Title: Nationwide surveillance of macrolide-resistant Mycoplasma pneumoniae infection in pediatric patients

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Running title: Surveillance of macrolide-resistant M. pneumoniae
ABSTRACT

We conducted nationwide surveillance to investigate regional differences in macrolide-resistant (MR) *Mycoplasma pneumoniae* strains in Japan. The prevalence of MR *M. pneumoniae* has increased gradually in pediatric patients between 2008 and 2012. Although regional differences were observed, high levels of MR genes were detected in all seven surveillance areas throughout Japan, ranging from 50% to 93%. These regional differences were closely related to the previous administration of macrolides.
Mycoplasma pneumoniae is a common causative pathogen of respiratory tract infections (RTIs) in children. During 2010 and 2012, epidemics of M. pneumoniae infection, especially among children, occurred throughout Japan, and the incidences were the highest observed during the past decade (1). Macrolides are generally considered to be the first-choice agents for the treatment of M. pneumoniae infection. In 2000, however, M. pneumoniae showing resistance to macrolides was isolated from clinical samples obtained from Japanese pediatric patients with pneumonia, and macrolide resistance has become widespread in Japan and China (2-7). Macrolide-resistant (MR) M. pneumoniae is also emerging in pediatric populations in other countries (8-13). However, data on MR M. pneumoniae has mostly been reported for limited areas (2-5), and there are reports on regional differences in MR M. pneumoniae throughout Japan. The purpose of our study was to investigate regional differences in the prevalence, resistance mechanism, and drug susceptibility of MR M. pneumoniae strains by means of the first nationwide surveillance of MR M. pneumoniae Japan.

All pediatric patients with RTIs who visited 65 institutions located in seven areas of Japan (A. Kyushu [13 million people], B. Chugoku [7 million people], C. Shikoku [3 million people], D. Kinki [20 million people], E. Tokai [15 million people], F. Kanto [42 million people], and G. Hokkaido [5 million people]; Fig. 1), participating in the Atypical Pathogen Study Group from January 2008 to December 2012 were enrolled in this study. The coverage in each area was based on collaborating physicians only. Four areas (A, B, C, and D) participated from 2008, area G participated from 2010, area E from 2011, and area F from 2012. A complete list of participating facilities is provided in the appendix. Nasopharyngeal swab specimens and sputum samples, if available, were collected by
pediatricians at the facilities from patients with RTIs. Informed consent was obtained from
the parents of all patients, and the study protocol was approved by the Ethics Committee at
Kawasaki Medical School.

Cultivation of \textit{M. pneumoniae} was carried out with pleuropneumonia-like organism
broth (PPLO; Oxoid, Franklin, NJ, 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) using sputum samples or
nasopharyngeal swab specimens (14). \textit{M. pneumonia} DNA was detected by real-time PCR
targeting a conserved part of the gene coding for the P1 adhesin gene (14). A search for
mutations at sites 2063, 2064, and 2617 in the \textit{M. pneumoniae} 23S rRNA domain V gene
region was performed using a direct sequencing method in isolates or samples with a
positive PCR result, as reported previously (3, 15, 16). The minimum inhibitory
concentrations (MICs) of 11 antimicrobial agents for the isolates were determined by
micro-dilution methods (17). The antimicrobial agents used for MIC determination were as
follows: erythromycin, clarithromycin, azithromycin, rokitamycin, clindamycin,
minocycline, tetracycline, tosufloxacin, garenoxacin, levofloxacin, and moxifloxacin.

Samples from a total of 2,120 patients with RTIs were sent to our hospital. Of these,
there were 769 positive cases by culture or real-time PCR for \textit{M. pneumoniae}. A total of
484 cases were classified as pneumonia, and the remaining 285 cases were classified as
bronchitis. The prevalence of MR \textit{M. pneumoniae} sequences in seven areas throughout
Japan is shown in Table 1. Five hundred and sixty-one (73%) of 769 patients with
\textit{M. pneumoniae} infection were determined to have a MR sequence. The resistance rate
varied in each area, and the highest resistance rate in 2012 was observed in area C at 100%,
then area D at 88%, and area B at 85%. In areas B and C, which both have small populations, there were many patients with MR \textit{M. pneumoniae}. In contrast, in area F, there were few patients with MR \textit{M. pneumoniae} although it is the most highly populated region in Japan. Figure 2 shows year-by-year changes in MR \textit{M. pneumoniae} observed in all areas from 2008 to 2012. The frequency of MR genes increased gradually each year: 56% (9/16) in 2008, 69% (9/13) in 2009, 71% (79/110) in 2010, 63% (176/281) in 2011, and 82% (288/349) in 2012.

