1-3]. Most cases of MRMp have been reported in children [4]. Mycoplasma's 170-kDa P1 protein is a adhesin protein that triggers a potent immune response. Mutations in the 23S rRNA for the 50S ribosome that alter the affinity for all macrolides cause mycoplasma to be resistant to this group of antibiotics. Most of the MRMp isolates demonstrate point mutations in domain V of 23S rRNA at positions 2058 or 2059 (*Escherichia coli* numbering) causing resistance to macrolides and lincosamides. Infection with macrolide resistant mycoplasma can cause clinical significant disease and worser patient outcomes when compared to Macrolide-Susceptible *M. pneumoniae* (MSMp) infections [5,6].

Children with macrolide-resistant *M. pneumoniae* infection, experienced considerably higher fever and cough durations, as well as hospital stays and antibiotic administration, as reported by Cardinale F et al., [6]. In the presence of macrolide resistance, tetracyclines or fluoroquinolones like levofloxacin, for 7-14 days are administered but these antibiotics are contraindicated in children less than 8 years old due to their side effect profiles [7]. Surveillance studies done in several countries have shown gradually increasing resistance to macrolides in mycoplasma over the past decade [7]. Such study is lacking in our country although anecdotal reports of resistant mycoplasma pneumonia have been published [8].

The clinic epidemiological features of mycoplasma pneumonia observed in the study have been published earlier [9]. Given the widespread usage of macrolides in the community, it's critical to assess the status of macrolide sensitivity in community-acquired pneumonia in our population. Hence, this study was done to study the prevalence of genomic resistance to macrolides in mycoplasma isolates among children hospitalised with Community Acquired Pneumonia (CAP).

MATERIALS AND METHODS

This descriptive cross-sectional study was conducted in Department of Paediatrics at Institute of Child Health and Hospital for Children, Chennai, Tamil Nadu, India (tertiary care centre for children in South India), from September 2019 to August 2020. The ethical clearance was obtained from the Institute Ethics Committee (No.10092019). Informed consent was obtained from the parents. Pneumonia was defined as the presence of fever and cough along with tachypnoea or lower chest wall indrawing or both, plus presence of new infiltrate on chest radiography.

Sample size calculation: With a prevalence of mycoplasma among CAP of 22% and 95% confidence interval, sample size was calculated as 268 [10].

Inclusion criteria: Children between 2 months to 12 years of age who were hospitalised with CAP were included in the study.

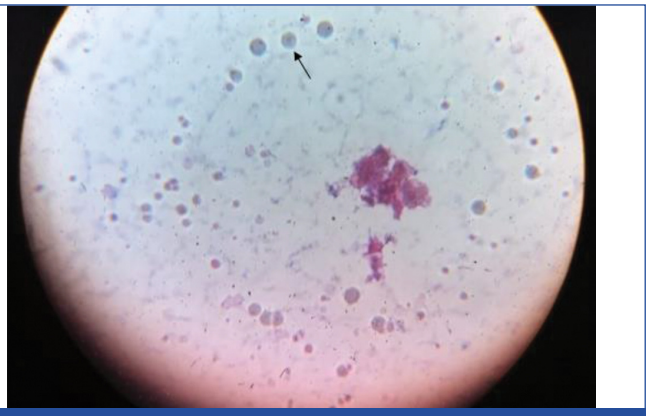
Exclusion criteria: Children who had received prior treatment with antimycoplasma agents (azithromycin, clarithromycin, erythromycin and fluroquinolones-doxycycline) and any child with contraindications to sputum induction like nasal bleeding, bleeding

tendencies and reduced level of consciousness were also excluded from the study.

Sample collection: The induced sputum was preferred for sample collection method because it is representative of the lower respiratory tract, yields a considerable amount of material for molecular analysis, and is relatively non invasive [11]. In children who were ventilated with community acquired pneumonia, mini bronchoalveolar lavage done via the endotracheal tube was performed for sampling.

