Introduction

Macrolides are oral antibiotics commonly used to treat respiratory infections. The increased use of macrolides has been accompanied by increased prevalence of macrolide-resistant strains of staphylococci, streptococci, pneumococci and enterococci. The mechanism underlying the development of resistance to macrolides has been investigated in detail with Staphylococcus spp. Resistant strains have been classified into constitutively resistant and inducibly resistant as a result of methylation of 23S rRNA. Another mechanism of resistance, involving efflux, has been reported recently. The efflux-type resistance to macrolides is encoded by msrA, and strains with this gene are resistant to 14-membered macrolides, but susceptible to rokitamycin, a 16-membered macrolide.

The incidence of erythromycin-resistant strains among Streptococcus pneumoniae isolates from patients with respiratory infection was approximately 10% until the early 1980s, but since the late 1980s erythromycin-resistant strains have been increasing, to >30% in some cases. These erythromycin-resistant S. pneumoniae are known to include strains susceptible to rokitamycin.

Recently, the so-called M phenotype of S. pneumoniae was identified. Isolates with this phenotype are resistant to 14- and 15-membered macrolides but susceptible to lincosamide and streptogramin B. They are characterized by the presence of the msrE gene, which is thought to encode an efflux pump. Various other macrolide-resistance genes have been investigated by the PCR method.

At the Seventh International Congress for Infectious Diseases in 1996, Fujii et al. reported the results of susceptibility tests to various macrolides in S. pneumoniae...
strains isolated and identified at Yamanashi Red Cross Hospital between February and October 1995. They reported that erythromycin-resistant strains of *S. pneumoniae* included strains with inducible resistance and those with non-inducible resistance to 14-membered macrolides.

We selected erythromycin-resistant strains from *S. pneumoniae* isolates at the hospital. These erythromycin-resistant strains were tested for susceptibility to macrolides and clindamycin, subjected to resistance induction testing by a disc diffusion method, and analysed for genes responsible for macrolide resistance, in order to investigate the relationship between these genes and resistance.

### Materials and methods

#### Test strains

Sixty-two erythromycin-resistant *S. pneumoniae* isolates, identified at the Department of Pediatrics, Yamanashi Red Cross Hospital in 1995, were used.

#### Antimicrobial drugs

The following eight antimicrobial drugs with known potency were used: penicillin G, erythromycin, clarithromycin, roxithromycin, oleandomycin, rokitamycin, josamycin and clindamycin.

#### Sensitivity tests

The isolates were cultured on trypto-soya agar (Nissui, Tokyo, Japan) supplemented with 5% defibrinated equine blood at 37°C for 18–20 h. The colony was harvested and then used to prepare a 1 × 10^6 cfu/mL bacterial suspension in 0.9% NaCl.

MICs were determined by an agar dilution method in accordance with the standard method specified by the Japan Society of Chemotherapy. Susceptibility disc medium-N (SDA, Nissui) containing 5% defibrinated equine blood was used.

A series of two-fold agar dilutions (0.0063–100 mg/L) of each antimicrobial drug was prepared, and the bacterial suspension was inoculated by using a microinoculator (Sakuma Co. Ltd, Tokyo, Japan). After overnight culture at 37°C, the lowest concentration of the drug in which bacterial growth was not observed was taken to be the MIC.

#### Resistance induction test (disc method)

Fifty microlitres of the bacterial suspension (1 × 10^6 cfu/mL) was applied to tryptone soya agar containing 5% defibrinated equine blood. A thin paper disc, 8 mm in diameter (Advantec, 49005020), containing 20 μL of a 20 mg/L or 100 mg/L solution of each macrolide was prepared. A rokitamycin disc was placed at the centre of the plate, with discs containing erythromycin, clarithromycin and oleandomycin placed 15–20 mm away. The plate was incubated overnight at 37°C.

If a zone of inhibition was detected around the rokitamycin disc, the isolate was considered to have non-inducible resistance to macrolides. If there was a defect in the zone of inhibition around the rokitamycin disc, as a result of the 14-membered macrolides, the isolate was considered to have macrolide-induced resistance.

