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Differences in the Frequency of 23S rRNA Gene Mutations in *Mycoplasma pneumoniae* between Children and Adults with Community-Acquired Pneumonia: Clinical Impact of Mutations Conferring Macrolide Resistance

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We investigated the frequency and clinical significance of macrolide resistance in adult and pediatric patients with community-acquired pneumonia from a *Mycoplasma pneumoniae* infection. The frequency of the A2063G mutation in the 23S rRNA gene was significantly higher in children than in adults (61.3% [19/31] and 13.3% [8/60], respectively; $P < 0.001$). Patients with macrolide-resistant *M. pneumoniae* infections showed a longer duration of fever ($P = 0.021$) and required a longer duration of antibiotic treatment ($P = 0.007$).

Mycoplasma pneumoniae is a major cause of community-acquired pneumonia (CAP) in both children and adults. While quinolones are frequently used to treat adults with *M. pneumoniae* pneumonia, macrolides are considered the drug of choice for pediatric patients with this condition due to the potential side effects of quinolones in children. An increase of macrolide-resistant *M. pneumoniae* (MRMP) has been observed in pediatric pneumonia patients in several countries (7, 11, 15, 20). Pneumonia caused by MRMP shows an increased duration of symptoms and requires a longer antibiotic treatment course compared to pneumonia caused by macrolide-susceptible *M. pneumoniae* (MSMP) in pediatric patients (2, 8, 16). Even though some findings for the frequencies of MRMP in adult patients have been reported, the clinical significance of macrolide resistance in adult pneumonia remains open for debate, since *M. pneumoniae* infection typically produces mild symptoms that spontaneously diminish (2, 3, 9, 10, 13). In terms of the frequencies and clinical significance of MRMP, simultaneous comparative findings between adults and children have not been reported previously.

In this study, we compared the prevalence and clinical significance of macrolide resistance in adult and pediatric patients with pneumonia during a short *M. pneumoniae* epidemic that arose in 2011 (21). Between September and December 2011, consecutive patients diagnosed with CAP, based on clinical symptoms and chest X-ray images, were enrolled in two hospitals in Seoul, Republic of Korea. Both hospitals are tertiary care centers affiliated with medical colleges and are located approximately 18 km apart. The patients who had other respiratory pathogens were excluded on the basis of their sputum culture, a urinary pneumococcal antigen test, a multiplex PCR assay for 15 respiratory viruses, and a multiplex PCR assay for common respiratory bacteria, including *Chlamydophila pneumoniae* and *Legionella pneumophila*. This study did not interfere with patient management decisions, which includes the use and duration of an antipyretic agent. Fever was defined as a body temperature of above 38°C. The initiation of fever was determined by objectively recording fever upon admission or a history of fever checked in-house or at a primary care clinic. The endpoint of fever was defined as a temperature below 38°C for at least 24 h without the use of an antipyretic.

Nasopharyngeal aspirate (NPA) specimens from pediatric patients and sputum specimens from adult patients were collected for PCR confirmation using Seeplex PneumoBacter ACE detection assays (Seegene, Seoul, Republic of Korea). Sputum specimens (350 μl) were treated with proteinase K, and DNA was extracted using QIAamp DNA stool minikits (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. NPA specimens were vortexed briefly, and 500-μl aliquots of the 3-ml samples of universal transport medium were processed using NucliSens easyMAG kits (bioMérieux, Marcy l'Etoile, France). DNA samples were stored at −70°C prior to sequencing analysis. Domain V of the 23S rRNA gene (GenBank accession no. X68422) was amplified using previously described primer pairs (19). The nested PCR primers and conditions described by C. E. Oh et al. were used for the specimens (14), which yielded negative results using the single PCR method mentioned above. PCR products were purified using a Power Gel Extraction kit (TaKaRa Bio Inc., Shiga, Japan). The purified templates were sequenced using an ABI Prism BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and were analyzed on an ABI 3730xl DNA analyzer (Applied Biosystems). For comparisons of clinical characteristics and outcomes, SPSS for Windows, version 18.0, was used as follows. Comparisons of frequencies were performed using the Pearson chi-square test or Fisher's exact test (if the expected frequency was less than 5). Comparisons of means (durations of illnesses or therapies) were performed using the Mann-Whitney U test.