Procedure

Culture: The sputum samples were inoculated in Pleuropnemonia Like Organism (PPLO) agar medium with supplements and incubated at 37°C with 5% CO_2. The PPLO agar plates were microscopically analysed for *M.pneumoniae* for 21 days, evaluating the morphological characteristics of PPLO colonies (fried egg appearance) as well as their staining reaction with Diene's dye [Table/Fig-1]. The mycoplasma isolates were then subjected to Deoxyribonucleic Acid (DNA) extraction and real time Polymerase Chain Reaction (PCR) analysis to detect the presence of mutation conferring P1 gene.



[Table/Fig-1]: Microscopic view of fried egg colonies of mycoplasma viewed under 100X magnification of culture plate (arrow).

DNA extraction: QIAamp DNA Mini Kit (Qiagen, Germany) was used to extract genomic DNA from sputum samples. The extracted DNA was quantified using NanoDrop UV Visible Spectrophotometers at 260 nm and stored at -20ºC. Those specimens that showed growth were subjected to real time polymerase chain reaction to identify the nucleotide sequences that confer macrolide resistance to the mycoplasma.

Real time PCR: Real-time PCR targeting the P1 cytadhesin gene was done using Quantstudio 6 (Applied Biosystem) real-time PCR system under the following conditions: initial activation at 95°C for 2 minutes, followed by 45 cycles of 95°C for 10 s and 60°C for 30 seconds. PCR reaction mixture comprised the following components per reaction: 10 µL of probe master mix, 0.5 uM of forward and reverse primer, 0.1 uM of probe, 5 µL of genomic DNA and nuclease-free water. Macrolide resistance among the *M.pneumoniae* was detected using previously published probe and temperature conditions [12].

High resolution melt analysis was performed between 81ºC and 84ºC. Melting temperature for the macrolide resistant *M.pneumoniae* strain was higher than the macrolide susceptible strain. Appropriate positive and negative controls were included in the assay.

STATISTICAL ANALYSIS

The association between culture and demographic parameter, clinical feature, physical examination, antibiotic and laboratory findings was assessed by cross tabulation and comparison of percentages. Chi-square test was used to test statistical significance. A p-value <0.05 was considered statistically significant. Statistical Package for Social Sciences (SPSS); IBM version 21.0 was used for statistical analysis.

RESULTS

A 268 children {147 (54.9%) male and 121 (45.1%) female} were eventually included in the study out of 312 children admitted for CAP who were examined for eligibility. There were 33 (12.3%) cases of mycoplasma pneumonia. *Mycoplasma pneumoniae* was most common in children aged 1 to 5 years old (51.5%), followed by infants (36.4%) and children aged 5 to 12 (12.1%). There was no significant difference in distribution among males (39.4%) and females (60.6%) (p-value=0.08). Clinical characteristics of children with mycoplasma pneumonia is given in [Table/Fig-2]. Macrolide sensitivity; *M.pneumoniae* isolated from the 33 cases by culture were subjected to PCR to detect presence of resistance conferring mutation. None of the mycoplasma isolates in the present study showed mutation for this resistance conferring gene.

Signs and symptoms	Children with community acquired pneumonia (N=268)		p-value (Chi-square test)
	M.pneumoniae culture positive (n=33)	M.pneumoniae culture negative (n=235)	
Fever	31 (93.9%)	227 (96.6%)	0.451
Cough	20 (60.6%)	190 (80.9%)	0.008
Fast breathing	16 (48.5%)	154 (65.5%)	0.057
Chest indrawing	9 (27.3%)	101 (43.0%)	0.086
Crepitations	14 (42.4%)	148 (63.0%)	0.024
Wheeze	7 (21.2%)	55 (23.4%)	0.780
Hypoxia {Arterial Oxygen Saturation (SaO_2v<92%)}	0	28 (11.9%)	0.036

[Table/Fig-2]: Clinical characteristics of children with Mycoplasma pneumonia.

