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Original Article

Persistent high macrolide resistance rate and increase of macrolide-resistant ST14 strains among *Mycoplasma pneumoniae* in South Korea, 2019–2020

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Abstract *Background:* Expansion of the single sequence type 3 (ST3) was associated with a high macrolide resistance rate among *Mycoplasma pneumoniae* in Korea during the 2014–2016 epidemic. This study investigates the macrolide resistance rate and genetic diversity of the subsequent epidemic of *M. pneumoniae* pneumonia in 2019–2020.
Methods: The culture for *M. pneumoniae* was developed from 1228 respiratory samples collected from children with pneumonia in four hospitals in Korea between January 2019 and January 2020. Determination of macrolide resistance and multilocus sequence typing analysis were performed on *M. pneumoniae* isolates. eBURST analysis was applied to estimate the relationships among strains and to assign strains to a clonal complex.
Results: *M. pneumoniae* was cultured in 93 (7.6%) of 1228 clinical samples. The overall macrolide resistance rate of *M. pneumoniae* strains was 78.5% (73/93). Of the nine STs identified,

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three were novel. The most common ST was ST3 (66 [71.0%]) followed by ST14 (18 [19.4%]) and ST7/ST15 (2 [2.2%] each). Three STs (ST3, ST14, and ST17) exhibited macrolide resistance. The macrolide resistance rates of ST3 and ST14 were 98.5% (65 of 66) and 38.9% (7 of 18), respectively.

Conclusion: Compared to the previous outbreak in 2014—2016, the overall macrolide resistance remained high; however, an increasing proportion of macrolide resistance was observed within ST14 strains in 2019—2020.

Introduction

Mycoplasma pneumoniae is a major pathogen of community-acquired pneumonia in children and adolescents.[1] Macrolides are recommended as the first-line therapy for pneumonia caused by *M. pneumoniae*.[2] Alternative antibiotics such as tetracyclines or fluoroquinolones are also active against *M. pneumoniae*.[3] However, due to the potential side effects of these alternative antibiotics, especially to children, their use is generally limited.

The macrolide resistance of *M. pneumoniae* plays a significant role in clinical settings; however, the precise nature of this role is debated.[4] Several studies have reported that individuals infected with macrolide-resistant *M. pneumoniae* and who receive macrolide treatment can experience a longer febrile period and extended antibiotic therapy than those infected with macrolide-susceptible strains.[5,6] However, these studies fail to report that the end-result of the treatment (such as mortality) differs considerably between the two groups, as macrolides are switched to alternative drugs when the clinical course is not favorable. Moreover, there have been reports of more frequent extrapulmonary complications and serious radiological findings in patients with macrolide-resistant *M. pneumoniae*, which emphasize the importance of macrolide resistance among *M. pneumoniae*.[7] A recent investigation of patients with 14 (2.8%) macrolide-resistant isolates and 485 (97.2%) macrolide-susceptible isolates reported nearly five-fold greater odds of pediatric intensive care unit admission in patients with macrolide-resistant *M. pneumoniae*.[8]

Macrolide resistance among children with *M. pneumoniae* pneumonia has been reported throughout the world.[4] Specifically, high rates of macrolide-resistant *M. pneumoniae* in several Asian countries are of great concern when treating children with this type of pneumonia.[9,10] In Japan, the highest macrolide resistance rate (86.2%) in 2010—2013 decreased to 56.3% in 2015—2016 and fell even further to 11.3% in 2018—2019.[11] This declining trend was closely associated with two dominant sequence types (STs). Conversely, in South Korea, the macrolide resistance rate remained high at 84.4% during the 2014—2016 epidemic, which was explained by the expansion of a single ST, ST3.[12] However, it is uncertain whether such resistance rates will remain high or begin to decrease. In 2019, a new *M. pneumoniae* epidemic began to develop among children with pneumonia. Therefore, this study investigates the macrolide resistance rate and distribution of STs for the most recent *M. pneumoniae* epidemic (2019—2020) in South Korea.

