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ORIGINAL ARTICLE

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In vitro activities of new ketolide, telithromycin, and eight other macrolide antibiotics against *Streptococcus pneumoniae* having *mefA* and *ermB* genes that mediate macrolide resistance

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Abstract The comparative in vitro activity of a new ketolide, telithromycin (TEL), and eight other macrolide-lincosamide antibiotics (MLS) against 215 strains, of *Streptococcus pneumoniae* including penicillin-resistant isolates (PRSP), was determined by the agar dilution method. These strains were isolated from patients with pneumonia, otitis media, and purulent meningitis between 1995 and 1997. Two genes, *mefA* and *ermB*, that encode MLS resistance in the strains were identified by polymerase chain reaction (PCR). Of the strains, 30.2% (n = 65) had the *mefA* gene, 37.7% (n = 81) had the *ermB* gene, and 1.4% (n = 3) had both resistant genes. The minimum inhibitory concentration (MIC_{90s}) of TEL and 16-membered ring MLS for strains having the *mefA* gene were 0.063–0.25 μg/ml, which were the same level as those for MLS-susceptible strains. On the other hand, the strains with the *mefA* gene showed low-level resistance to 14- and 15-membered ring MLS, with MIC_{90s} ranging from 1 to 4 μg/ml. Only the MIC_{90} of TEL at 0.5 μg/ml, for strains having the *ermB* gene was superior to that of the 14-, 15-, and 16-membered ring MLS (MIC_{90}, ≥64 μg/ml). TEL also showed excellent activity against PRSP having abnormal *pbp1a*, *pbp2x*, and *pbp2b* genes. Most strains having the *mefA* and *ermB* genes were serotyped to 3, 6, 14, 19, and 23. These results suggest that TEL may be a useful chemotherapeutic agent for respiratory tract infections caused by *S. pneumoniae*.

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Introduction

Penicillin-resistant *Streptococcus pneumoniae* (PRSP) are the cause of community-acquired infections worldwide.[1] PRSP are often resistant to 14- and 16-membered ring macrolide-lincosamide antibiotics (MLS).[2–4]

Recently, it has been reported that *S. pneumoniae* have a resistance mechanism[3,5–7] which differs from that of the high-level MLS resistance induced by 23S rRNA methylase encoded by the *ermB* gene.[8] Unlike the ribosomes in *S. pneumoniae* having the *ermB* gene, the ribosomes in the strains having the *mefA* gene are sensitive to erythromycin (EM). These strains are resistant to 14-membered-ring and azalide macrolides, but not to 16-membered ring MLS. The characteristic resistant gene, *mefA*, has been sequenced[9] and shown to be highly homologous to the *mefA* gene detected in *Streptococcus pyogenes*.[10] The function of the product encoded by the *mefA* gene is assumed to be the efflux pump system, because labeled EM accumulates in the cells upon the addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).[9] These findings indirectly suggest that the resistance mechanism mediated by the *mefA* gene also has the same function.

The antimicrobial activities of β-lactams, MLS, and new quinolones were studied for PRSP strains (n = 1283) collected in an epidemiological survey between 1993 and 1996[11] in Japan. In terms of EM susceptibility, *S. pneumoniae* were classified into three groups: (1) highly resistant strains, having a minimum inhibitory concentration (MIC) ≥32 μg/ml (28.9%); (ii) moderately resistant strains, having an MIC of 0.5–8 μg/ml (30.1%); and (iii) susceptible strains, having an MIC ≤0.25 μg/ml (42.0%).

Recently, a new class of macrolides with 14-membered lactone rings, the ketolide antibiotics, which are derivatives of erythromycin A characterized by a 3-keto function instead of the cladinose moiety, has been shown to be active

against most EM-resistant gram-positive organisms.[12] TEL, one of the most active ketolides, shows activity against streptococci and pneumococci that are cross-resistant to EM, spiramycin, and clindamycin.[13,14] This activity was attributed to lack of induction of resistance to MLS by this compound.[15]

Here, we describe the relationships between the results of polymerase chain reaction (PCR) identification of the *ermB* and *mefA* genes and the susceptibilities of TEL and eight other MLS against *S. pneumoniae*, including PRSP.

