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Phenotypic and Molecular Characterization of Tetracycline- and Erythromycin-Resistant Strains of *Streptococcus pneumoniae*

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Sixty-five clinical isolates of *Streptococcus pneumoniae*, all collected in Italy between 1999 and 2002 and resistant to both tetracycline (MIC, ≥8 μg/ml) and erythromycin (MIC, ≥1 μg/ml), were investigated. Of these strains, 11% were penicillin resistant and 23% were penicillin intermediate. With the use of the erythromycin-clindamycin-rokitamycin triple-disk test, 14 strains were assigned to the constitutive (cMLS) phenotype of macrolide resistance, 44 were assigned to the partially inducible (iMcLS) phenotype, 1 was assigned to the inducible (iMLS) phenotype, and 6 were assigned to the efflux-mediated (M) phenotype. In PCR assays, 64 of the 65 strains were positive for the tetracycline resistance gene *tet*(M), the exception being the one M isolate susceptible to kanamycin, whereas *tet*(K), *tet*(L), and *tet*(O) were never found. All cMLS, iMcLS, and iMLS isolates had the erythromycin resistance gene *erm*(B), and all M phenotype isolates had the *mef*(A) or *mef*(E) gene. No isolate had the *erm*(A) gene. The *int-Tn* gene, encoding the integrase of the Tn*916*-Tn*1545* family of conjugative transposons, was detected in 62 of the 65 test strains. Typing assays showed the strains to be to a great extent unrelated. Of 16 different serotypes detected, the most numerous were 23F ($n = 13$), 19A ($n = 10$), 19F ($n = 9$), 6B ($n = 8$), and 14 ($n = 6$). Of 49 different pulsed-field gel electrophoresis types identified, the majority ($n = 39$) were represented by a single isolate, while the most numerous type included five isolates. By high-resolution restriction analysis of PCR amplicons with four endonucleases, the *tet*(M) loci from the 64 *tet*(M)-positive pneumococci were classified into seven distinct restriction types. Overall, a Tn*1545*-like transposon could reasonably account for tetracycline and erythromycin resistance in the vast majority of the pneumococci of cMLS, iMcLS, and iMLS phenotypes, whereas a Tn*916*-like transposon could account for tetracycline resistance in most M phenotype strains.

In *Streptococcus pneumoniae*, tetracycline resistance is predominantly due to ribosomal protection, i.e., the production of cytoplasmic proteins—-encoded by *tet*(M) or, less often, other *tet* genes—capable of interacting with the ribosome and making it insensitive to tetracycline inhibition (3, 38). An efflux-mediated mechanism reducing the intracellular tetracycline concentration to subtoxic levels is less common in streptococci, where this mechanism is due to membrane proteins encoded by the gene *tet*(K) or *tet*(L) (3).

Pneumococcal resistance to macrolides is due to either target site modification or active efflux (40). The former, prevalent mechanism usually depends on a posttranscriptional, methylase-mediated modification of 23S rRNA encoded by the *erm*(B) gene (19, 40). *erm*(B)-associated coresistance to macrolide, lincosamide, and streptogramin B (MLS) antibiotics can be expressed either constitutively, with high-level resistance to all MLS antibiotics (cMLS phenotype), or inducibly (iMLS phenotype). More often, pneumococcal strains appear inducibly resistant to only 16-membered macrolides and constitutively resistant to lincosamides (iMcLS phenotype) (22). Another methylase first described (31) and then found to be extensively present (15, 17) in *Streptococcus pyogenes*, mediated by a gene originally called *erm*(TR) and now designated *erm*(A) according to current nomenclature (30), has only occasionally been reported in *S. pneumoniae* (2, 35). Recently, mutations in 23S rRNA or ribosomal proteins leading to macrolide resistance in clinical isolates of *S. pneumoniae* have also been described (10, 37). A macrolide efflux mechanism, first demonstrated with *S. pneumoniae* and *S. pyogenes* and then with other streptococci, is associated with a resistance pattern (M phenotype) characterized by low-level resistance to only 14- and 15-membered macrolides among MLS antibiotics (34). M phenotype resistance is mediated by *mef* genes, two variants of which, *mef*(A) and *mef*(E)—originally discovered in *S. pyogenes* (4) and *S. pneumoniae* (36), respectively—have 90% identity (36) and have been regarded as a single gene class designated *mef*(A) (30). However, the two variants have recently been shown to be carried by different genetic elements in *S. pneumoniae* (9), and due to a number of important differences in the properties of *mef*(A)- and *mef*(E)-carrying pneumococci (9, 22), it has been recommended that the distinction between the two genes be maintained. A novel erythromycin efflux system, not associated with *mef*(A) or with other known macrolide efflux genes, has lately been described in *erm*(A)-positive strains of *S. pyogenes* with inducible, high-level resistance to erythromycin (14). However, its presence in *S. pneumoniae* and other streptococci has not yet been addressed.

