

Phenotypic Differentiation of Macrolide Resistance among *Streptococcus pneumoniae* Carrying *mefA* and/or *ermB* Genes

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We examined whether 55 isolates of erythromycin-resistant *Streptococcus pneumoniae* ($MIC \geq 1 \mu\text{g/mL}$) carrying *mefA* and/or *ermB* genes would develop resistance to telithromycin. Fifteen isolates, carrying only the *mefA* gene, had the M resistance phenotype by their susceptibility pattern and their susceptibility to rokitamycin did not change after exposure to $0.1 \mu\text{g/mL}$ of erythromycin. Of the other 40 isolates, 25 carried the *ermB* gene and 15 carried the both *mefA* and *ermB* genes, all of them showed high resistance to clindamycin ($MIC \geq 128 \mu\text{g/mL}$) and were resistant to macrolides, lincosamides and streptogramin B (MLS_B resistance phenotype). Twenty six isolates with the MLS_B resistance phenotype showed decreased sensitivity to telithromycin after exposure to erythromycin, although the elevated MICs of telithromycin remained below $1 \mu\text{g/mL}$, the remaining 14 isolates did not show an obvious decrease of their sensitivity to telithromycin (only two-fold dilution). However, by adding the telithromycin disk to the erythromycin-rokitamycin double disk diffusion test, the zone of inhibition around the telithromycin disk was blunted proximal to the erythromycin disk in all isolates with the MLS_B resistance phenotype including the 25 isolates which were considered to have a true cMLS_B phenotype because of their constitutive resistance to rokitamycin ($MIC \geq 4 \mu\text{g/mL}$). Furthermore, in 16 isolates, highly resistance to rokitamycin ($MIC \geq 64 \mu\text{g/mL}$), the zone of inhibition around the telithromycin disk was blunted to both erythromycin and rokitamycin disks. These results indicate that the expression of telithromycin resistance was induced even in *S. pneumoniae* isolates with a true cMLS_B resistance phenotype.

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Introduction

Streptococcus pneumoniae is a major causative pathogen of lobar pneumonia, it also causes numerous other serious acute pyogenic disorders such as septicemia, meningitis, and peritonitis, respectively. This microorganism has recently attracted attention because of its resistance to penicillin and other antimicrobial agents. The incidence of macrolide-resistance has risen to approximately 50% among clinical isolates of *S. pneumoniae*.¹⁻⁵

Erythromycin-resistant *S. pneumoniae* has been divided into two phenotypes based on their patterns of susceptibility to macrolides, lincosamide, and streptogramin B (MLS); the M phenotype, due to an active efflux pump encoded by the *mefA* gene and characterized by resistance to 14- and 15-membered macrolides but susceptibility to 16-membered macrolides, lincosamides, and streptogramin B,^{6,8}

and the MLS_B phenotype which shows a ribosomal modification due to adenine-dimethylase encoded by the *ermB* gene and is characterized by cross-resistance to all macrolides, lincosamides, and streptogramin B.⁹

The expression of M resistance is constitutive, while that of MLS_B resistance may be inducible (iMLS_B) or constitutive (cMLS_B). In general, clindamycin is used to discriminate between the iMLS_B and cMLS_B phenotypes as an indicator, compared to 14- and 15-membered macrolides.¹⁰⁻¹² However, although this is possible for staphylococci and streptococci, discrimination between iMLS_B and cMLS_B phenotypes is still unclear in the case of *S. pneumoniae*. Only recently, Montanari et al. tried to discriminate the inducible macrolides but constitutive lincosamides, and streptograminB (iMcLS_B) resistance from true cMLS_B resistance using the triple-disk diffusion test, set up by adding a rokitamycin disk, which is considered to be a weak

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inducer, to the erythromycin-clindamycin double disk diffusion test. However, they also concluded that discrimination between inducible and constitutive MLS_B resistance is far more uncertain and is strongly affected by the inducibility of resistance by the test antibiotic.¹³

Telithromycin (ketolide) is a new class of semi-synthetic 14-membered macrolide that does not induce resistance.¹⁴ As many previous investigators reported, no telithromycin-resistant isolate has been isolated among *S. pneumoniae* with the cMLS_B phenotype, while staphylococci or *Streptococcus pyogenes* with the cMLS_B phenotype becomes resistant even to this drug.^{5,11,12,14-16}

In this study, we tried to investigate whether telithromycin resistance could be induced in 55 clinical isolates of erythromycin-resistant *S. pneumoniae*, especially in isolate with the MLS_B phenotype.

