Regional Differences in Prevalence of Macrolide Resistance among Pediatric Mycoplasma pneumoniae Infections in Hokkaido, Japan

Nobuhisa Ishiguro*, Naoko Koseki†, Miki Kaiho†, Hideaki Kikuta‡, Takehiro Togashi†, Koji Oba§, Keisuke Morita‖, Naoko Nagano¶, Masanori Nakamichi**††, Kyosuke Hazama‡‡, Toru Watanabe‡‡, Satoshi Sasaki‡‡‡, Atsuko Horino†††, Tsuyoshi Kenri†††, Tadashi Ariga, and Hokkaido Pediatric Respiratory Infection Study Group**

1Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo; 2Pediatric Clinic, Toei Hospital, Sapporo; 3Sapporo City University School of Nursing, Sapporo; 4Department of Biostatistics, School of Public Health, Graduate School of Medicine, and Interfaculty Initiative in Information Studies, Graduate School of Interdisciplinary Information Studies Library, The University of Tokyo, Tokyo; 5Department of Pediatrics, Japanese Red Cross Asahikawa Hospital, Asahikawa; 6Nagano Pediatric Clinic, Asahikawa; 7Department of Pediatrics, Kushiro Red Cross Hospital, Kushiro; 8Hazama Pediatric Clinic, Muroran; 9Watanabe Pediatric Allergy Clinic, Sapporo; 10Department of Pediatrics, Aiiku Hospital, Sapporo, Hokkaido; and 11Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo, Japan

SUMMARY: Recently, macrolide-resistant (MR) Mycoplasma pneumoniae appeared, and prevalence of macrolide resistance among M. pneumoniae infections varies by country. However, reports on regional differences in the prevalence of MR M. pneumoniae within a country are scarce. In this study, 617 nasopharyngeal swab samples were collected from 617 pediatric patients, and DNA of M. pneumoniae was identified in 95 samples. In 51 of the 95 M. pneumoniae positive samples, we detected the presence of mutation A2063G mutation (conferring macrolide resistance) in the 23s rRNA gene. The overall macrolide resistance rate was 53.7%, but there were regional differences: 0.0% in Muroran, 5.3% in Asahikawa, 55.3% in Sapporo, and 100.0% in Kushiro. Statistically significant pairwise differences in the prevalence of MR M. pneumoniae were observed among these cities except for the pair of Muroran and Asahikawa. After exclusion (in order to avoid the influence of macrolides) of patients who were prescribed macrolides before collection of nasopharyngeal swab samples, statistically significant differences persisted: 0.0% in Muroran, 5.6% in Asahikawa, 38.5% in Sapporo, and 100.0% in Kushiro.

INTRODUCTION

Mycoplasma pneumoniae is a common causative pathogen of community-acquired respiratory tract infections mainly in children and young adults (1). Macrolides are generally considered the drugs of choice for treatment of children with M. pneumoniae infection (2). Since approximately the year 2000, macrolide-resistant (MR) M. pneumoniae has been appearing in Asia, Europe, Canada, and the USA (3–14). The rate of macrolide resistance among M. pneumoniae infections ranges from 3 to 26% in Europe (9,15), from 63 to 97% in China (16–19), and from 25 to 93% in Japan (20–22). The total number of febrile days and the number of febrile days during macrolide administration are greater in patients infected with MR M. pneumoniae than in patients infected with macrolide-sensitive (MS) M. pneumoniae (23). Therefore, it is important to understand the regional prevalence of MR M. pneumoniae infections to predict the duration of fever caused by M. pneumoniae. Differences in the prevalence of MR M. pneumoniae among 7 surveillance areas throughout Japan were recently reported (21), but such differences among cities are not known. The purpose of this study was to determine the differences in prevalence of MR M. pneumoniae among 4 cities in Hokkaido (83,457 km²), the northernmost island of Japan.

MATERIALS AND METHODS

Clinical samples: Nasopharyngeal swab samples were collected from pediatric patients who were suspected of having a respiratory tract infection associated with M. pneumoniae from December 1, 2012, to July 31, 2014, at 8 pediatric clinics and in the department of pediatrics of 6 hospitals in the cities of Sapporo, Asahikawa, Kushiro, and Muroran, in Hokkaido, Japan (Fig. 1). The nasopharyngeal swab samples were suspended in 3 ml of BD universal viral transport medium (Becton Dickinson, Sparks, MD, USA) before extraction of DNA.

