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Short Communication

In Vitro Activities of 11 Antimicrobial Agents against Macrolide-Resistant *Mycoplasma pneumoniae* Isolates from Pediatric Patients: Results from a Multicenter Surveillance Study

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SUMMARY: Macrolide-resistant *Mycoplasma pneumoniae* is emerging in several countries, and it is mainly observed in children. To our knowledge, we conducted the first multicenter prospective epidemiological study of macrolide-resistant *M. pneumoniae* in order to investigate regional differences in the susceptibility of macrolide-resistant *M. pneumoniae* to antibacterial agents. The in vitro activities of 11 antimicrobial agents against macrolide-resistant *M. pneumoniae* isolates from 5 different areas of Japan were investigated. Among 190 *M. pneumoniae* isolates from pediatric patients, 124 (65.2%) isolates showed macrolide resistance and possessed an A2063G transition in domain V of the 23S rRNA. These isolates showed high resistance to erythromycin, clarithromycin, and azithromycin with minimum inhibitory concentrations (MICs) $\geq 16\ \mu g/ml$. Conversely, quinolones such as garenoxacin, moxifloxacin, tosufloxacin, and levofloxacin exhibited potent antimycoplasmal activity. No regional differences were observed with respect to the MICs among the 5 areas in Japan.

Mycoplasma pneumoniae is a major causative pathogen of respiratory tract infections, and it is found mainly in children and younger adults. Although the 14- and 15-membered macrolides are generally considered as the first-choice agents against *M. pneumoniae* infections in children, it has been reported that more than 40% of *M. pneumoniae* isolates are resistant to macrolides in Japan (1,2). Macrolide-resistant *M. pneumoniae* is also emerging in several other countries, primarily in children (3–9). During an outbreak of *M. pneumoniae* infections in Italy in 2010, 11 (25.5%) of the 43 hospitalized pediatric patients with *M. pneumoniae* were macrolide resistant (4). In particular, the reported incidence of macrolide-resistant *M. pneumoniae* is more than 90% in China (6). Thus, the increasing resistance of *M. pneumoniae* to commonly used 14- and 15-membered macrolides is a growing concern.

Specific point mutations in domain V of the 23S rRNA gene of *M. pneumoniae* define the macrolide-resistant phenotypes. The mutations that induce a high level of macrolide resistance are an A to G transition at position 2063 and an A to C transition at position 2064; whereas, low-level resistance is induced by a C to A or G transition at position 2617 and an A to T transition at position 2063 (1,9). All of the macrolide-resistant *M. pneumonia* isolates had mutations in domain V of the 23S rRNA gene (1,2,10,11). Several studies indicated that macrolide resistance in *M. pneumoniae* may have clinical significance in terms of diminished response to treatment with drugs in this class (2,12,13). In Japan, reports of macrolide-resistant *M. pneumoniae* were from limited areas, and there have been no large-scale epidemiological studies conducted throughout Japan. The purpose of the present study was to investigate regional differences in the susceptibility of macrolide-resistant *M. pneumoniae* to antibacterial agents. Thus, to our knowledge, this is the first multicenter prospective epidemiological study of macrolide-resistant *M. pneumoniae*.

We enrolled all pediatric patients with acute respiratory tract infections who visited 62 institutions located in 5 areas of Japan (20 institutions in Kyushu, 25 in Chugoku, 3 in Shikoku, 11 in Kinki, and 3 in Hokkaido), participating in the Atypical Pathogen Study Group from November 2009 to August 2011 (Fig. 1). Pediatricians at the facilities collected samples from patients with suspected *M. pneumoniae* infections. Informed consent was obtained from the parents of all patients. The Ethics Committee at Kawasaki Medical School approved the study protocol.

M. pneumoniae isolates were obtained by cultivation of specimens. The medium used for isolation and determination of the minimum inhibitory concentration (MIC) was pleuropneumonia-like organism broth (PPLO; Oxoid, Franklin, N.J., USA) supplemented with 0.5% glucose (Wako Pure Chemicals Inc., Osaka, Japan), 20% Mycoplasma supplement-G (Oxoid), and 0.0025% phenol red (Sigma-Aldrich Co. LLC., St. Louis, Mo., USA).

