Development of macrolide resistance-associated mutations after macrolide treatment in children infected with *Mycoplasma pneumoniae*

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**Abstract**

**Purpose.** To determine the timing of the emergence of macrolide-resistant mutations after macrolide treatment in individuals with *Mycoplasma pneumoniae* infections.

**Methodology.** Between October 2011 and December 2013, serial pharyngeal swab specimens were collected before and after macrolide treatment from 21 otherwise healthy children infected with *M. pneumoniae* without macrolide-resistant mutations. The copy numbers of a *M. pneumoniae* gene and the proportion of clones showing macrolide-resistance mutations were determined for each specimen.

**Results.** After macrolide treatment (10–15 mg kg\(^{-1}\) day\(^{-1}\) clarithromycin for 5–10 days or 10 mg kg\(^{-1}\) day\(^{-1}\) azithromycin for 3 days), fever resolved in 19 (90 %) of 21 children within 1 to 2 days, and the *M. pneumoniae* gene copy number decreased in all but one specimen in the second set of specimens relative to the number in the corresponding initial specimens. None of the second specimens, which were collected 2–4 days after initiation of macrolide treatment, showed mutations in the 23S rRNA gene. However, the proportion of mutant clones with A2063G and A2064G mutations in the specimens collected 7–24 days after initiation of treatment increased to 100 %. We identified a family in which three members had *M. pneumoniae* infections. The analysis of transmission in this household indicated that the *M. pneumoniae* harbouring a macrolide-resistant mutation that developed in the index patient after macrolide treatment was not transmitted to the family members.

**Conclusion.** A macrolide-resistant population might develop in individual patients up to 24 days after initiation of macrolide treatment. However, the decrease in *M. pneumoniae* load after macrolide administration effectively reduces interpersonal transmission.

**INTRODUCTION**

*Mycoplasma pneumoniae* is an important cause of upper and lower respiratory tract infections, mainly in children and young adults. *M. pneumoniae* infections in children can be treated with 14- or 15-membered-ring macrolides, which are generally considered to be first-line antibiotics [1, 2]. A macrolide-resistant *M. pneumoniae* strain harbouring a 23S rRNA gene mutation was first isolated in 2001 [3]. Since then, macrolide-resistant *M. pneumoniae* has become common worldwide [4]. In Japan, the rates of macrolide-resistant *M. pneumoniae* infections have increased annually: 5.0 % in 2003, 30.6 % in 2006, 59.1 % in 2009 and 89.5 % in 2011 [5–7]. However, regional differences in the prevalence of macrolide-resistant *M. pneumoniae* were recently reported [8, 9]. In Yamagata, Japan, the rates were 48.2 % in 2012 and 32.3 % in 2013 [10].

*M. pneumoniae* acquires its resistance to macrolides through point mutations at nucleotides 2063, 2064 and 2617 (*M. pneumoniae* numbering) in domain V of the 23S rRNA gene, which interfere with the binding of macrolides to rRNA. Mutations at positions 2063 and 2064 result in high macrolide resistance, whereas the mutation at position 2617 produces weak macrolide resistance in *M. pneumoniae* [3, 10, 11]. The A2063G transition is the most frequently
reported mutation, followed by A2063T and A2064G [4, 5, 8, 10]. Previous in vitro experiments showed that M. pneumoniae with macrolide-resistant mutations could be selected from a macrolide-susceptible reference strain or a clinical isolate by serial passaging in a sub-inhibitory concentration of macrolide [3, 11–13]. Therefore, the high prevalence of macrolide resistance in Asian countries may be associated with selective pressure from frequent macrolide use [2, 4]. Some case reports have documented the acquisition of resistance after macrolide treatment in individual patients, including an immunocompromised child [14–19]. However, little is known about whether macrolide-resistant mutants are initially present as a minor population in untreated patients or whether such mutants develop de novo via replication during macrolide therapy. The subsequent selection and transmission of resistant mutants are also unstudied.

