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Master of Philosophy

Emerging macrolide-resistant strains during an outbreak of *Mycoplasma pneumoniae* infections in Korea

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Abstract

Background: *Mycoplasma pneumoniae* is a pathogen causing community-acquired pneumonia, and macrolides basically play an important role in *M. pneumoniae* treatment. Since the first report of emerging macrolide resistant *M. pneumoniae* (MRMP) in Japan, the prevalence of MRMP has been steadily increased worldwide and it would be the significant threat to use of macrolide in *M. pneumoniae* infection especially in children. The aim of this study was to determine of subtype of *M. pneumoniae* strains and investigate its association with macrolide resistance in Korea

Methods: During an outbreak of *M. pneumoniae* infections in Korea between 2014 and 2016, 249 clinical specimens were analyzed for molecular genotyping determination and macrolide resistance. *M. pneumoniae* subtypes determined by genotyping of *p1* gene DNA, and confirmed the mutations associated with resistance (A2063G and A2064G) by sequencing the targeted domain V regions of the 23S ribosomal RNA gene. We analyzed the relationship between subtype and the presence of macrolide resistance.

Results: Two hundred one (80.7%) were classified as subtype 1 and 48 (19.3%) as subtype 2. Macrolide resistance genotype occupied 180 (72.3%) among the whole clinical specimens. One hundred sixty nine (80.7%) of the subtype 1 were macrolide-resistant, and of these, the A2063G mutation was identified in 167 (98.8%) and the A2064G mutation was identified in 2 (1.2%) in the remaining. On the other hand, only 11 (19.3%) of the subtype 2 were a macrolide-resistant genotype, which was all the A2063G mutation. We found that the emergence of macrolide-resistant genotypes were more frequent in the subtype 1 ($p<0.001$).

Conclusion: This is the first study assessing whether genotyping is related to MRMP the outbreak of *M. pneumoniae* in Korea. Our findings revealed subtype 1 was predominant and a macrolide resistance-associated *M. pneumoniae* genotype. Given that the current increasing trend of the incidence of MRMP over the world, the epidemiological monitoring of

macrolide resistance has become necessary in order to effective antibiotic treatment.

Keywords. *Mycoplasma pneumonia*, Macrolide Resistance, Genotyping, Outbreak

Contents

Lists of figures, tables & abbreviations

Introduction

Mycoplasma pneumoniae is a main pathogen causing community-acquired pneumonia (CAP) accounting for as many as 10-30% of causes in children and young adults[1-4, 13, 17, 20]. It cause upper and lower respiratory infections, and also involve extrapulmonary complications such as encephalitis[3, 13, 20]. Although *M. pneumoniae* infections are generally self-limiting, the disease can progress into severe pneumonia or systemic infections depending on the host immune status[3, 20].

Epidemic outbreaks occur every 3-7 years[5, 6, 7], while *M. pneumoniae* infection is endemic over the world[36]. Cyclic epidemics occur every 3-4 years in South Korea[36]. Outbreak peaks are observed in summer or early fall in eastern Asia[14], but there were no seasonal variation in other areas[12, 19, 35]. Previous studies have shown that the predominant subtype of *M. pneumoniae* have been changed over time during each outbreak, like in seasonal influenza[35]. According to a study of *M. pneumoniae* epidemiology between 1995 and 2005, subtype 2 strains were predominant between 1995 and 2001, but subtype 1 strains were increased after the late 1980s and dominated after 2003[33, 35]. Likewise, the reports in France between 1994 and 2006 shows that subtype 1 strains were predominant before 1997, but type 2 had been increased[34]. Finally, both types were present with the same proportion and subtype 2 predominated in 2000[34]. Although the periodic outbreak could not be explained clearly for now, these changes in P1 adhesin type may play an important role for the development of outbreaks[17, 20, 35]. Some reports suggest the hypothesis that the difference of antigenicity between two types of *M. pneumoniae* would influence on interaction with host immune responses [36, 34, 20]. Once the host population develops immunity against the dominant type of *M. pneumoniae,* the other type could successfully get out of the host inflammatory response and next outbreak can occur[35].

M. pneumoniae is intrinsically resistant against to β-lactam antibiotics due to the lack of a cell wall[9, 21, 23]. Because the clinical course of *M. pneumoniae* infection is generally mild and uncommonly develops severe pneumonia, the clinical relevance of antibiotic treatment for *M. pneumoniae* pneumonia is questionable for some physicians[24, 34]. However, it is mostly accepted that early usage of antibiotics for *M. pneumoniae* infection are effective for more rapid resolution of fever, and especially for those involving lower respiratory tract systemic antibiotics should be administered[36, 20]. The guidelines of the infectious Diseases Society of America and American Thoracic Society recommend macrolides as the first choice antibiotics for *M. pneumoniae* pneumonia in children[35]. Because tetracycline administration is associated with side effects like permanent tooth discoloration for pediatric patients, it is recommended only for children over 8-year-old[34-36, 20]. With belief that prompt macrolide treatment is useful during the outbreaks of *M. pneumoniae*, which reduces the duration of symptoms, macrolides has been frequently used for treatment of *M. pneumoniae* infection in Korea[20].

Since the first report of emerging macrolide resistant *M. pneumoniae* (MRMP) in the 2001 in Japan[6, 8, 10, 15], MRMP rapidly and broadly spread after 2000[35], though its prevalence of differ from country to country[1, 5, 6]. MRMP infection has been increasingly reported in eastern Asian countries, whereas western countries including France (9.85), Netherlands (0.0%), Canada (12.1%), Germany (3.0%), and the USA (8.3%) showed relatively low incidence rates of MRMP[1-2, 5-6, 16, 19, 22-27, 28-29, 36]. At present, the incidence of MRMP is estimated at 50-90% in Japan[15, 20, 35], and 92% of the macrolide resistance rate was recorded in China[11]. In addition, MRMPs are isolated more frequently from pediatric patients than from adults[34, 36, 37], in particular, in the place where macrolides are widely used[35]. Considering the potential side effects of drugs like fluoroquinolones or doxycyclines for children, it is obvious that the increase of MRMP infection would be a growing problem. However, the clinical relevance of MRMP has not been clarified yet [8, 34-35, 37].