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**Members of the Atypical Pathogen Study Group are listed in the Appendix.

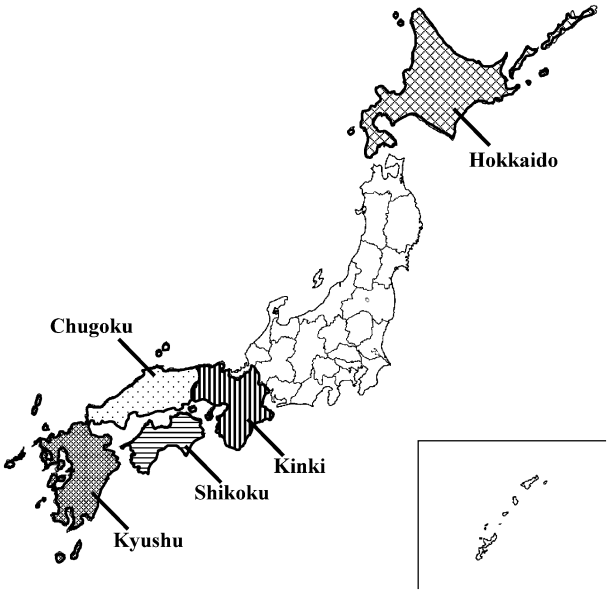

Fig. 1. Samples were collected from pediatric patients with acute respiratory tract infections who visited 62 institutions located in 5 areas of Japan, i.e., Kyushu area, Chugoku area, Shikoku area, Kinki area, and Hokkaido area.

Table 1. Prevalence of *Mycoplasma pneumoniae* isolates in 5 areas of Japan

Area	No. of samples tested	No. of isolates (%)	No. of resistant isolates (%)
Kyushu	160	37 (23.1)	28 (75.6)
Chugoku	550	73 (13.2)	39 (53.4)
Shikoku	57	19 (33.3)	19 (100)
Kinki	104	20 (19.2)	5 (25.0)
Hokkaido	161	41 (27.3)	33 (80.4)
Total	1,032	190 (18.4)	124 (65.2)

Table 2. Baseline characteristics of the patients with *Mycoplasma pneumoniae* infections

	Macrolide-resistant *M. pneumoniae*	Macrolide-sensitive *M. pneumoniae*	*P*
No.	124	66	
Age, mean ± SD (y)	8.5 ± 4.3	7.1 ± 4.4	0.098
Gender, male	74 (59)	41 (62)	0.758
Disease classification			
Pneumonia	75 (60)	42 (63)	0.754
Bronchitis	49 (40)	24 (37)	

Data represent the numbers of patients, and numbers in parentheses are percentages.

The MICs of the 11 antimicrobial agents for the isolates were determined by micro-dilution methods (14). Briefly, medium containing 10^5–10^6 CFU/ml of *M. pneumoniae* was placed in 96-well microplates and incubated at 37°C for 6–8 days. The MIC was defined as the lowest concentration of antimicrobial agents at which the metabolism of the organism was inhibited as evidenced by a lack of color change in the medium when the drug-free control first showed a color. Reference strain FH was used as drug-susceptible control. The antimicrobial agents used for MIC determination were as follows: erythromycin, clarithromycin, azithromycin, rokitamycin, clindamycin, minocycline, tetracycline, tosufloxacin, garenoxacin, levofloxacin, and moxifloxacin.

Specific point mutations in domain V of the 23S rRNA gene of *M. pneumoniae* define the macrolide-resistance phenotypes. We performed full-length sequencing of the 23S rRNA gene in *M. pneumoniae* isolates showing macrolide resistance, as reported previously (11).

Statistical analysis was performed using Stat View version 5.0. (SAS Institute Inc., Cary, N.C., USA). The number of patients per gender and disease classification was compared using the Fisher's exact test. We used the Wilcoxon rank-sum test to compare the mean age of patients. *P* values less than 0.05 were statistically significant.