Materials and methods

Bacterial strains

S. pneumoniae clinical isolates (215 strains) were used in this study. These strains were isolated between 1995 and 1997 in Japan, from the nasopharynx ($n = 106$), sputum ($n = 18$), and blood ($n = 12$) of patients with respiratory tract infections (including pneumonia); from otorrhea ($n = 56$) of patients with acute otitis media; and from cerebrospinal fluid ($n = 23$) of patients with purulent meningitis.

Blood agar medium containing 5% sheep blood (Trypticase Soy Agar Modified; Nippon Becton Dickinson, Tokyo, Japan) was used for the cultivation of these isolates at 37°C in a humidified atmosphere supplemented with 5% CO_2. *S. pneumoniae* were identified by the detection of an autolysin (*lytA*) gene by previously described PCR methods.[16,17]

Antibiotic susceptibility testing

The MICs of the MLS and β-lactam antibiotics were determined by the agar plate dilution method, using cation-adjusted Mueller Hinton agar (MH agar; Eiken, Tokyo Japan) supplemented with 5% defibrinated sheep blood. Bacteria preserved in skim milk were spread on the blood agar plate and incubated with CO_2 at 37°C for 18 h, and these procedures were repeated. Bacteria grown on the agar plate were suspended in 2 ml of MH broth to adjust the McFarland value to 0.1. After the addition of 100 μl sheep blood, further incubation at 37°C was done for 6 h. Each bacterial isolate was inoculated on an agar plate containing each antibiotic, using a microplanter (Sakuma, Tokyo, Japan) to determine susceptibility. The inoculum size on the agar plate was adjusted to 10^5 cells per spot. The MICs were determined after incubation at 37°C for 18 h. The following antibiotics were provided by their manufacturers: EM (Shionogi, Osaka, Japan); cefotaxime (CTX; Pfizer Pharmaceutical, Tokyo, Japan), clarithromycin (CAM; Taisho Pharmaceutical, Tokyo, Japan), roxithromycin (RXM; Aventis Pharma, Tokyo, Japan), TEL (Aventis Pharma); azithromycin (AZM; Pfizer Pharmaceutical); josamycin (JM; Yamanouchi Pharmaceutical, Tokyo, Japan); midecamycin (MDM) and penicillin G (PG) (Meiji Seika, Tokyo, Japan); rokitamycin (RKM; Asahi Chemical Industry, Tokyo, Japan); and clindamycin (CLDM; Pharmacia, Tokyo, Japan).

PCR primers

Based on the DNA sequences of the *ermB*[8,18–20] and *mefA*[10] genes, the genes in MLS-resistant *S. Pneumoniae* stocked in our laboratory were sequenced. Two sets of oligonucleotide primers, designed on the basis of our sequence results, were as follows: ermB1, 5′-$_{721}$CGTACCTTGGATATTCACC G$_{740}$-3′ and ermB2, 5′-$_{944}$GTAAACAGTTGACGATATT CTCG$_{922}$-3′ for the *ermB* gene; and mefA1, 5′-$_{288}$CTGT ATGGAGCTACCTGTCTGG$_{309}$-3′ and mefA2, 5′-$_{581}$CCC AGCTTAGGTATACGTAC$_{562}$-3′ for the *mefA* gene. The DNA fragments amplified by these primers were 224 bp for the *ermB* and 294 bp for the *mefA* gene. The nucleotide sequences were determined according to previously described methods.[21] The data will appear in the DDBJ, EMBL, and Gene Bank nucleotide sequence databases with the accession numbers AB011258 and AB011259.

PCR conditions

A single colony on the blood agar medium was suspended in a microtube containing 30 μl of a lysis solution.[17] The tube was set in a thermal cycler (Gene Amp PCR System 9600-R; Perkin-Elmer, Norwalk, CT, USA), and bacterial cells were lysed under the reactive conditions of 60°C for 10 min followed by 94°C for 5 min. Next, 2 μl of the bacteriolytic solution was placed in a PCR tube containing 25 μl of a reaction mixture. One ml of the reaction mixture consisted of 60 ng of a primer for each of *ermB* and *mefA*, 80 μl of 25 mM dNTP mixture, 25 U of *Taq* DNA polymerase, and 100 μl of 10× PCR buffer. The PCR conditions were 94°C for 20 s, 52°C for 20 s, and 72°C for 15 s, with 30 cycles in total. Total time for the PCR procedure was within 2.5 h.