Materials and methods

M. pneumoniae strains

A total of 1228 respiratory samples collected from children with pneumonia in four hospitals in South Korea were cultured for *M. pneumoniae* during January 2019 and January 2020. The hospitals were Seoul National University Children's Hospital (Seoul), Seoul National University Bundang Hospital (Seongnam), Chungnam National University Hospital (Daejeon), and Jeju National University Hospital (Jeju). Samples were cultured using a pleuropneumonia-like organism broth and agar for nasopharyngeal aspirates obtained from the patients, as described in a previous study.[13]

Macrolide resistance and MLST analysis

For the cultured *M. pneumoniae* isolates, the macrolide resistance and multilocus sequence typing (MLST) were determined, as described previously.[12,13] Mutations responsible for macrolide resistance were confirmed by sequence analysis of the amplified polymerase chain reaction (PCR) products targeting domain V of the 23S rRNA.[13] MLST was performed on extracted DNA samples from *M. pneumoniae* isolates.[12] Unreported STs and alleles were submitted for new assignments (http://pubmlst.org/mpneumoniae/). goeBURST software (http://www.phyloviz.net/goeburst/) was employed to estimate the associations and links among the STs.[14]

Statistical analyses

Statistical analyses were performed using SPSS Statistics for Windows version 25.0 (IBM Corp.) where applicable. Categorical data were analyzed using the chi-squared test or Fisher's exact test. $P < 0.05$ was considered statistically significant.

Ethics statement

The institutional review board of Seoul National University Hospital approved the study protocol (IRB no. H-

1012—007—341). Informed consent was exempted because nasopharyngeal aspirates were obtained as part of standard patient care.

Results

M. pneumoniae isolation

M. pneumoniae was cultured in 93 (7.6%) of 1228 clinical samples. Of the 93 isolates, macrolide resistance was noted in 73 isolates (78.5%). The rates for positive culture and macrolide resistance differed among the hospitals, ranging from 6.6% to 13.8% and from 50.0% to 84.6%, respectively (Table 1).

MLST and macrolide resistance of *M. pneumoniae*

During the study period, nine STs (ST2, ST3, ST7, ST14, ST15, ST17, ST38, ST39, and ST40) were identified (Table 2). Of the nine STs, ST38, ST39, and ST40 were novel STs. The most common ST was ST3 (66 [71.0%]), followed by ST14 (18 [19.4%]) and ST7/ST15 (2 [2.2%] each).

The overall macrolide resistance rate was 78.5% (73 of 93). Of the nine STs, three (ST3, ST14, and ST17) exhibited macrolide resistance. The macrolide resistance rates of ST3 and ST14 were 98.5% (65 of 66) and 38.9% (7 of 18), respectively. The distribution of STs differed according to macrolide susceptibility (Fig. 1). Among 73 macrolide-resistant isolates, ST3 (65 [89.0%]) was predominant, followed by ST14 (7 [9.6%]) and ST17 (1 [1.4%]). Among the 20 macrolide-susceptible isolates, ST14 (11 [55.0%]) was the major ST, followed by ST7/ST15 (2 [10.0%] each) and ST2/ST3/ST38/ST39/ST40 (1 [5.0%] each). The mutation identified by 23S rRNA sequencing was determined to be the A2063G mutation, which is common to all macrolide-resistant isolates, except for one ST17 isolate, which harbored an A2063T mutation.

Three novel STs were identified in this study. ST40 contained a new *pgm9* allele that harbored a single-nucleotide point mutation when aligned to a previously known allele (*pgm3*). ST38 and ST39 were new combinations of previously reported alleles. All three new STs were macrolide susceptible.

Table 2 Distribution of *Mycoplasma pneumoniae* and macrolide susceptibility within the STs, South Korea, 2019—2020.