Materials and Methods

Bacterial strains

Fifty-five clinical isolates of erythromycin-resistant *S. pneumoniae*, isolated from the sputum of patients with lower respiratory tract infections from 1998 to 2000, were used. All isolates were identified by their susceptibility to optochin and bile solubility test¹⁷ and by PCR amplification of the *lyt A* gene.¹⁸ *S. pneumoniae* ATCC 6305 was used as the quality control strain.

Antibiotics

Reference samples of antimicrobial agents of known potency were kindly supplied as powder. Erythromycin was from Shionogi Pharmaceutical (Osaka, Japan); clarithromycin was from Taisho Pharmaceutical (Tokyo, Japan); azithromycin was from Pfizer Laboratories (Groton, CT, USA); rokitamycin was from Asahi Kasei (Tokyo); telithromycin was from Nippon Hoechst Marion Roussel (Tokyo); and clindamycin was from Upjohn (Tokyo).

Determination of MICs

All isolates were tested for their susceptibility against the 6 antibiotics described above at concentrations between ≤ 0.015 and 128 $\mu\text{g/mL}$. MICs were determined by the two-fold agar dilution method in Sensitivity Test Agar (Mueller-Hinton agar medium; Eiken Chemicals, Tokyo) with 8% Strepto Haemo supplement (SHS; Eiken Chemicals). The bacteria were grown overnight in Sensitivity Test broth (Eiken Chemicals) with supplemented 8% SHS at 35°C. The culture was diluted to a final concentration of 10⁶ CFU/mL with buffered saline containing gelatin (BSG). The bacterial suspensions were delivered by an inoculator (Sakuma Seisaku, Tokyo) with an inoculum size of 5×10⁴ CFU/spot on agar plates containing the test drug at various concentrations. The plates were then incubated for 18 h at 35°C. The MIC was defined as the lowest concentration of the compound that prevented visible growth. MIC break points suggested by the National Committee of Clinical Laboratory Standards (NCCLS)¹⁹ were ≤ 0.25 $\mu\text{g/mL}$ (susceptible), 0.5 $\mu\text{g/mL}$

(intermediate), ≥ 1 $\mu\text{g/mL}$ (resistant) for erythromycin, clindamycin and clarithromycin; and ≤ 0.5 $\mu\text{g/mL}$ (susceptible), 1 $\mu\text{g/mL}$ (intermediate), ≥ 2 $\mu\text{g/mL}$ (resistant) for azithromycin; those suggested by the French Society for Microbiology²⁰ for rokitamycin were ≤ 1 $\mu\text{g/mL}$ (susceptible), 2 $\mu\text{g/mL}$ (intermediate), ≥ 4 $\mu\text{g/mL}$ (resistant). In the absence of an established standard, the MIC break points for telithromycin were ≤ 1 $\mu\text{g/mL}$ (susceptible), 2 $\mu\text{g/mL}$ (intermediate), ≥ 4 $\mu\text{g/mL}$ (resistant).

DNA isolation and PCR experiments

DNA was obtained as previously reported.²¹ The presence of macrolide resistance genes was investigated by PCR using a commercially available PCR kit (Gene Amp PCR Reaction kit with Ampli TaqDNA Polymerase, Takara Kyoto, Japan) and a DNA Thermal Cycler PH2000 (Perkin-Elmer Cetus Instruments, Emeryville, CA). Primer sets were 5'-GAAAAGGTACTCAACCAAATA-3' and 5'-AGTAACGGTACTTAAATTGTTTAC-3' for *ermB*, and 5'-AGTATCATTAATCACTAGTGC-3' and 5'-TTCTTCTGGTACTAAAA GTGG-3' for *meA* based on a previously published report.²¹ The expected PCR products size was 639 bp for the *ermB* gene and 348 bp for the *meA* gene. The reaction mixture contained 72.5 μL of H₂O, 10 μL of 10×buffer, 2 μL each of 10 mM dATP, dCTP, dGTP, and dTTP, 0.5 μL of Taq polymerase (5 units/ μL), 1 μL each of primers (20 μM), and 10 μL of template DNA. The PCR involved 35 cycles of denaturation at 94°C for 60 sec, annealing at 52°C for 60 sec, and elongation at 72°C for 60 sec, followed by heating at 72°C for 7 min. Five microliters of the PCR product were subjected to electrophoresis on 1.2% agarose gels to identify the amplified DNA fragment.