There was no significant difference in gender or disease classification between the seven surveillance areas, but the mean age was significantly higher in area E than in area A \((p = 0.0139)\) (Table 1). The number of patients in the >6-years-old group was significantly higher in area E than in area A \((p = 0.0321)\), area B \((p = 0.0364)\), and area C \((p = 0.0459)\).

Among 561 patients with MR \textit{M. pneumoniae}, 538 had an A-to-G transition at position 2063 (A2063G), 18 had an A-to-T transition at position 2063 (A2063T), three had an A-to-G transition at position 2064 (A2064G), one had an A-to-C transition at position 2063 (A2063C), and one had a C-to-G transition at position 2617 (C2617G).

The prior prescription of antibiotics before visiting the study clinic or hospital and effective antibiotics against MR \textit{M. pneumoniae} are shown in Table 1. Macrolides were administered to 352 (62%) patients before study enrollment, 168 patients received clarithromycin and 184 received azithromycin, and to 219 (39%) patients after enrollment, 121 patients received clarithromycin and 98 received azithromycin. The resistance rate was closely related to the previous administration of macrolides before visiting the study clinic or hospital. The highest rates of prior prescription of macrolides were observed in areas C and E at rates of 84%. The resistance rates were 93% in area C and 84% in area E. The
majority of attending physicians treated patients with MR *M. pneumoniae* with minocycline
or tosufloxacin in accordance with the Japanese guidelines for the management of
respiratory infectious diseases in children (18).

A total of 252 isolates of *M. pneumoniae* were obtained by cultivation of samples from
these patients and 183 isolates had a mutation. The Japanese Society for Mycoplasmology
has proposed resistance breakpoints for the compounds employed against *M. pneumoniae*
isolates (19). The criteria for drug-resistant *M. pneumoniae* are MIC $\geq 16$ μg/ml for
erthyromycin, clarithromycin, and azithromycin. Among the 183 isolates of MR
*M. pneumoniae*, the MIC$_{90}$ values for 14- and 15-membered macrolides erythromycin,
clarithromycin, and azithromycin were $>128$ μg/ml, $>128$ μg/ml, and 128 μg/ml,
respectively, which were higher than 69 isolates with macrolide-susceptible (MS)
*M. pneumoniae* with MIC$_{90}$ values of 0.0078 μg/ml for erythromycin, 0.0039 μg/ml for
clarithromycin and 0.0005 μg/ml for azithromycin. Conversely, tetracycline, minocycline,
tosufloxacin, garenoxacin, levofloxacin, and moxifloxacin showed good antimycoplasmal
activity, with MIC$_{90}$ values of 0.5 μg/ml, 2.0 μg/ml, and 0.5 μg/ml, 0.0625 μg/ml,
0.5 μg/ml, and 0.125 μg/ml, respectively, against MR *M. pneumoniae* isolates, which were
equal to MS *M. pneumoniae* isolates. No regional differences in the drug susceptibility of
MR *M. pneumoniae* were observed.

The increase in MR *M. pneumoniae* has become a serious issue in Japan.

*M. pneumoniae* pneumonia is specified for weekly reporting by specially designated
sentinel clinics in accordance with the Japanese Infectious Diseases Control Law (1).
However, most of the reported cases with *M. pneumoniae* pneumonia are diagnosed by
serology, not culture or PCR. The National Institute of Infectious Disease does not perform
surveillance for mutations in *M. pneumoniae* isolates. Thus, we carried out the first
nationwide surveillance of MR *M. pneumoniae*. The prevalence of MR *M. pneumoniae* is
increasing gradually in pediatric patients. Although regional differences were observed,
high levels of MR genes were detected in all areas of Japan, with levels of 50–93% during
2008–2012. These regional differences were closely related to the previous administration
of macrolides.