ST	No. (%)	Clonal complex	No. (%) isolates	
			Macrolide resistant[b]	Macrolide susceptible
ST2	1 (1.1)	2		1 (100)
ST3	66 (71.0)	1	65 (98.5)	1 (1.5)
ST7	2 (2.2)	2		2 (100)
ST14	18 (19.4)	2	7 (38.9)	11 (61.1)
ST15	2 (2.2)	2		2 (100)
ST17	1 (1.1)	1	1 (100)	
ST38[a]	1 (1.1)	NA		1 (100)
ST39[a]	1 (1.1)	1		1 (100)
ST40[a]	1 (1.1)	2		1 (100)
Total	93		73 (78.5)	20 (21.5)

[a] Newly identified ST in this study.
[b] Mutation site (23S rRNA): A2063G (ST3 and ST14); A2064G (ST17).
ST, sequence type; NA, not applicable.

eBURST analysis

The eBURST diagram was examined along with all known STs to determine the topological position of the STs identified in this study (Fig. 2). In general, STs from this study were divided into two clonal complexes (CCs) with one singleton (ST38). The majority of STs were categorized as CC1 (73.1%). The rate of macrolide resistance was significantly higher in CC1 than in CC2 (98.5% vs. 33.3%, $P < 0.001$).

Discussion

The macrolide resistance rate among *M. pneumoniae* isolates has been continuously increasing in South Korea, rising from 0% in 2000 to 84.4% in 2014—2016.[12] In this study, we investigated changes in the genetic characteristics of *M. pneumoniae* to determine whether the resistance rate will

Table 1 Detection rate and macrolide susceptibility of *Mycoplasma pneumoniae* among participating hospitals, South Korea, 2019—2020.

Site	No. cultured samples	No. *M. pneumoniae* isolated (%)	No. (%) isolates	
			Macrolide resistant	Macrolide susceptible
SNUCH[a] (Seoul)	978	65 (6.6)	55 (84.6)	10 (15.4)
SNUBH[b](Seongnam)	146	18 (12.3)	12 (66.7)	6 (33.3)
CNUH[c] (Daejeon)	75	6 (8.0)	4 (66.7)	2 (33.3)
JNUH[d] (Jeju)	29	4 (13.8)	2 (50.0)	2 (50.0)
Total	1228	93 (7.6)	73 (78.5)	20 (21.5)

[a] SNUCH, Seoul National University Children's Hospital.
[b] SNUBH, Seoul National University Bundang Hospital.
[c] CNUH, Chungnam National University Hospital.
[d] JNUH, Jeju National University Hospital.

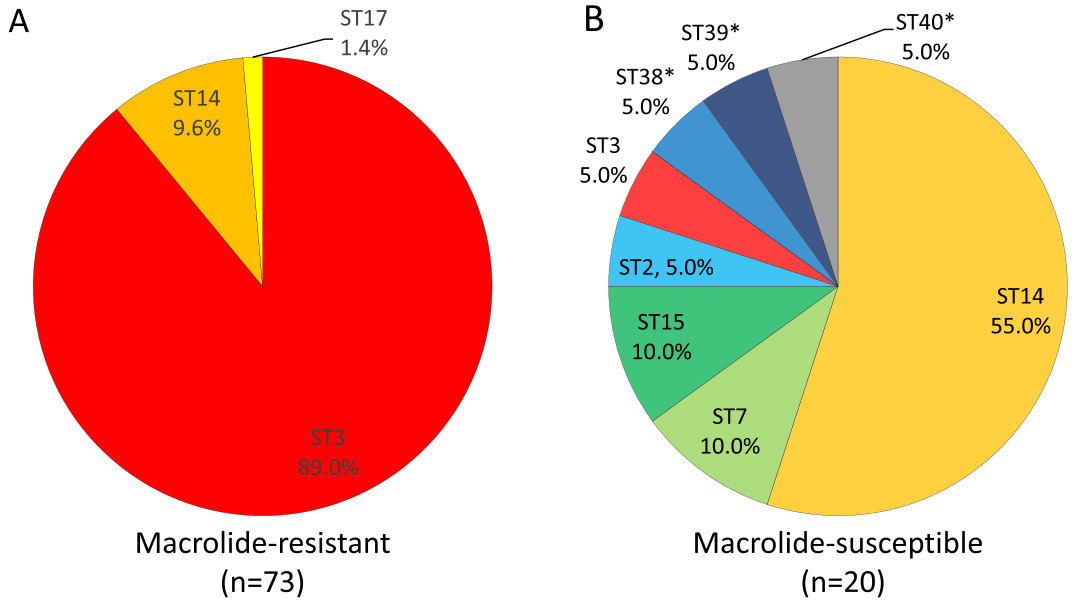

Figure 1. Distribution of *Mycoplasma pneumoniae* among 73 macrolide-resistant and 20 macrolide-susceptible isolates from South Korea, 2019–2020. A) macrolide-resistant isolates; B) macrolide-susceptible isolates. Values are percentages. *Newly identified ST in this study. ST, sequence type.