During the study period, a total of 91 CAP patients were confirmed as having *M. pneumoniae* pneumonia by the PCR method (Table 1). The median age of the patients was 27 years (range, 1 to

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TABLE 1 Characteristics of community-acquired pneumonia in patients infected with *Mycoplasma pneumoniae* with and without an A2063G mutation

Characteristic	Value for characteristic[a]			
		Patients infected with *M. pneumoniae*		
	All patients ($n = 91$)	With the A2063G mutation ($n = 27$)	Without the A2063G mutation ($n = 64$)	*P* value
Age, yr [median (range)]	27 (1–84)	6 (2–58)	37 (1–84)	
No. of males (%)	39 (42.9)	11 (40.7)	28 (43.8)	0.791
No. of patients (%) with the following symptom or characteristic				
Cough	89 (97.8)	27 (100)	62 (96.9)	0.492
Sputum	84 (92.3)	25 (92.6)	59 (92.2)	0.657
Coryza	7 (7.7%)	4 (14.8)	3 (4.7)	0.190
Fever (>38°C) at presentation	58 (63.7)	23 (85.2)	35 (54.7)	0.008
Parapneumonic effusion	17 (18.7)	5 (18.5)	12 (18.8)	0.615
Elevated liver enzymes[b]	29 (31.9)	8 (29.6)	21 (32.8)	0.484
No. of patients (%) with the following initial antibiotic therapy				
β-Lactam + macrolide	56 (61.5)	22 (81.5)	34 (53.1)	
β-Lactam + fluoroquinolone	18 (19.8)	1 (3.7)	17 (26.6)	
Macrolide	5 (5.5)	1 (3.7)	4 (6.3)	
Fluoroquinolone	10 (11.0)	3 (11.1)	7 (10.9)	
β-Lactam only	2 (2.2)	0 (0)	2 (3.1)	
No. of patients (%) hospitalized	80 (87.9)	26 (96.3)	54 (84.4)	0.164
Durations (no. of days), mean ± SD				
Duration of hospital stay (IQR)[c]	6.0 ± 5.4 (3–7)	7.4 ± 6.9 (3–10)	5.4 ± 4.6 (3–6)	0.242
Duration of fever after start of antibiotics	3.8 ± 3.4	5.6 ± 4.6	2.7 ± 1.4	0.001
Total duration of fever	8.2 ± 3.8	9.6 ± 4.2	7.4 ± 3.3	0.021
Total duration of antibiotic use	12.0 ± 5.4	14.5 ± 6.3	10.9 ± 4.6	0.007

[a] The values for the characteristics are shown. Most values are the number of patients with the percentage in parentheses. For the various duration characteristics, the values are means ± standard deviations.
[b] Twofold increase above normal cutoff values.
[c] IQR, interquartile range.

84 years). Thirty-one patients (34.1%) were children less than 16 years old. Thirty-nine patients (42.9%) were male. Four pediatric (12.9%) and five adult (8.3%) patients were transferred from other clinics under macrolide and/or quinolone therapy for 1 to 5 days. All patients had infiltrations showing on chest X-rays. Eighty patients (87.9%) were hospitalized for 2 to 32 days. A β-lactam antibiotic in addition to a macrolide antibiotic (61.5%, $n = 56$) was the most frequently used regimen within 24 h of admission, followed by a β-lactam plus fluoroquinolone (19.8%, $n = 18$), fluoroquinolone (11.0%, $n = 10$), macrolide (5.5%, $n = 5$), and β-lactam (2.2%, $n = 2$). All of the pediatric patients were initially treated with a macrolide antibiotic plus a β-lactam antibiotic. Antipyretics were used in almost all of the pediatric patients (28/31 [90.3%]) and half of the adult patients (30/60 [50.0%]). Among the pediatric patients, two (2/31 [6.5%]) had asthma. Among the adult patients, 12 patients (12/60 [20.0%]) had underlying disorders, including end-stage renal disease ($n = 3$), diabetes ($n = 3$), solid cancer ($n = 2$), chronic obstructive pulmonary disease ($n = 2$), and transplantation recipient status ($n = 2$).