In streptococci, drug resistance determinants occur more frequently on conjugative transposons than on plasmids. In particular, in *S. pneumoniae* the association of erythromycin resistance and tetracycline resistance may be due to Tn*1545* and related conjugative transposons, which encode erythromycin resistance via *erm*(B) and tetracycline resistance via *tet*(M)

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and also kanamycin resistance via *aphA3* (8). These transposons belong to a larger class of conjugative transposons, typically represented by Tn*916*, which encode *tet*(M)-mediated resistance to tetracycline but not resistance to erythromycin or kanamycin (6). An integrase gene usually called *int-Tn*, related to the second of the 24 open reading frames of Tn*916*, is characteristic of the Tn*916*-Tn*1545* family of conjugative transposons (6).

In this study, a collection of clinical *S. pneumoniae* isolates resistant to both tetracycline and erythromycin were typed and investigated for a number of phenotypic and genotypic characteristics inherent to either resistance.

MATERIALS AND METHODS

Bacterial strains. Sixty-five clinical isolates of *S. pneumoniae*, all resistant to both tetracycline (MIC, ≥8 μg/ml) and erythromycin (MIC, ≥1 μg/ml), were tested. All strains, collected from several Italian laboratories between 1999 and 2002, were isolated from upper respiratory tract specimens (the vast majority), sputum, blood, or cerebrospinal fluid. Strain identification was confirmed in our laboratory by conventional tests, such as susceptibility to optochin and solubility in bile, and by the API system (Biomérieux, Marcy-l'Etoile, France).

Antibiotics and susceptibility tests. Tetracycline, erythromycin, minocycline, and penicillin were purchased from Sigma Chemical Co., St. Louis, Mo. Broth microdilution MICs were determined and MIC breakpoints of tetracycline and erythromycin were interpreted as recommended by the National Committee for Clinical Laboratory Standards (24). Mueller-Hinton II broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 3% lysed horse blood was used as the test medium. The inoculum was 5×10^5 CFU/ml, and *S. pneumoniae* ATCC 49619 was used for quality control. Kanamycin susceptibility was determined by a standard agar diffusion test (25) using 30-μg commercial disks (Oxoid Ltd., Basingstoke, United Kingdom) with the following zone diameter breakpoints: susceptible, ≥18 mm; intermediate, 14 to 17 mm; resistant, ≤13 mm.

Determination of the macrolide resistance phenotype. Test strains were assigned to the constitutive (cMLS), the partially inducible (iMcLS), the inducible (iMLS), or the efflux-mediated (M) macrolide resistance phenotype on the basis of the triple-disk (erythromycin plus clindamycin and rokitamycin) test, as described previously (23).

Gene detection by PCR. Tetracycline resistance genes *tet*(K) and *tet*(L) were detected by using the primer pairs described by Trzcinski et al. (39), and *tet*(M) and *tet*(O) were detected by using those described by Corso et al. (7) and Olsvik et al. (26), respectively. Erythromycin resistance genes *erm*(A) and *erm*(B) were detected by using the oligonucleotide primers designated III_8 and III_{10} by Seppälä et al. (31) and the primer pair developed by Sutcliffe et al. (33), respectively. The *mef* gene was detected by using the primer pair described by Sutcliffe et al. (33); *mef*(A) and *mef*(E) were then distinguished by PCR restriction fragment length polymorphism analysis of the 348-bp amplicon with *Bam*HI (New England Biolabs, Beverly, Mass.), which has no restriction site in *mef*(E) and one in *mef*(A), generating two fragments of 284 and 64 bp (22). The integrase gene *int-Tn*, associated with the Tn*916*-Tn*1545* family of conjugative transposons, was detected by using the primer pair described by Poyart-Salmeron et al. (27). DNA preparation and amplification and electrophoresis of PCR products were carried out by adapting established methods (16) to the procedures described for the individual primer pairs.

Serotyping. All isolates were serotyped by the capsular swelling test using specific antisera (Statens Seruminstitut, Copenhagen, Denmark). Serotypes were indicated with conventional capsular type designations.

PFGE. *Sma*I macrorestriction fragment patterns were analyzed by pulsed-field gel electrophoresis (PFGE); macrorestriction and PFGE were performed, and the relevant patterns were analyzed and compared as recently described elsewhere (29). For clusters with at least two isolates, types were designated with lowercase letters in order of size.