Since the summer of 2011, new *M. pneumoniae* epidemics have spread in Korea, but strain typing has not yet been established. In this study, we investigated the dominant *M. pneumoniae* strains by amplification of *p1* gene with nested-PCR methods and described overall epidemiology during the recent epidemic in Korea in terms of macrolide resistance and subtypes. We also evaluated the clinical features of subtype 1 and subtype 2, and if there is association between specific type and macrolide resistance.

Patients and methods

Clinical specimens and patient data

During an outbreak of *M. pneumoniae* infections between October 2014 and December 2016 in Asan Medical Center, 632 respiratory samples of patients under suspicion of CAP tested by polymerase chain reaction (PCR) for *M. pneumoniae* using AmpliSens *Mycoplasma pneumoniae/Clamydia pneumoniae*-FRT PCR kit (InterLabService Ltd., Moscow, Russia) at admission. Of these, 249 *M. pneumoniae* isolates with positive results by PCR for *M. pneumoniae* were included in this study. Ages, genders, febrile duration, clinical symptoms, laboratory data, radiologic findings, and pre-administration of macrolide treatment for all study population were retrospectively collected from electronic medical records. All of the chest radiographs were reviewed by an experienced radiologist. Infiltration on the chest radiography was defined as poorly defined opacity in the lung fields and consolidation was defined as the presence of air-space opacity. Pleural effusion or abnormalities of lymph nodes were classified as other symptoms. Fever was defined as a body temperature above 38°C. This work was approved by the Research Ethics Committee at Asan Medical Center, Seoul, Republic of Korea. Informed consent was waived by the Institutional Review Board of Asan Medical Center because this study performed retrospectively and did not require any extra clinical specimens.

***M. pneumoniae* genotyping**

Genomic DNA of all 249 *M. pneumoniae* isolates was extracted by the QIAamp DNA minikit (Qiagen, USA) according to manufacturer's recommendation. DNA was tested using a nested PCR targeting RepMP4 and RepMP2/3 polymorphic region for the P1 adhesin of *M. pneumoniae* (Figure 1-(A)). The synthetic DNA primers used for genotyping *M. pneumoniae* were listed in Table 1. Typing of all 249 *M. pneumoniae* isolates was performed by targeting the *p1* cytoadhesion type 1 and type 2 with primer pairs as previously documented. For detection of the RepMP4 region of the p1 gene, the ADH2F and ADF2R primer pair was used for the first PCR, and the ADF3F and ADF3R primer pair for the second PCR. Finally, the PCR primer set consisting of ADH4F, N1 and 2N2C was used for typing the RepMP4 region. The agarose gel (2%) electrophoresis pattern of the final products from *p1* genes gives a 343 or 560 bp amplification product from subtype 1 or 2 *p1* gene DNA, respectively (Figure 1-(B)). For detection of the RepMp 2/3 region, the ADH3 and MP2/3-R1 primer pair was used for the first PCR, and the MP2/3-F2 and MP2/3-R2 primer pair was used for the second PCR (Figure 1-(A)). The PCR primer set consisting of MP2/3-F3, R3-1, R3-2 and R3-2V was used for typing of the RepMP2/3 region. This PCR product on the agarose gel electrophoresis gives a 394 or 617 bp amplification product from subtype 1 or subtype 2 (Figure 1-(B)). The reaction conditions used in the nested PCR were 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. Premix Ex *Taq* (TaKaRa Bio Inc., Shiga, Japan) was used for the reaction, in accordance with the manufacturer's instructions.