Induction of macrolide resistance

1) Disk diffusion method

Two milliliters of the bacterial suspension (containing approximately 10⁸ cfu/mL) were transferred onto 10 mL of Sensitivity Test agar containing 8% SHS and spread onto the surface. Excess bacterial suspension was removed and then paper disks (8 mm diameter; "thick"; Tokyo Roshi Kaisha, Tokyo) containing erythromycin or rokitamycin at the dose of 20 $\mu\text{g/disk}$, or telithromycin at the dose of 5 $\mu\text{g/disk}$ were placed on the surface of the agar plate. The plates were incubated overnight at 35°C. The induction of telithromycin resistance was assessed based on the shape of the zone of inhibition around the telithromycin disk proximal to either erythromycin or rokitamycin disk.

2) Agar dilution method

Induction of macrolide resistance was evaluated by comparing the MICs of telithromycin in the presence and absence of subinhibitory concentration (0.1 $\mu\text{g/mL}$) of erythromycin as the inducer. When determining the MIC of telithromycin, we prepared two sensitivity agar plates: one contained only telithromycin and the other contained telithromycin plus erythromycin (0.1 $\mu\text{g/mL}$). The MIC was determined in accordance with the susceptibility test.

3) Broth culture method

Two isolates of *S. pneumoniae*, the iMLS_B phenotype (KU 3898) and cMLS_B phenotype (KU 3902), were cultured in Sensitivity Test broth containing 8% SHS to the log growth phase (OD₆₀₀ ± 0.3). Erythromycin, rokitamycin, and telithromycin were added to the broth to final concentrations of 0.1 and 1 µg/mL to induce telithromycin resistance. After incubation at 35°C for 2 h, 0.25 mL of each culture was transferred to L-tubes containing 4.75 mL of Sensitivity Test broth supplemented 8% SHS, to have 5 mL of the cell-drug mixtures which contained 0.06 µg/mL of telithromycin (MICs of telithromycin against these two isolates). These cell suspensions were shake-cultured at 35°C. The OD of the culture was measured at 600 nm after 0, 1, 2, 4, 6, and 8 h of culture in a spectrophotometer (SPECTRONIC 20, Milton Roy Japan, Tokyo). The experiments were performed independently in triplicate.

Results

PCR identification of macrolide resistance gene in clinical isolates of *S. pneumoniae*

The genes encoding macrolide resistance were subjected to PCR amplification and agarose gel electrophoresis. PCR amplified products were obtained from all 55 isolates. The *ermB* gene was identified in 40 of the 55 isolates (72.7%), and 25% of the isolates carried this gene in combination with *mefA* gene. The remaining 15 isolates (27.3%) carried the *mefA* gene only.

MIC values of macrolide antibiotics and clindamycin for clinical isolates of *S. pneumoniae*

Table 1 presents the MICs of antibiotics tested against *S. pneumoniae* isolates carried macrolide resistance genes. Fifteen isolates carried only *mefA* gene and were considered to have the M phenotype; that is low

level resistance to erythromycin, azithromycin, and clarythromycin but susceptible to the other three antibiotics. By contrast, the other 40 isolates carried the *ermB* gene or both the *ermB/mefA* genes had the MLS_B phenotype and showed marked resistance to clindamycin (MIC range was ≥ 128 µg/mL). The MICs of rokitamycin against these 40 isolates ranged from 0.5 to ≥ 128 µg/mL, and allowed further discrimination between 15 iMLS_B (MIC range was from 0.5 to 2 µg/mL) and 25 isolates with the cMLS_B (MIC range was from 4 to 128 µg/mL) phenotype. All 55 erythromycin-resistant isolates were inhibited with ≤ 0.25 µg/mL of telithromycin regardless of their resistance phenotype.