Similar PCR techniques, described previously,[17] were applied to classify *S. pneumoniae* as penicillin-susceptible (PSSP), penicillin-intermediate (PISP), and PRSP at the molecular level. The PCR methods identified abnormal *pbp1a*, *pbp2x*, and *pbp2b* genes having amino acid substitution(s) on each *pbp* gene that apparently decreases susceptibility.

Serotyping

S. pneumoniae strains were serotyped or serogrouped by capsular swelling reaction with antisera obtained commercially from the Statens Serum Institut (Copenhagen, Denmark). Strains grown overnight on a blood agar plate were suspended in 100 μl of saline buffer and an equal volume of 5× diluted methylene blue stain was added. Each 5 μl aliquot of the suspension was spotted onto a glass slide, and made to react with omni and pooled antisera. Strains showing evidence of capsular swelling under light microscopy were tested with the individual specific antisera contained in the reactive pool.

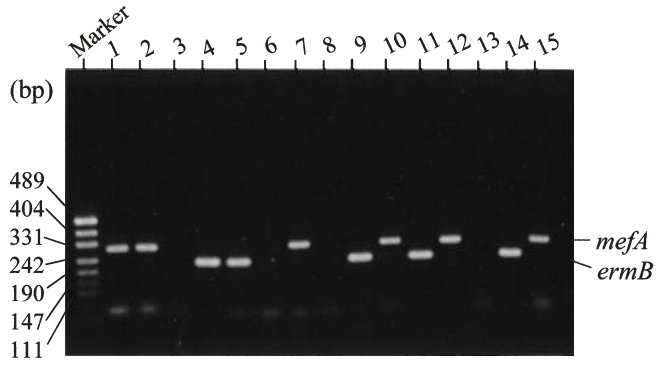

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR)-amplified DNA fragments of the *mefA* (294 bp) and *ermB* (224 bp) genes in 15 clinical isolates of *Streptococcus pneumoniae*. A single colony of *S. pneumoniae* grown on a blood agar plate was suspended in a microtube containing 30 µl of lysis solution. The tubes were put in a thermal cycler, and the bacterial cells were lysed for 10 min at 60°C and for 5 min at 94°C. Next, 2 µl of the lysate was added to reaction mixture containing two sets of primers. The PCR cycling conditions were 30 cycles at 94°C for 20 s, 52°C for 20 s, and 72°C for 15 s. Amplified DNA fragments were analyzed using gel electrophoresis with 3% agarose

Statistical analysis

Data analysis was performed using the SAS Institute (Cary, NC, USA) statistical software. We used the χ^2 test to test for bivariate null hypotheses of independence.

Results

Amplified DNA profile

Figure 1 shows the PCR-amplified DNA profile obtained from 15 strains of *S. pneumoniae* selected from the strains stored at Teikyo University. Two DNA fragments, 294 bp and 224 bp, correspond to the fragments of the *mefA* and *ermB* genes, respectively. Two genes were thus identified for the 215 *S. pneumoniae* strains.

Relationships between MLS susceptibility and *mefA/ermB* genes

Figure 2 shows the relationships between the susceptibilities to EM, AZM, RKM, and CLDM in the 215 strains of *S. pneumoniae* and the presence of the *mefA* and *ermB* genes.

