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Mutations Associated with Ketolide Resistance in *Streptococcus pneumoniae* Collected in the 2009 SENTRY Antimicrobial Surveillance Program

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SENTRY ANTIMICROBIAL SURVEILLANCE

Abstract

Background: Ketolide resistance (R) is very rare in *S. pneumoniae* (SPN) and is usually associated with a variety of ribosomal mutations and/or mutations in the region upstream of the *erm*(B) gene that control expression. We investigated the mechanisms of resistance in 5 telithromycin (TELI) -R SPN found in the SENTRY Program (2009) and assessed the activity of solithromycin (CEM-101), a new fluoroketolide in clinical development.

Methods: 2,123 SPN isolates obtained from patients with community-acquired bacterial pneumonia in 23 countries were tested for susceptibility to TELI by CLSI methods (M07-A8 and M100-S20-U). Only 5 (0.2%) isolates were observed to be TELI-R. Strains were screened for *erm*(B) and *mef*(A/E) resistance genes by PCR, and mutations in the 23S rRNA, L22 and L4 proteins, and the *erm*(B) promoter region by PCR and DNA sequencing.

Results: All TELI-R strains were from the Peoples Republic of China and had TELI MIC values of 8 µg/ml, however the CEM-101 MICs were only 0.06-0.25 µg/ml. Significant 23S rRNA, L4 and L22 mutations were not present in any strains. Novel amino acid substitutions in the *erm*(B) leader peptide were detected in 4/5 strains and an identical pattern of mutations were found in all 5 strains in the region between the *erm*(B) and leader peptide genes.

Conclusion: Ketolide-R in SPN continues to be rare (<1%) globally. R was found to be associated with a variety of mutations upstream of *erm*(B) and appear to result in increased production of ermB with subsequent increases in the rate of dimethylation of A2058 in domain V of the 23S rRNA. CEM-101 remained very active against these strains and hence appeared refractory to the effect of these resistance mechanisms and a good clinical candidate.

Introduction

Macrolide resistance in *Streptococcus pneumoniae* is the result of either target-site modification, active drug efflux, or both. Target-site modification is commonly caused by the acquisition of the *erm*(B) gene which methylates nucleotide A2058 in domain V of 23S rRNA and prevents protein elongation. In a smaller proportion of strains, macrolide resistance mutations in the peptidyl transferase center (nucleotides A2058 and A2059 most commonly) and/or ribosomal proteins L4 and L22 are responsible for macrolide resistance.

Telithromycin is a ketolide antimicrobial agent and is a semi-synthetic derivative of erythromycin A. Ketolides bind to 23S at domain II as well as domain V and retain good activity against macrolide resistant strains, hence telithromycin resistance is rare. Ketolide resistance is known to be caused by mutations in the leader sequence of the *erm*(B) gene which result in increased expression of the gene and a subsequent increase in the ratio of di- to mono-methylation of A2058. Resistance is also associated with a combination of mutations in L4, L22 and 23S domains II and V.

Solithromycin (CEM-101) is the first fluoroketolide selected as a candidate for oral and/or parenteral therapy of community-acquired bacterial pneumonia (CABP) and other infections, such as urethritis and *Mycobacterium avium* infections. Solithromycin has demonstrated excellent activity (MIC_{90}, 0.12 µg/ml) against geographically varied *S. pneumoniae* isolates and is typically two- to four-fold more active than telithromycin.

In this study, we investigate the mechanisms leading to elevated ketolide (telithromycin) MIC values found in five *S. pneumoniae* strains isolated during the global 2009 SENTRY Antimicrobial Susceptibility Surveillance Program and describe the activity of solithromycin against these strains.

Methods

Bacterial Strain Collection. During 2009, a total of 2,123 *S. pneumoniae* isolates were obtained from patients with CABP in 23 countries. Species identifications were performed by the submitting laboratories with confirmation by the central monitoring laboratory (JMI Laboratories, North Liberty, Iowa, USA).

Susceptibility Test Methods. All isolates were tested for susceptibility to solithromycin and comparators by reference broth microdilution methods using the Clinical Laboratory Standards Institute recommendations (CLSI; M07-A8, 2009). Susceptibility testing was performed by using validated broth microdilution panels manufactured by TREK Diagnostics Systems (Cleveland, Ohio, USA). Further validation of the minimum inhibitory concentration (MIC) values was performed by concurrent testing of CLSI-recommended (M100-S20-U, 2010) quality control (QC) strains, including *S. pneumoniae* ATCC 49619. Categorical interpretation of comparator MIC values was performed according to CLSI (M100-S20-U, 2010) criteria, when available.

Molecular Test Methods.Telithromycin resistant strains (MIC ≥4 µg/ml) were screened for *erm*(B) and *mef*(A/E) resistance genes by PCR, and for mutations in the 23S rRNA, L22 and L4 proteins, and the *erm*(B) promoter region by PCR and DNA sequencing. Sequence data was compared to *erm*(B) Genbank accession No. X52632.