Real-time PCR assay: DNA was extracted using the QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands) from 1 ml of BD universal viral transport medium and was finally resuspended in 50 µl of buffer. DNA of M. pneumoniae was identified by real-time PCR using Mp181-F and Mp181-R primers and the Mp181-P probe with 1 µl of DNA as described elsewhere (24).
Detection of a resistance-associated point mutation in domain V of 23S rRNA: Mutations associated with resistance to macrolides at sites 2063, 2064, and 2617 in the *M. pneumoniae* 23S rRNA gene (domain V region) were detected by a sequencing method described elsewhere (25). *M. pneumonia* with a point mutation in domain V of the 23S rRNA gene was defined as MR *M. pneumoniae*.

Isolation and molecular typing of *M. pneumoniae* strains: The modified Hayflick medium was used for the isolation of *M. pneumoniae* from patients (26). The *p1* gene, encoding P1 cytadhesin, an essential pathogenic factor of *M. pneumoniae*, was subtyped by a PCR-based method (27).

Statistical analysis and ethics: All statistical analyses were performed in the JMP software, version 11.0.0 (SAS Institute, Cary, NC, USA). The prevalence rates of MR *M. pneumoniae* were compared by Fisher's exact test. The multiplicity was adjusted by Bonferroni’s correction method (adjusted significance level was 0.008 if all combinations of 4 cities were compared). All of the necessary ethics approvals for this study were obtained from the Institutional Review Board of Hokkaido University Hospital for Clinical Research.

RESULTS

A total of 617 nasopharyngeal swab samples were collected from 617 patients, and DNA of *M. pneumoniae* was identified in 95 samples. The average age of the patients was 8.4 years, and the ratio of men to women was 50:45. In 51 of the 95 *M. pneumoniae*-positive samples, the presence of the A2063G mutation in the 23S rRNA gene, a point mutation known to confer macrolide resistance to *M. pneumoniae*, was detected, but other mutations (A2063C, A2063T, A2064G, or C2617G) were not detected. In the remaining 44 *M. pneumoniae*-positive samples, these mutations were not detected. The overall macrolide resistance rate was 53.7% (51 of 95), whereas the municipal resistance rate varied: 0.0% (0 of 9) in Muroran, 5.3% (1 of 19) in Asahikawa, 55.3% (21 of 38) in Sapporo, and 100.0% (29 of 29) in Kushiro (Table 1). Table 1 shows the *P* values of Fisher’s exact test for pairwise comparisons of the prevalence rates of macrolide resistance among *M. pneumoniae* infections between two cities. Statistical significance was observed for pairwise differences in the prevalence of MR *M. pneumoniae* except for the pair Asahikawa and Muroran. Differences in prevalence of MR *M. pneumoniae* were also observed between patients visiting hospitals and those visiting clinics and between outpatients and inpatients (Table 2). Statistical significance was observed for the differences in the prevalence of MR *M. pneumoniae* between patients with and without macrolide pre-administration: 92.0% (23 of 25) and 40.0% (28 of 70), respectively (Table 3). Macrolides had been administered to 25 (26.3%) of the patients before collection of nasopharyngeal swab samples. After exclusion of the nasopharyngeal swab samples from 25 patients who had received macrolides, statistical significance of the differences was still observed between regions (Table 1) and between patients visiting hospitals and those visiting clinics, whereas the statistical significance of the difference between outpatients and inpatients disappeared (Table 4).

Twenty-three strains of *M. pneumoniae* were found in 23 randomly selected samples, and these were genotyped. Four of 6 strains of MR *M. pneumoniae* from the Kushiro samples were found to be subtype 1, and 2 were variant 2c (Table 5).

DISCUSSION

In the present study, DNA of *M. pneumoniae* was identified in 95 of 617 nasopharyngeal swab samples from patients who were suspected of having respiratory
Table 1. Prevalence of macrolide-resistant *M. pneumoniae* in 4 cities