It has been reported that more than half of attending physicians felt that macrolides
were clinically effective even in patients infected with MR *M. pneumoniae* (3). Our
previous study demonstrated that six of 21 patients with MR *M. pneumoniae* responded
clinically to macrolide therapy (15). In addition, no apparent treatment failure or serious
illness was reported for patients with MR *M. pneumoniae*. Experimental and clinical
evidence supports the idea that the pathogenesis of lung injuries caused by *M. pneumoniae*
infection is associated with cell-mediated immunity rather than with direct cell damage by
the pathogen itself (20-23). Numerous studies have documented that certain macrolides
have a wide spectrum of immunomodulatory effects on mammalian cells both in vivo and
in vitro (24). Thus, it is possible that the immunomodulatory effects of macrolides can
improve clinical symptoms in these patients with MR *M. pneumoniae*. It is important to
determine the anti-inflammatory effect for the management of MR *M. pneumoniae*
infections. To evaluate the immunomodulatory effects, it will be necessary to compare
microbiological and clinical outcomes among patients with MR *M. pneumoniae* treated
with or without macrolides.

Our study had several limitations. In some areas and years, the number of patients was
too low to clarify the prevalence of resistance. In addition, not all areas participated
simultaneously in the epidemiological study. Consequently, this nationwide surveillance
program to investigate the prevalence of MR *M. pneumoniae* cases will be continuing.

In conclusion, the prevalence of MR *M. pneumoniae* in Japanese pediatric patients was
very high and regional differences were observed. Physicians should evaluate patients for a
history of macrolide treatment before selecting antimicrobials.

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References


Figure legends

Figure 1. Samples were collected from pediatric patients with acute respiratory tract infections who visited 65 institutions located in ten areas of Japan (shaded areas). A. Kyushu, B. Chugoku, C. Shikoku, D. Kinki, E. Tokai, F. Kanto, G. Hokkaido, H. Hokuriku, I. Koshinetsu, and J. Tohoku.

Figure 2. Year-by-year increases in the frequency of macrolide-resistant *Mycoplasma pneumoniae* cases from 2008 to 2012.
TABLE 1. Prevalence of macrolide-resistant *M. pneumoniae* cases, and categorical variables age, gender, disease classification, mutation type, prior prescription of antibiotics before visiting the study clinic or hospital, and effective antibiotics of 561 pediatric patients with MR *M. pneumoniae* infection in seven areas

<table>
<thead>
<tr>
<th>Areas</th>
<th>A. Kyushu</th>
<th>B. Chugoku</th>
<th>C. Shikoku</th>
<th>D. Kinki</th>
<th>E. Tokai</th>
<th>F. Kanto</th>
<th>G. Hokkaido</th>
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</thead>
<tbody>
<tr>
<td>No. (%) of resistant cases / <em>M. pneumoniae</em> patients from 2008 to 2012</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>0/1</td>
<td>3/7</td>
<td>5/6</td>
<td>1/2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2009</td>
<td>1/3</td>
<td>5/6</td>
<td>0/1</td>
<td>3/3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2010</td>
<td>18/21 (85)</td>
<td>27/35 (77)</td>
<td>6/6</td>
<td>3/17 (17)</td>
<td>ND</td>
<td>ND</td>
<td>25/31 (80)</td>
</tr>
<tr>
<td>2011</td>
<td>24/49 (49)</td>
<td>55/119 (46)</td>
<td>40/42 (95)</td>
<td>35/42 (83)</td>
<td>11/11 (100)</td>
<td>ND</td>
<td>11/18 (61)</td>
</tr>
<tr>
<td>2012</td>
<td>19/27 (70)</td>
<td>106/124 (85)</td>
<td>10/10 (100)</td>
<td>68/77 (88)</td>
<td>76/92 (82)</td>
<td>9/18 (50)</td>
<td>0/1</td>
</tr>
<tr>
<td>5-year total (2008–2012)</td>
<td>62/101 (61)</td>
<td>196/291 (67)</td>
<td>61/65 (93)</td>
<td>110/141 (78)</td>
<td>87/103 (84)</td>
<td>9/18 (50)</td>
<td>36/50 (72)</td>
</tr>
</tbody>
</table>

| No. of patients | 62 | 196 | 61 | 110 | 87 | 9 | 36 |
| Mean age (range), yr | 7.1 (0–14) | 7.9 (0–15) | 7.5 (0–14) | 7.6 (1–15) | 8.8 (3–15) | 7.6 (3–13) | 7.6 (2–13) |