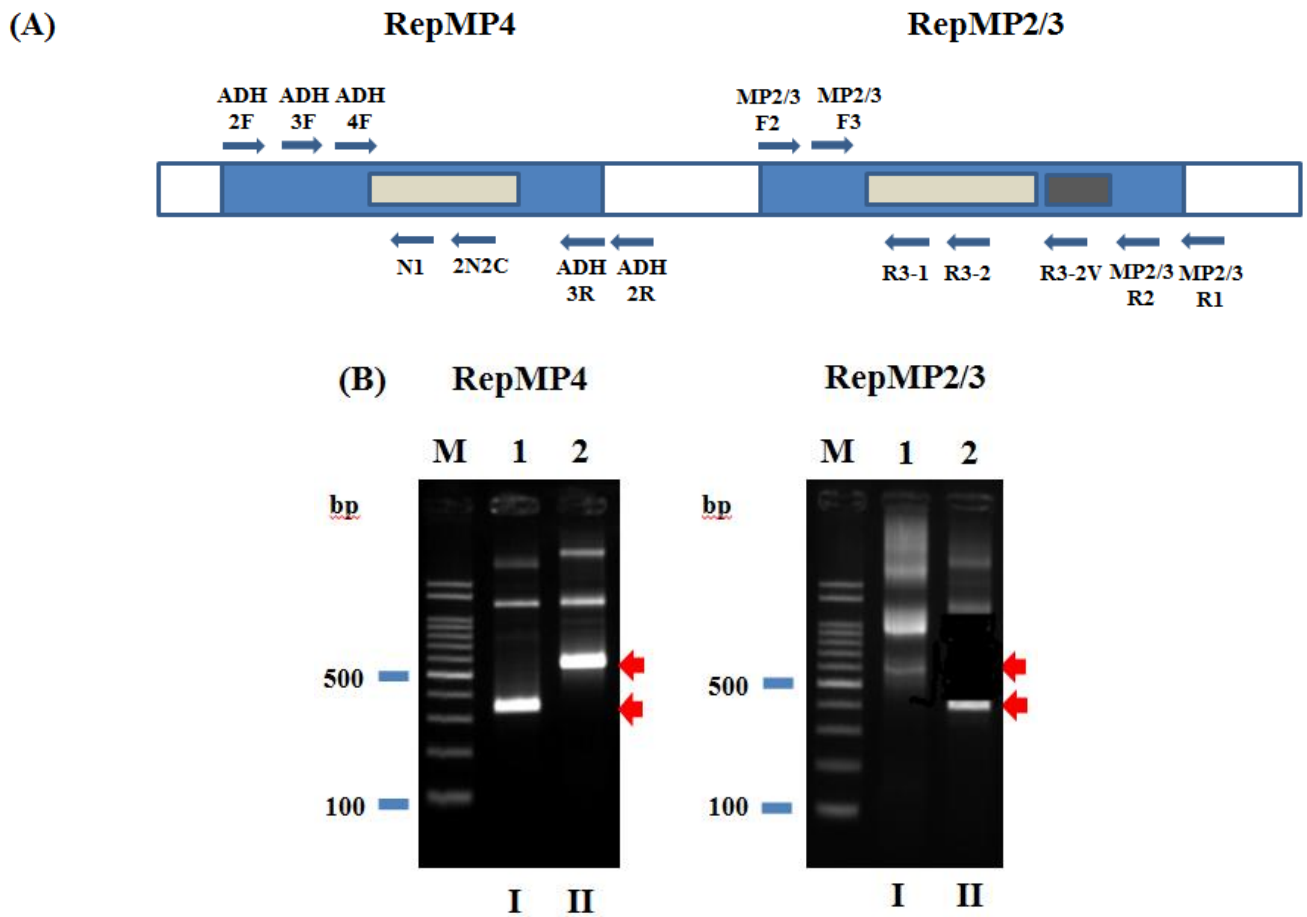

Figure 1. Schematic picture of the *p1* gene and agarose gel (2%) electrophoresis pattern

(A) Location of the primer for nested PCR and genotyping of The RepMP4 region and the RepMP2/3 region by PCR. (B) The agarose gel (2%) electrophoresis pattern of the final products from *p1* genes

Table 1. PCR primers used for the detection and typing of *M. pneumoniae*

Primer	Sequence (5′→3′)
ADH2F	GGCAGTGGCAGTCAACAAACCACGTAT
ADH2R	GAACTTAGCGCCAGCAACTGCCAT
ADH3F	GAACCGAAGCGGCTTTGACCGCAT
ADH3R	GTTGACCATGCCTGAGAACAGTAA
ADH4F	GACCGCATCAACCACCTTTGCGTTACG
N1	CCCGGTGGTGGAAGTATTTT
2N2C	TGCCTTGGTCACCGGAGTTG
MP2/3-R1	AGATTGACCTGAGCCTGAAG
MP2/3-F2	CACAAGTGGTTCGCGTTCCT
MP2/3-R2	GGCTGGGTGGAATGGTCTGT
MP2/3-F3	TCGACCAAGCCAACCTCCAG
R3-1	TTGGAATCGGACCCACTTCG
R3-2	CGACGTTGTGTTTGTGCCAC
R3-2V	CGGTATAGCTAATTTGGTAC

Determination of macrolide resistance genotypes

For identifying the mutations associated with resistance (A2063G and A2064G), we amplified the domain V regions of the 23S ribosomal RNA gene by methods described previously. Specific primers were designed for the detection of the point mutations of domain V of 23S rRNA. Domain V of the 23S rRNA gene was amplified using previously described primer pairs (Table 2). After the first PCR with the 23s rRNA specific PCR MP23-333F and MP23-638R primers, the second PCR was performed with the MP23-V-F and MP23-638R primers. Premix Ex *Taq* (TaKaRa) was used for the reaction, in accordance with the manufacturer's instructions. The reaction conditions used in the nested PCR were 35 cycles of 95 °C for 30 s, 55-60 °C for 40s-1 min and 72 °C for 40s-1 min. PCR products were confirmed by electrophoresis on 2.0 % agarose gel. PCR products were purified using a Power Gel Extraction kit (TaKaRa Bio Inc., Shiga, Japan), and the purified products were sequenced by an ABI Prism BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and after that, they were analyzed with an ABI 3730xl DNA analyzer (Applied Biosystems).

Table 2. PCR primers for Determination of macrolide resistance genotypes

Primer	Sequence (5′→3′)
Domain V of 23S rRNA gene	
MP23-333F	CGCAAGCGAAGCTTTTAACT
MP23-638R	ATTCCACCTTTCGCATCAAC
MP23S V-F	TAACTATAACGGTCCTAAGG
MP23-638R	ATTCCACCTTTCGCATCAAC

Statistical analysis

Descriptive statistics were performed in terms of quantitative and qualitative data and absolute frequency. Comparisons were conducted with chi-square test or Fisher's exact test for categorical variables, and Student's *t* test or Mann-Whitney test for continuous variables as appropriate. A p value <.05 was defined as statistically significant. SPSS version 18.0 for window (SPSS Inc., Chicago, IL, USA) was used for data analysis.

Results

Overall epidemiology during an outbreak of *M. pneumoniae* pneumonia in Korea

The number of cases of *M. pneumoniae* pneumonia between October 2014 and December 2016 are shown in the following graph (Figure 2). The number of cases was higher from early autumn to winter. During an outbreak of *M. pneumoniae*, the number of MRMP cases was increased with the increase of M. *pneumoniae* pneumonia. The number of cases of *M. pneumoniae* pneumonia was the highest in toddler group (1≤ <3) and the second most common in the infant group (<1) (Figure 3-(A)). More than 85% of MRMP were isolated from patients under the age of 5. Of the whole strains, the subtype 1 accounted for about 84% before school age group (5≤ <12), but the subtype 1 strains occupied about 55.2% in the patients order than 12 years (Figure 3-(B)). Over the study period, the distribution of subtype 1 and subtype 2 was as follows (Figure 4). According to *p1* typing study, the dominant *M. pneumoniae* was the subtype 1 stains. Also, we did not observe the subtype 2 variants. In our study population, there was no clear type-shift pattern during the whole research period.