In the present study, to determine the timing of the emergence of M. pneumoniae with macrolide-resistant mutations in individuals receiving macrolide treatment, we examined specimens serially collected before and after macrolide treatment from 21 otherwise healthy children infected with M. pneumoniae without macrolide-resistant mutations. Moreover, we analysed household transmission among the family members of an index patient who acquired a macrolide-resistant mutant after the end of macrolide treatment.

**METHODS**

**Study design**

Initial specimen collection was performed at Yamanobe Pediatric Clinic during routine diagnosis in collaboration with the Yamagata Prefecture health authorities as part of the National Epidemiological Surveillance of Infectious Diseases, Japan (NESID). Between October 2011 and December 2013, a total of 294 pharyngeal swab specimens were obtained from children (<16 years old) who were clinically suspected of M. pneumoniae infection and from 152 patients who tested positive for M. pneumoniae infection using culture methods. The second specimen collection for this study was conducted at the same clinic, in accordance with the guidelines of the Declaration of Helsinki. Among the 107 M. pneumoniae–positive children, after excluding 45 patients with macrolide-resistant mutations in their initial specimens, a total of 21 children, from whom subsequent pharyngeal swab specimens were obtained on days 2–24 after the beginning of macrolide treatment, were included in this study. An overview of the study participants is presented in Fig. 1. The clinical characteristics of participants were retrospectively obtained from their medical records. Patients’ guardians provided written informed consent for participation in this study. The study protocol was approved by the ethics committees of the Yamagata University Faculty of Medicine (H26-35) and the Yamagata Prefectural Institute of Public Health (YPHIPHEC H26-02).

**Isolation of M. pneumoniae and molecular typing**

Clinical specimens were transported to the Yamagata Prefectural Institute of Public Health. The cultivation of M. pneumoniae was performed with pleuropneumonia-like organism (PPLO) broth (Difco, Detroit, MI) as previously described [10, 20]. When the broth colour changed from red to yellow, DNA was extracted, and polymerase chain reaction (PCR) assays using two sets of primers for both the 23S rRNA gene and molecular typing were performed as described by Matsuoka et al. [22]. The sequences of the PCR products were subsequently compared to the sequence of M. pneumoniae M129 (GenBank accession no. X68422).

To detect mutations conferring macrolide resistance, amplification and sequencing of domain V of the 23S RNA gene in M. pneumoniae were performed as described by Matsuoka et al. [22]. The sequences of the PCR products were subsequently compared to the sequence of M. pneumoniae M129 (GenBank accession no. X68422).

P1 genotyping for M. pneumoniae was performed using extracted DNA in accordance with a previously described method based on PCR-restriction fragment length polymorphism [22, 23]. Multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) was performed on all...
isolates and on two reference strains (M129: ATCC29342; FH: ATCC15531) according to previously described methods [24] and international guidelines [25]. The sequences of the VNTR loci in the *M. pneumoniae* genome were compared with those of M129 and FH, and the MLVA pattern of each isolate was identified by counting each VNTR number.

**Real-time PCR for quantification of an *M. pneumoniae* gene in clinical specimens**

To quantify the copy number of the *M. pneumoniae* community-acquired respiratory distress syndrome (CARDS) toxin gene in clinical specimens, bacterial DNA was extracted from 200 µl of clinical specimens using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) and eluted in 100 µl of DNase-free buffer. Real-time PCR targeting the *M. pneumoniae* CARDS toxin gene was performed using previously reported primers and probes [26]. The copy number of the *M. pneumoniae* CARDS toxin gene was calculated based on a standard curve generated from the threshold cycle value as previously described [27].

**Sequencing of the *M. pneumoniae* 23S rRNA gene in clinical specimens**

The DNA samples extracted from clinical specimens were used as templates to amplify the *M. pneumoniae* 23S rRNA gene by PCR with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA, USA). The first PCR was performed using primers MN23SDVnestF (5'-AC TGAAACAGCTACATTCCGG-3') and MN23SDVnestR (5'-GAGAACAGAACACGTTTGC-3') under the following conditions: 1 cycle of 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 58 °C for 20 s and 72 °C for 45 s. The second PCR was performed with the previously reported primers MN23SDVF and MN23SDVR under the following conditions: 1 cycle of 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 69 °C for 20 s and 72 °C for 45 s [22]. The resulting amplicons were cloned into the pCR4 Blunt-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and then used to transform TOP10 Chemically Competent Cells (Invitrogen). At least 16 culture-positive clones of each specimen were obtained, and their nucleotide sequences were determined using the previously reported primer MN23SF1937 [22] and the newly developed primer MN23SF2528 (5’-TCGAGCTGAAGCACGGTTTCG-3’). This experiment was approved by the Safety Committee for Gene Recombination Experiments of Yamagata University (26-34).

