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Streptococcus pneumoniae Isolates Resistant to Telithromycin

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The telithromycin susceptibility of 210 erythromycin-resistant pneumococci was tested with the agar diffusion method. Twenty-six *erm*(B)-positive isolates showed heterogeneous resistance to telithromycin, which was manifested by the presence of colonies inside the inhibition zone. When these cells were cultured and tested, they showed stable, homogeneous, and high-level resistance to telithromycin.

Telithromycin (TEL), the first ketolide drug, was developed to overcome macrolide resistance and introduced into clinical use a few years ago. The antimicrobial spectrum of TEL covers the most important respiratory pathogens, including macrolide-resistant pneumococci, but it is inactive against macrolide-lincosamide-streptogramin B-resistant *Streptococcus pyogenes* and constitutively macrolide-lincosamide-streptogramin B-resistant *Staphylococcus aureus* strains (1, 2, 6, 15, 18).

According to data collected over 3 years in the international PROTEKT study, only 10 TEL-resistant (TEL R) isolates were detected from among over 13,000 clinical pneumococci (9). A low occurrence of TEL-nonsusceptible pneumococci has also been reported in other studies (3, 17, 19, 24). However, there is some evidence that TEL resistance might be emerging among pneumococci (8, 11, 13, 21). Pneumococci with a macrolide resistance mechanism usually have elevated TEL MICs compared to macrolide-susceptible wild-type isolates (3, 5, 15). However, the TEL MICs of these isolates do not usually exceed the CLSI breakpoint for nonsusceptible isolates (≥2 μg/ml) (10, 14). Mutations at macrolide and ketolide binding sites, such as domains II and V of 23S rRNA and ribosomal proteins L4 and L22, have been reported to associate with an elevated TEL MIC. Mutations in the resistance determinant *erm*(B) have also been suggested to confer TEL resistance (13).

In our previous study carried out in Finland, 2.8% of pneumococci (n = 1,007) had TEL MICs of ≥2 μg/ml, and nearly all such isolates carried *erm*(B). However, due to the lack of breakpoints for the agar dilution method used in the study, the proportion of telithromycin nonsusceptibility could not be determined (20). The objectives of this study were to investigate the TEL susceptibility of isolates with a known macrolide resistance determinant using an approved CLSI disk diffusion method (4) and to compare these results with those obtained by the agar dilution (5) and CLSI broth microdilution (4) methods.

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Bacterial isolates and susceptibility testing. Two hundred ten erythromycin-resistant (MIC ≥ 0.5 μg/ml) pneumococci with a known macrolide resistance determinant and 47 randomly selected erythromycin-susceptible pneumococci were investigated from among 1,007 *Streptococcus pneumoniae* isolates that were collected in 2002 in Finland for macrolide resistance surveillance purposes (20).

TEL susceptibility was tested with 15-μg TEL disks (Oxoid Ltd., Basingstoke, Hampshire, England) by use of the CLSI disk diffusion technique in 5% CO_2. Susceptibility testing was repeated at least five times for isolates showing resistance to TEL. If colonies were detected inside the growth inhibition zone (i.e., heterogeneous resistance to TEL), one such colony was isolated (the zone isolate) and TEL susceptibility was determined by use of the disk diffusion method. In addition, TEL MICs of isolates showing heterogeneous TEL resistance in the disk diffusion test, as well as of their respective zone isolates, were concurrently determined by the agar dilution method in 5% CO_2 and by the CLSI broth microdilution method in ambient air. Finally, the stability of TEL resistance was tested in three zone isolates by the serial passage method. Isolates were cultured on 5% sheep blood agar plates and after overnight incubation were subcultured again onto new blood agar plates. The procedure was repeated five times on consecutive days. The TEL susceptibilities of all five subcultures were tested by the disk diffusion method. *S. pneumoniae* ATCC 69419 was used as the quality control strain.

