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Mechanisms of Macrolide Resistance among *Streptococcus pneumoniae* Isolates from Russia▽

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Among 76 macrolide-nonsusceptible *Streptococcus pneumoniae* isolates collected between 2003 and 2005 from Central Russia, the resistance mechanisms detected in the isolates included *erm*(B) alone (50%), *mef* alone [*mef*(E), *mef*(I), or a different *mef* subclass; 19.7%], or both *erm*(B) and *mef*(E) (30.3%). Isolates with dual resistance genes [*erm*(B) and *mef*(E)] belonged to clonal complex CC81 or CC271.

Antimicrobial resistance in *Streptococcus pneumoniae*, especially to macrolides, has been a matter of growing concern in the last two decades due to increasing numbers of reports on the failures of treatment of infections caused by macrolide-resistant pneumococcal isolates (4, 10). The most prevalent mechanisms of macrolide resistance in *S. pneumoniae* are target modification due to ribosomal methylation [associated with the *erm*(B) or the *erm*(A) gene] and a macrolide-specific efflux mechanism encoded by closely related *mef* genes (15). A new subclass of *mef* genes, *mef*(I), has recently been recognized in *S. pneumoniae* (2). The emergence and dissemination of macrolide-resistant *S. pneumoniae* strains with both *erm*(B) and *mef* genes are also new challenges (6). The goals of this study were to analyze the phenotypic characteristics, mechanisms of resistance, and clonal relationships among macrolide-resistant *S. pneumoniae* isolates from Russia.

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A total of 823 isolates of *S. pneumoniae* from the central and northwestern regions of Russia (Moscow, $n = 618$; St. Petersburg, $n = 174$; Yaroslavl, $n = 31$) were obtained from 2003 to 2005. Only one isolate per patient was included in the study. Isolates were from sputum and other lower respiratory tract specimens (56.6%), upper respiratory tract specimens (37.9%), sterile body sites (2.2%), and other or unspecified sources (3.3%). The patient age groups were ≤3 years (15.6%), 4 to 16 years (32.5%), 17 to 65 years (23.4%), >65 years (12.9%), and no age data available (15.7%). Among all patients, 56% were males, 39.5% were females, and no data were available for 4.5%.

MIC testing was performed by the broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute (1). *S. pneumoniae* ATCC 49619 was used as a control strain. Ninety-one (11.1%) isolates were nonsusceptible to erythromycin (MICs ≥ 1 μg/ml). Only three isolates were resistant to macrolides alone; all other isolates were multidrug resistant. Associated resistance to penicillin G, tetracycline, chloramphenicol, co-trimoxazole, and levofloxacin was observed in 64.5%, 84.2%, 50.0%, 80.2%, and 3.3% of the macrolide-nonsusceptible isolates, respectively. Resistance to three or more classes of antibacterials was detected in 81.5% of macrolide-nonsusceptible isolates. Seventy-six erythromycin nonsusceptible isolates were sent to the National Reference Center for Streptococci (Aachen, Germany), where the results of susceptibility testing were confirmed, detection of macrolide resistance genes was carried out, and serotyping and multilocus sequence typing (MLST) were performed. Macrolide resistance phenotypes were characterized by the triple-disk diffusion test with erythromycin, clindamycin, and rokitamycin disks (14).

Genomic DNA from clinical isolates was used as the template for PCR assays. The oligonucleotide primers used are described in Table 1. DNA sequence analysis of the *mef* genes was performed with the same primers used for PCR and an ABI Prism 3100 genetic analyzer (Applied Biosystems). The original database entries for *mef*(A), *mef*(E), and *mef*(I) (GenBank accession numbers U70055, U83667, and AJ971089, respectively) were used for the alignment of the nucleotide sequences and the discrimination of *mef* genes.

The resistance genes detected and the associated phenotypes and serotypes are summarized in Table 2. Only *mef* genes were detected in 15 isolates (19.7%). Twelve of these were positive by PCR with the MCP-L and MCP-S sets of primers. The 1,218-bp amplicons obtained with the MCP-L primers were used as templates for discrimination of the *mef* genes by DNA sequence analysis. Eight of the amplicons were *mef*(E); two were *mef*(I); and two amplicons had >99% similarity with a *mef* gene from *Bacteroides ovatus* (GenBank accession number AJ557257) but less than 95% similarity with *mef*(A), *mef*(E), and *mef*(I) subclass sequences. The sequences of the *mef*(E), *mef*(I), and the similar *Bacteroides ovatus mef* genes were similar to each other. The remaining three isolates had positive PCR results only with the MCP-S set of primers.

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TABLE 1. Oligonucleotide primers used for conventional PCR in the study

Primer use and name	Sequence	Position	Product size (bp)
Detection of *erm*(B)			
F-ECP	5′-GAAAAGGTACTCAACCAAATA-3′	43–62	639
R-ECP	5′-AGTAACGGTACTTAAATTGTTTAC-3′	658–681	
Detection of *mef* genes			
F-MCP-S	5′-ATGGAAAAATACAACAATTGGAAA-3′	1–24	263
R-MCP-S	5′-CCAGCTGCTGCGATAATTAA-3′	245–263	
Detection of *mef* genes			
F-MCP-L	5′-ATGGAAAAATACAACAATTGGAAA-3′	1–24	1,218
R-MCP-L	5′-TTATTTTAAATCTAATTTTCTAACCTC-3′	1191–1218	

Sequence analysis of the 263-bp amplicons from these isolates revealed the highest level of similarity (>99%) with the corresponding fragment of the *mef*(I) gene. These data support the hypotheses that the diversity of *mef* genes in *S. pneumoniae* may be much greater than is currently known and that the recently described *mef*(I) subclass may have spread in different geographical regions. We did not detect any relevant difference in susceptibility to macrolides between the *mef*(E) and the *mef*(I) isolates. The discrimination of *mef* genes may be more important when their association with different genetic elements (Tn*1207.1*, mega element [8] and the 5216IQ complex [13]) and the potential for horizontal transfer are considered. All *mef*-only isolates were of the macrolide resistance (M) phenotype. These *mef*-positive isolates belonged to different serotypes, with no serotype obviously predominating (Table 2).