**Statistical analysis**

We examined differences among the three study groups. The Kruskal-Wallis test and multiple comparisons were employed for continuous variables using SPSS version 20 (IBM, USA). Fisher’s exact test was used for comparisons of male-to-female ratio using R version 3.3.2 (The R Foundation, Vienna, Austria). A *P*-value<0.05 was regarded as statistically significant.

**RESULTS**

**Patient information**

To determine the timing of the emergence of macrolide-resistant mutations, the 21 participants were divided into three groups based on the culture results of their second specimens (Fig. 1): group A (*n*=8), culture-negative second specimen; group B (*n*=7), *M. pneumoniae* without macrolide-resistant mutations in the second specimen; and group C (*n*=6), *M. pneumoniae* with macrolide-resistant mutations in the second specimen. First, to confirm that the *M. pneumoniae* isolates present in individuals before and after macrolide treatment were identical, molecular typing methods were applied to *M. pneumoniae* strains isolated from both the initial and second specimens. P1 genotyping and MLVA typing confirmed that the isolates from every participant in group B and group C were the same before and after macrolide treatment (Table S1, available in the online Supplementary Material).

Patient information about prescribed antibiotics is presented in Table 1. Administration of antibiotics began on the same day that the initial specimen was collected. Of the 21 children that showed *M. pneumoniae* without macrolide-resistant mutations in their initial specimens, 18 received clarithromycin, and 3 received azithromycin. These medicines were prescribed according to the recommendations of the Japan Pediatric Society, namely, clarithromycin at 10–15 mg kg⁻¹ day⁻¹ for 10 days or azithromycin at 10 mg kg⁻¹ day⁻¹ for 3 days [2]. The clinical diagnosis of eight patients (patients 3, 5, 8, 10, 11, 14, 15 and 17) was *M. pneumoniae*-associated pneumonia, whereas the remaining 13 patients were diagnosed with upper respiratory tract infections. All the participants were otherwise healthy, and none of them except for Patient 17 required hospital admission. A comparison of patient characteristics according to the study group is shown in Table 2. There was no statistically significant difference among the three groups in terms of age distribution, body temperature at first visit, and duration of fever from onset. However, the maximum temperatures of the patients in group C were significantly higher than those of the patients in group B (*P*=0.005), according to the multiple-comparison test.

**Effect of macrolides on clinical course**

For each patient, the periods from the beginning of macrolide treatment to resolution of fever and cough are presented in Table 1. No statistically significant difference among the three groups was found in fever duration after the beginning of macrolide treatment (Table 2). The difference in the duration of cough after the beginning of macrolide treatment could not be compared, as cough was not yet resolved for most children over the time during which their medical records could be followed.

The copy number of a *M. pneumoniae*-specific gene in all second specimens, which were collected after the beginning of macrolide treatment, decreased compared to the number in the corresponding initial specimens, except for in Patient 18.
Table 1. Information about prescribed antibiotics and testing of clinical specimens obtained from 21 children infected with *M. pneumoniae* without macrolide-resistant mutations