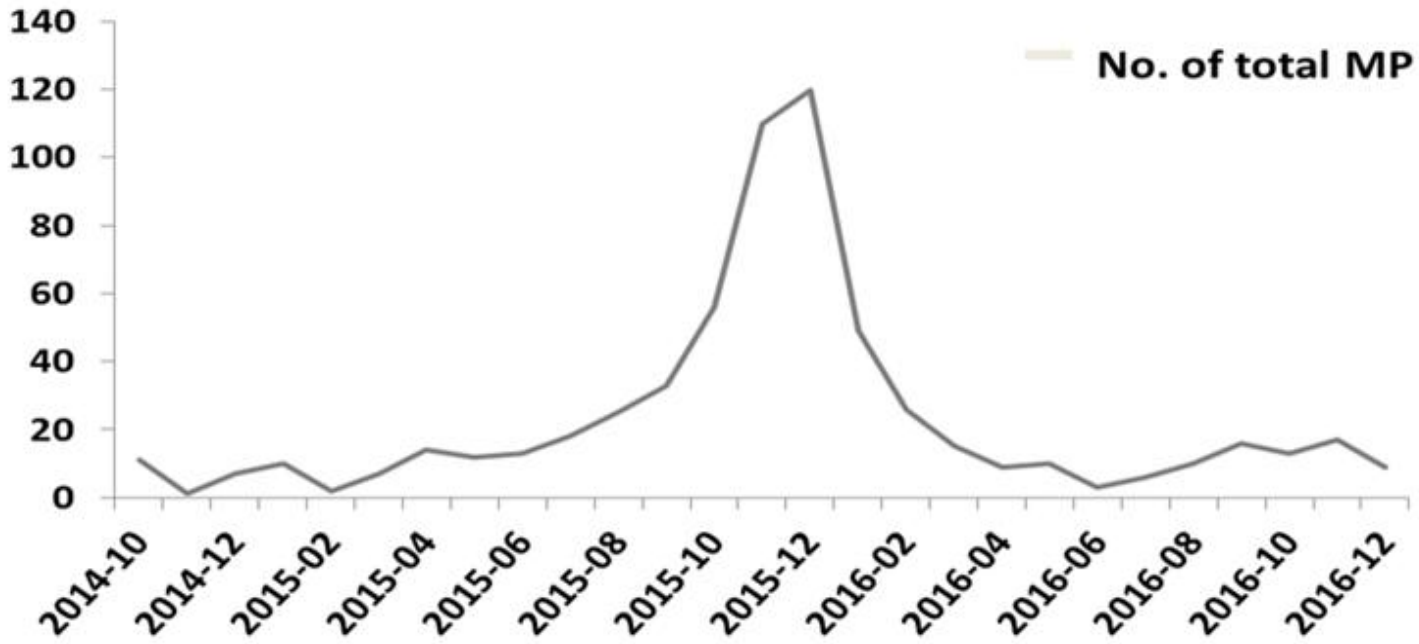

Figure 2. Monthly distribution of cases for *Mycoplasma pnumoniea* pneumonia patients and that for MRMP between October 2014 and December 2016.