Effect of erythromycin against the MICs of telithromycin

Table 1 also presents the effect of subinhibitory concentration of erythromycin on the MICs of telithromycin tested by the agar dilution method. The MICs of telithromycin against all 15 isolates with the M phenotype carrying only the *mefA* gene showed no change regardless of the presence of erythromycin. While among the 40 isolates with the MLS_B phenotype, 26 isolates (8 iMLS_B and 18 true cMLS_B phenotype) showed a decreased of their susceptibility to telithromycin in the presence of erythromycin, although the MICs of telithromycin below 1 µg/mL. The other 14 isolates (7 iMLS_B and 7 true cMLS_B phenotype) did not show an obvious decreased of their susceptibility to telithromycin (only two-fold dilution).

Detection of induced resistance to telithromycin by the disk diffusion test

All isolates used in this study were tested for induction of telithromycin resistance by the disk diffusion method. As shown in Figure 1, M type inhibition was observed in all isolates carrying the *mefA* gene (Figure 1 A, D). By contrast, all of the isolates carrying the *ermB* gene showed antagonism between erythromycin and

Table 1 Susceptibility to macrolides of 55 clinical isolates of *S. pneumoniae* carrying macrolide genes

| Phenotype (Number of strains) | Antibiotic | MIC (µg/mL) | | | |
|---------------------------------------|-------------------------------|-------------------------------|-------------------|-------------------|------|
| | | MIC range | MIC ₅₀ | MIC ₉₀ | |
| M resistance (n=15) | Erythromycin | 1~4 | 2 | 4 | |
| | Clarithromycin | 1~2 | 1 | 2 | |
| | Azithromycin | 1~4 | 2 | 4 | |
| | Rokitamycin | 0.06~0.25 | 0.13 | 0.25 | |
| | Clindamycin | 0.03~0.25 | 0.06 | 0.13 | |
| | Telithromycin | ≤0.015~0.13 | 0.03 | 0.06 | |
| | Telithromycin ^{ind*} | ≤0.015~0.13 | 0.03 | 0.06 | |
| MLS _B resistance (n=40) | iMLS _B (n=15) | Erythromycin | 8~>128 | 128 | >128 |
| | | Clarithromycin | 4~>128 | >128 | >128 |
| | | Azithromycin | 32~>128 | >128 | >128 |
| | | Rokitamycin | 0.5~2 | 1 | 2 |
| | | Clindamycin | 128~>128 | >128 | >128 |
| | | Telithromycin | ≤0.015~0.13 | 0.03 | 0.06 |
| | | Telithromycin ^{ind*} | 0.06~0.5 | 0.25 | 0.25 |
| | cMLS _B (n=25) | Erythromycin | 64~>128 | >128 | >128 |
| | | Clarithromycin | 64~>128 | >128 | >128 |
| | | Azithromycin | >128 | >128 | >128 |
| | | Rokitamycin | 16~>128 | >128 | >128 |
| | | Clindamycin | >128 | >128 | >128 |
| | | Telithromycin | ≤0.015~0.13 | 0.03 | 0.06 |
| | | Telithromycin ^{ind*} | 0.06~0.5 | 0.25 | 0.5 |

*Induction was performed by incubation with 0.1 µg/mL of erythromycin.