As a single resistance determinant, the *erm*(B) gene was detected by PCR in 38 (50.0%) of the erythromycin-nonsusceptible isolates. A majority of the *erm*(B) isolates ($n = 27$) were indistinguishable on the basis of their inducible macrolide-lincosamide-streptogramin B resistance (iMcLS) phenotype by the triple-disk diffusion test (no significant zone of inhibition appeared around either the erythromycin or the clindamycin disk, and a zone of inhibition appeared around the rokitamycin disk). Serotype 6B predominated among isolates with the iMcLS phenotype. Two *erm*(B)-positive isolates had phenotypes similar to iMcLS, but without blunting of the zone of inhibition around the rokitamycin disk. The remaining nine isolates had the constitutive macrolide-lincosamide-streptogramin B resistance (cMLS) phenotype, and all of these were highly resistant to macrolides and clindamycin.

The dual combination of the *ermB* and the *mef* genes was detected in 23 isolates (30.3%), and among these, all *mef* genes were determined to be *mef*(E), as was reported for dual-mechanism isolates from the PROTEKT study (7). Twenty-one of the isolates from this group had the cMLS phenotype and two had the iMcLS-like phenotype, without blunting of the inhibition zone around rokitamycin. Serotype 23F predominated among isolates with the cMLS phenotype.

Eight epidemiologically unrelated isolates carrying both *erm*(B) and *mef*(E), obtained from different hospitals from 2003 to 2005, were randomly selected and analyzed by MLST, as described by Enright and Spratt (5). *S. pneumoniae* isolates carrying both the *erm*(B) and the *mef*(E) genes belonged to two clonal complexes (CCs), CC81 and CC271. CC81 was represented by four sequence type (ST) variants: ST81 (one isolate) and three new single-locus variants of ST81, ST2032 (two isolates), ST2033 (two isolates), and ST2576 (one isolate). Two CC271 isolates belonged to ST320. To our knowledge,

TABLE 2. Macrolide resistance genes, phenotypes, and serotypes of erythromycin-nonsusceptible isolates

Gene(s)	*mef* subclass	Phenotype	No. of isolates	MIC range (μg/ml)		Serotype(s) (no. of isolates)
				Erythromycin	Clindamycin	
mef (15 [19.7])[a]	*mef*(E)	M	8	1–8	0.015–0.06	6A (1), 6B (2), 19A (1), 19F (1), 23F (1), 35F (1), NT[b] (1)
	mef(I)[c]	M	5	2–8	0.015–0.06	6A (2), 11B (1), NT (2)
	mef[d]	M	2	8–16	0.03	9A (1), 13(1)
erm(B) (38 [50.0])		iMcLS	27	2–≥32	2–≥32	3 (3), 6A (2), 6B (15), 14 (2), 19F (2), 35C (1), NT (2)
		iMcLS (NB[e])	2	≥32	≥32	6A (1), 6B (1)
		cMLS	9	≥32	≥32	6A (1), 19A (1), 19F (3), 23F (2), 35F (1), NT (1)
erm(B) + *mef*(E) (23 [30.3])		iMcLS (NB)	2	≥32	≥32	23F (2)
		cMLS	21	≥32	8–≥32	19F (4), 23F (16), NT (1)

[a] Data in parentheses represent the number (percent) of isolates.
[b] NT, nontypeable.
[c] For three of these isolates, *mef*(I) was assigned on the basis of sequencing of an internal fragment.
[d] Similarity of 99% with *mef* from a *Bacteroides ovatus* strain with GenBank accession number AJ557257.
[e] NB, no blunting of inhibition zone around rokitamycin.

this is the first description of a high prevalence of CC81 isolates carrying both *erm*(B) and *mef*(E) outside Asia or Africa. Such isolates of *S. pneumoniae* are usually reported from regions with a high prevalence of resistance to macrolides (30% or more) worldwide (3, 6, 11, 12), but in Russia the rate of resistance to macrolides is relatively low. From 1998 to 2003 the rates of resistance to macrolides in Moscow varied from 7.9% to 19% (9). The independent emergence in Russia of dual isolates seems to be unlikely among isolates with the *erm*(B) genotype, which (at least at present) is significantly more prevalent than the *mef* genotype among macrolide-resistant isolates of *S. pneumoniae* (50% and 19.7%, respectively). It is possible that isolates with both *erm*(B) and *mef*(E) reached Central Russia from Southeast Asia through the Russian Far East.

Nucleotide sequence accession numbers. The sequences of the *mef*(E), *mef*(I), and the similar *Bacteroides ovatus mef* genes have been deposited in the GenBank database under accession numbers EU486997, EU486999, and EU486995, respectively. A representative of the 263-bp amplicon sequence was deposited in the GenBank database under accession number EU487001.

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