JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2003, p. 428–431 Vol. 41, No. 1
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.1.428–431.2003

Phenotypes and Genotypes of Erythromycin-Resistant Pneumococci in Italy

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Received 5 August 2002/Returned for modification 23 September 2002/Accepted 6 October 2002

Of 120 erythromycin-resistant pneumococci isolated in Italian hospitals, 39 (32.5%) were M-type isolates, carrying the *mef* gene alone. The *mef* gene was also detected, together with *erm*(AM), in one constitutively resistant isolate and in five isolates of the partially inducible phenotype. Among the 45 *mef*-positive isolates, 25 (55.6%) carried *mef*(A) and 20 (44.4%) carried *mef*(E) as observed from PCR-restriction fragment length polymorphism analysis of a 1,743-bp amplicon. The same result was obtained by a similar method applied to a more common 348-bp amplicon.

Macrolide resistance in *Streptococcus pneumoniae* is due to either target site modification—generally depending on a post-transcriptional modification of 23S rRNA mediated by *erm*-class methylases (39) or less often on mutations in 23S rRNA or ribosomal proteins (7, 37, 38)—or active efflux. In this study, 120 erythromycin-resistant pneumococci were investigated for macrolide resistance phenotypes and genotypes, with emphasis on the discrimination between *mef*(A) and *mef*(E).

Efflux-mediated resistance. The efflux mechanism, which reduces the intracellular antibiotic concentration to subtoxic levels (40), is associated with a resistance pattern (M phenotype) characterized by resistance, among macrolides-lincosamides-streptogramin B (MLS), only to 14- and 15-membered macrolides, usually at a low level (34). M-type resistance is mediated by the *mef* gene, two variants of which are conventionally described: one, *mef*(A), originally discovered in *Streptococcus pyogenes* (5), and the other, *mef*(E), originally discovered in *S. pneumoniae* (36). Considering that the *mef* genes are detected mostly by a PCR method unable to distinguish between the two variants (33) and that *mef*(A) and *mef*(E) have 90% identity (36), they are regarded as a single gene class, designated *mef*(A) (28). However, *mef*(A) and *mef*(E) have recently been shown to be carried by different genetic elements in *S. pneumoniae*, which are inserted at different sites in the chromosome. Both elements carry, adjacent to *mef*, an open reading frame showing homology to the *msr*(A) gene associated with macrolide efflux in *Staphylococcus aureus* (6, 12, 29). Due to a number of important differences in the properties of *mef*(A)- and *mef*(E)-carrying pneumococci, it has been recommended that the distinction between the two genes be maintained (6).

Bacterial strains and typing. The 120 erythromycin-resistant pneumococci studied (for which the MIC of erythromycin was $\geq$1 μg/ml) were independent isolates, collected from several laboratories throughout Italy between 1999 and 2002. All were clinical strains, isolated from a variety of clinical specimens (upper respiratory tract material, sputum, blood, cerebrospinal fluid). Multiple isolates from the same patient were excluded. Strain identification was confirmed in our laboratory by conventional tests such as susceptibility to optochin and solubility in bile and by employing the API system (BioMérieux, Marcy-l'Etoile, France). Serotyping, done by the capsular swelling test using specific antisera from the Statens Seruminstitut, Copenhagen, Denmark, showed that the 120 isolates were distributed over 24 serotypes, with the majority (80 isolates) belonging to five serotypes with more than 10 isolates each (23F, 14, 19A, 19F, and 6B). Typing by random amplified polymorphic DNA (RAPD) analysis as described previously (15) showed that the 120 isolates were distributed over 59 and 53 RAPD types by using primers M13 and ERIC1 (18), respectively.

Macrolide resistance phenotypes. The M phenotype associated with efflux-mediated resistance has been described above. Methylase-mediated coresistance to MLS antibiotics can be expressed either constitutively, with high-level resistance to all MLS antibiotics (cMLS phenotype), or inducibly (iMLS phenotype); most often, however, inducibility is in regard to macrolides, particularly 16-membered ones, but not lincosamides, to which these strains are usually resistant without induction (iMcLS phenotype) (23). The macrolide resistance phenotype was determined for all isolates by the triple-disk (erythromycin plus clindamycin and rokitamycin) test and macrolide MIC induction tests as described previously (23) (Table 1). MICs were determined by the broth microdilution method according to the procedure recommended by the National Committee for Clinical Laboratory Standards (25). Compared with the distribution of strains into macrolide resistance phenotypes reported in previous studies, the rate of cMLS-type isolates rose further, from <2% among the erythromycin-resistant pneumococci isolated from 1996 to 1999 (13) and 12% among those isolated from 1998 to 2000 (23) to 17% in this study of strains isolated from 1999 to 2002. M-type isolates, accounting for approximately one-third of the erythromycin-resistant isolates in this study, were also apparently on the increase compared with rates reported in previous Italian studies and ranging from <10% (21) to 20 to 26% (6, 13, 20, 23). Moreover, we observed (for the first time in our experience) two true iMLS-type isolates, i.e., isolates showing inducibly expressed resistance not only to macrolides but also to clindamycin.