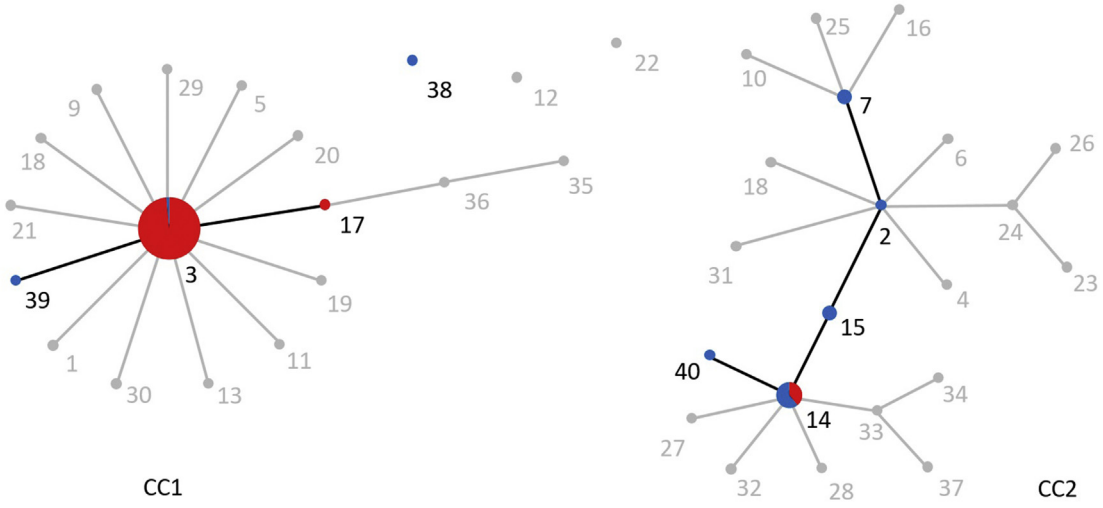

Figure 2. *Mycoplasma pneumoniae* ST (sequence type) relationship between 93 isolates from South Korea, 2019–2020, and previously reported STs (in gray) from PubMLST (http://pubmlst.org/mpneumoniae/). Two main clonal complexes (CCs) were defined with one singleton (ST38). The size of each circle correlates to the number of isolates of each ST. Red and blue indicate macrolide-resistant and macrolide-susceptible isolates, respectively.

continue to increase or begin to decline in South Korea. We demonstrated that the macrolide resistance rate of a recent outbreak of *M. pneumoniae* pneumonia in 2019–2020 was 78.5%. The high macrolide resistance rate in this study period was similar to that (84.8%) during the preceding outbreak (Fig. 3). Although the high resistance rate persisted across the two consecutive outbreaks, we identified more diverse STs among the *M. pneumoniae* isolates in 2019–2020 than in the previous epidemic, when only two STs (ST3 and ST14) circulated. Furthermore, compared to the 2014–2016 epidemic, when ST3 was the only ST representing macrolide resistance, we identified three STs (ST3, ST14, and ST17) harboring macrolide resistance in 2019–2020.

ST analysis by eBURST enables the clustering of STs into related strains.[14] As shown in Table 2 and Fig. 2, each CC comprises distinct STs: CC1 (ST3, ST17 and ST39); CC2 (ST2, ST7, ST14, ST15, and ST40). In general, ST3 is the mainstay (founder) of CC1 and the majority of STs are single-locus variants of ST3. On the other hand, the STs in CC2 are more diversely distributed with single- and multilocus variants. The association of a specific ST to either CC1 or CC2 has been repeatedly reported since the initial development of the MLST scheme.[15–17] Moreover, genotyping methods

