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Prevalence and Molecular Genetics of Macrolide Resistance among *Streptococcus pneumoniae* Isolates Collected in Finland in 2002

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The prevalence and mechanisms of macrolide resistance among 1,007 clinical pneumococcal isolates collected in Finland were investigated. Of these, 217 (21.5%) were resistant to erythromycin and 11% to clindamycin. Among the erythromycin-resistant isolates, *mef*(E) was present in 95 isolates (44%), *mef*(A) was present in 12 isolates (6%), and *erm*(B) was present in 90 isolates (41%). A double mechanism, *mef*(E) and *erm*(B), was detected in five isolates (2%). Ribosomal mutation was detected in 14 (6%) macrolide-resistant isolates in which no other determinant was found. Based on the telithromycin MICs, two groups of isolates were formed: 83.3% of the isolates belonged to a major group for which the telithromycin MIC range was ≤0.008 to 0.063 μg/ml, and 16.7% belonged to a minor group for which the telithromycin MIC range was 0.125 to 8 μg/ml. All except three isolates in the minor population carried a macrolide resistance gene.

Increasing resistance to macrolides among *Streptococcus pneumoniae* isolates is a worldwide problem. The proportion of resistant isolates ranges from 3 to 80% in different countries (2, 7, 20, 22, 23, 26, 33). Macrolide resistance is mediated by two main mechanisms in pneumococci: target site modification and drug efflux. The former is most often mediated by methylases encoded by the *erm*(B) gene, which is the most common methylase gene, or *erm*(A) [subclass *erm*(TR)], which is only infrequently found in pneumococci. Drug efflux is mediated by *mef*(A), which codes for an efflux pump (27). Two subtypes of *mef* efflux genes, *mef*(A) and *mef*(E), have been found in pneumococci (32, 40). These are variants of the same gene but are carried by different genetic elements (8, 36). An additional efflux mechanism, mediated by the *msr*(D) or the *mel* gene, has been found in genetic elements containing the *mef* gene (17, 38), but the significance of simultaneously carrying two efflux mechanisms is unknown. *msr*(D) and *mel* are homologues of the *msr*(A) gene found in staphylococci (38). Other possible mechanisms responsible for macrolide resistance in pneumococci include mutations in domain V or II of 23S rRNA or in genes coding for 50S ribosomal proteins L22 and L4 (27).

Telithromycin was the first ketolide introduced into clinical use. It is a semisynthetic derivative of erythromycin A composed of a 14-membered lactone ring, but the neutral sugar L-cladinose has been replaced by a keto group at position C-3. A C-11–C-12 carbamate side chain improves the affinity to ribosomes (1). According to present knowledge, telithromycin is effective against macrolide-resistant pneumococci, although some isolates may have elevated MICs to telithromycin (11, 12, 19, 24). Depending on the breakpoints and methods, the proportion of telithromycin nonsusceptibility has been reported to be 0.2% to 3.6% among macrolide-resistant pneumococci (11, 30).

The objectives of this study were to determine the prevalence of macrolide resistance in clinical isolates and the activity of telithromycin against clinical isolates and to investigate the molecular mechanisms of macrolide-resistant pneumococci.

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MATERIALS AND METHODS

Pneumococcal isolates and susceptibility testing. Pneumococal isolates (n = 1,007) were collected between May and December 2002 by a network of 24 Finnish Study Group for Antimicrobial Resistance (FiRe) laboratories, each of which was requested to send 50 consecutive pneumococcal isolates to the National Public Health Institute. Isolates were from both invasive sites (n = 129) and noninvasive sites (n = 878). The MICs for erythromycin, azithromycin, spiramycin, telithromycin, and clindamycin were determined by an agar plate dilution technique in a 5% CO_2 atmosphere (35). Telithromycin was kindly provided by Sanofi Aventis (Romainville, France), while the other antimicrobials were purchased from their respective manufacturers. *S. pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 were used as quality controls. CLSI (formerly NCCLS) breakpoints were used (31) for all antimicrobials except azithromycin, for which, due to the effect of the CO_2 atmosphere, we used the following breakpoints: susceptibility, ≤1 mg/liter; intermediate, 2 mg/liter; and resistant, ≥4 mg/liter. Both intermediate and resistant isolates were taken into account when resistance percentages were calculated.