Using the sequencing results for the 23S rRNA genes, 27 (29.7%) of the patients were diagnosed with A2063G mutations. The prevalence of A2063G mutations was significantly higher in children (19/31 patients [61.3%]) than in adults (8/60 patients [13.3%]) ($P < 0.001$). There were no other known mutations such as A2063C, A2064G, or A2067G found in domain V of the 23S rRNA gene in our patient series. Twenty-nine (35.8%) samples needed nested PCR for amplification of 23S rRNA gene.

Compared with MSMP patients, MRMP patients showed significantly longer durations of antibiotic treatments (14.5 ± 6.3 days versus 10.9 ± 4.6 days; $P = 0.007$), longer febrile periods from the onset of illness (9.6 ± 4.2 days versus 7.4 ± 3.3 days; $P = 0.021$), and longer febrile periods from the start of any antibiotic regimen (5.6 ± 4.6 days versus 2.7 ± 1.4 days; $P = 0.001$) (Table 1). Longer durations of illness were observed in both age groups, although the differences were not statistically significant (Table 2).

In our current analysis, children showed significantly higher proportions of macrolide resistance (61.3%) compared with adult and adolescent patients of ≥16 years old (13.3%). Presumptive causes of this finding would include more frequent use of macrolides in children for the treatment of acute lower respiratory symptoms. Data from the Korean Health Insurance Review and Assessment Service showed higher frequencies of antibiotic treatment in children compared with adults for acute lower respiratory infections in 2007 (65.9 to 68.4% in children versus 46.1 to 50.8% in adults) (http://kosis.kr/abroad/abroad_02List.jsp?paretID=1211035,354). Toddlers and school-age children with symp-

TABLE 2 Durations of illness and treatments in pediatric and adult patients infected with *Mycoplasma pneumoniae* with and without an A2063G mutation

Duration	Mean no. of days ± SD of illness or treatment in patients <16 years old			Mean no. of days ± SD of illness or treatment in patients ≥16 years old		
	Infected with *M. pneumoniae* with the A2063G mutation ($n = 19$)	Infected with *M. pneumoniae* without the A2063G mutation ($n = 12$)	*P* value	Infected with *M. pneumoniae* with the A2063G mutation ($n = 8$)	Infected with *M. pneumoniae* without the A2063G mutation ($n = 52$)	*P* value
Duration of hospital stay	6.3 ± 5.2	4.1 ± 1.8	0.269	10.3 ± 10.1	5.8 ± 5.0	0.162
Total duration of fever	9.2 ± 4.6	7.1 ± 3.0	0.241	11.3 ± 1.7	7.5 ± 3.6	0.023
Duration of fever after start of antibiotics	5.6 ± 2.7	2.7 ± 1.8	0.103	5.8 ± 1.0	2.6 ± 1.2	0.002
Total duration of antibiotic use	15.4 ± 5.4	12.1 ± 2.6	0.039	12.6 ± 8.1	10.7 ± 4.9	0.861

toms and signs of pneumonia are usually treated empirically with macrolide antibiotics, because *M. pneumoniae* is a leading cause of CAP in these age groups. Moreover, children are usually not treated with quinolones due to the risk of side effects, whereas adults are frequently treated with quinolones for acute lower respiratory symptoms. A remarkable and continuous increase in the production of new macrolides has been noted in the market in the Republic of Korea (6). Presumptive and often inappropriate use of macrolides contributes to the increase of resistant pathogens, including macrolide-resistant *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Streptococcus pyogenes*, and *M. pneumoniae* (4). In addition, noncompliance in children to macrolide use might contribute to bacterial resistance during the course of infection.

In our current study, patients with MRMP showed significantly longer durations of fever, antibiotic treatment, and fever while undergoing antibiotic treatments. These differences were also observed in each age group, although they did not reach statistical significance (Table 2). Reports on the clinical presentation of MRMP infections are rare in adults with pneumonia (3, 10). In our series, four adult patients with MRMP were initially treated with macrolides (with or without β-lactam antibiotics). Three of the patients failed to improve for 2 to 6 days while being treated with a macrolide antibiotic until the antibiotic regimen was modified to include a fluoroquinolone antibiotic. One study in China (2) has reported differences in the clinical features of MRMP and MSMP in pneumonia patients aged ≥14 years. In that study, patients with MRMP showed significantly longer durations of antibiotic therapy compared with patients with MSMP (mean, 9 days versus 7 days, respectively), as well as prolonged fever after the initiation of antibiotics (mean, 4 days versus 3 days, respectively), but the duration of fever from the onset of illness showed no significant difference (2).