<table>
<thead>
<tr>
<th>Group*</th>
<th>Patient</th>
<th>Age/sex</th>
<th>Antibiotics:</th>
<th>Period (day) from initiation of antibiotic treatment to:</th>
<th>Copy number of <em>M. pneumoniae</em> gene (copies µl⁻¹) in:</th>
<th>Proportion of macrolide-resistant mutant in†:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Drug used</td>
<td>Dosage, medication days</td>
<td>Resolution of fever</td>
<td>Resolution of cough</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>4 y/M</td>
<td>Clarithromycin</td>
<td>14.0 mg kg⁻¹ day⁻¹, 10 days</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5 y/F</td>
<td>Clarithromycin</td>
<td>15.0 mg kg⁻¹ day⁻¹, 10 days</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7 y/M</td>
<td>Clarithromycin</td>
<td>9.0 mg kg⁻¹ day⁻¹, 10 days</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7 y/M</td>
<td>Clarithromycin</td>
<td>13.5 mg kg⁻¹ day⁻¹, 10 days</td>
<td>2</td>
<td>14§</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8 y/F</td>
<td>Clarithromycin</td>
<td>10.8 mg kg⁻¹ day⁻¹, 10 days</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10 y/F</td>
<td>Clarithromycin</td>
<td>9.8 mg kg⁻¹ day⁻¹, 7 days</td>
<td>1</td>
<td>9§</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>10 y/M</td>
<td>Clarithromycin</td>
<td>10.3 mg kg⁻¹ day⁻¹, 5 days</td>
<td>1</td>
<td>7§</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>13 y/M</td>
<td>Azithromycin</td>
<td>10.0 mg kg⁻¹ day⁻¹, 3 days</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>4 y/M</td>
<td>Clarithromycin</td>
<td>10.0 mg kg⁻¹ day⁻¹, 3 days</td>
<td>1</td>
<td>3§</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7 y/M</td>
<td>Clarithromycin</td>
<td>9.9 mg kg⁻¹ day⁻¹, 7 days</td>
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<td>3§</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>8 y/M</td>
<td>Clarithromycin</td>
<td>10.4 mg kg⁻¹ day⁻¹, 7 days</td>
<td>2</td>
<td>2§</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>8 y/M</td>
<td>Clarithromycin</td>
<td>10.0 mg kg⁻¹ day⁻¹, 5 days</td>
<td>1</td>
<td>2§</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>9 y/F</td>
<td>Clarithromycin</td>
<td>10.7 mg kg⁻¹ day⁻¹, 7 days</td>
<td>1</td>
<td>4§</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>9 y/F</td>
<td>Azithromycin</td>
<td>9.2 mg kg⁻¹ day⁻¹, 3 days</td>
<td>1</td>
<td>3§</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>11 y/F</td>
<td>Azithromycin</td>
<td>9.0 mg kg⁻¹ day⁻¹, 3 days</td>
<td>1</td>
<td>3§</td>
</tr>
<tr>
<td>C</td>
<td>16</td>
<td>3 y/M</td>
<td>Clarithromycin</td>
<td>14.8 mg kg⁻¹ day⁻¹, 10 days</td>
<td>1</td>
<td>24§</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>5 y/F</td>
<td>Clarithromycin</td>
<td>9.4 mg kg⁻¹ day⁻¹, 3 days§</td>
<td>3</td>
<td>7§</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>5 y/M</td>
<td>Clarithromycin</td>
<td>15.2 mg kg⁻¹ day⁻¹, 10 days</td>
<td>1</td>
<td>14§</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>6 y/F</td>
<td>Clarithromycin</td>
<td>12.0 mg kg⁻¹ day⁻¹, 10 days</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6 y/F</td>
<td>Clarithromycin</td>
<td>13.2 mg kg⁻¹ day⁻¹, 10 days</td>
<td>1</td>
<td>7§</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>8 y/F</td>
<td>Clarithromycin</td>
<td>11.4 mg kg⁻¹ day⁻¹, 10 days</td>
<td>9</td>
<td>8§</td>
</tr>
</tbody>
</table>

ND, not determined; –, not available.

*Group A, culture-negative second specimen; Group B, *M. pneumoniae* without macrolide-resistant mutations in the second specimen; Group C, *M. pneumoniae* with macrolide-resistant mutations in the second specimen.

†For each specimen, the nucleotide sequences of the 23S rRNA genes of at least 16 clones were determined. The numbers of mutated clones/total number of analysed clones are shown in parentheses.

‡Period (day) from onset of fever to initial specimen collection (first day of antibiotic treatment) is shown in parentheses.