Molecular analysis. The *erm*(B) gene and its promoter region (beginning 280 base pairs upstream from the start of the ErmB methylase protein-coding region) was sequenced for three TEL R isolates and their respective zone isolates (6). The following primers were used to amplify the *erm*(B) gene: 5′-GAAGCAAACTTAAGAGTGTG-3′ and 5′-GCTAGGGACCTCTTTAGCTT-3′. Sequencing primers of *erm*(B) were 5′-CAGTGATTACGCAGATAAATA-3′, 5′-GACACGAATGTTCAGTTTTA-3′, 5′-CCTAAACCAAAAGTAAACAG-3′, and 5′-TCTCGATTGACCCATTTTGA-3′. Pyrosequencing was used to detect mutations at the macrolide and ketolide binding sites (12, 22). Primers for detecting mutations in positions 2058 to 2059 and 2611 of domain V have previously

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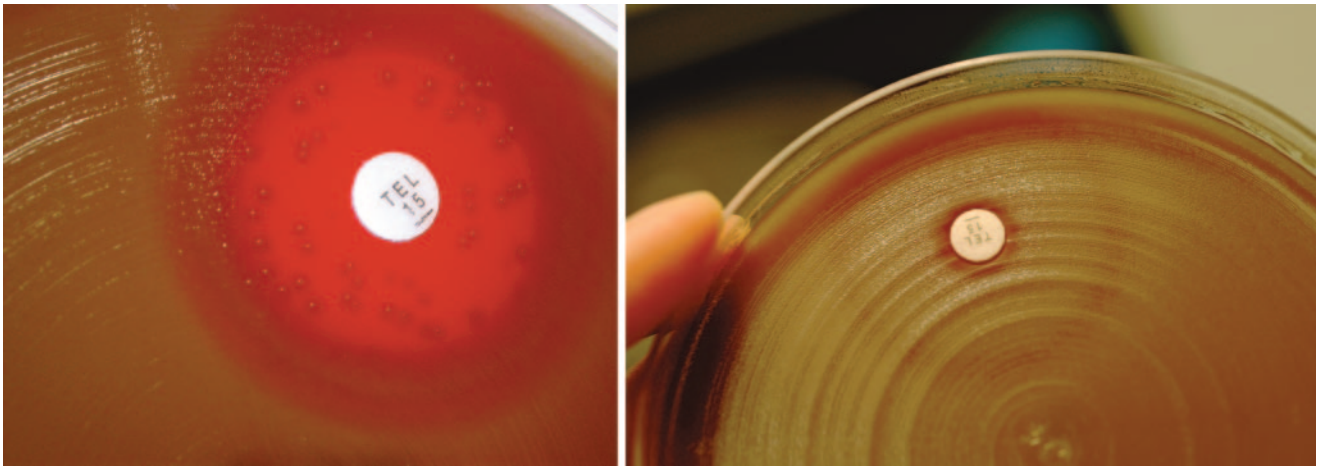

FIG. 1. On the left is the typical growth pattern of an isolate showing heterogeneous resistance to TEL. A few colonies can be observed inside the inhibition zone. On the right, a small inhibition zone and homogeneous growth pattern can be seen around the TEL disk of a zone isolate that has been derived from a colony growing inside the inhibition zone.

been published (12, 20). Primers for amplifying loop 35 of domain II were 5′-GCGCCTTAGTATCATGACGTAGA-3′ (biotinylated), 5′-AATGTCGACGCTAGCCCTAAAG-3′, and, for pyrosequencing, 5′-CGCTACCCACAAGTCA-3′. In addition, genes coding for 50S ribosomal proteins L4 and L22 of these isolates, as well as the whole 23S rRNA gene of one TEL R isolate and the respective zone isolate and two other zone isolates, were sequenced (16, 23). The identities of one TEL R isolate and its respective zone isolate were confirmed with pulsed-field gel electrophoresis (7).

Altogether, 26/210 (13%) erythromycin-resistant isolates showed heterogeneous resistance to TEL, i.e., one to several clearly visible colonies grew inside the inhibition zone (Fig. 1). All of these isolates harbored the *erm*(B) determinant, and two of them also carried the *mef*(E) gene. In addition, two other *mef*(E)-positive isolates for which no growth was detected inside the zone were classified as being intermediately susceptible. All 47 erythromycin-susceptible isolates were susceptible to TEL and had wide inhibition zones. The majority of the zone isolates showed a high level of resistance to TEL without any detectable inhibition zones in the disk diffusion test (Table 1). TEL resistance among zone isolates was observed to be constant and did not decline during five serial passages.

TEL MICs of the majority of TEL-resistant isolates were ≥2 μg/ml when the agar dilution method was used but well below the resistance breakpoint when determined by the CLSI broth microdilution method (Table 2). Zone isolates had high MICs (4 to 64 μg/ml) by the agar dilution method, and the majority of them were also clearly resistant according to the CLSI broth microdilution method (Table 2). It should be noted, however, that some isolates failed to grow in ambient air when the broth microdilution method was used.