Detection of macrolide resistance mechanisms. All erythromycin-resistant isolates (n = 217), 4 clindamycin-resistant isolates, and 41 randomly selected macrolide-susceptible isolates were investigated for the presence of the macrolide resistance genes *mef*(A/E), *erm*(B), and *erm*(TR) by a multiplex PCR method (16) with the primers described previously (16, 34, 39). Separate PCRs were run to differentiate efflux gene subclasses *mef*(A) and *mef*(E) in all *mef*-positive isolates, as well as to detect the presence of *msr*(D). The primers used for the detection of *mef*(A) and *mef*(E) have been described previously (5, 8). A modified primer pair was used for the detection of *msr*(D): 5′-CAGTTGGACGAA GTAACTCTG-3′ (forward primer) and 5′-CTCTTACGTTCTTCCTCTTTC-3′ (5). Testing for the detection of *msr*(D) was performed with 53 randomly selected isolates: 30 isolates with *mef*(E), 12 isolates with *mef*(A), 6 isolates with

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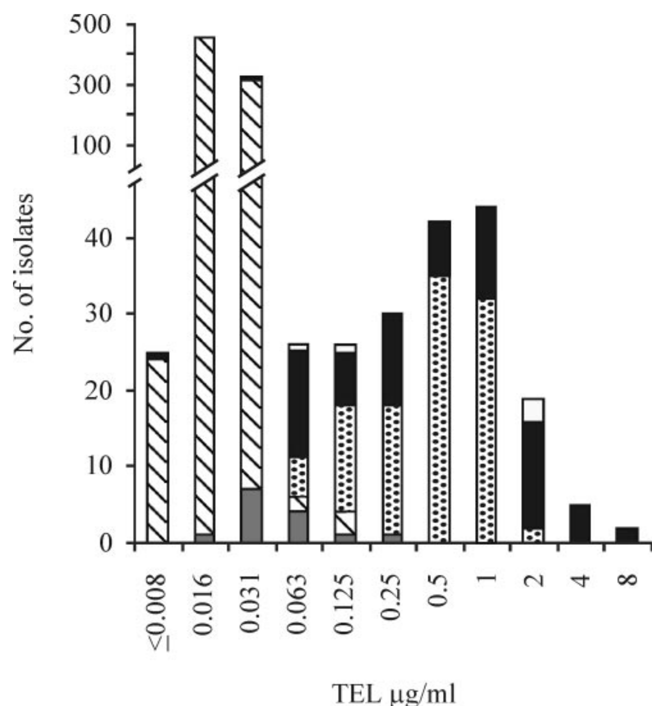

FIG. 1. Distribution of telithromycin MICs of macrolide-susceptible and -resistant pneumococci harboring a macrolide resistance mechanism. Of the susceptible pneumococci, 41 isolates were randomly tested for the presence of a macrolide resistance determinant; none of them carried any macrolide resistance mechanism. Bars with slashes, isolates susceptible to erythromycin and azithromycin (n = 787); gray bars, isolates with mutations or undetermined mechanism (n = 17); spotted bars, isolates with *mef*(A) or *mef*(E) (n = 107); black bars, isolates with *erm*(B) or *erm*(TR) (n = 91); white bars, isolates with double mechanism *erm*(B) and *mef*(E) (n = 5).

erm(B), and 5 susceptible isolates. The PCR run for *mef*(A), *mef*(E), and *msr*(D) included initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for *mef*(A) and *msr*(D) or 58°C for *mef*(E), and elongation at 72°C for 1 min. The magnesium concentration was 1.5 mM. All PCRs were run with a Whatman Biometra thermocycler (Biometra, Goettingen, Germany). Positive and negative controls were included in every run. Ribosomal mutations at positions 2058-2059 and 2611 of domain V of 23S rRNA (*Escherichia coli* numbering) and mutations in genes coding for 50S ribosomal proteins L4 and L22 were sought if the isolate was nonsusceptible to any of the antimicrobials tested and no known resistance gene was present. In addition, mutations were investigated in 13 randomly selected isolates: 7 with *erm*(B), 2 with *mef*(E), 2 with both *erm*(B) and *mef*(E), and 2 that showed a macrolide-susceptible phenotype. Mutations at positions 2058-2059 and 2611 of domain V of 23S rRNA were detected by a pyrosequencing technique (18, 37), and mutations in L4- and L22-coding genes were detected by sequencing (25, 42) with known primers (42). Primers for the detection of mutations at positions 2058 and 2059 have been described previously (18). The following primers were used for the detection of mutations at position 2611: for PCR, primers 5′-TGG GTTCAGAACGTCGTGAGA-3′ (forward primer) and 5′-GCGGTAAGTCC ACTCTGGTC-3′ (reverse primer), and for pyrosequencing, primer 5′-CGTGA GACAGTTCGGTC-3′ (EMBL accession number AE0088386).