DISCUSSION

Surveillance studies on macrolide resistance mycoplasma prevalence done in children have shown resistance rates of 46-93% in Japan, 69-97% in China, 12.3-23% in Taiwan, 61.3% in South Korea, 30% in Israel, 9.8% in France, and 8.2% in the United States [13]. Because its incidence decreased with the use of macrolides being curtailed, the rising prevalence of macrolide-resistant *M. pneumoniae* may be ascribed to this practise [14].

Point mutations in domain V of the 23S rRNA gene is associated with a 10^3-fold to $>10^5$ fold elevation of the minimum inhibitory concentration of erythromycin when compared to susceptible mycoplasma strains [15]. Substitution of adenine with guanine at base position 2063 was present in all 26 mutant sequences from a study from Singapore and is the only study on the prevalence of genotypic macrolide resistance in *M. pneumoniae* from South-East Asia in current literature [16]. The prevalence of genomic resistance in the mycoplasma isolates was not observed in the present study. This also correlated clinically as children in the *M. pneumoniae* positive group universally improved clinically. Less common mutations occurring at positions 2063 (A→T, A→C), 2064 (A→G, A→C), 2067 (A→G) and 2617 (not studied in the present study) have been reported [1,17].

Although no macrolide resistance was observed in our study, given the widespread use of macrolide in lower respiratory tract infection, we may be in the initial stages in development of macrolide resistance which will likely increase in the next decades. The low prevalence in the study population may be due to the low prevalence of macrolide exposure. Similar low prevalence of macrolide resistance has been reported in some countries but there may be an underestimation of the spread of macrolide-resistant *M. pneumoniae* in countries that lack national surveillance systems for monitoring the same. [18,19] Hence, unwarranted use of macrolides should be avoided in children with community acquired pneumonia. Continued vigilance is also required to detect the development of resistance to macrolides.

To the best of authors' knowledge, this is the first study from India studying the prevalence of genomic resistance of mycoplasma isolates from children with community acquired pneumonia. The study's sampling method of induced sputum is more representative

of the lower respiratory tract than nasopharyngeal aspirates, which could indicate a carrier state.

Point mutations conferring macrolide resistance, in less common sites of the P1 protein genome may need to be further be assessed in India. Guo DX et al., have reported missense mutation (K27N) in L4 ribosomal protein, mutation M144V in L4 ribosomal protein and S170P in L22 which can also confer macrolide resistance. Further studies are needed to detect such mutations [20]. About 90% of *M. pneumoniae* infections in Asian countries like China and Japan are caused by resistant strains [21]. On the other hand, in Western countries, the proportion of resistant bacteria has been relatively low, ranging from 1% to 10% [22]. This variation in resistance pattern has been attributed to the judicious use of macrolides in the western countries.

Approximately 25% of patients with *M. pneumoniae* infection may be hospitalised due to extrapulmonary complications or severe pneumonia [23]. Additionally, the prevalence and incidence of *M. pneumoniae* may vary in different seasons and across different geographical regions [24].

Limitation(s)

There were several limitations in the present study. First, only the most common mutation conferring genomic resistance in the P1 protein gene was evaluated. There may be other mutations that are prevalent in the studied community which could alter the resistance pattern. Secondly, the genomic resistance was not correlated with in-vitro microbiological resistance in the study. So, the degree of in-vivo resistance to macrolides could not be reliably assessed. Third, the seasonal data could not be obtained.

CONCLUSION(S)

Genomic resistance of mycoplasma to macrolides is currently not demonstrable in children with community acquired pneumonia in the study population. Surveillance of *M. pneumoniae* infection is particularly important in the monitoring and treatment of community acquired pneumonia. So, authors recommend further research in this regard to ease the burden on the health care system in our country. More number of researches to be done in this area to get overall data of genomic resistance.

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