Macrolide resistance was detected using a genotype method instead of a phenotype method. Sequence analysis of domain V of the 23S rRNA gene was performed because mutations in this area are known to be dominant in conferring macrolide resistance in *M. pneumoniae* (19, 22). The A2063G mutation was found to be the most prevalent in MRMP isolates, followed by the A2064G mutation. Mutations in ribosomal proteins L4 and L22 were very rare, and the role of these mutations in resistance to macrolides remains uncertain (1, 17). In previous publications, most cases of MRMP with a high MIC harbored a 23S rRNA gene mutation, and all of the clinical isolates with mutations at positions A2063 and A2064 showed high resistance to all types of macrolide antibiotics tested (7, 12, 19, 22). Sequence analysis of the 23S rRNA gene for dominant mutations (A2063G, A2064G) conveying macrolide resistance was performed on DNA isolated directly from sputum and nasal aspirate specimens without employing time-consuming phenotypic assays. These assays may also enable clinicians to select appropriate treatment options more rapidly and may provide a convenient method for conducting surveillance for genetic mutations that confer antibiotic resistance.

Our study has some limitations that need to be addressed. Some of the information available to us regarding the onset of illness and initial treatment in our patient cohorts was insufficient, since several patients were transferred from primary care hospitals under antibiotic treatment. Inaccurate data were considered missing values in the analysis. The total duration of symptoms could not be investigated, because most patients were discharged after improvement of respiratory symptoms and the alleviation of fever. Changes in the antibiotic regimen, from macrolides to quinolones, could not be statistically analyzed, since it was not performed in children. Since our current study was a noninterventional observational study, diagnostic work-ups or outpatient department follow-ups were completed as part of the routine clinical practice by the attending physicians. Hence, we could not obtain sequential antibody titers from most of our patients. Although four pediatric and five adult patients were transferred from other clinics while being treated by macrolide and/or quinolone therapy for 1 to 5 days, we were still able to amplify the *M. pneumoniae* DNA in those samples. PCR systems can be applied to specimens containing *M. pneumoniae* organisms that are not viable (18). We may have included patients of an *M. pneumoniae* carrier state. Since the current study was focused on the impact of mutations in *M. pneumoniae* that conferred macrolide resistance, we did not include cases of coinfection or perform more-thorough diagnostic tests.

The target for the detection of *M. pneumoniae* is the ITS1 gene from the PneumoBacter ACE detection kit. *M. pneumoniae* contains one rRNA operon and, therefore, only one ITS1 gene and one 23S rRNA gene (5). All samples were positive for the ITS1 gene by single PCR, which suggests that all of the samples contained a sufficient amount of *M. pneumoniae* DNA. Meanwhile, the presence of a nonamplified specimen after 23S rRNA PCR implies that there could be other reasons for unsuccessful PCR amplification such as nucleotide variation in primer binding sites.

In summary, we have demonstrated a higher frequency of macrolide resistance in children with *M. pneumoniae* pneumonia

compared with adults during a brief epidemic of *M. pneumoniae* pneumonia in a city. Our clinical data suggest that MRMP infection takes a more serious clinical course due to a prolonged duration of fever despite antibiotic treatment in both adults and children.