RESULTS

The prevalences of erythromycin, azithromycin, and clindamycin resistance were 21.5%, 22.3%, and 11.0%, respectively. The proportion of telithromycin-nonsusceptible isolates was not determined because of the lack of breakpoints for the method used here. Based on the telithromycin MICs, two groups of isolates were formed: a major group (83.3% of isolates) with an MIC range of ≤0.008 to 0.063 μg/ml and a minor group (16.7% of isolates) with an MIC in the range 0.125 to 8 μg/ml. All except three isolates in the minor group carried a macrolide resistance gene (Fig. 1). Of the 217 erythromycin-resistant isolates, 95 (44%) had *mef*(E), 12 (6%) had *mef*(A), and 90 (41%) had *erm*(B). Only one isolate had *erm*(TR). Five (2%) isolates carried both the *erm*(B) and the *mef*(E) genes.

TABLE 1. Isolates with ribosomal mutations (including three isolates whose resistance mechanism remained unresolved) and their respective MICs to erythromycin, azithromycin, spiramycin, telithromycin, and clindamycin

Isolate no.	Mutation in 23S rRNA gene, Domain V		Mutation in 50S ribosomal proteins		Resistance gene	MIC (μg/ml)[a]				
	Type and position	No. of mutated alleles total no.	L4	L22		ERY	AZM	SPI	TEL	CLI
45	A2059G	1/4	Wild	Wild		32	>128	>128	0.031	2
560	A2059G	1/4	$E_{30}\rightarrow K$	Wild		8	>128	2	0.031	0.5
561	A2059G	1/4	$E_{30}\rightarrow K$	Wild		32	>128	128	0.031	1
588	A2059G	2/4	$T_{94}\rightarrow I$	Wild		32	>128	>128	0.016	1
904	A2059G	3/4	Wild	Wild		128	>128	>128	0.063	2
1166	A2059G	4/4	Wild	Wild		>128	>128	>128	0.031	4
5	C2611T	4/4	Wild	Wild		0.25	8	4	0.063	4
152	C2611T	4/4	$V_{205}\rightarrow G$	$A_{101}\rightarrow P$		0.125	4	2	0.031	2
522	C2611T	4/4	Wild	Wild		0.125	8	4	0.031	2
48	Wild		$_{68}E_{69}$ insertion	Wild		1	4	4	0.031	0.25
438	Wild		$_{68}GQK_{69}$ insertion	Wild		1	8	32	0.25	0.063
156	Wild		$S_{20}\rightarrow N$	$R_{22}\rightarrow C$		8	32	0.5	0.125	0.125
551	Wild		$S_{20}\rightarrow N$	Wild		2	64	32	0.063	>128
545	Wild		$S_{20}\rightarrow N$	Wild	*mef*(E)	32	128	0.5	2	0.125
837	Wild		$S_{20}\rightarrow N$	Wild	*mef*(E) + *erm*(B)	>128	>128	>128	2	>128
843	Wild		$E_{30}\rightarrow K$	Wild		>128	>128	>128	0.063	>128
354	Wild		Wild	Wild		0.125	8	0.5	0.031	0.125
695	Wild		Wild	Wild		1	4	2	0.031	0.5
965	Wild		Wild	Wild		2	4	1	0.031	0.25

[a] ERY, erythromycin; AZM, azithromycin; SPI, spiramycin; TEL, telithromycin; CLI, clindamycin.

TABLE 2. Macrolide resistance mechanisms and MIC data for erythromycin, azithromycin, spiramycin, telithromycin, and clindamycin

Mechanism (no. of isolates)	MIC (μg/ml)														
	Erythromycin			Azithromycin			Spiramycin			Telithromycin			Clindamycin		
	Range	50%	90%	Range	50%	90%	Range	50%	90%	Range	50%	90%	Range	50%	90%
mef(E) (95)	0.5–>128	8	64	1–>128	32	128	0.125–1	1	1	0.031–2	0.5	1	0.031–0.25	0.125	0.25
mef(A) (12)	16–64	32	64	32–>128	32	128	1	1	1	0.125–0.5	0.5	0.5	0.125–0.25	0.125	0.125
erm(B) (90)	2–>128	>128	>128	2–>128	>128	>128	1–>128	>128	>128	0.008–8	0.25	2	0.125–>128	>128	>128
erm(B) + *mef*(E) (5)	>128			>128			>128			0.063–2	2	>128			
erm(A) (1)	>128			>128			>128			0.25			>128		
Mutation only (14)[a]	0.25–>128	8	>128	4–>128	32	>128	0.5–>128	32	>128	0.016–0.25	0.031	0.125	0.063–>128	2	>128

[a] The individual genotypes and phenotypes of mutated isolates are presented in Table 1.

The erythromycin MICs in the *mef*(A)-positive isolates were higher than those in the *mef*(E) isolates ($P = 0.002$, Mann-Whitney U test). *msr*(D) was present in all *mef*-positive isolates tested but not in those with *erm*(B) or in susceptible isolates. Fourteen isolates (6%) in which no other mechanism was found had a mutation in domain V of 23S rRNA or in ribosomal protein L4 or L22 (Table 1). No macrolide resistance mechanisms were detected in susceptible isolates. The resistance mechanism remained unresolved in three isolates (Table 1). MIC data for isolates with different resistance mechanisms are summarized in Table 2.