### Preparation of probe for hybridization

*S. pneumoniae* strain NAS172, a representative strain with inducible resistance to macrolides, and strain NAS208, which has non-inducible resistance, were cultured on 2 mL of brain heart infusion medium containing 10% equine serum at 37°C for 6 h. The resulting colonies were harvested, and suspended in 500 μL of TESS buffer (30 mM Tris–HCl pH 7.5, 5 mM EDTA, 50 mM NaCl and 25% sucrose). This bacterial suspension was treated with phenol–chloroform to extract the genomic DNA, which was then precipitated using ethanol.

The DNA derived from *S. pneumoniae* strain NAS172 was amplified with the *ermB* primer (shown below) by PCR. The 550 bp fragment was integrated into pT7 vector, creating permB550. In the same way, the DNA from *S. pneumoniae* strain NAS208 was amplified with the *mefE* primer and the 1.5 kb fragment was integrated into pT7 to produce pemefE1.5. For PCR, 10 μL DNA solution, 2.5 U Taq polymerase (TaKaRa, Tokyo, Japan), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM NTP and 0.5 μM forward and reverse primer were mixed in a final volume of 100 μL. PCR comprised 30 cycles each of 94°C for 60 s, 50°C for 30 s, and 74°C for 30 s.

Primer sequences were as follows:

- **mefE forward**: 5'-ATG-GAA-AAA-TAC-AAC-AAT-TGG-AAA-CGA-3'
- **mefE reverse**: 5'-TTA-ATT-TAA-ATC-TAA-TTT-TCT-AAC-CTC-3'
- **ermB forward**: 5'-GAA-AAG-GTA-CTC-AAC-CAA-ATA-3'
- **ermB reverse**: 5'-ATG-GGT-AGT-ATT-GTT-TAC-3'

permB550 and pemefE1.5 were transformed into *E. coli* HB101, and each plasmid was isolated and purified. In the resultant permB550, the insert region was cut with *EcoRI* and *PstI*, while in pemefE1.5, the insert region was cut with *SpeI*. The resulting fragments were purified with agarose gel electrophoresis. These fragments (100 ng of DNA) derived from the insert regions were labelled using a digoxigenin random primer labelling kit (Boehringer–Mannheim, Tokyo, Japan). To these probes, 5 × SSC, 50% formamide, 0.5% SDS and 2% blocking reagent (Boehringer–Mannheim) were added to make hybridization fluid.
Hybridization
A loop of culture of the erythromycin-resistant isolates of *S. pneumoniae* incubated on agar containing 5% equine serum was transferred to nylon membrane (Hybond-N; Amersham plc, Amersham, UK). The membrane was processed by denaturation (in 1.5 M NaCl, 0.5 N NaOH), neutralization (in 1 M Tris–HCl pH 7.4, 1.5 M NaCl) and washing (in 3 × SSC), and then baked at 80°C for 2 h. It was then prehybridized at 42°C for 2 h in hybridization fluid without probe, and then hybridized at 42°C for 16 h in hybridization fluid with probe. The membrane filter was washed with 2 × SSC and 0.1% SDS at room temperature for 5 min twice, and with 0.1 × SSC and 0.1% SDS at 68°C for 1 h twice.

Hybridization was detected with a digoxygenin detection kit (Boehringer–Mannheim) by the usual method. Exposure time was 12 h at room temperature.

Results

**Determination of MIC**
The susceptibility of 62 erythromycin-resistant strains of *S. pneumoniae* and four representative macrolide-sensitive strains of *S. pneumoniae* to various macrolides and penicillin G is shown in Table I.

The MICs of erythromycin and clarithromycin (14-membered macrolides) showed two peaks, being distributed between 0.2 and 6.25 mg/L and >25 mg/L. The MIC of roxithromycin and oleandomycin (14-membered macrolides) also showed two peaks, at 0.39–50 mg/L and 100 mg/L.

Rokitamycin (a 16-membered macrolide) had a wide range of MICs, from <0.05 mg/L to 100 mg/L. Of the macrolides tested, rokitamycin had the greatest antimicrobial activity against erythromycin-resistant strains.

Clindamycin MICs were >100 mg/L for half of the isolates tested.

**The inhibitory zone of macrolides and distribution of drug-resistance genes**
The typical ‘D’-shaped zone of inhibition seen around the rokitamycin disc with strains with inducible resistance is shown in Figure 1a. In such cases there is a defect in the zone of inhibition of rokitamycin, caused by resistance to 14-membered macrolides (erythromycin, oleandomycin). Strains with non-inducible resistance to macrolides typically showed a non-'D'-shaped zone of inhibition around the rokitamycin disc, as shown in Figure 1b.