HRRA. High-resolution restriction analysis (HRRA) of the *tet*(M) gene was carried out essentially as described by Doherty et al. (12). Briefly, a 10-μl aliquot of the PCR product obtained by using the primer pair described by Corso et al. (7) from each *tet*(M)-positive isolate was digested with the following restriction endonucleases: *Aci*I, *Mse*I, *Rsa*I, and *Taq*I (New England Biolabs). Restriction fragments were separated by agarose (4%) gel electrophoresis and visualized by staining with ethidium bromide. The molecular size marker (100-bp DNA ladder) was from M-Medical Genenco, Florence, Italy. Each restriction pattern yielded by each endonuclease was assigned a number; restriction genotypes were determined on the basis of the combined restriction patterns of all four enzymes and lettered with capitals in order of size.

TABLE 1. Distribution of MICs of tetracycline, erythromycin, and penicillin for 65 tetracycline- and erythromycin-resistant isolates of *S. pneumoniae*

Antibiotic	No. of strains inhibited by MIC (μg/ml) of[a]:											
	<0.12	0.12	0.25	0.5	2	4	8	16	32	64	128	>128
Tetracycline							5	13	15	13	2	17
Erythromycin					1	3	1	5	2	4	7	42
Penicillin	43	5	9	1	5	2						

[a] No strain was inhibited by a MIC of 1 μg/ml.

RESULTS

Antibiotic susceptibility. The distribution of MICs of tetracycline, erythromycin, and penicillin for the 65 tetracycline- and erythromycin-resistant isolates of *S. pneumoniae* is summarized in Table 1. Forty-three strains were susceptible to penicillin (MIC, <0.12 μg/ml), 15 were intermediate, and seven were resistant (the MIC for five of these strains was 2 μg/ml and that for the other two was 4 μg/ml). All the strains but one were resistant to kanamycin according to the results of the disk tests.

Macrolide resistance phenotypes. On the basis of the erythromycin-clindamycin-rokitamycin triple-disk test, 14 of the 65 test strains were assigned to the cMLS phenotype, 44 were assigned to the iMcLS phenotype, 1 was assigned to the iMLS phenotype, and 6 were assigned to the M phenotype. These findings, together with the ranges of MICs of erythromycin, are summarized in Table 2.

Genotypic strain characterization. As shown in Table 3, the tetracycline resistance genes *tet*(K), *tet*(L), and *tet*(O) were not found. In contrast, 64 of the 65 test strains were positive for *tet*(M), the exception being one M phenotype isolate (MIC of penicillin, 0.25 μg/ml; MIC of tetracycline, 16 μg/ml; MIC of minocycline, 0.25 μg/ml; MIC of erythromycin, 16 μg/ml), the only isolate susceptible to kanamycin, which was negative for all four tetracycline resistance genes tested. All cMLS and iMcLS isolates as well as the iMLS isolate had the *erm*(B) gene, and all six M phenotype isolates had the *mef* gene [four with *mef*(E) and two with *mef*(A)]. The *mef* gene was also found in six isolates with the *erm*(B) gene: five [four with *mef*(E) and one with *mef*(A)] of the iMcLS phenotype and one [with *mef*(A)] of the cMLS phenotype. No isolate had the *erm*(A) gene. Of the 59 strains carrying both *tet*(M) and *erm*(B), i.e., all the strains of the cMLS, iMcLS, and iMLS phenotypes, 57 were also positive for the *int-Tn* gene (all ex-

TABLE 2. Macrolide resistance phenotypes of 65 tetracycline- and erythromycin-resistant isolates of *S. pneumoniae*

Macrolide resistance phenotype	No. (%) of strains	Range of MICs (μg/ml) of erythromycin
cMLS	14 (21.6)	≥128
iMcLS	44 (67.7)	4–>128
iMLS	1 (1.5)	>128
M	6 (9.2)	2–16

TABLE 3. Macrolide resistance phenotypes and their association with tetracycline and erythromycin resistance genes, the *int-Tn* gene, serotypes, PFGE types, and restriction types of the *tet*(M) gene in 65 tetracycline- and erythromycin-resistant isolates of *S. pneumoniae*

Macrolide resistance phenotype (*n*)	Resistance gene[a]				*int-Tn* gene	No. of strains	Serotype(s) (no. of strains)[b]	PFGE type(s) (no. of strains)[b,c]	Restriction type of *tet*(M) loci (no. of strains)[b,d]
	tet(M)	*erm*(B)	*mef*(A)	*mef*(E)					
cMLS (14)	+	+	−	−	+	12	23F (3), 19F (3), 14 (2), 3, 6B, 9A, 15A	*b* (2), *d* (2), *c*, Ost (7)	A (4), E (3), G (3), B (2)
	+	+	+	−	+	1	19A	Ost	C
	+	+	−	−	−	1	6B	Ost	A
iMcLS (44)	+	+	−	−	+	38	19A (8), 23F (7), 19F (5), 6B (3), 3 (2), 6A (2), 10A (2), 10F (2), 14 (2), 15A, 33F, 36	*a* (4), *c* (2), *e* (2), *f* (2), *i* (2), *b*, *h*, *j*, Ost (23)	A (22), C (5), D (4), B (3), F (3), E
	+	+	−	+	+	4	6B, 7F, 19F, 23F	*a, b, g, h*	A, B, C, D
	+	+	+	−	+	1	3	*g*	A
	+	+	−	−	−	1	6B	Ost	A
iMLS (1)	+	+	−	−	+	1	23F	Ost	A
M (6)	+	−	−	+	+	4	6B, 11A, 19A, 23F	*j*, Ost (3)	B (3), D
	+	−	+	−	+	1	14	Ost	B
	−	−	+	−	−	1[e]	14	Ost	