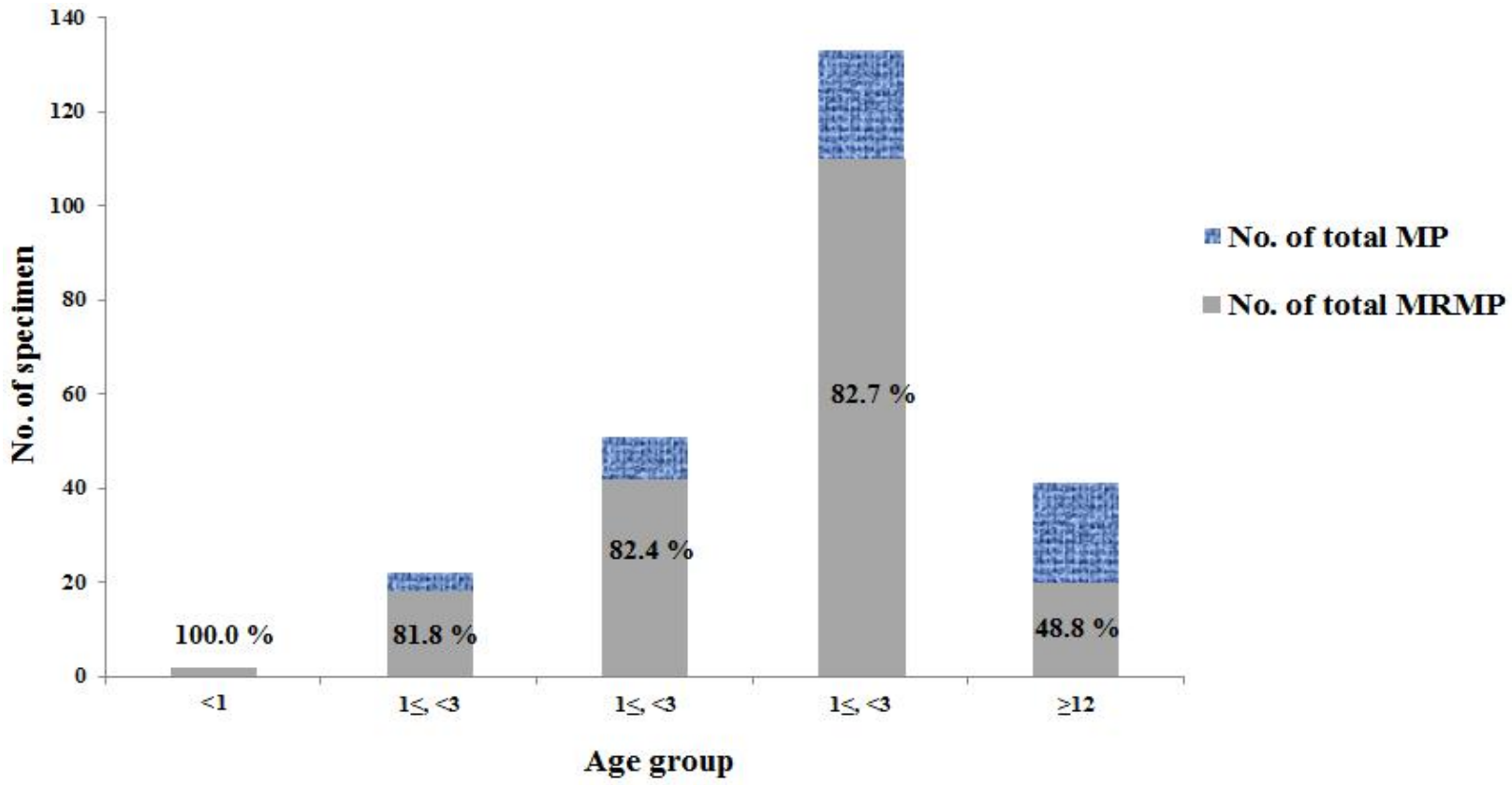

Figure 3. Distribution of MSMP and MRMP according to the age groups

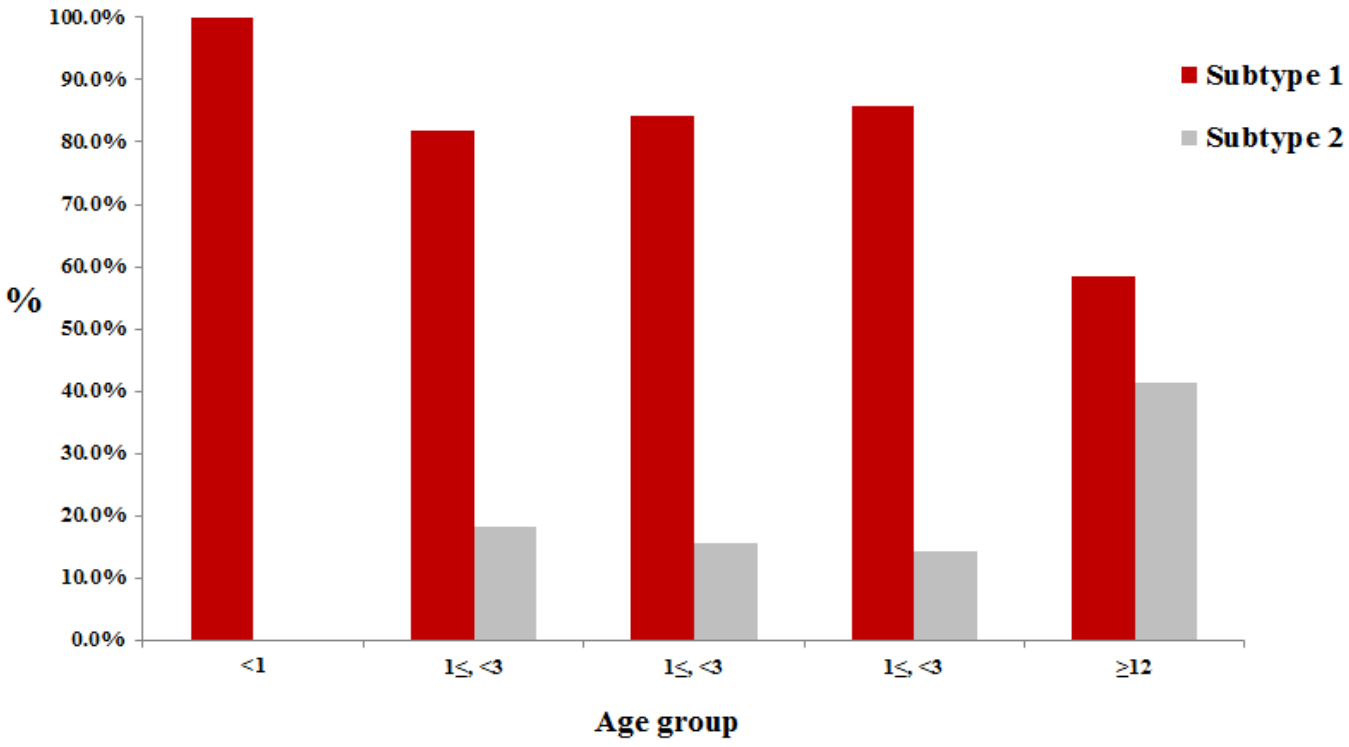

Figure 4. Distribution of *Mycoplasma pnumoniea* subtypes according to the age group