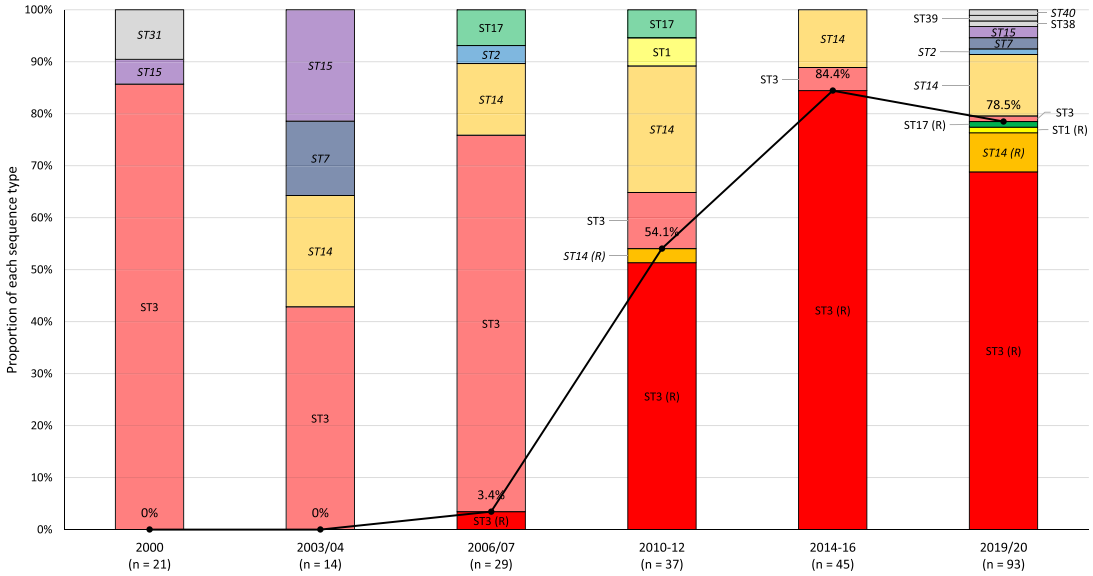

Figure 3. *Mycoplasma pneumoniae* ST (sequence type) distribution for each outbreak and the macrolide resistance within specific STs, South Korea, 2000—2021. The broken line represents the macrolide resistance rate within each outbreak. The STs from the clonal complex 2 strains are italicized. The y-axis indicates the proportion of each ST. (R) designates macrolide resistance. ST, sequence type.

for *M. pneumoniae* have continued to evolve since the introduction of P1 typing using restriction fragment length polymorphism or pulsed-field gel electrophoresis.[18] As the MLST scheme is assumed to be more discriminatory than previous molecular methods, the results of MLST and CC were previously compared to those of P1 typing. The results showed that the known P1 type 1 and type 2 strains are closely related to CC1 and CC2, respectively.[15] This association has since been reported in other studies investigating both CC and P1 types.[11,17]

The propensity for high macrolide resistance in CC1 was clearly demonstrated in a study of 419 *M. pneumoniae* isolates collected between 2011 and 2017 in the Osaka prefecture of Japan.[19] The majority of CC1 harbored macrolide resistance mutations (204/223 isolates, 91.5%). In contrast, the total number of isolates with macrolide resistance mutations in CC2 was only 3.1% (6/193). Similarly, macrolide-resistant *M. pneumoniae* in South Korea has been shown to have a strong association with P1 type 1.[20] Detailed ST analysis has shown that strains with ST3 in CC1 are responsible for macrolide resistance in most Asian countries.[12,16] Moreover, a recent study on 266 *M. pneumoniae* isolates in Taiwan also showed the clonal spread of macrolide-resistant ST3 and ST17.[6]

In a study of isolates collected during 2002—2016 in Japan, two STs in CC2 (ST14 and ST7) and ST19 in CC1 were most common among the macrolide-susceptible isolates.[16] As the resistance rate declined rapidly during 2018—2019 in Japan, both ST7 and ST33 (CC2) were dominant among the susceptible strains.[11] Furthermore, the overall macrolide resistance rate was 12.0% among the 75 strains with ST14 during the period from 2000 to 2016 in Japan.[16] However, there was only one ST14 isolate (macrolide-susceptible) identified from 33 isolates during the 2018—2019 epidemic, when the macrolide resistance rate was markedly reduced.[11] Although ST14 almost disappeared in that epidemic, ST33, a single-locus variant of ST14, represented a substantial proportion of macrolide-susceptible strains.