Results

- In 2009, worldwide, against all 2,123 *S. pneumoniae* isolates, solithromycin (MIC_{90}, 0.25 µg/ml) was two-fold more active than telithromycin (MIC_{90}, 0.5 µg/ml; Table 1). In the subset of 459 isolates obtained from the Peoples Republic (P.R.) of China, while the MIC_{50} (0.25 µg/ml) of telithromycin was at least four-fold higher than the global rate (≤0.06 µg/ml), the $MIC_{50/90}$ (≤0.06/0.25 µg/ml) for solithromycin remained unchanged (Table 1).
- Only five strains were found to be resistant to telithromycin by CLSI interpretive criteria (≥4 µg/ml) and the solithromycin MIC range for these strains was only 0.06 to 0.25 µg/ml (Table 2). All five strains were isolated from P.R. China.
- All five strains contained *erm*(B) and one strain (2099B) also contained *mef*(A/E). No mutations were detected in the 23S rRNA and L4/L22 riboprotein genes for any of the five strains (Table 2).
- Amino acid substitutions were found in the leader peptide in four of the five strains (Table 2). Three strains had an A25T substitution and the remaining strain had a Q5L substitution. The contribution of these substitutions to increase *erm*(B) expression is unknown, but any modifications in the leader peptide have potential to influence expression.
- All five strains had an identical four nucleotide mutation pattern in the non-coding region between the leader peptide and *erm*(B) (Table 2 and Figure 1). This region forms stem-loop secondary structures that influence ribosome binding and hence *erm*(B) expression, consequently even single nucleotide changes can greatly modify this secondary structure. Of interest is the proximity of two of the mutations to the ribosome binding (Shine-Delgarno [SD]) site. The consensus sequence for the SD site is AG rich and typically AGGAGG; the two mutations (318 and 319) increase the SD region AG content and likely increase ribosome binding affinity and hence expression.

Table 1. MIC frequency and cumulative percent inhibited distribution of solithromycin and telithromycin against 2,123 *S. pneumoniae* isolated in 2009

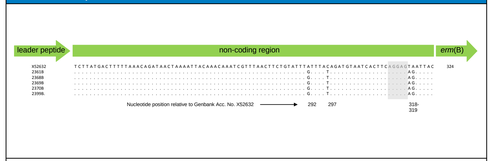
	MIC (µg/ml)									
	≤0.06	0.12	0.25	0.5	1	2	4	8	MIC_{50}	MIC_{90}
All countries (n=2123)										
Telithromycin	1488 (71)	142 (77)	111 (82)	339 (98)	36 (99.7)	2 (99.8)	0 (99.8)	5 (100)	≤0.06	0.5
Solithromycin	1655 (78)	125 (84)	299 (98)	41 (99.9)	3 (100)				≤0.06	0.25
P.R. China subset (n=459)										
Telithromycin	204 (44)	25 (49.9)	47 (60)	158 (95)	19 (98.7)	1 (98.9)	0 (98.9)	5 (100)	0.25	0.5
Solithromycin	232 (51)	47 (61)	159 (95)	20 (99.8)	1 (100)				≤0.06	0.25

Table 2. Summary of phenotypic and genotypic characteristics of telithromycin-resistant *S. pneumoniae*

	MIC µg/ml			Genes found		Mutations found	
Strain	TEL[a]	SOL[b]	ERY[c]	*mef*(A/E)	*erm*(B)	Leader peptide (amino acid)	*erm*(B) upstream (nucleotides)[d]
2099B	8	0.25	>256	+	+	wt	T318A, A319G, A292G, C297T
2361B	8	0.12	>256	-	+	Q5L	T318A, A319G, A292G, C297T
2368B	8	0.12	>256	-	+	A25T	T318A, A319G, A292G, C297T
2369B	8	0.06	>256	-	+	A25T	T318A, A319G, A292G, C297T
2370B	8	0.12	>256	-	+	A25T	T318A, A319G, A292G, C297T

a. TEL = telithromycin
b. SOL = solithromycin
c. ERY = erythromycin
d. Nucleotide positions are relative to Genbank accession No. X52632

Figure 1. Nucleotide sequence alignment of the non-coding region between the leader peptide codon and *erm*(B) for the five telithromycin-resistant *S. pneumoniae*[a]



a. Dots (.) indicate identical nucleotides to the reference sequence X52632 and shaded area is the Shine-Delgarno sequence – the ribosomal binding site for *erm*(B)

Conclusions

- When tested against 2,123 *S. pneumoniae* collected worldwide in 2009, telithromycin susceptibility was 99.8% with only five resistant isolates found. Solithromycin (MIC_{90}, 0.25 µg/ml) was two-fold more active than telithromycin (MIC_{90}, 0.5 µg/ml; Table 1). In the subset of 459 isolates obtained from the P.R. China, while the MIC_{50} (0.25 µg/ml) of telithromycin was at least four-fold higher than the global result (≤0.06 µg/ml), the $MIC_{50/90}$ (≤0.06/0.25 µg/ml) for solithromycin remained unchanged (Table 1).
- Only five strains were found to be resistant to telithromycin by CLSI interpretive criteria (≥4 µg/ml) and the solithromycin MIC range for these strains was only 0.06 to 0.25 µg/ml (Table 2).
- Amino acid substitutions in the leader peptide (4/5 strains) and nucleotide mutations in the non-coding region between the leader peptide and *erm*(B) (same pattern in all 5 strains) were found and appear to result in increased expression of *erm*(B) and hence telithromycin (not solithromycin) resistance. However, this hypothesis requires further confirmatory studies (in progress).
- These results are in agreement with the X-ray crystallographic analysis of solithromycin bound to the bacterial ribosome. This showed that solithromycin was differentiated from telithromycin by having additional binding sites in domain II and also having a third interactive site on the floor of the peptide tunnel of the 23S ribosome, via the fluorine at position 2 of the macrolide ring of solithromycin. It is believed that the three binding sites help overcome macrolide and ketolide resistance mechanisms.

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