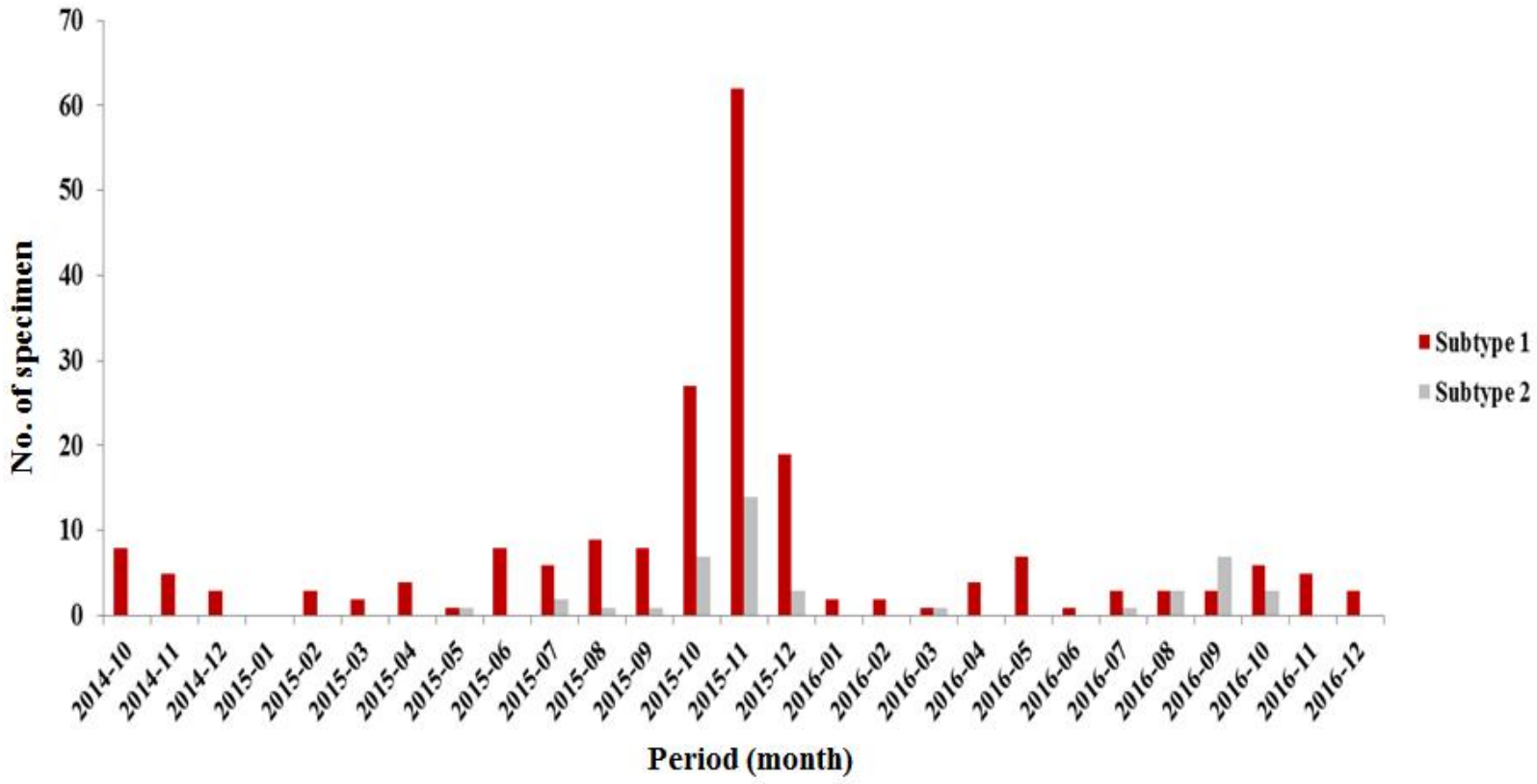

Figure 5. Monthly distribution of the type of P1 adhesin gene of 249 *Mycoplasma pnumoniea* between October 2014 and December 201

Clinical characterization of each type of *M. pneumoniae* strains

Two hundred one (80.7%) were classified as subtype 1 and 48 (19.3%) as subtype 2. The demographic and clinical characteristics of patients are summarized in Table 3. Patients infected with subtype 1 were younger (8.9 years vs. 17.2 years, respectively, $p<0.001$), and more likely to have longer fever (temperature ≥ 38° C) duration (6.7 ± 3.6 days vs. 5.3 ± 4.7 days, respectively, $p<0.027$), compared with patients infected with subtype 2. The most common clinical symptoms were cough, fever and sputum in both groups (98.4%, 98.0%, and 79.5%, respectively). There were no significant differences in clinical manifestations. Chest radiographs of all patients were available, and lobar consolidation patterns were most common without statistically significant differences between two types (60.7% in type 1 vs. 70.8% type 2, $p<0.133$). Laboratory findings except for platelets counts were not different between type 1 and type 2 of *M. pneumoniae*. Patients with subtype 1 *M. pneumoniae* were more frequently pre-administered with macrolides ($p<0.001$). One hundred sixty nine (80.7%) of the type 1 were macrolide-resistant, and of these, the A2063G mutation was identified in 167 (98.8%) and the A2064G mutation was identified in 2 (1.2%) in the remaining. On the other hand, only 11 (19.3%) of the type 2 were a macrolide-resistant genotype, which was all the A2063G mutation. The emergence of macrolide-resistant genotypes were more frequent in the type 1 ($p<0.001$). We found no significant differences in hospitalization rates between the patients with subtype 1 and subtype 2.

Patients having mixed population of wild type and mutant type simultaneously.

Four *M. pneumoniae* strains showing a mixed population of wild type and mutant at position 2063 of 23S rRNA were detected (Table 4). Three pediatric patients were prescribed with macrolide before determination of macrolide-resistance genotyping. Although only adult patient had no history of macrolide treatment, a mix of wild type and A2063G mutant type were observed. The patient treated with colistin and piperacillin/tazobactam against carbapenem-resistant *Acinetobacter baumannii* pneumonia at the detection of mixed population of wild type and mutant type *M. pneumoniae* strains.

Comparison of hospitalization rates, hospital days and rate of pre-administration of macrolides between MSMP and MRMP in patients with *M. pneumoniae*

The overall macrolide resistance rate occupied 180 (72.3%) among the whole clinical specimens. There were no significant differences in admission rates between MSMP and MRMP groups (Table 5). The hospital days of patients with MRMP appears to be prolonged compared to those with MSMP (4.8 ± 2.6 days in non-MRMP vs. 6.9 ± 4.0 days in MRMP, $p<0.001$; data not shown), and had a higher rate of pre-administration of macrolides than patients with MSMP (64.2%, $p=0.002$; Table 6).

Table 3. Patients' demographics and clinical features according to the sequencing type of P1 gene

Characteristics	Patients with type 1 *M. pneumoniae* (n=201)	Patients with type 2 *M. pneumoniae* (n=48)	*P*-value
Age, years	8.85 (± 11.0)	17.2 (± 20.3)	<0.001
Sex			
Male	91 (45.3%)	18 (37.5%)	0.329
Female	110 (54.7%)	20 (62.5%)	
Febrile duration	6.7 (± 3.6)	5.3 (± 4.7)	0.027
Symptoms duration			
Fever	197 (98.0%)	47 (97.9%)	1.000
Cough	197 (98.0%)	48 (100.0%)	1.000
Sputum	163 (81.1%)	35 (72.9%)	0.207
Dyspnea	28 (13.9%)	7 (14.6%)	0.907
Wheezing	40 (19.9%)	9 (18.8%)	0.875
Gastrointestinal signs	50 (24.9%)	10 (20.8%)	0.556
Laboratory findings			
Leukocytes	8592.5 (± 4132.1) (n=186)	8819.1 (± 4063.8)	0.748
Hemoglobin	12.3 (± 1.1) (n=185)	12.3 (± 1.5) (n=42)	0.887

Platelet	284.1 (± 106.3) (n=186)	247.1 12.3 (± 95.8) (n=42)	0.039
Absolute neutrophil count	5497.8 (± 3471.5) (n=186)	5990.1 (± 3673.4) (n=42)	0.431
C-reactive protein	7.4 (± 29.7) (n=183)	6.7 (± 6.5) (n=41)	0.882
Radiologic findings			
Consolidation	122 (60.7%)	34 (70.8%)	0.133
Interstitial infiltration	55 (27.4%)	6 (12.5%)	
Consolidation + Interstitial pattern	12 (6.0%)	4 (8.3%)	
Others	11 (5.5%)	4 (8.4%)	
Previous macrolide treatment	130 (64.7%)	11 (35.4%)	< 0.001
Detection of 23S rRNA mutation	169 (80.7%)	11 (19.3%)	< 0.001
A2063G	167 (98.8%)	11 (100.0%)	< 0.001
A2064G	2 (1.2%)	0	
Hospitalization required			0.069
Yes	133 (66.2%)	25 (52.1%)	
No	68 (33.8%)	23 (47.9%)	

The significance of differences between groups was evaluated using the Mann-Whitney U test or Pearson's χ^2 test. All data are expressed as means ± SDs with ranges for continuous variables and numbers with percentages for categorized variables unless indicated otherwise.

