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Identification of an *erm*(A) Erythromycin Resistance Methylase Gene in *Streptococcus pneumoniae* Isolated in Greece

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In a serotype 11A clone of erythromycin-resistant pneumococci isolated from young Greek carriers, we identified the nucleotide sequence of *erm*(A), a methylase gene previously described as *erm*(TR) in *Streptococcus pyogenes*. The *erm*(A) pneumococci were resistant to 14- and 15-member macrolides, inducibly resistant to clindamycin, and susceptible to streptogramin B. To our knowledge, this is the first identification of resistance to erythromycin in *S. pneumoniae* attributed solely to the carriage of the *erm*(A) gene.

Resistance of *Streptococcus pneumoniae* to erythromycin and the other macrolides is increasing in many parts of the world (1, 5, 7, 18). Strains resistant to erythromycin are also resistant to azithromycin, clarithromycin, and roxithromycin (25). Recently, it has been shown that pneumococci resistant to erythromycin have mainly one of two distinct resistance determinants, *erm*(B) or *mef*(A) (15, 17, 19, 20, 23; A. Tait-Kamradt, T. Davies, F. Brennan, F. Depardieu, P. Courvalin, J. Duignan, J. Petitpas, L. Wondrack, M. Jacobs, P. Appelbaum, and J. Sutcliffe, Addendum Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. LB-8, p. 15, 1999). *mef*(A) encodes an efflux pump that appears to be specific for 14- and 15-member macrolides. The remainder of the resistant strains carry an *erm*(B) methylase. In this case, an adenine residue in 23S rRNA is methylated, leading to reduced binding of 14-, 15-, and 16-member macrolides, lincosamides, and streptogramin B (MLS_B) to their shared target site in the 50S ribosomal subunit. *erm* synthesis can be inducible or constitutive.

The nasopharynx is the main reservoir of antibiotic-resistant pneumococci in children, and carriage usually precedes infection (11). From 10 February 1997 to 10 February 1999, nasopharyngeal cultures for *S. pneumoniae* were performed for 2,448 Greek infants and toddlers who were enrolled in the Hellenic Antibiotic-Resistant Respiratory Pathogens Study. Children 2 to 23 months of age were enrolled from the outpatient clinics of four hospitals, as well as from the private offices of 14 practicing pediatricians in different areas of central and southern Greece (22). At the time the nasopharyngeal culture was obtained, the children were healthy and were brought to the pediatrician to be vaccinated or had signs and symptoms of an acute respiratory tract infection. Isolation, identification, susceptibility testing, and serotyping of the *S. pneumoniae* strains were performed as described previously (21, 22). Of a total of 781 pneumococcal isolates recovered from the 2,448 children studied, 137 (18%) were erythromycin resistant, with 67.9% of them carrying the *erm*(B) gene and 29.2% having *mef*(A) gene products (22). In 4 (2.9%) of the 137 erythromycin-resistant pneumococcal isolates, neither the *erm*(B) gene nor the *mef*(A) gene was identified. The present study was undertaken to investigate the phenotype, genotype, and mechanism of resistance of isolates carrying neither *erm*(B) nor *mef*(A).

The susceptibility of the four erythromycin-resistant *S. pneumoniae* isolates that carried neither *erm*(B) nor *mef*(A) to erythromycin, azithromycin, josamycin, streptogramin A and B, penicillin, and tetracycline was tested. MICs were determined in ambient air in microtiter trays with Mueller-Hinton broth supplemented with 2.5% lysed horse blood following recommendations by the National Committee for Clinical Laboratory Standards (12). All compounds were purchased from Sigma or made by published methods at Pfizer, Inc. Double disk diffusion analysis was performed as previously described (19). Induction was present when the zone of inhibition around the clindamycin or streptogramin B disk was blunted on the side next to the erythromycin disk.

Determination of erythromycin resistance mechanisms. Primers for internal regions of *erm*(A), *erm*(B), *erm*(C), *erm*(TR), *msr*(A), *mef*(A), *mph*(A), *mph*(B), *ere*(A), and *ere*(B) have been described previously (20, 24). Primers designed from the *S. pyogenes erm*(TR) sequence (16) to amplify the entire class A gene were also used in this study: 5′-AAGATTAGTTCAT TATAACC-3′ [−38 to −18 bp upstream of the start codon for *erm*(TR)] and 5′-TTATTGAAATAATTTGTAAC-3′ [anneals to the terminal 20 bases of *erm*(TR)]. Primers for *mph*(C) are based on the sequence of a putative macrolide phosphorylase from *Staphylococcus aureus* clinical strains (10; J. Cheng, T. Grebe, L. Wondrack, P. Courvalin, and J. Sutcliffe, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 837, p. 114, 1999) and are described in reference 24. Amplified PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, Calif.) and sequenced on an ABI 373XL automated sequencing apparatus with stretch upgrade (PE Biosystems, Foster City, Calif.) as described previously (24).

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TABLE 1. Origin of the *Streptococcus pneumoniae* isolates carrying the *erm*(A) gene[a]

Strain no.[b]	Date of isolation	Age (mo)	No. of siblings (age in yr)	Clinical condition
16	22 February 1997	11	2 (5 and 9)	Healthy
96	12 April 1997	16	2 (16 and 19)	Acute otitis media
215	31 October 1997	13	1 (4)	Acute otitis media
357	28 January 1998	16	None	Healthy

[a] Note that none of the children attended a day care center.
[b] All strains were serotype 11A.