| City    | Medical institution | Hospital/Clinic | No. of MR/total isolates (%) | No. of MR/total in the city (%) | No. of MR/total in the city (%) |
|---------|---------------------|-----------------|-----------------------------|--------------------------------|--------------------------------|
| Sapporo | A Hospital          | 3/5 (60.0)      | 2/4 (50.0)                  |                                |                                |
|         | B Hospital          | 1/6 (16.7)      | 1/5 (20.0)                  |                                |                                |
|         | C Hospital          | 9/11 (81.8)     | 4/6 (66.6)                  |                                |                                |
|         | D Hospital          | 5/6 (83.3)      | 1/2 (83.3)                  |                                |                                |
|         | E Clinic            | 2/3 (66.7)      | 2/3 (66.7)                  | 10/26 (38.5)                   |                                |
|         | F Clinic            | 0/1 (0.0)       | 0/1 (0.0)                   |                                |                                |
|         | G Clinic            | 0/1 (0.0)       | 0/1 (0.0)                   |                                |                                |
|         | H Clinic            | 0/1 (0.0)       | 0/1 (0.0)                   |                                |                                |
|         | I Clinic            | 1/2 (50.0)      | 0/1 (0.0)                   |                                |                                |
|         | J Clinic            | 0/2 (0.0)       | 0/2 (0.0)                   |                                |                                |
| Asahikawa| K Hospital         | 1/5 (20.0)      | 1/5 (20.0)                  |                                |                                |
|         | L Clinic            | 0/14 (0.0)      | 0/13 (0.0)                  | 1/18 (5.6)                     |                                |
| Kushiro | M Hospital          | 29/29 (100.0)   | 17/17 (100.0)               |                                |                                |
| Muroran | N Clinic            | 0/9 (0.0)       | 0/9 (0.0)                   |                                |                                |
|         | E Clinic            | 0/14 (0.0)      | 0/13 (0.0)                  |                                |                                |
|         | F Clinic            | 0/9 (0.0)       | 0/9 (0.0)                   |                                |                                |
|         | G Clinic            | 0/1 (0.0)       | 0/1 (0.0)                   |                                |                                |
|         | H Clinic            | 0/1 (0.0)       | 0/1 (0.0)                   |                                |                                |
|         | I Clinic            | 1/2 (50.0)      | 0/1 (0.0)                   |                                |                                |
|         | J Clinic            | 0/2 (0.0)       | 0/2 (0.0)                   |                                |                                |
| TOTAL   |                      | 51/95 (53.7)    | 28/70 (40.0)                |                                |                                |

MR, macrolide resistant.
1): Samples from patients in whom macrolides were not pre-administered.
2): Similar statistically significances were observed in total cases and cases not pre-administered.
*, P value by Fisher’s exact test, < 0.05. **, P value by Fisher’s exact test, < 0.008.

Table 2. Prevalence of macrolide-resistant *M. pneumoniae* case Total No. Macrolide P value1) Sensitive (z) Resistant (z)

| Case      | Total No. | Macrolide Sensitive (%) | Resistant (%) | P value1) |
|-----------|-----------|--------------------------|---------------|-----------|
| Hospitals | 62        | 14 (22.6)                | 48 (77.4)     | < 0.0001* |
| Clinics   | 33        | 30 (90.9)                | 3 (9.1)       |           |
| Outpatients | 70  | 39 (55.7)              | 31 (44.3)     | 0.0024*   |
| Inpatients | 25  | 5 (20.0)                | 20 (80.0)     |           |

1): P value by Fisher’s exact test. *, statistically significant.

Table 3. Prevalence of macrolide-resistant *M. pneumoniae* in patients pre-administered macrolide

| Pre-administration | Macrolide | P value1) |
|--------------------|-----------|-----------|
| Total No. | Sensitive (%) | Resistant (%) | |
| Yes               | 25        | 2 (8.0)    | 23 (92.0) | < 0.0001* |
| No                | 70        | 42 (60.0)  | 28 (40.0) |           |

1): P value by Fisher’s exact test. *, statistically significant.

Table 4. Prevalence of macrolide-resistant *M. pneumoniae* in whom macrolides were not pre-administered

| Case      | Total No. | Macrolide Sensitive (%) | Resistant (%) | P value1) |
|-----------|-----------|--------------------------|---------------|-----------|
| Hospitals | 39        | 13 (33.3)                | 26 (66.7)     | < 0.0001* |
| Clinics   | 31        | 29 (93.6)                | 2 (6.4)       |           |
| Outpatients | 60  | 38 (63.3)              | 22 (36.7)     | 0.1833    |
| Inpatients | 10  | 4 (40.0)                | 6 (60.0)      |           |

1): P value by Fisher’s exact test. *, statistically significant.