Table 4. Clinical findings of patients who had wild type and mutant type clones simultaneously.

Patients	Sex	Age (yr)	Diagnosis	Chest Radiography	Antibiotic treatment	P1 type	23S rRNA mutation
1	F	5	Pneumonia	Patchy consolidation in the right middle lobe	Clarithromycin for 3days before admission Azithromycin for 3 days	1	A2063G
2	M	4	Pneumonia	Improved atelectasis of the right middle lobe	Azithromycin for 6 days	2	A2063G
3	F	7	Pneumonia	Ill-defined patchy opacity in left Lower Lobe	Azithromycin for 4 days	2	A2063G
4	M	63	Lung cancer, Malignant pleural effusion	Patchy consolidation in left mid to lower lobe. Loculated right pleural effusion	Doxycycline for 6 days	2	A2063G

Table 5. Comparison of macrolide resistance between hospitalized patients and non-hospitalized patients.

Hospitalization	No. of patients	No. (%) of macrolide resistant mutation detected	*P*-value
Yes	158	126 (79.7)	0.265
No	91	67 (73.6)	
Total	249	193 (77.5)	

Table 6. Comparison of macrolide resistance between patients with and without previous history of macrolide treatment

Previous macrolide treatment	No. of patients	No. (%) of macrolide resistant mutation detected	*P*-value
Yes	147	124 (84.4)	0.002
No	102	69 (67.6)	
Total	249	193 (77.5)	

Discussion

Our findings indicated more than 80.7% of subtype 1 was detected among sequenced strains during the co-circulation of type 1 and type 2 during an epidemic. We do not find the type shift phenomenon of *M. pneumoniae* over the study period. So far, there have been epidemiological studies on *M. pneumoniae* infection. Because fastidious growth of *M. pneumoniae* requires a great deal of to obtain a sufficient number of isolates, we tried to detect and genotype the *p1* gene without isolating *M. pneumoniae* itself.

In this study, we compared the characteristics of patients harboring *M. pneumoniae* subtype 1 and subtype 2 for the first time. There is no difference between the two groups in clinical manifestations, laboratory findings and radiologic findings. In radiologic findings, lobar patterns were most frequently observed, unlike the fact that interstitial pattern is generally common in typical *M. pneumoniae* pneumonia[13, 37, 20)]. Also notable is the point that patients with *M. pneumoniae* subtype 1 were significantly younger, and they got early treatment of macrolide compared to those with subtype 2 strains. Given the clinical features of hospitalized patients are usually assumed to be more severe, the proportion of hospitalized patients in each type seems to reflect disease severity. The rate of inpatients was 66.2% in type 1 and 52.1% in type 2 (p=0.069). This indicates that the disease severity is not determined depending on *M. pneumoniae* genotypes.

In recent years, emerging of MRMP has become a problem, in particular in the East Asian region including China, Japan and Korea[3, 20, 34, 30)]. According to Korean investigation group reported the substantially increased prevalence of macrolide resistance of *M. pneumoniae* in children from 2.9 % in 2003 to 62.9% in 2011[20, 23, 37-38)]. Total macrolide resistance rate in this study was 72.3%. Altogether, the MRMP have been increasing since 2003 in South Korea[23, 37-38)]. Previously, several studies have compared the clinical

characteristics between MSMP and MRMP[7, 10, 15, 26]. Patients harboring MRMP were younger than those with MSMP, and showed longer febrile days[7, 10, 15, 26]. But, it has been found that there were no significantly differences in terms of respiratory symptoms, radiologic fndings and disease severity between two groups[7, 10, 15, 26]. Of note, physicians were likely to perform early administration of macrolides in patients with MRMP [35, 37, 38]. Our results indicated 59.0 % of *M. pneumoniae* pneumonia patients had previous macrolide treatment before diagnosis of *M. pneumoniae* pneumonia infection. Macrolide resistance could be induced with few days after starting macrolide treatment and under early exposure to macrolides in particular, for young children, which would eventually lead to antibiotic selective pressure in children[35, 18, 28, 33]. Based on this, MRMP seem to be a significant threat to treatment during period of extensive macrolide use worldwide[28, 29, 31, 37], especially for young ages under 12 years as our data showed. However, clinical relevance of MRMP has not been clarified for now. In this context, we raised three important issues concerning MRMP; 1) is it worth to impose on clinical relevance of MRMP?, 2) does the associations between the subtype and macrolide resistance exist?, 3) is MRMP natural occurring throughout the population or does undergo clonal spreading?.

Clinical relevance of resistant strains has not been definitely established because it is not clarified whether resistant strains can cause more severe or prolonged disease[3, 13, 20]. *M. pneumoniae* acquires macrolide resistance basically as a result of nucleotide substitutions at macrolide hot spots in the V domain of the 23S rRNA gene[3, 20, 37]. Mutations at nucleotide 2063 (A2063T/G), 2064 (A2064G), and 2617 (C2617A/G) have been shown to be associated with increased MICs up to of 32 to 64 mg/L to macrolides including erythromycin, azithromycin, and clarithromycin [3-4, 7, 9, 21]. However, some studies documented that in actual clinical setting, drug resistance of MRMP does not directly lead to clinical severity[3, 4]. In fact, most MRMP cases showed rarely treatment failure with macrolide treatment. MP pneumonia is a host immune-mediated and self-limited disease, and macrolide had anti-

inflammatory effect itself so that corticosteroid or macrolide may redece clinical symptoms[3-5]. Narita postulated that that MRMP strains may have less efficient protein synthesis because of ribosomal mutations, and that would be why the drug resistance of MP itself does not directly result in clinical severity[18]. Because there no reported study comparing clinical outcomes in patients with MRMP treated with and without macrolides, it would be necessary further research regarding to clinical relevance of MRMP in clinical practice.