<table>
<thead>
<tr>
<th>No. (%) of patients</th>
<th>&lt;1 yr old</th>
<th>2–5 yr old</th>
</tr>
</thead>
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<tr>
<td>2008</td>
<td>3 (5)</td>
<td>23 (37)</td>
</tr>
<tr>
<td>2009</td>
<td>9 (5)</td>
<td>51 (26)</td>
</tr>
<tr>
<td>2010</td>
<td>2 (3)</td>
<td>23 (38)</td>
</tr>
<tr>
<td>2011</td>
<td>1 (1)</td>
<td>36 (33)</td>
</tr>
<tr>
<td>2012</td>
<td>0 (0)</td>
<td>22 (25)</td>
</tr>
<tr>
<td>5-year total (2008–2012)</td>
<td>0 (0)</td>
<td>32 (33)</td>
</tr>
</tbody>
</table>
>6 yr old 36 (58) 136 (69) 36 (59) 73 (66) 65 (75) 6 (67) 26 (72)

No. of males/females 31/31 113/83 36/25 55/55 39/48 5/4 18/18

No. (%) of diseases

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<th>Disease</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumonia</td>
<td>36 (58) 116 (59) 41 (67) 76 (69) 58 (67) 5 (56) 23 (64)</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>26 (42) 80 (41) 20 (33) 34 (31) 29 (33) 4 (44) 13 (36)</td>
</tr>
</tbody>
</table>

No. (%) of point mutation in domain V of 23S rRNA

<table>
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<th>No. (%)</th>
</tr>
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<tbody>
<tr>
<td>A2063G</td>
<td>52 (84) 191 (98) 59 (96) 107 (97) 84 (97) 9 (100) 36 (100)</td>
</tr>
<tr>
<td>A2063T</td>
<td>10 (16) 4 (2) 1 (2) 3 (3) 0 0 0</td>
</tr>
<tr>
<td>A2063C</td>
<td>0 0 1 (2) 0 0 0 0</td>
</tr>
<tr>
<td>A2064G</td>
<td>0 1 (0.1) 0 0 2 (2) 0 0</td>
</tr>
<tr>
<td>C2617G</td>
<td>0 0 0 0 1 (1) 0 0</td>
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</tbody>
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No. (%) of patients with prior prescription

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<th>antibiotic</th>
<th>No. (%)</th>
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<tr>
<td>Macrolides</td>
<td>22 (35) 101 (52) 51 (84) 79 (72) 73 (84) 4 (44) 22 (61)</td>
</tr>
<tr>
<td>Minocycline</td>
<td>0 3 (1) 0 1 (1) 0 0 0</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>0 3 (1) 0 0 0 0 0</td>
</tr>
<tr>
<td>Tosufloxacin</td>
<td>3 (5) 14 (7) 0 1 (1) 1 (1) 0 0</td>
</tr>
<tr>
<td>β-lactams</td>
<td>14 (22) 28 (14) 4 (6) 4 (4) 5 (6) 0 7 (19)</td>
</tr>
</tbody>
</table>

No. (%) of patients with effective antibiotics
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<tr>
<th>Antibiotic</th>
<th>14 (23)</th>
<th>28 (14)</th>
<th>7 (11)</th>
<th>15 (14)</th>
<th>7 (8)</th>
<th>3 (33)</th>
<th>14 (39)</th>
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<tbody>
<tr>
<td>Macrolides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minocycline</td>
<td>18 (29)</td>
<td>118 (60)</td>
<td>34 (56)</td>
<td>31 (28)</td>
<td>52 (60)</td>
<td>1 (11)</td>
<td>6 (17)</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>0</td>
<td>2 (1)</td>
<td>0</td>
<td>1 (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tosufloxacin</td>
<td>30 (48)</td>
<td>48 (25)</td>
<td>20 (33)</td>
<td>63 (57)</td>
<td>28 (32)</td>
<td>5 (56)</td>
<td>16 (44)</td>
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<tr>
<td>β-lactams</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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</tbody>
</table>
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