The nucleotide sequences of *erm*(B) and its promoter area, as well as of genes coding for ribosomal proteins L4 and L22, were identical for heterogeneously TEL-resistant isolates and zone isolates. No mutations known to confer macrolide resistance were detected in 23S rRNA genes or in genes coding for L4 or L22. No previously published deletions in the *erm*(B) leader peptide sequence were detected in these isolates (13). 23S rRNA genes of four isolates were identical and

TABLE 1. Comparison of TEL disk diffusion test results for pneumococcal isolates according to macrolide resistance determinant

Parameter[a]	Value of parameter for type of isolate					
	erm(B) with heterogeneous resistance (n = 26)[b]	Zone (n = 26)[c]	Other with *erm*(B) (n = 66)[d]	*mef*(A/E) (n = 104)	With mutation (n = 14)[e]	Macrolide susceptible (n = 47)
Zone size (mm) around 15-μg TEL disk						
Mean (SD)	20.6 (1.9)	8.3 (NC)	26.9 (2.2)	22.5 (2.9)	28.5 (2.8)	31.5 (2.3)
Min–max	15–24	6–19	21–33	18–30	24–34	28–37
Mode	20	6	25	21	29	32
Median	21	6	26	22	28	32
No. of isolates						
TEL I	0	2	0	2	0	0
TEL R	26	23	0	0	0	0

[a] TEL I, TEL intermediate; TEL R, TEL resistant.
[b] Isolates having a visible inhibition zone but individual colonies detected inside the inhibition zone; two had the double mechanism *erm*(B) plus *mef*(E).
[c] Isolates derived from individual colonies detected inside the inhibition zone. NC, not calculated.
[d] Including two isolates with the double mechanism *erm*(B) plus *mef*(E).
[e] Including three erythromycin-resistant isolates with unknown mechanism.

TABLE 2. Comparison of TEL MICs of isolates showing heterogeneous resistance and their respective zone isolates, determined with the agar dilution and CLSI broth microdilution methods[a]

Statistic[b]	*erm*(B) isolates with heterogeneous resistance[c]		Zone isolates[d]	
	Agar dilution (n = 26)	CLSI broth (n = 24)[e]	Agar dilution (n = 26)	CLSI broth (n = 23)[e]
Geometric mean MIC (μg/ml)	1.89	0.15	33.8	2.87
Min–max MIC (μg/ml)	0.063–8	0.063–2	4–64	0.125–8
MIC_{50} (μg/ml)	2	0.125	32	4
MIC_{90} (μg/ml)	4	1	64	8
No. of isolates with MIC ≥ 2 μg/ml (%)	21 (81)	1 (4)	26 (100)	21 (91)

[a] Agar dilution was in 5% CO_2; CLSI broth microdilution was in a normal atmosphere.
[b] Min–max, range from minimum value to maximum value; MIC_{50}, 50% MIC; MIC_{90}, 90% MIC.
[c] Isolates having a visible inhibition zone but individual colonies detected inside the inhibition zone.
[d] Zone isolates were derived from individual colonies from inside the inhibition zone.
[e] Some isolates did not grow with the microdilution method in a normal atmosphere.

showed 99.97% and 99.83% similarities to published genomic 23S rRNA sequences of *S. pneumoniae* strains TIGR4 and R6, respectively. Pulsed-field gel electrophoresis profiles of a TEL-resistant isolate and of its respective zone isolate were identical.

In conclusion, the main finding of this study was the existence of heterogeneous TEL resistance among pneumococci carrying *erm*(B), manifested by the presence of bacterial colonies inside the inhibition zone around the TEL disk. Isolates derived from inside the inhibition zone showed stable, homogeneous, and high-level resistance to TEL. These results indicate that the presence of TEL nonsusceptibility among macrolide-resistant pneumococci may be more frequent than previously reported. Furthermore, we suggest further investigation into whether susceptibility testing of pneumococci in ambient air with existing breakpoints underestimates the occurrence of telithromycin resistance. We recommend that TEL susceptibility testing by the disk diffusion technique should be performed routinely at least for those pneumococcal isolates that are resistant to erythromycin. The clinical significance of and genetic mechanism underlying this heterogeneous TEL resistance pattern should be investigated.

Nucleotide sequence accession numbers. Accession numbers for the *erm*(B) sequence and 23S rRNA sequences derived from this study are AM180135 and AM180136, respectively.

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