Sequence comparisons were carried out with Vector NTI sequence analysis software (InforMax, Inc., North Bethesda, Md.).

Genotypic analysis of erythromycin-resistant *S. pneumoniae*. Molecular analysis of the genotype of the four erythromycin-resistant *S. pneumoniae* isolates that carried neither *erm*(B) nor *mef*(A) was performed by pulsed-field gel electrophoresis (PFGE) as described previously (13).

Presence of the *erm*(A) gene in erythromycin-resistant pneumococci. Genomic DNA from the four resistant isolates which possessed neither *erm*(B) nor *mef*(A) was isolated and subjected to PCR analysis with primers specific for macrolide esterases [*ere*(A) and *ere*(B)], phosphotransferases [*mph*(A), *mph*(B), and *mph*(C)], an ABC-binding transporter [*msr*(A)], and rRNA methylases [*erm*(TR), *erm*(A), and *erm*(C)] (16, 19, 20, 23, 24). Each isolate had a PCR product only when primers specific for the *erm*(TR) determinant were used. The use of primers encompassing the entire *erm*(TR) gene plus 38 bases upstream revealed that the nucleotide sequences from the four pneumococci were identical to the *erm*(TR) gene from a clinical strain of *Streptococcus pyogenes* (16). However, based on a recent classification of the MLS_B resistance genes, *erm*(TR) has been assigned to class A as an *erm*(A) determinant (15).

The four *S. pneumoniae* isolates carrying the *erm*(A) gene were recovered from the nasopharynges of four children during an 11-month period (Table 1). These children were heavily colonized with pneumococcus, because colony counts revealed $> 10^5$ CFU/ml. The four children were living in unrelated parts of the city of Patras and its surroundings in southwestern Greece, and we were not able to identify any close contact among them.

The MIC ranges of the antimicrobial agents tested were as follows: erythromycin, 0.78 to 3.12 μg/ml; azithromycin, 6.25 to 25 μg/ml; josamycin, 0.20 to 0.78 μg/ml; streptogramin A, 25 μg/ml; streptogramin B, 0.78 to 1.56 μg/ml; penicillin G, 0.1; and tetracycline, 6.25 μg/ml. The *erm*(A) pneumococcal isolates were inducibly resistant to clindamycin. Due to the large zone of inhibition around the erythromycin disk for the *erm*(A) strains, it was necessary to increase the spacing between disks beyond 12 to 16 mm to adequately identify blunting.

Molecular analysis by PFGE showed that the four serotype 11A *erm*(A) strains had a clonal relationship sharing an identical genotype. The PFGE patterns of two serotype 11A pneumococci are shown in Fig. 1.

To our knowledge, this is the first identification of resistance to erythromycin in *S. pneumoniae* attributed solely to carriage of the *erm*(A) gene. There has been one report of an erythromycin-resistant *S. pneumoniae* strain, which carried *erm*(A) [subclass *erm*(TR)] in addition to the *erm*(B) gene (2). *erm*(A) is an erythromycin resistance methylase gene which was recently described as *erm*(TR) in *S. pyogenes* strains in Finland (9, 16). Other studies have expanded the finding of *erm*(A)$^+$ strains of *S. pyogenes* to Greece (our unpublished data), Italy (6), France (3), Spain (14), and Canada (4). In addition, the majority of group G, but not group C, streptococci, harbor *erm*(A) (8).

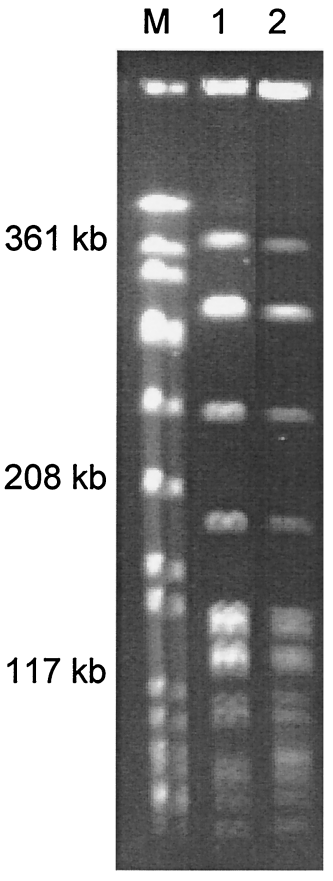

FIG. 1. *Sma*I PFGE patterns of erythromycin-resistant pneumococci carrying the *erm*(A) gene. Lane 1, strain 16; lane 2, strain 215. An *Sma*I digest of genomic DNA from *S. aureus* ATCC 8325 was used as the molecular weight standard (M).

At the level of the clinical laboratory, data from the MIC and disk analysis of strains harboring *erm*(A) could possibly be interpreted as representing an M phenotype (macrolide resistant, but susceptible to clindamycin and streptomycin B), especially since streptogramin B is not routinely used in the disk analysis. The zone sizes for clindamycin in the *erm*(A) strains are intermediate, and the zones around the erythromycin disk can be intermediate. Because of the larger zones, it may be easy to miss the blunt that occurs between the erythromycin and clindamycin zones. The intermediate zones for the *erm*(A) strains translate to an equivocal result for clindamycin. However, given that the strain carries a methylase, it is highly likely these strains would be resistant to clindamycin therapy, unlike strains carrying *mef*(A).

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