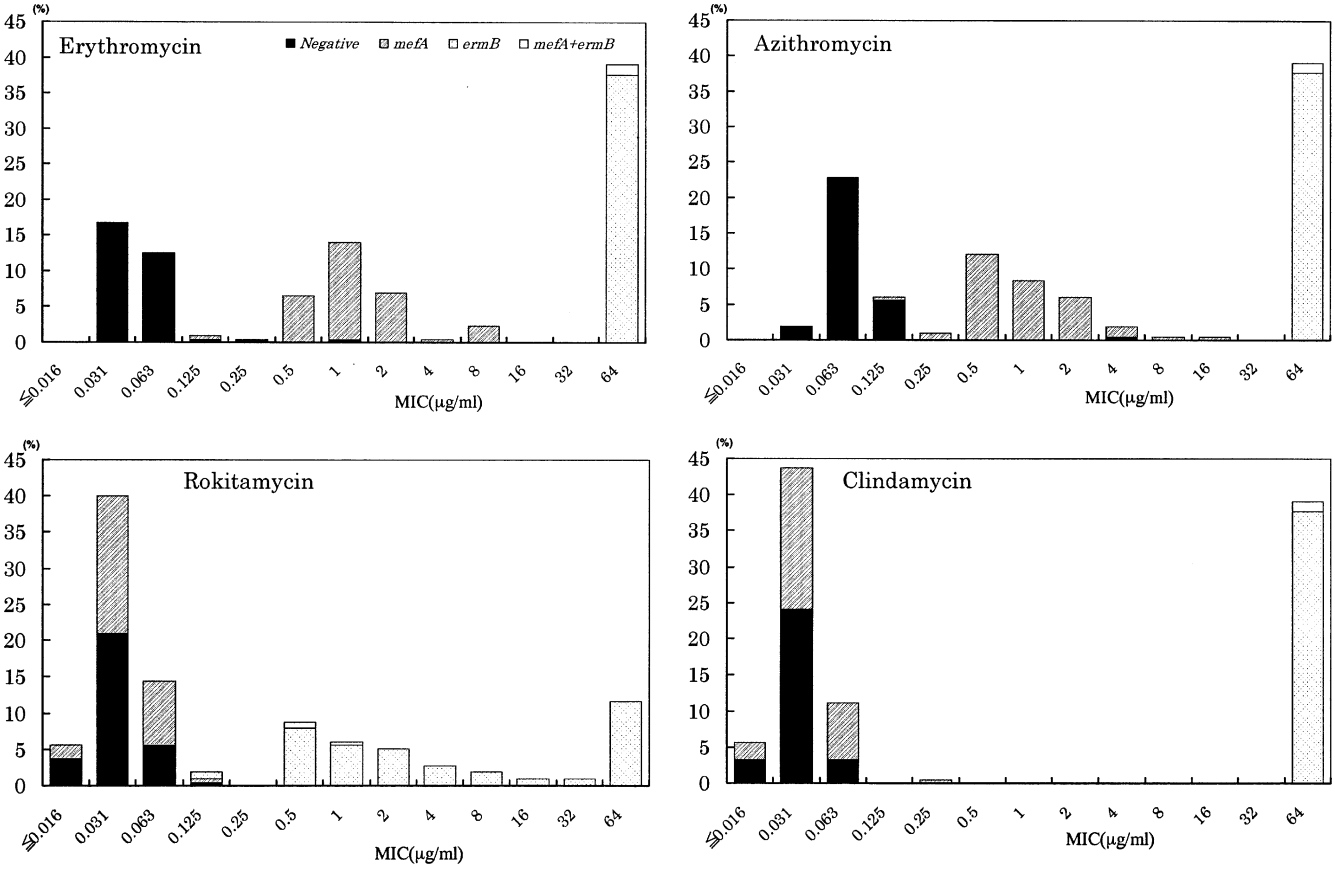

Fig. 2. Correlation between susceptibility to erythromycin, azithromycin, rokitamycin, and clindamycin of 215 strains of *Streptococcus* pneumonial, and the presence of the *mefA* and *ermB* genes. *Black bars*, *mefA* and *ermB* genes-negative ($n = 66$); *striped bars*, *mefA* gene-positive ($n = 65$); *dotted bars*, *ermB* gene-positive ($n = 81$); *white bars*, *mefA* and *ermB* genes-positive ($n = 3$)

Table 1. *Streptococcus pneumoniae* strains (n = 212) characterized according to their macrolide-resistant genes and MICs[a]

Antibiotic	Macrolide-resistant gene					
	Negative (n = 66)		*mefA* (n = 65)		*ermB* (n = 81)	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Erythromycin	0.031	0.063	1	2	>64	>64
Roxithromycin	0.063	0.063	1	4	>64	>64
Clarithromycin	0.031	0.063	0.25	1	>64	>64
Azithromycin	0.063	0.125	1	2	>64	>64
Josamycin	0.125	0.125	0.125	0.25	32	>64
Midecamycin	0.125	0.25	0.125	0.25	32	>64
Rokitamycin	0.031	0.063	0.031	0.063	2	>64
Clindamycin	0.031	0.063	0.031	0.063	>64	>64
Telithromycin	0.016	0.016	0.063	0.125	0.063	0.5