§Period (day) from beginning of antibiotic treatment to second specimen collection is shown in parentheses.

||Not resolved when second specimen was collected or medical records could be followed.

¶Clarithromycin was stopped after prescription for 3 days because of vomiting.
in group C (Table 1). The M. pneumoniae loads in initial specimens according to the time after the onset of fever and in second specimens according to the time after the beginning of antibiotic treatment are shown in Fig. 2. Statistical analysis (Table 2) revealed no significant differences among the three groups with respect to M. pneumoniae gene copy number in initial specimens or in the period from the onset of fever to initial specimen collection. Among second specimens, although there was no statistically significant difference in M. pneumoniae gene copy number among the three groups, the period from the beginning of antibiotic treatment to second specimen collection was significantly shorter in group B patients than in group A (P=0.014) or group C (P=0.001) patients, according to the multiple comparison test. As shown in Fig. 2(b), all second specimens from group B were collected 2–4 days after the beginning of antibiotic treatment, while the second specimens from group A and group C were collected at later times. The M. pneumoniae loads appeared to decrease over the 10-day course of macrolide treatment. Among the six specimens of group C, five were collected after 10 days of macrolide treatment, and one specimen from Patient 17 was collected 7 days after macrolide treatment began. For Patient 17, medication was discontinued after administration of clarithromycin for 3 days, because the patient vomited whenever she took the medicine. Therefore, six of the specimens with M. pneumoniae harbouring macrolide-resistant mutations (group C) were collected after the end of macrolide treatment.

### Proportion of M. pneumoniae harbouring macrolide-resistant mutations in clinical specimens

To determine whether M. pneumoniae organisms with macrolide-resistant mutations were initially present as a minor population in untreated individuals, we sequenced the 23S rRNA gene in 10 initial specimens collected from patients in groups B and C. This analysis detected no mutant clones of the 23S rRNA gene in any of the samples (Table 1). The second specimens of group B that were collected 2–4 days after macrolide administration also revealed 0% (0/16) mutant clones of the 23S rRNA gene. However, the proportion of mutant clones in the second specimens of group C, which were collected 7–24 days after the start of macrolide treatment, increased to 100% (18/18) (Table 1). Among the 6 specimens with a mutation, 2 and 3 specimens were composed entirely of A2063G mutants and A2064G mutants, respectively, whereas the remaining specimen was composed of 89% (16/18) A2063G and 11% (2/18) A2064G.

### Transmission of M. pneumoniae among family members

We identified a family in which three members had M. pneumoniae infections. The three members were Patient 16, Patient 20, and their sister, who was aged 3 months. All the strains isolated from their specimens had the same P1 genotype (2a) and MLVA type (3-5-6-2), providing evidence of household transmission. As shown in Fig. 3, the symptom onset intervals between the index case (Patient 16) and the family contacts (Patient 20 and the sister) ranged between 24 and 25 days. A M. pneumoniae strain with the A2064G mutation was isolated from the second specimen of the index case, which was collected 24 days after the start of macrolide treatment. Nevertheless, no mutation in the 23S rRNA gene was detected in M. pneumoniae isolates from the initial specimens of either contact, which were collected on the same day that the second specimens were collected from the index case. A second specimen from Patient 20 (a contact) collected 17 days after the start of macrolide treatment showed a