A total of 190 (18.4%) isolates of *M. pneumoniae* were obtained by cultivation of samples from 1,032 pediatric patients with respiratory tract infections (37 samples were obtained in Kyushu, 73 in Chugoku, 19 in Shikoku, 20 in Kinki, and 41 in Hokkaido) (Table 1). Of the 190 isolates, 124 (65.2%) were classified as macrolide-resistant *M. pneumoniae* by genetic analysis, and all had the point mutation A2063G in domain V of the 23S rRNA gene. Patient characteristics are shown in Table 2. There were no statistically significant differences in terms of age, gender, or disease classification between the macrolide-resistant and macrolide-sensitive *M. pneumoniae* groups. Table 3 shows the MIC range, MIC_{50}, and MIC_{90} for the 11 agents according to the presence or absence of a mutation in the 23S rRNA gene in the 190 *M. pneumoniae* isolates. Among the 124 isolates that were with macrolide resistant, the MIC_{50} and MIC_{90} values for 14- and 15-membered macrolides (e.g., erythromycin, clarithromycin, and azithromycin) were 64 μg/ml to >128 μg/ml and 128 μg/ml to >128 μg/ml, respectively. Compared to the 14- and 15-membered macrolides, the 16-membered macrolide, rokitamycin, had moderate-level resistance, with MIC_{50} and MIC_{90} values of 0.25 μg/ml and 0.5 μg/ml, respectively. Higher MIC values are reported for rokitamycin in the other mutations such as A2064G and A2063C in domain V of the 23S rRNA gene (1). Conversely, all quinolones, especially garenoxacin and moxifloxacin, showed excellent antimycoplasmal activity, with MIC_{50} values of 0.0313 μg/ml and 0.0625 μg/ml, respectively, and MIC_{90} values of 0.0625 μg/ml and 0.125 μg/ml, respectively, against macrolide-resistant *M. pneumoniae* isolates; these values were equal to that against macrolide-sensitive *M. pneumoniae* isolates. Tosufloxacin, the only quinolone approved for treatment of pneumonia in pediatric patients in Japan, also showed good antimycoplasmal activity against the macrolide-resistant isolates. The MIC_{50} and MIC_{90} values were 0.25 μg/ml and 0.5 μg/ml, respectively. There were no regional differences among the 5 areas in Japan with respect to the MIC values.

A2063G in domain V of the 23S rRNA is the most frequent mutation association with macrolide resistance, followed by A2064G. The A2063C, C2617G, C2617A, and A2063T mutations are rare (1,2,9). All macrolide-resistant isolates in the present study had the same mu-

Table 3. In vitro antimycoplasmal activity against clinical isolates of *Mycoplasma pneumoniae* according to the presence or absence of a mutation in the 23S rRNA gene

Antimicrobial agent	MIC (μg/ml) for:						
	FH[1)]	Macrolide-sensitive *M. pneumoniae* (n = 66)			Macrolide-resistant *M. pneumoniae* (n = 124)		
		Clinical isolate			Clinical isolate		
		Range	50%	90%	Range	50%	90%
Erythromycin	0.0039	0.001–0.0078	0.0039	0.0078	128–>128	>128	>128
Clarithromycin	0.002	0.001–0.0156	0.002	0.0039	128–>128	>128	>128
Azithromycin	0.00025	0.000125–0.001	0.00025	0.0005	16–>128	64	128
Rokitamycin	0.0156	0.0039–0.0313	0.0078	0.0156	0.0625–1	0.25	0.5
Clindamycin	2	0.25–4	1	2	32–>128	128	>128
Minocycline	1	0.25–2	1	2	0.25–4	1	2
Tetracycline	0.5	0.25–1	0.5	0.5	0.25–1	0.5	0.5
Tosufloxacin	0.25	0.125–0.5	0.25	0.5	0.125–0.5	0.25	0.5
Garenoxacin	0.0313	0.0156–0.0625	0.0313	0.0625	0.0156–0.125	0.0313	0.0625
Levofloxacin	0.5	0.5–1	0.5	0.5	0.25–1	0.5	0.5
Moxifloxacin	0.0625	0.0625–0.125	0.125	0.125	0.0313–0.125	0.0625	0.125