Resistance genes. While efflux-mediated resistance is encoded by the above-mentioned *mef* genes, methylase-mediated

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TABLE 1. Macrolide resistance phenotypes and correlations with erythromycin resistance genes and serotypes in 120 clinical isolates of erythromycin-resistant *S. pneumoniae*

Phenotype of macrolide resistance	No. (%) of strains	Antibiotic[a]	MIC (μg/ml)[b]			No. of strains with gene:			Serotype (no. of strains)
			Range	50%	90%	*erm*(AM)	*mef*(A)	*mef*(E)	
cMLS	20 (16.7)	Erythromycin	64–>128	128	>128	20	1		23F (5), 19F (4), 6B (3), 14 (3), 3 (2), 9A (1), 15A (1), 19A (1)
		Clindamycin	128–>128	>128	>128				
		Clindamycin (ind.)	>128	>128	>128				
		Rokitamycin	4–>128	32	>128				
		Rokitamycin (ind.)	>128	>128	>128				
iMLS	2 (1.7)	Erythromycin	64–>128			2			7F (1), 23F (1)
		Clindamycin	0.06–0.12						
		Clindamycin (ind.)	>128						
		Rokitamycin	≤0.03–0.25						
		Rokitamycin (ind.)	>128						
iMcLS	59 (49.2)	Erythromycin	4–>128	128	>128	59	1	4	19A (11), 23F (10), 19F (9), 6B (6), 3 (5), 6A (3), 10F (3), 14 (3), 10A (2), 23A (2), 9V (1), 15A (1), 20 (1), 33F (1), 36 (1)
		Clindamycin	2–>128	64	>128				
		Clindamycin (ind.)	32–>128	>128	>128				
		Rokitamycin	≤0.03–1	0.25	1				
		Rokitamycin (ind.)	4–>128	>128	>128				
M	39 (32.5)	Erythromycin	2–16	8	16		23	16	14 (14), 23F (2), 19A (2), 3 (2), 6B (1), 15B (1), 33A (1)[c]
		Clindamycin	≤0.03–0.12	≤0.03	0.12				
		Clindamycin (ind.)	≤0.03–0.25	0.06	0.12				23A (3), 23F (3), 1 (2), 6B (1), 9V (1), 10F (1), 11A (1), 12F (1) 18F (1), 19A (1), 35 (1)[d]
		Rokitamycin	≤0.03–0.25	≤0.03	0.25				
		Rokitamycin (ind.)	≤0.03–0.25	≤0.03	0.25				

[a] ind., after induction by pregrowth in 0.05 μg of erythromycin per ml.
[b] 50% and 90%, MICs at which 50 and 90% of isolates, respectively, are inhibited.
[c] Isolates carrying the *mef*(A) gene.
[d] Isolates carrying the *mef*(E) gene.

resistance is usually encoded by the conventional *erm*(AM) gene, which belongs to gene class *erm*(B) (28). Another methylase, mediated by the *erm*(TR) gene, belonging to gene class *erm*(A) (28), was first described (31) and then found to be extensively present (14, 19) in *S. pyogenes*, whereas its presence in *S. pneumoniae* has been reported only occasionally (4, 35). The presence of erythromycin resistance genes was investigated by PCR. Primer pairs specific for the detection of *erm*(AM) and *erm*(TR) were as reported by Sutcliffe et al. (expected amplicon size, 639 bp) (33) and by Seppälä et al. (primers III_8 and III_{10}; expected amplicon size, 208 bp) (31), respectively. Two primer pairs were used to detect the *mef* gene: the one described by Sutcliffe et al. (expected amplicon size, 348 bp) (33), and the one described by Del Grosso et al. [primers MEF3 and MEF4, derived from Tait-Kamradt et al. (36); expected amplicon size, 1,743 bp] (6). All cMLS, iMLS, and iMcLS isolates had the *erm*(AM) gene, and all M-type isolates had the *mef* gene. The latter gene was also detected, besides *erm*(AM), in one cMLS isolate and in five iMcLS isolates (Table 1). No isolate had the *erm*(TR) gene.

Discrimination between *mef*(A) and *mef*(E). *mef*(A) and *mef*(E) were distinguished by PCR-restriction fragment length polymorphism (RFLP) analysis as suggested by Del Grosso et al. (6), i.e., by digesting the 1,743-bp amplicon with restriction endonucleases (New England Biolabs, Beverly, Mass.) *Bam*HI [which has no restriction site in *mef*(E) and one in *mef*(A), generating two fragments of 1,340 and 403 bp] and *Dra*I [which has two restriction sites in *mef*(E), generating three fragments of 782, 711, and 250 bp, and one in *mef*(A) generating two fragments of 1,493 and 250 bp]. In this way, of the 39 M-type isolates, 23 were found to carry *mef*(A) and 16 were found to carry *mef*(E); of the five *mef*-positive iMcLS-type isolates, four carried *mef*(E) and one carried *mef*(A); and the *mef*-positive cMLS-type isolate carried *mef*(A) (Table 1). In additional experiments, we attempted to apply PCR-RFLP analysis to the 348-bp amplicon yielded by the primers described by Sutcliffe et al. (33). Complete overlap with the above-reported results of the discrimination between *mef*(A) and *mef*(E) was obtained by digesting the 348-bp amplicon with *Bam*HI, which had no restriction site in *mef*(E) and one in *mef*(A) generating two fragments of 284 and 64 bp; *Dra*I cut neither *mef*(A) nor *mef*(E) (Fig. 1). Aliquots of 10 μl of the PCR product were digested with 1 U of enzyme following the instructions of the manufacturer.