<table>
<thead>
<tr>
<th>Variables</th>
<th>Study group*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A (n=8)</td>
<td>Group B (n=7)</td>
</tr>
<tr>
<td>No. of males/females</td>
<td>5/3</td>
<td>4/3</td>
</tr>
<tr>
<td>Age of patients, year, median (range)</td>
<td>7.5 (4.0–13.0)</td>
<td>8.0 (4.0–11.0)</td>
</tr>
<tr>
<td>Temperature at the first visit, °C, median (range)</td>
<td>38.5 (37.5–39.2)</td>
<td>38.0 (37.5–38.8)</td>
</tr>
<tr>
<td>Maximum temperature, °C, median (range)</td>
<td>38.8 (38.2–40.1)</td>
<td>38.5 (37.5–39.0)</td>
</tr>
<tr>
<td>Duration of fever, day, median (range)</td>
<td>4.5 (3.0–6.0)</td>
<td>4.0 (2.0–6.0)</td>
</tr>
<tr>
<td>Duration of fever after initiation of antibiotic treatment, day, median (range)</td>
<td>1.0 (1.0–2.0)</td>
<td>1.0 (1.0–2.0)</td>
</tr>
<tr>
<td>Period from onset of fever to collection of initial specimen, day, median (range)</td>
<td>3.5 (1.0–5.0)</td>
<td>3.0 (1.0–5.0)</td>
</tr>
<tr>
<td>Period from beginning of antibiotic treatment to collection of second specimen, day, median (range)</td>
<td>9.5 (7.0–15.0)</td>
<td>3.0 (2.0–4.0)</td>
</tr>
<tr>
<td>Copy numbers in initial specimen, copies µl⁻¹, median (range)</td>
<td>4796 (232–28893)</td>
<td>12087 (518–26015)</td>
</tr>
<tr>
<td>Copy numbers in second specimen, copies µl⁻¹, median (range)</td>
<td>ND (ND–105)</td>
<td>37 (ND–945)</td>
</tr>
</tbody>
</table>

ND, not determined.

*Group A, culture-negative second specimen; Group B, M. pneumoniae without macrolide-resistant mutations in the second specimen; Group C, M. pneumoniae with macrolide-resistant mutations in the second specimen.

†P<0.05 for comparisons between group B and group C.

‡P<0.05 for comparisons between group A/C and group B.
The predominance of the A2063G mutant population (100%), in stark contrast to the 100% A2064G mutant population observed in the second specimen of the index case.

**DISCUSSION**

In the present study, we analysed the timing of the emergence of *M. pneumoniae* harbouring macrolide-resistant mutations in individuals who received macrolide treatment. The most striking observation was that the macrolide-resistant mutant population is more likely to develop after the end of macrolide treatment than during macrolide treatment. All second specimens containing macrolide-resistant mutations (group C) were collected after the end of macrolide treatment. In addition, no mutant clones of the 23S rRNA gene were detected in the corresponding initial specimens collected before the beginning of treatment. If macrolide-resistant mutants were present in individuals before the

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**Fig. 2.** *M. pneumoniae* load as quantified by real-time PCR in (a) initial specimens according to time after the onset of fever and (b) second specimens according to time after the initiation of antibiotic treatment. Open triangles indicate group A, with a culture-negative second specimen; open circles indicate group B, *M. pneumoniae* without macrolide-resistant mutations in the second specimen; filled circles indicate group C, *M. pneumoniae* with macrolide-resistant mutations in the second specimen.

**Fig. 3.** Emergence of *M. pneumoniae* harbouring a macrolide-resistant mutation in an index child and onset of symptoms in household contacts among family members. MS+, *M. pneumoniae* without macrolide-resistant mutations; MR+, *M. pneumoniae* with macrolide-resistant mutations; CAM, clarithromycin.
beginning of treatment as a minor population, selection for macrolide resistance by the prescribed medicine would progress more rapidly, and macrolide-resistant mutants would likely come to predominate during the period of macrolide treatment. Our observations that the second specimens collected during the period of macrolide treatment resulted in culture negativity or no mutant population suggest that *M. pneumoniae* in individuals can be eradicated by macrolide treatment or that the macrolide-resistant population had not yet developed.