Table 5. pl1 gene typing of *M. pneumoniae* isolates

| City    | pl1 gene typing |
|---------|-----------------|
|         | subtype 1 | subtype 2 | variant 2a | variant 2c |
| Sapporo | S          | 3         | 1         | 0         | 0         | 2         |
|         | R          | 5         | 5         | 0         | 0         |
| Asahikawa | S         | 3         | 3         | 0         | 0         |
|         | R          | 0         | 0         | 0         | 0         |
| Kushiro | S          | 0         | 0         | 0         | 0         |
|         | R          | 6         | 4         | 0         | 0         | 2         |
| Muroran | S          | 6         | 0         | 1         | 3         | 2         |
|         | R          | 0         | 0         | 0         | 0         |
| TOTAL   | 23         | 13        | 1         | 3         | 6         |

tract infections associated with *M. pneumoniae*. Although the overall prevalence of macrolide resistance among *M. pneumoniae* infections was 53.7%, substantial regional differences were observed: high MR rate in Kushiro (100.0%), and low MR rates in Asahikawa (5.3%) and Muroran (0.0%). Differences in the prevalence of MR *M. pneumoniae* among prefectures in Japan have been reported previously (21). In the present report, we showed for the first time the differences in the prevalence of macrolide resistance among *M. pneumoniae* infections across cities in one prefecture of Japan. Information on the prevalence of MR *M. pneumoniae* in each city is important for clinicians because the mean prevalence of 53.7% is far from local prevalence rates in Kushiro, Asahikawa, and Muroran.

The fact that the number of clinics and hospitals that participated in this study was limited raises the following question: does the difference in macrolide resistance rates arise from differences in regions or differences in
medical institutions? In Sapporo, both MS and MR *M. pneumoniae* were detected at medical institutions where *M. pneumoniae* was detected in more than 3 samples. In Asahikawa, there are 14 pediatric clinics and 5 outpatient pediatric clinics in hospitals. K Hospital and L Clinic, therefore, cover at least 10% of pediatric patients in Asahikawa. K Hospital, located in the western part of Asahikawa, is a secondary medical care center, and people living anywhere in the city visit this hospital. L Clinic is located in the eastern part of Asahikawa, and people living in the eastern part of the city visit this clinic. M Hospital in Kushiro, which participated in this study, is a secondary medical care center, and people living anywhere in the city visit this hospital.

In Muroran, there are 2 pediatric clinics and 2 outpatient pediatric clinics in hospitals. N Clinic covers approximately 30% of pediatric patients living anywhere in Muroran (Table 1). Therefore, it is reasonable to assume that the bias toward MR (Kushiro) or MS (Muroran and Asahikawa) derives from regional differences, not from institutional differences.

MR *M. pneumoniae* was detected in 77.4% (48 of 62) of patients visiting hospitals and only in 9.1% (3 of 33) of patients visiting clinics; this pathogen was detected in 80.0% (20 of 25) of inpatients and only in 44.3% (31 of 70) of outpatients (Table 2). These differences can be at least partially explained by the fact that patients infected with MR *M. pneumoniae* have a fever of longer duration than do patients infected with MS *M. pneumoniae* (5.1 vs. 1.7 days, manuscript in preparation).

In conclusion, there are regional differences in the prevalence of MR *M. pneumoniae* infections in pediatric patients in Hokkaido. After exclusion of the patients who received macrolides before collection of nasopharyngeal swab samples, statistical significance of regional differences was still observed.

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**Appendix** The members of the Hokkaido Pediatric Respiratory Infection Study Group are as follows: Nobuhisa Ishiguro, Naoko Koseki, Miki Kaiho, Hideaki Kikuta, Takehiro Togashi, Keisuke Morita, Naoko Nagano, Masanori Nakanishi, Kyosuke Hazama, Toru Watanabe, Satoshi Sasaki, Tadashi Ariga, Akiko Okamura, Shigeru Yamazaki, Satoru Shida, Naofumi Kaji, Tetsuo Nagashima, Mikio Yoshio, Yutaka Takehashi, Mutsumo Komno, Akihito Ichiizaka, Takeyasu Takebayashi, Mutsumo Shibata, Hiteto Furuyama, Hiroyuki Sawada, Yoshio Matsuozono, Mari Murashita, Tatsuru Yamanaka, Hiroyuki Naito, Yasushi Akutsu, Hayato Aoyagi, Katuyuki Tobise, Chie Tobise, and Katsumi Azuma.

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