Correlations between *mef* genes and serotypes. Fourteen of the 23 isolates carrying *mef*(A) belonged to serotype 14, the

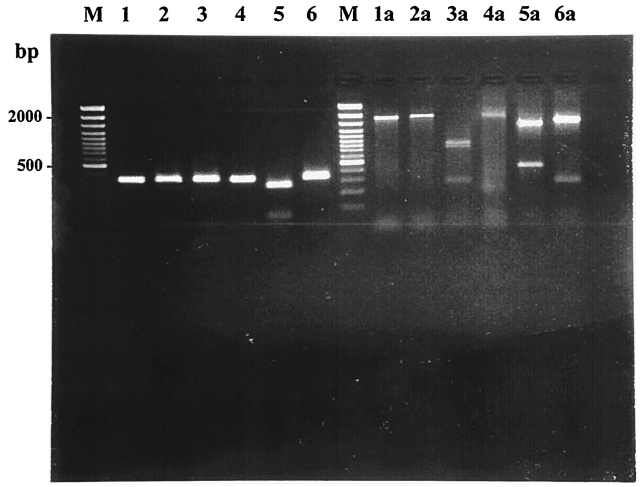

FIG. 1. PCR-RFLP analysis of two M-type erythromycin-resistant pneumococci, one carrying the *mef*(E) gene (lanes 1 to 3 and 1a to 3a) and the other carrying the *mef*(A) gene (lanes 4 to 6 and 4a to 6a). Lanes 1 and 4, PCR amplicon (348 bp) from the *mef* primers designed by Sutcliffe et al. (34); lanes 1a and 4a, PCR amplicon (1,743 bp) from primers MEF3 and MEF4 designed by Del Grosso et al. (6); lanes 2, 5, 2a, and 5a, restriction patterns from *Bam*HI enzyme; lanes 3, 6, 3a, and 6a, restriction patterns from *Dra*I enzyme; lanes M, 100-bp DNA ladder.

remaining 9 being distributed over six other serotypes (Table 1). The 16 isolates carrying *mef*(E) were more scattered (over 11 serotypes), with no more than 3 isolates in each serotype (23A and 23F). In another recent Italian study of 20 M-type pneumococci, all of the 17 isolates carrying *mef*(A) belonged to serotype 14, whereas the 3 isolates carrying *mef*(E) belonged to different serotypes (6).

Distribution of M-type pneumococci. The distribution of phenotypes and genotypes of erythromycin-resistant pneumococci may vary considerably from area to area. As regards M-type isolates, recent Italian rates ranging between one-third (in the present study) and one-fifth (6, 13, 20, 23) of erythromycin-resistant isolates contrast with the complete absence of M-type pneumococci in recent surveys carried out in a country as close as France (1, 10). Rates of M-type isolates similar to those found in Italy have been reported in Greece (35), in a multinational European study (30), in Georgia (United States) (11), and in Taiwan (17); lower rates have been reported in Spain (32), Belgium (8), Central and Eastern European countries (24), and South Africa (22); higher rates have been reported in Germany (27), Japan (26), Canada (16), and in a nationwide study in the United States (9).

***mef*(A) and *mef*(E) genes in M-type pneumococci.** Far fewer data are available to assess the actual and different contributions of the *mef*(A) and *mef*(E) genes to M-type erythromycin resistance in pneumococci. Our finding of a prevalence of *mef*(A) over *mef*(E) is consistent with the results of another recent investigation in Italy (6): in our study, however, the *mef*(A)-to-*mef*(E) ratio was lower, and the *mef*(A)-positive strains were distributed over seven serotypes rather than belonging to serotype 14 only (6).

Conclusions. The custom of designating the *mef* gene as *mef*(A) or *mef*(E) depending on its detection in *S. pyogenes* or *S. pneumoniae* rather than on the basis of specific, molecular differentiation should be reconsidered. The discrimination of *mef*(A) from *mef*(E) by RFLP analysis of the 348-bp PCR fragment (33) using a single endonuclease (*Bam*HI) suggested herein is simple and rapid and should be recommended for future studies of M-type pneumococci. It would be interesting to see whether a similar mixed occurrence of *mef*(A) and *mef*(E) is also found in the M-type strains of *S. pyogenes*, which are usually considered to be associated with the *mef*(A) gene but without any specific attempt at discriminating between the two *mef* variants. In at least one study (2), both *mef*(A) and *mef*(E) have been separately detected in M-type strains of *S. pyogenes*. The same investigators also separately detected both *mef*(A) and *mef*(E) in M-type strains of *Streptococcus agalactiae* (3).

This work was supported in part by a grant from the Italian Ministry of Education, University and Research.

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