Among the 6 specimens collected 7–24 days after the start of macrolide treatment (group C), the A2063G or A2064G mutants were found to compose 100% of the population in 5 specimens collected within 4 to 14 days of the end of treatment. The one remaining specimen from Patient 21, which was composed of 89% A2063G and 11% A2064G mutants, was collected 2 days after the 10 days of macrolide treatment had ended. The *M. pneumoniae* gene copy number in the second specimen of Patient 21 was the highest among the second specimens of group C, and this patient also had a mild fever that persisted for 9 days. Therefore, the macrolide-resistant population may have already developed in Patient 21 during the 10 days of treatment. Two previous reports have described the acquisition of macrolide resistance using serial specimens; the molecular typing method was used to confirm that the *M. pneumoniae* strains present before and after treatment were identical [17, 19]. Mixed populations of wild-type and mutant *M. pneumoniae* were detected in both specimens after macrolide treatment; one was collected on day 55 after the beginning of treatment from an immunocompromised child [17], and another was collected 16 days after the end of azithromycin treatment from a boy with severe co-morbidities [19]. Our study reveals the possibility that otherwise healthy children infected with *M. pneumoniae* without macrolide-resistant mutations can carry a macrolide-resistant population for up to 24 days after the beginning of macrolide treatment or for 14 days after the end of macrolide treatment.

Among the 14 specimens collected after more than 7 days after the start of macrolide treatment, 8 specimens were culture-negative (group A), and 6 specimens possessed a 100% macrolide-resistant population (group C). The medication for patients in group A, including prescribed antibiotics, dosage, and medication days, was similar to those for patients in group C. In addition, there was no significant difference between group A and group C with respect to the *M. pneumoniae* gene copy number in initial specimens, the period from the initiation of antibiotic treatment and the second specimen collection, or the demographic characteristics of patients. Therefore, we cannot explain why the macrolide-resistant population developed after the end of macrolide treatment only in specimens from group C. *In vitro* data suggest that resistant strains of *M. pneumoniae* can be induced by sub-inhibitory concentrations of macrolides [3, 11–13]. Indeed, the development of macrolide resistance in patient 17 is likely to be the result of treatment with sub-inhibitory concentrations due to the vomiting that occurred whenever the patient took the medicine, as previously reported [18]. However, the remaining 5 patients in group C were treated with the proper dosage and course of macrolides. It may be impossible to prevent a macrolide-resistant population from developing within individual patients after the end of macrolide treatment, even when macrolides are prescribed and administered with an appropriate dosage and duration. However, we cannot conclude that acquisition of resistance after macrolide administration is associated with macrolide treatment failure, as macrolide therapy was effective for reducing fever within 1–2 days and for decreasing the *M. pneumoniae* load. A previous report showed that the median time for carriage of *M. pneumoniae* DNA in the throat was 7 weeks after disease onset, irrespective of adequate antibiotic treatment [28]. The macrolide-resistant population may develop due to drug selection pressure on *M. pneumoniae*, which may not be eradicated completely. Future work using more sensitive methods such as deep sequencing, which can detect extremely minor populations, will more precisely resolve the course of drug selection during macrolide treatment.

In the present study, the absence of a macrolide-resistant population in initial specimens from family contacts indicates that transmission of *M. pneumoniae* to family members occurred before the macrolide-resistant population developed within the index patient. As macrolide treatment certainly reduced *M. pneumoniae* loads in infected patients, transmission to family members may have occurred soon after symptom onset in the index patient. *M. pneumoniae* can be transmitted through aerosols from person to person. In view of the close contact needed for droplet transmission and the slow (6 h) generation time of *M. pneumoniae*, 1 to 3 weeks of incubation time is typical [1]. A previous study reported that *M. pneumoniae* infections spread slowly in the family setting, with a median case-to-case interval of 23 days [29]. Such incubation periods are comparable to our case, which showed a symptom onset interval of 24–25 days. Even if close contact among family members occurs after the end of macrolide treatment for the index patient, the smaller number of *M. pneumoniae* organisms harbouring macrolide-resistant mutations compared with the initial load may weaken transmission. In addition to bacterial loads, cough surely facilitates the spread of *M. pneumoniae* from person to person through aerosols. Indeed, the fever decreased within 1 to 2 days in most participants who received macrolide treatment. However, cough tended to be persistent even after the end of macrolide treatment. Although the macrolide-resistant population developed in individual patients, continued administration of antitussive drugs after macrolide treatment could be effective for reducing the transmission of *M. pneumoniae* from person to person.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study was approved by the ethics committees of the Yamagata University Faculty of Medicine (H26–35) and the Yamagata Prefectural Institute of Public Health (YPIPHEC H26–02).

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