Table I shows the classification of resistance patterns of the 62 erythromycin-resistant strains of *S. pneumoniae*. Thirty-seven (59.7%) of these 62 strains were classified as having inducible resistance, and 25 (40.3%) as having non-inducible resistance; the ratio of inducible to non-inducible resistance was thus 3:2.

All of the 62 erythromycin-resistant strains of *S. pneumoniae* contained *ermB* or *mefE* or both (Table II). The *mefE* gene alone was found in 25 strains (40.3%), *ermB* alone in 27 strains (43.5%), and both *mefE* and *ermB* in ten strains (16.1%). Those strains with inducible resistance (as judged by the disc diffusion method) were found to have *ermB* or *ermB + mefE*, while all strains with non-inducible resistance had only *mefE*. In macrolide-sensitive strains, neither *ermB* nor *mefE* was detected.

*Is there any connection between presence of macrolide-resistance genes and resistance to penicillin G?*
According to NCCLS criteria, six of the 62 (9.7%) macrolide-resistant strains of *S. pneumoniae* were classified as penicillin-susceptible (PSSP; MIC < 0.05 mg/L), 39 (62.9%) as penicillin-intermediately susceptible (PISP; MIC = 0.1–1 mg/L) and 17 (27.4%) as penicillin-resistant (PRSP; MIC < 1 mg/L). Thus PISP strains predominated.

No correlation was seen between the presence of the

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**Figure 1.** Appearance of the zone of inhibition around rokitamycin (RKM) discs in the presence of the macrolides erythromycin (EM) and clarithromycin (CAM) on plates of *S. pneumoniae* strains NAS208 (a) and NAS185 (b).
Table 1. MIC distribution of macrolides and penicillin G for erythromycin-resistant clinical isolates of *S. pneumoniae*

<table>
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<th>Antimicrobial</th>
<th>ES(^a)</th>
<th>(\leq 0.05)</th>
<th>0.1</th>
<th>0.39</th>
<th>0.78</th>
<th>1.56</th>
<th>3.13</th>
<th>6.25</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>(&gt;100)</th>
<th>MIC (mg/L)</th>
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<td></td>
<td>25</td>
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<td>7</td>
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<tr>
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<td>10</td>
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<td>12</td>
<td>9</td>
<td>7</td>
<td>8</td>
<td>1</td>
<td></td>
<td></td>
<td>&gt;100</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) ES, erythromycin sensitivity; S, typical strains sensitive to erythromycin (\(n = 4\)); R, resistant (\(n = 62\)).
mefE and ermB genes in macrolide-resistant S. pneumoniae

Table II. Relationship between presence of macrolide-resistance genes and the phenotype of erythromycin-resistant S. pneumoniae isolates

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>number of strains</th>
<th>mefE</th>
<th>ermB</th>
<th>mefE + ermB</th>
<th>total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inducible</td>
<td>0</td>
<td>25</td>
<td>10</td>
<td></td>
<td>37 (59.7)</td>
</tr>
<tr>
<td>Non-inducible</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td></td>
<td>25 (40.3)</td>
</tr>
<tr>
<td>Total (%)</td>
<td>25 (40.3)</td>
<td>27 (43.5)</td>
<td>10 (16.1)</td>
<td>62 (100)</td>
<td></td>
</tr>
</tbody>
</table>

Table III. Presence of macrolide-resistance genes and penicillin G susceptibility of S. pneumoniae

<table>
<thead>
<tr>
<th>Group</th>
<th>number of strains</th>
<th>mefE</th>
<th>ermB</th>
<th>mefE + ermB</th>
<th>total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSSP</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td></td>
<td>6 (9.7)</td>
</tr>
<tr>
<td>PISP</td>
<td>15</td>
<td>17</td>
<td>7</td>
<td></td>
<td>39 (62.9)</td>
</tr>
<tr>
<td>PRSP</td>
<td>8</td>
<td>7</td>
<td>2</td>
<td></td>
<td>17 (27.4)</td>
</tr>
</tbody>
</table>

ermB and/or mefE gene and resistance to penicillin G (Table III).