DISCUSSION

The prevalence of macrolide resistance among pneumococci in Finland doubled between 1999 and 2002 (www.ktl.fi/extras/fire). Currently, there are no signs that this worrying trend is slowing, despite recommendations to avoid the overuse of macrolides, and more effective measures such as encouraging the use of vaccines should be considered.

In this study we used a 5% CO_2 supplement to confirm the proper growth of resistant isolates (15). The CO_2 supplement may elevate macrolide, ketolide, and clindamycin MICs (6, 15). Despite the CO_2 supplement, the results of this study can be considered reliable since 99% of the isolates with erythromycin MICs $\geq$0.05 μg/ml harbored a macrolide resistance determinant or had a mutation, thus reflecting the resistance category and genotype well. Moreover, none of the susceptible isolates carried macrolide resistance genes or mutations.

Two groups of pneumococci were formed on the basis of telithromycin MICs: a highly susceptible major group and a minor group of isolates in which the presence of macrolide resistance genes was associated with elevated telithromycin MICs. Nevertheless, the MICs were not constant among isolates with the same macrolide resistance determinant. This was especially true for *erm*(B)-positive isolates. It is not yet clear why some isolates carrying the same macrolide resistance determinant are fully susceptible to telithromycin but others are not. It may also be possible that true telithromycin resistance in pneumococci evolves in macrolide-resistant isolates that have a moderately elevated MIC to telithromycin.

The proportions of different macrolide resistance determinants recorded here were similar to those from a previous Finnish study on invasive pneumococci (33) and resemble those in North America and Scotland, where the efflux mechanism is the most prevalent (2, 13, 21). This is in contrast to the situation in Europe, where *erm*(B) dominates (7, 21, 29, 30). There have recently been reports of pneumococci carrying a double mechanism, both *erm*(B) and *mef*(E) (3, 14, 28). The spread of similar strains is considered of great concern, since they are often multiresistant and are clonally related (14, 28). In a recent report on the global situation, the prevalence of isolates having both the *erm*(B) and the *mef*(E) genes was 7% (14). In our study, only 2% of isolates carried a double mechanism; and in those isolates, the *mef*(E) subtype was always present together with *erm*(B).

mef(A) and *mef*(E) have 90% similarity at the nucleotide level and are considered variants of the same gene, *mef*(A) (36); but because they are carried in different genetic elements in pneumococci, they should be differentiated (8). In addition,

there are epidemiological and phenotypic differences between these subtypes (2, 8, 17). For instance, it has been reported that *mef*(A) isolates have higher MICs to erythromycin than *mef*(E) isolates (2). A similar observation was recorded in this study. *mef*(E) is the prevailing efflux gene subtype in the United States, Asia, and South Africa (5) and, according to this study, also in Finland. *mef*(A) has been more frequently reported in other parts of Europe (2, 5, 29, 32).

The proportion of isolates with mutations in this study was relatively high (6%) compared to that indicated in a recent report on the global prevalence (10). The most frequent mutation in our study was an A2059G change in domain V of 23S rRNA, which has been reported to be one of the most common mutations in pneumococci (9, 10). An A2059G transition leads to modification at the erythromycin binding site, which causes resistance to 14-, 15-, and 16-membered macrolides and elevated MICs to clindamycin but not to telithromycin (12, 41, 42). Position C2611 is another common site where mutations have been found in the pneumococcus (10, 42). The C2611U mutation has been described in laboratory strains of pneumococci obtained after serial passage on azithromycin or clindamycin (4), and it was only recently found in clinical isolates of *S. pneumoniae* (9). In our study, the isolates with this mutation shared a similar phenotype, being susceptible to erythromycin but not to azithromycin or clindamycin. The MICs to these agents were only slightly elevated, however.

Six new mutations that, to the best of our knowledge, have not previously been described were found in this study. Two of these mutations ($_{68}E_{69}$ and $_{68}GQK_{69}$ insertions) were located in the highly conserved region $_{63}LPWRQKGTGRAR_{74}$ of the L4 protein, where mutations conferring macrolide resistance have been described previously (9, 35, 42). The possible role of these mutations, as well as the role of other new mutations ($T_{94}I$ and $V_{205}G$ in L4 or $R_{22}C$ and $A_{101}P$ in L22), in conferring macrolide resistance awaits experimental confirmation.

In conclusion, the level of erythromycin resistance is increasing in Finland. The dominant macrolide resistance mechanism is an efflux mechanism caused by either *mef*(E) or *mef*(A). Although telithromycin has good activity against pneumococci, the significance of macrolide-resistant isolates having an elevated MIC to telithromycin should be further investigated.

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