MIC, minimum inhibitory concentration
[a] Values are in μg/ml

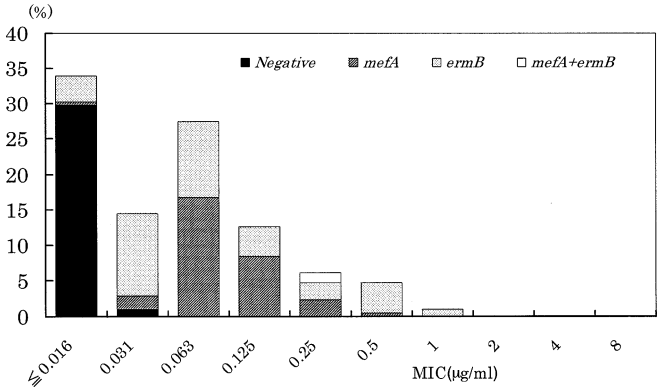

Fig. 3. Correlation between susceptibility to a new ketolide (telithromycin) of 215 strains of *Streptococcus pneumoniae*, and the presence of the *mefA* and *ermB* genes. *Black bars*, *mefA* and *ermB* genes-negative (n = 66); *striped bars*, *mefA* gene-positive (n = 65); *dotted bars*, *ermB* gene-positive (n = 81); *white bars*, *mefA* and *ermB* genes-positive (n = 3)

Figure 3 shows similar results for TEL. These strains were classified into four groups; group 1, without a resistant gene (n = 66; 30.7%), group 2, with the *mefA* gene (n = 65; 30.2%), group 3, with the *ermB* gene (n = 81; 37.7%); and group 4, with both the *mefA* and *ermB* genes (n = 3; 1.4%).

Table 1 shows the MIC_{50} and MIC_{90} values of the nine MLS, including TEL, according to the different resistant genes. While the strains having the *mefA* gene showed low-level resistance to 14-membered-ring MLS and AZM, with MIC_{90} values ranging from 1 to 4 μg/ml, these strains were susceptible to the 16-membered-ring MLS and CLDM, with MIC_{90} values ranging from 0.063 to 0.25 μg/ml, these MIC levels being the same as those of MLS-susceptible strains. Almost all the strains having the *ermB* gene showed high resistance to EM, RXM, and CAM (14-membered ring macrolides); AZM (an azalide); JM, MDM, and RKM (16-membered ring macrolides); and CLDM, with MIC_{90} values of ≥64 μg/ml.

In contrast, the antimicrobial activity of TEL was obviously superior against MLS-susceptible strains without resistant genes, with an MIC_{90} of 0.016 μg/ml; against strains having the *mefA* gene, with an MIC_{90} of 0.125 μg/ml, and against strains having the *ermB* gene, with an MIC_{90} of 0.5 μg/ml. The MIC of TEL for the three strains having both resistant genes was 0.25 μg/ml for each strain.

Relationship between MLS-resistant genes and abnormal PBP genes

Table 2 shows the relationship between MLS-resistant genes and the abnormal PBP genes that mediate β-lactam antibiotic resistance. Abnormal *pbp1a*, *pbp2x*, and *pbp2b* genes in the strains used were identified by PCR. In the statistical analysis, a significant correlation was observed between MLS-resistant genes and abnormal PBP genes (χ^2 = 79.6536; P = 0.0000). Of note, most of the strains identified molecularly as PRSP that possessed three abnormal PBP genes, *pbp1a*, *pbp2x*, and *pbp2b*, were also found to have a high frequency of MLS-resistant genes. Strains possessing only the abnormal *pbp2x* gene, which affects the MICs of cepharlosporins rather than penicillins, also had the *mefA* or *ermB* gene. These strains have a higher prevalence in Japan than in the United States.

Relationship between MLS-resistant genes and serotype

Table 3 shows the presence or absence of the *mefA* and *ermB* genes and the serotypes of the tested strains. Miscellaneous serotypes were observed in strains having no MLS-resistant genes. In contrast, most of the strains with the *mefA* gene were serotyped as 19, 6, and 14, and most of those with the *ermB* gene were serotyped as 6, 23, 3, and 19.