Unlike Japan, our study results revealed no significant reduction in macrolide resistance rate in South Korea during 2019—2020. However, an increasing proportion of macrolide resistance was observed within ST14. During the previous five epidemics spanning from 2000 to 2016, only one (4.5%) macrolide-resistant isolate among the 22 isolates involved ST14.[12] Interestingly, the ST14 macrolide resistance rate observed in this study was 38.9%, suggesting the emergence of macrolide resistance among ST14 strains. The high macrolide resistance rate of ST14 also resulted in an increase of macrolide-resistant strains within the CC2 group (also known as P1 type 2 in other studies) from that observed in other studies conducted in Japan, Taiwan, China, and the United States.[6,16,21,22] This finding is partly consistent with a previous report on the antimicrobial susceptibility and genotype of *M. pneumoniae* isolates from 2014 to 2016, Beijing.[21] The authors also found that, despite a modest decrease in the overall macrolide resistance among *M. pneumoniae* isolates, the macrolide resistance rate of P1 type 2 isolates increased synchronously with an increase in the proportion of type 2 isolate. However, as the current study did not observe a substantial increase in the proportion of type 2 isolates, we suggest that the causal relationship of macrolide resistance and changes in the distribution of genetic types cannot be generalized over different communities or countries; this topic demands further exploration. The macrolide resistance rate among ST14 strains was 30.0% during 2017—2019 in Taiwan, where the macrolide resistance rate was relatively high (77.0%), and predominantly comprised ST3 and ST17.[6] Although the macrolide resistance rate of ST14 was

not as high as that in the current study, it showed a consistent trend.

Notably, all macrolide-resistant ST3 strains found in Taiwan harbored an A2063G mutation, whereas macrolide-resistant ST17 isolates harbored either an A2063G or A2063T mutation. As the current study found a single ST17 with an A2063T mutation, this raises the possibility that genotypes have a preference for a specific macrolide-resistant mutation, irrespective of regional differences in macrolide resistance or the distribution of genetic types. Furthermore, differences may exist in the mechanism of acquiring point mutations (or in the mechanism of protection from acquiring point mutations) according to the P1 type or ST, which also requires further exploration.

Although the consumption of macrolides in Japan was actively minimized by replacing them with tosufloxacin, the overall macrolide consumption in South Korea, which was estimated by statistics provided by the Health Insurance Review and Assessment Service, remained consistent from 2015 to 2019 because fluoroquinolones were not approved for pediatric use.[23] This situation is similar to the antibiotic prescription pattern in Taiwan.[6] Considering these trends and the current study results, antibiotic pressure may partly influence macrolide resistance among *M. pneumoniae*. However, the reasons for alternate increases and decreases in STs and changes in macrolide resistance are not fully understood. In addition, except for a few STs, the causal relationship of ST and macrolide resistance remains unknown. Considering only the results of this study, a proportional increase of P1 type 2 strains may not be a sign of decreasing macrolide resistance, regardless of the variability within strains.

There are a few limitations to this study. First, the number of *M. pneumoniae* strains collected for analysis was limited. Although we utilized the PCR results in the clinical management of patients, only the clearly cultured isolates were included in this study. Second, the sensitivity of antimicrobials other than macrolides could not be measured due to the nature of the study, which relies on the known 23S rRNA mutations to confirm macrolide resistance. Finally, despite the concern regarding clinical manifestations of macrolide resistance, including various complications, clinical information was not included in this study. Nevertheless, we believe that this study provides one of the most current comprehensive regional investigations of *M. pneumoniae* genetics with macrolide resistance, which has substantial value for comparative and/or future studies.

In conclusion, in the most recent *M. pneumoniae* epidemic in South Korea (2019—2020), the overall macrolide resistance remained high, despite increased genetic variability among the isolates. Most notably, ST14 and the macrolide resistance pattern within ST14 have emerged as possible predictors of the macrolide resistance rate in future epidemics.

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Declaration of competing interest

The authors declare that there is no conflict of interest.

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