Interestingly, the strong association between macrolide resistance and *M. pneumoniae* type 1 were observed in this study. Previously, several studies tried to clarify the associations between the type and macrolide resistance, but most of them failed to find out the association between subtype and MRMP[5, 12, 19]. Only one study in China documented *M. pneumoniae* type 1 showed strong association with MRMP[9]. According to the epidemiologic study by multiple locus variable number tandem repeat analysis (MLVA) genotype in China over 13 years, there was a close association between macrolide resistance and genotype 4-5-7-2/P1 which of all were belong to subtype 1[29, 38]. Also, other investigator found that patients infected with genotype 5-4-5-7-2/P1 and genotype 3-4-5-7-2/P1, which of both were also subtype 1, had strong association with macrolide resistance, had significantly higher PSI scores ($p<0.001$) and longer total duration of cough ($p=0.011$)[31]. In most previous studies, it is suggested that the macrolide resistance for *M. pneumoniae* is not associated with a specific p1 genotype but the current predominant strains[5, 12, 19]. Therefore, more studies using pulsed field gel electrophoresis and MLVA should be undertaken to investigate associations between the presence of macrolide resistance and specific *M. pneumoniae* molecular characteristics.

Concerning the mechanism of *M. pneumoniae* spreading, it is largely debated whether resistance is occurring frequently throughout the population or if clonal spread of resistance strains is occurring. Any extrachromosomal element has not been described, and only target alteration by acquired mutations has been associated with antibiotic resistance in *M. pneumoniae* infection[18]. MRMP could be induced by exposure of sub-lethal concentration of

macrolides in *in vitro* setting as described previously[3]. In some cases, MSMP isolated at initial admission, but MRMP isolated a few days after macrolide treatment, thus possibly meaning that the mutations could be induced by administration of macrolides *in vivo*[32]. This explains the coexistence of mixed population of MRMP and MSMP in this study. On the other hand, clonal spreading of a MRMP strain within a single family in Italy reported by Maria *et al.*[32]. Several studies regarding MLVA genotypes strongly suggested the possibility of clonal spreading during an MRMP epidemic[29, 31, 38]. The most commonly detected genotype 5-4-5-7-2/P1 of MRMP were belong to type 1, and we found that type 1 was related to MRMP[29, 31, 38], thereby it is suggest that subtype 1 *M. pneumoniae* would play an important role for acquisition of resistance clone. Taken together, it is assumed that both of mechanisms may attribute to increasing propagation of MRMP during an outbreak of *M. pneumoniae.* According to our data, 67.6 % of *M. pneumoniae* infections were macrolide resistant strains among the patients without previous macrolide treatment. This means that significant proportion of resistance strains are already spread throughout the community. Thus monitoring of epidemic trend for *M. pneumoniae* strains and their resistance seems to be necessary in order to recognize early changes in the antibiotic resistance patterns in human respiratory infection.

Conclusions

This is the first study assessing whether *M. pneumoniae* type is related to macrolide resistance during the outbreak of *M. pneumoniae* in Korea. For the times, subtype 1 was predominant, which is more likely to be infected in patient with younger ages rather than subtype 2. Given that the current rapidly increasing trend of the incidence of MRMP over the world, to treat pediatric respiratory infections, judicious use of antimicrobial drug should be emphasized. Also the epidemiological monitoring of macrolide resistance has become necessary in order to establish the effective antibiotic treatment against those infections.

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국문초록

배경 : *Mycoplasma pneumoniae* 은 지역 사회 획득 폐렴을 일으키는 주요 병원균이며, macrolides 는 기본적으로 *M. pneumoniae* 치료에 중요한 역할을 한다. 일본에서 2001 년 macrolides resistant 기본적으로 *M. pneumoniae* 첫 번째 보고 이후, MRMP 의 유행은 전 세계적으로 꾸준히 증가 해 왔다. 특히 소아에서 MRMP 감염은 치료실패로 이어질 수 있어 문제가 된다. 본 연구의 목적은 국내 유행 동안 우세한 *M. pneumoniae* subtype 및 유전자형과 macrolide 내성과의 관련성을 분석하고자 한다.

방법 : 2014 년에서 2016 년 사이에 한국에서 *M. pneumoniae* 감염 유행 동안 확보 가능한 균주 249 주를 분석하였다.macrolide 내성에 대해 249 주를 분석 하였다. *M. pneumoniae* subtype 은 p1 유전자 DNA 의 genotyping 방법으로 분석하고, 23S ribosomal RNA 유전자의 target domain V 영역을 sequencing 함으로써 내성관련 돌연변이 (A2063G 와 A2064G)를 확인 하였다.

결과 : 전체 균주에서 제 1 형 type (Macroride resistance genotype)은 전체 임상 검체 중 180 주 (72.3 %)를 차지 하였다. 제 1 형 type 196 주 (80.7 %)는 macrolide 내성이었으며, 이 중 A2063G 돌연변이는 167 주 (98.8 %), A2064G 돌연변이는 2 개 (1.2 %) 확인되었다. 제 2 형 type 은 11 (19.3 %) 주만이 A2063G 돌연변이 인 macrolide 내성유전자형이었다. 우리는 MRMP 발생이 제 1 형 type 에서 더 빈번하다는 것을 발견했다 ($p < 0.001$).

결론 : 본 연구는 국내에서 *M. pneumoniae* 의 MRMP 발생과 유전자형이 관련이 있는지를 평가 한 최초의 연구이다. 우리의 결과에 의하면 최근 국내 유행 동안 제 1 형 type 우세하였고 MRMP 는 제 2 형보다 제 1 형에서 좀 더 빈번하게 나타났다. 세계적으로 MRMP 발생이 증가하고 있는 추세이므로 효과적인 항생제 치료를 위해서는 MRMP 대한 꾸준한 역학 적 감시가 필요하다.