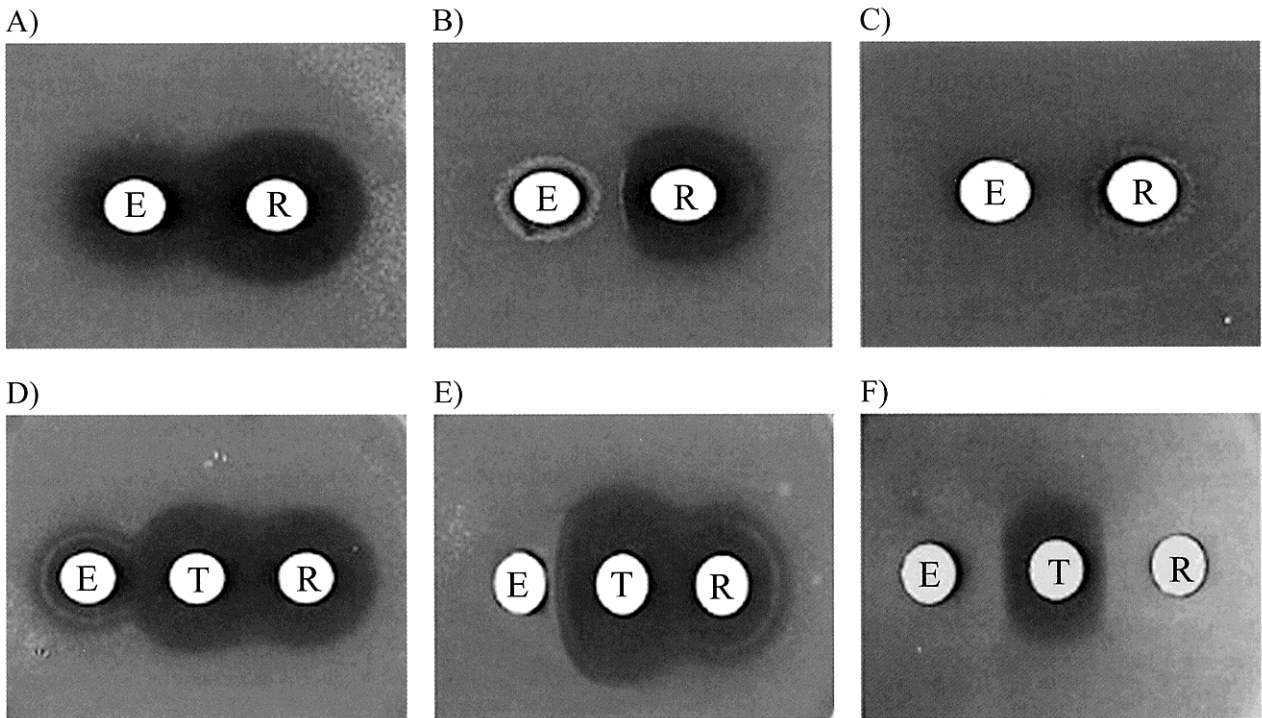


Figure 1. Phenotype expression of macrolide-resistant *S. pneumoniae* isolates determined by double and triple disk diffusion methods. In the double-disk method (A to C), erythromycin (20 μg) and rokitamycin (20 μg) placed on the right and the left side respectively. In the triple-disk method (D to F), telithromycin (5 μg) placed at the center on each panel, with erythromycin on the right and rokitamycin on the left. A) and D), M phenotype; B), C) and E), F), MLS_B phenotype. E, erythromycin; T, telithromycin; R, rokitamycin.

telithromycin (Figure 1 B), and erythromycin, rokitamycin, and telithromycin (Figure 1 E, F). Furthermore, the 16 isolates which showed high-level resistance to rokitamycin ($\text{MIC} \geq 64 \mu\text{g/mL}$) also showed antagonism between rokitamycin and telithromycin (Figure 1 F). In the absence of telithromycin, the induced resistance of these isolates could not be detected because of the absence of an inhibition zone around both the erythromycin and rokitamycin disks (Figure 1 C).

Induction of macrolide resistance as assessed by the broth method

We investigated the possibility of erythromycin, rokitamycin and telithromycin to induce resistance against telithromycin in *S. pneumoniae* KU 3898 which has an iMLS_B phenotype and susceptible to rokitamycin ($\text{MIC}=1 \mu\text{g/mL}$). Pre-exposure to 0.01 and 0.1 $\mu\text{g/mL}$ of rokitamycin and telithromycin did not induce elevated resistance to telithromycin, while the bacterial calls preexposed to erythromycin at the dose of 0.1 and 1 $\mu\text{g/mL}$ clearly showed resistance to telithromycin (Figure 2 A). We also investigated the possibility of these 3 drugs to induce resistance against telithromycin in *S. pneumoniae* KU 3902 which has a true cMLS_B phenotype and shows high resistance to rokitamycin ($\text{MIC} \geq 128 \mu\text{g/mL}$). Both erythromycin and rokitamycin but not telithromycin induce resistance to telithromycin in KU 3902 (Figure 2 B). These findings strongly suggested that telithromycin has no activity to induce macrolide resistance in *S. pneumoniae* isolate with the MLS_B phenotype.