[1)]: Reference strain.

tation of A2063G. These isolates were highly resistant to 14- and 15-membered ring macrolides and clindamycin with MIC_{90} values greater than or equal to 128 μg/ml. A recent study found that the microbiological and clinical efficacy of the 14- and 15-membered ring macrolides for treating patients with macrolide-resistant *M. pneumoniae* was low, at 9.5% and 28.5%, respectively (2). These data indicate correlation of in vitro susceptibility and the microbiological and clinical efficacy. Our results, together with that of previous studies, indicate that 14- and 15-membered ring macrolides are less effective against macrolide-resistant *M. pneumoniae* patients (2,12,13).

The optimal treatment for serious infection caused by macrolide-resistant *M. pneumoniae* remains unclear. In healthy children with severe infections caused by macrolide-resistant *M. pneumoniae*, ciprofloxacin showed a favorable clinical and microbiological response without any adverse effects (3,15). The 2011 version of the Japanese guidelines for the management of respiratory infectious diseases in children recommends the use of tosufloxacin instead of macrolides when a macrolide-resistant *M. pneumoniae* infection is suspected (16). Tosufloxacin, a fluoroquinolone antimicrobial agent that has been reported to have a broader spectrum and potent activity against Gram-positive and Gram-negative bacteria including *M. pneumoniae*, has been approved for the treatment of pneumonia in pediatric patients since 2010 (17,18). In our study, tosufloxacin showed good in vitro activity against clinical isolates of *M. pneumoniae*, both with or without the A2063G mutation. Further, we demonstrated that the number of *M. pneumoniae* isolates in the nasopharynx of patients with macrolide-resistant *M. pneumoniae* decreased promptly after treatment with tosufloxacin for 48 h, and this was found to be related to the clinical outcome (19). To investigate the clinical efficacy of tosufloxacin against macrolide-resistant *M. pneumoniae* strains, multicenter clinical trials will be needed. After tosufloxacin obtained authority's approval of indication, guidelines should recommend the use of tosufloxacin for the treatment of macrolide-resistant *M. pneumoniae* strains. Furthermore, physicians should select antibiotics based on the pharmacokinetics/pharmacodynamics. A nationwide surveillance may provide important information regarding empirical therapy against *M. pneumoniae* infections.

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Conflict of interest None of declare.

Appendix The Atypical Pathogen Study Group comprised Akiko Maki (Hashima Children's Clinic), Hiroshi Sakata (Hokkaido P.W.F.A.C Asahikawa-Kosei General Hospital), Hidekazu Nakajima (Kojima Central Hospital), Hitoshi Ochiai (Ochiai Pediatric Clinics), Humitaka Hirata (Hirata Clinic), Kazuyo Nomura (Kama Red Cross Hospital), Kanetsu Okura (Okura Clinics), Kazumi Hiraba (Mokubo Pediatric Clinics), Kensuke Nagai (Nagai Pediatric Clinics), Masakazu Umemoto (Umemoto Children's Clinics), Makoto Obata (Kobuchi Hospital), Makoto Kuramitsu (Aoba Children's Clinics), Naohiko Moriguchi (Sakai Hospital, Kinki University Faculty of Medicine), Nobuaki Takeda (Takeda Children's Clinics), Shigeru Mori (Momotaro Clinics), Takuya Inoue (Chayamachi Children's Clinic), Takashige Okada (Okada Children's Clinics), Tatsuo Koga (Koga Pediatric Clinics), Takashi Nakano (Kawasaki Hospital), Tetsu Sugimura (Sugimura Children's Clinics), Tomohiro Ichimaru (Saga Prefectural Hospital Koseikan), Toshiaki Ihara (National Mie Hospital), Yoshikuni Nakao (Mabi Memorial Hospital), and Yasuko Okamoto (Okamoto Clinics).

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