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REFERENCES

1. **Bebear CM, Pereyre S.** 2005. Mechanisms of drug resistance in *Mycoplasma pneumoniae.* Curr. Drug Targets Infect. Disord. **5**:263–271.
2. **Cao B, et al.** 2010. High prevalence of macrolide resistance in *Mycoplasma pneumoniae* isolates from adult and adolescent patients with respiratory tract infection in China. Clin. Infect. Dis. **51**:189–194.
3. **Isozumi R, Yoshimine H, Morozumi M, Ubukata K, Ariyoshi K.** 2009. Adult community-acquired pneumonia caused by macrolide resistant *Mycoplasma pneumoniae.* Respirology **14**:1206–1208.
4. **Jacobs MR, Johnson CE.** 2003. Macrolide resistance: an increasing concern for treatment failure in children. Pediatr. Infect. Dis. J. **22**:S131–S138.
5. **Klappenbach JA, Dunbar JM, Schmidt TM.** 2000. rRNA operon copy number reflects ecological strategies of bacteria. Appl. Environ. Microbiol. **66**:1328–1333.
6. **Koh E, Kim S.** 2010. Decline in erythromycin resistance in group A streptococci from acute pharyngitis due to changes in the *emm* genotypes rather than restriction of antibiotic use. Korean J. Lab Med. **30**:485–490.
7. **Liu Y, et al.** 2009. Antimicrobial susceptibility of *Mycoplasma pneumoniae* isolates and molecular analysis of macrolide-resistant strains from Shanghai, China. Antimicrob. Agents Chemother. **53**:2160–2162.
8. **Matsubara K, et al.** 2009. A comparative clinical study of macrolide-sensitive and macrolide-resistant *Mycoplasma pneumoniae* infections in pediatric patients. J. Infect. Chemother. **15**:380–383.
9. **Miyashita N, et al.** 2012. Macrolide-resistant *Mycoplasma pneumoniae* in adolescents with community-acquired pneumonia. BMC Infect. Dis. **12**: 126. doi:10.1186/1471-2334-12-126.
10. **Miyashita N, et al.** 2011. Community-acquired macrolide-resistant *Mycoplasma pneumoniae* pneumonia in patients more than 18 years of age. J. Infect. Chemother. **17**:114–118.
11. **Morozumi M, et al.** 2008. Increased macrolide resistance of *Mycoplasma pneumoniae* in pediatric patients with community-acquired pneumonia. Antimicrob. Agents Chemother. **52**:348–350.
12. **Morozumi M, Takahashi T, Ubukata K.** 2010. Macrolide-resistant *Mycoplasma pneumoniae*: characteristics of isolates and clinical aspects of community-acquired pneumonia. J. Infect. Chemother. **16**:78–86.
13. **Narita M.** 2011. Two unexpected phenomena in macrolide-resistant *Mycoplasma pneumoniae* infection in Japan and the unique biological characteristics of *Mycoplasma pneumoniae*. J. Infect. Chemother. **17**:735–736.
14. **Oh CE, Choi EH, Lee HJ.** 2010. Detection of genetic mutations associated with macrolide resistance of *Mycoplasma pneumoniae.* Korean J. Pediatr. **53**:178–183.
15. **Peuchant O, et al.** 2009. Increased macrolide resistance of *Mycoplasma pneumoniae* in France directly detected in clinical specimens by real-time PCR and melting curve analysis. J. Antimicrob. Chemother. **64**:52–58.
16. **Suzuki S, et al.** 2006. Clinical evaluation of macrolide-resistant *Mycoplasma pneumoniae.* Antimicrob. Agents Chemother. **50**:709–712.
17. **Waites KB, Balish MF, Atkinson TP.** 2008. New insights into the pathogenesis and detection of *Mycoplasma pneumoniae* infections. Future Microbiol. **3**:635–648.
18. **Waites KB, Taylor-Robinson D.** 2011. *Mycoplasma* and *Ureaplasma*, p 970–985. *In* Versalovic J, et al (ed), Manual of clinical microbiology, 10th ed, **vol 1**. ASM Press, Washington, DC.
19. **Wolff BJ, Thacker WL, Schwartz SB, Winchell JM.** 2008. Detection of macrolide resistance in *Mycoplasma pneumoniae* by real-time PCR and high-resolution melt analysis. Antimicrob. Agents Chemother. **52**:3542–3549.
20. **Xin D, et al.** 2009. Molecular mechanisms of macrolide resistance in clinical isolates of *Mycoplasma pneumoniae* from China. Antimicrob. Agents Chemother. **53**:2158–2159.
21. **Youn YS, Lee KY.** 2012. *Mycoplasma pneumoniae* pneumonia in children. Korean J. Pediatr. **55**:42–47.
22. **Zhao F, et al.** 2012. Antibiotic sensitivity of 40 *Mycoplasma pneumoniae* isolates and molecular analysis of macrolide-resistant isolates from Beijing, China. Antimicrob. Agents Chemother. **56**:1108–1109.