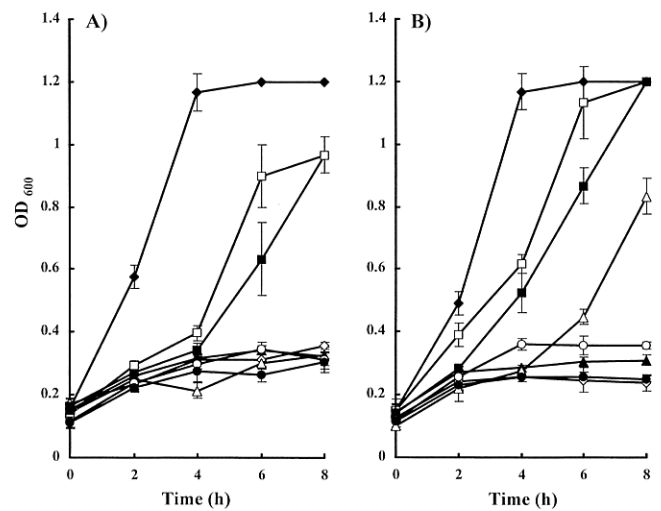


Figure 2. Kinetics of induced telithromycin resistance in A) KU3898 (iMLS_B phenotype) and B) KU3902 (true cMLS_B phenotype). Resistance was induced by pre-exposure to the antibiotics at several concentrations as follows; erythromycin 0.1 (\square) and 1 $\mu\text{g/mL}$ (\blacksquare), rokitamycin 0.01 (\triangle) and 0.1 $\mu\text{g/mL}$ (\blacktriangle), telithromycin 0.01 (\circ) and 0.1 $\mu\text{g/mL}$ (\bullet) and without pre-exposure (\diamond). Growth curves of each isolates in drug-free broth were shown as control (\blacklozenge). Only erythromycin in the case of KU3898 induced telithromycin resistance. Each point and bar represent the mean value \pm SD of experiments done independently in triplicate.

Discussion

Two major mechanisms are related to resistance to macrolides in *S. pneumoniae*, similar to staphylococci and other streptococci, an active efflux pump encoded by *mefA* and ribosomal modification due to adenine-dimethylase encoded by *ermB*. Recently, two additional mechanisms were described; alterations in the L4 ribosomal protein and mutation in the 23S rRNA.^{22,23}

Among macrolide-resistant isolates of *S. pneumoniae*, a predominance of isolates carrying the *ermB* gene over the *mefA* gene was observed in this study (72.3%, 27.3%). We also found that 25% (10 of 40) of the isolates carrying the *ermB* gene also carried the *mefA* gene, consistent with the results of other investigations in Europe^{1,4,14} and Japan.²⁴

From the results MIC determination, the 15 isolates carrying only the *mefA* gene and the 40 isolates carrying the *ermB* gene or *ermB/mefA* genes had the MLS_B phenotype. No isolate resistant to telithromycin was found in this study.

Montanari et al. tried to discriminate between *S. pneumoniae* with the iMLS_B phenotype and those with the true cMLS_B phenotype. They suggested that the isolate showed high-level resistance to rokitamycin without induction could be of a true cMLS_B phenotype.¹³ According to this discrimination, 25 isolates with the true cMLS_B phenotype were discriminated from 15 isolates with the iMLS_B phenotype. However, they also concluded that discrimination between the iMLS_B and cMLS_B phenotypes was far more uncertain and it was strongly affected by the induction of resistance to the test antibiotic.¹³ Indeed, constitutive expression of the *ermB* gene in *S. pneumoniae* has not been clearly understood or explained due to the instability of the stem-loop structure in the regulatory region of the *ermB* gene.²⁵ While, constitutive expression of *erm* gene in Staphylococci²⁶ and other Streptococci²⁷ has been characterized with some alterations, such as tandem duplication or deletion, in the regulatory sequence.

As for the induction of telithromycin resistance, triple disk diffusion test, set up by adding a telithromycin-disk to erythromycin-rokitamycin double-disk diffusion test, revealed that all 40 isolates with the MLS_B phenotype including the 25 isolates with the true cMLS_B phenotype were induced their resistant to telithromycin with erythromycin, a good inducer. Interestingly, rokitamycin, which is thought to be a very weak inducer, induced telithromycin resistance in 16 isolates highly resistant to rokitamycin. This is the first study to investigate the induction of telithromycin resistance in *S. pneumoniae* with the MLS_B phenotype. However, telithromycin inhibited all 40 isolates at concentrations below 1 µg/mL even in their induced state. This phenomenon may reflect the strong affinity of this drug to methylated 23S rRNA.

Only telithromycin showed no induction of telithromycin resistance in any of the isolates in this study, regardless of their resistance phenotype. Now we plan to investigate whether telithromycin can induce resistance to itself in telithromycin-sensitive *S. pneumoniae* isolates.

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