Discussion

Two main resistance mechanisms are known for MLS-resistant gram-positive cocci.[22,23] One is the production of methylase, which dimethylates 23S rRNA, a target for

Table 2. *Streptococcus pneumoniae* strains (n = 215) characterized according to their macrolide-resistant genes and according to PBP gene alterations

PBP gene (normal/abnormal)	No. of strains (%)	MIC_{90} (μg/ml)		Macrolide-resistant gene			
		Penicillin G	Cefotaxime	Negative	*mefA*	*ermB*	*mefA* + *ermB*
Normal	55 (25.6)	0.031	0.063	36	11	8	
pbp1a	2 (0.9)	–	–		2		
pbp2x	57 (26.5)	0.063	0.25	13	11	33	
pbp2b	5 (2.3)	0.125	0.063	1		4	
pbp1a + *pbp2x*	15 (7.0)	0.5	0.5	5	4	6	
pbp1a + *pbp2b*	5 (2.3)	0.25	0.125	2		3	
pbp2x + *pbp2b*	6 (2.8)	0.25	0.25	1	1	4	
pbp1a + *pbp2x* + *pbp2b*	70 (32.6)	2	1	8	36	23	3

Table 3. Serotype of *Streptococcus pneumoniae* strains characterized according to their macrolide-resistant genes

Macrolide-resistant gene	Capsule serotype															Total
	3	4	6	7	9	10	11	14	15	18	19	22	23	Other	NT	
Negative	6	2	10	1	4	2	4	9	5	5	7	3	7		1	66 (30.7%)
mefA			12	1				8	3		31		6	2	2	65 (30.2%)
ermB	16		25		2			4			15		17	1	1	81 (37.7%)
mefA + *ermB*													3			3 (1.4%)
Total	22	2	47	2	6	2	4	21	8	5	53	3	33	3	4	215

NT, not typed

MLS, resulting in the organism acquiring resistance to MLS. The genes encoding modifying enzymes are *ermA*,[24–26] *ermB*,[27] and *ermC*[28,29] in staphylococci; and *ermAM* (*ermB*) in *Enterococcus faecalis*[19,20] and in *S. sanguis*.[30] In *S. pneumoniae*, the *ermAM* (*ermB*) gene has been shown to encode methylase activity, and is highly homologous to *ermB* of *E. faecalis* and *S. sanguis*. Regulation of inducibility for dimethylase activity depends on the nucleotide sequences on the regulatory domain present on the upstream of the structural gene.[15]

Another resistance mechanism is an active efflux pump system for MLS that has been incorporated into cells. The mechanism has been observed in staphylococci, where membrane-binding proteins are encoded by the *erpA*[31,32] and *msrA*[28,33–37] genes.

Recently, a resistance mechanism similar to that of *msrA* has been reported for *S. pyogenes* and *S. pneumoniae*. Two genes, *mefA* in *S. pyogenes*[10] and *mefE* (*mefA*) in *S. pneumoniae*,[3,9,38] were found to be 94% homologous by analysis of amino acid sequences, in which the encoded hydrophobic membrane protein was estimated to have 12 membrane-penetrating sites (data not shown here).

In Japan, strains such as MLS-resistant *S. pneumoniae* are rapidly increasing, in parallel with increases in PISP and PRSP;[17] in particular, MLS low-level resistant *S. pneumoniae* isolates possessing the *mefA* gene are increasing. Most of the strains identified molecularly as PRSP that possessed three abnormal PBP genes, *pbp1a*, *pbp2x*, and *pbp2b*, and showed an MIC_{50} of 2 μg/ml against penicillin G, were found to have MLS-resistant genes at high frequency. Strains possessing only the abnormal *pbp2x* gene, which affects the MICs of cepharlosporins rather than penicillins (strains which are more prevalent in Japan than in the United States) also had the *mefA* or *ermB* gene. These strains showed resistance to β-lactam and MLS antibiotics and are termed multidrug-resistant *S. pneumoniae* (MDRSP). The rapid increase of these MDRSP isolates in the pediatric and otolaryngological fields is problematic in that there are few oral antibiotics that are good to use for empiric therapy.

As described above, only TEL displayed high activity against *mefA*- and *ermB*-positive MDRSP having three abnormal PBP genes, with its MIC_{90} values being 0.125 μg/ml and 0.5 μg/ml, respectively. These results suggest that TEL may be useful as a chemotherapeutic agent for infections caused by *S. pneumoniae*, including PRSP, in outpatients.

Last but not least, the proper use of antibiotics, based on molecularly analyzed evidence at the gene level, which can be acquired in a short time by PCR, as described here, is most important in preventing the increase of resistant microorganisms.

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