Identification of the genes for resistance to macrolides and susceptibility to various macrolides

The relationship between the identification of the genes in organisms and the MICs of various macrolides against the organisms is shown in Figure 2.

ermB type. Strains with this gene showed a wide range of MICs (0.78–100 mg/L) of 14-membered macrolides, except oleandomycin. The MICs of oleandomycin for strains with this gene were all ≥50 mg/L, which was obviously different from the MICs for strains with mefE. The MICs of rokitamycin, a 16-membered macrolide, were 0.39–>100 mg/L, while the MICs of josamycin were 1.56–>100 mg/L. The MIC range of clindamycin was 0.1–>100 mg/L, the MIC being ≥100 mg/L for nearly half of the strains.

ermB + mefE type. Patterns of MICs of macrolides for strains with ermB + mefE were generally the same as those for ermB, showing a wide range and frequently being >100 mg/L for 14-membered macrolides. The exceptions to this were oleandomycin and clindamycin, MICs of which were >100 mg/L for all strains with ermB + mefE.

mefE type. Against strains with only the mefE gene, the MICs of 14-membered macrolides (erythromycin, clarithromycin, roxithromycin and oleandomycin) ranged from 0.2 to 12.5 mg/L, indicating an intermediate level of resistance. The MICs of 16-membered macrolides against these strains were 0.05–0.2 mg/L for rokitamycin, and 0.1–1.56 mg/L for josamycin, showing susceptibility. Against these strains, the MICs of clindamycin and penicillin G were 0.025–0.39 mg/L and 0.05–6.25 mg/L, respectively.

Discussion

Macrolides are frequently used for treating infant respiratory infection because of their safety. S. pneumoniae, often isolated from patients with respiratory infection, has recently attracted attention because of its increasing resistance to penicillin. Macrolide-resistant strains have been reported to account for approximately 50% of clinical isolates of S. pneumoniae. Two types of mechanism of macrolide resistance have been noted in staphylococci: (i) inducible resistance associated with methylation of 23S rRNA by a methylation enzyme, and (ii) resistance associated with an efflux pump, whereby macrolides are pumped out of strains.

In the present study, 62 clinical isolates of erythromycin-resistant S. pneumoniae were examined for the genes (ermB and mefE) associated with macrolide resistance. Some isolates had just the ermB gene, some just the mefE gene, and some had both. Strains with ermB accounted for 43.5% of all macrolide-resistant strains, and most of these were resistant to 14-membered macrolides. However, against these strains, the MICs of rokitamycin, a 16-membered macrolide, ranged widely, from 0.39 to >100 mg/L.
Strains with *ermB*+*mefE* accounted for 16.1% of all macrolide-resistant strains, the smallest in percentage. These strains had the same distribution of susceptibility to each antibiotic as strains with *ermB* alone, which suggests that *ermB* is predominantly expressed over *mefE*. These strains with both *ermB* and *mefE* were highly resistant to oleandomycin and clindamycin, with MICs of these antibiotics being >100 mg/L. Testing resistance induction with the disc diffusion method confirmed that macrolides induced resistance to macrolides.

Strains with *mefE* accounted for 40.3% of erythromycin-resistant strains, showing an intermediate level of resistance to 14-membered macrolides. However, these types of strains were susceptible to rokitamycin and josamycin, which are 16-membered macrolides, and to clindamycin. Since resistance develops by the mechanism of efflux in

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**Figure 2.** Correlation of MICs (mg/L) of macrolides or penicillin G and the presence of macrolide-resistance genes (*mefE* alone, ■; *ermB* alone, □; or both *ermB* and *mefE*, ▼) for 62 erythromycin-resistant isolates of *S. pneumoniae*. 
these strains, 16-membered macrolides are probably more rarely excreted than 14-membered macrolides. Therefore, 16-membered macrolides may be useful clinically in the treatment of disease caused by this type of strain.

The resistance induction test with the disc diffusion method showed that macrolides did not induce resistance in strains with \textit{mefE}. Strains with \textit{mefE} had an intermediate level of resistance to oleandomycin, which is clearly different from strains with \textit{ermB}.

Strains with \textit{ermB} can be distinguished from those with \textit{mefE} using the disc diffusion method with rokitamycin and erythromycin discs, and by the testing susceptibility to oleandomycin. Because of their strong antimicrobial activity, 16-membered macrolides, especially rokitamycin, are most useful against strains with \textit{mefE}, which have non-inducible resistance.

References


Received 8 June 1998; returned 6 October 1998; revised 24 November 1998; accepted 21 December 1998