[a] *tet*(K), *tet*(L), *tet*(O), and *erm*(A) were not detected in any test strain. +, present; −, absent.
[b] The number of strains is indicated for types represented by more than one strain.
[c] Ost, one-strain type.
[d] Restriction profiles were as follows (different profiles yielded by the same endonucleases are distinguished by subscript numerals): A, *Rsa*I_1, *Mse*I, *Taq*I, *Aci*I_1; B, *Rsa*I_2, *Mse*I, *Taq*I, *Aci*I_2; C, *Rsa*I_2, *Mse*I, *Taq*I, *Aci*I_3; D, *Rsa*I_1, *Mse*I, *Taq*I, *Aci*I_3; E, *Rsa*I_1, *Mse*I, *Taq*I, *Aci*I_4; F, *Rsa*I_2, *Mse*I, *Taq*I, *Aci*I_1; G, *Rsa*I_1, *Mse*I, *Taq*I, *Aci*I_5.
[e] This strain was the only kanamycin-susceptible isolate and was also found to be susceptible to minocycline.

cept a cMLS isolate and an iMcLS isolate). Of the six M phenotype strains, which carried *mef*(A) or *mef*(E) as the only erythromycin resistance determinant, the five with the *tet*(M) gene were also *int-Tn* positive, whereas the isolate lacking *tet*(M) as well as the other tetracycline resistance genes tested was also *int-Tn* negative.

Typing. Sixteen different serotypes, of which 11 were represented by at least two isolates, were detected. The most numerous was 23F ($n = 13$), followed by 19A ($n = 10$), 19F ($n = 9$), 6B ($n = 8$), and 14 ($n = 6$).

Forty-nine PFGE types, of which 10 were represented by at least two isolates, were detected. Type *a*, the most numerous, included five isolates.

The distribution of serotypes and PFGE types among the 65 erythromycin- and tetracycline-resistant *S. pneumoniae* isolates and their associations with the characteristics described above (macrolide resistance phenotypes, tetracycline and erythromycin resistance genes, and the *int-Tn* gene) are summarized in Table 3.

Restriction types of the *tet*(M) gene. With HRRA of allelic variation within *tet*(M), endonucleases *Mse*I and *Taq*I each yielded consistently the same fingerprinting profiles from the PCR amplicons of all strains, whereas *Aci*I and *Rsa*I produced five and two distinct profiles, respectively (Fig. 1). Altogether, the *tet*(M) loci from the 64 *tet*(M)-positive pneumococci analyzed fell into seven restriction types (A to G). Their distribution is reported in Table 3. Over half (31 of 59) of the strains with the *erm*(B) gene (cMLS, iMcLS, and iMLS phenotypes) had restriction type A, whereas four of the five strains with the *mef* gene (M phenotype) exhibited restriction type B. Restriction types A and B are shown in Fig. 2.

DISCUSSION

In clinical isolates of *S. pneumoniae*, tetracycline resistance is frequently associated with erythromycin resistance. In one large U.S. survey carried out from 1999 to 2000, >60% of multiresistant pneumococci exhibiting erythromycin resistance were also resistant to tetracycline (11); in Europe, associations of >80% in erythromycin-resistant pneumococci isolated in Spain (32) and Italy (21) have recently been reported. This association may reflect the widespread presence in pneumococcal populations of transposons, typified by Tn*1545*, thought to result from the insertion over time of resistance determinants, such as *erm*(B) for erythromycin and *aphA3* for kanamycin, into primitive gram-positive conjugative transposons carrying *tet*(M) and the integrase gene *int-Tn*, typified by Tn*916* (3, 6).

Among the 65 tetracycline- and erythromycin-resistant clinical strains of *S. pneumoniae* investigated in this study, the presence of Tn*1545*-like transposons could reasonably account for tetracycline and erythromycin resistance in the vast majority (at least 57 of 59) of the strains of the cMLS, iMcLS, and iMLS phenotypes, i.e., those sharing *tet*(M), *erm*(B), kanamycin resistance, and the *int-Tn* gene. The remaining two isolates shared *tet*(M), *erm*(B), and kanamycin resistance but were *int-Tn* negative. However, *tet*(M) was also associated with *int-Tn* in five of the six strains of the M phenotype, i.e., strains lacking *erm* genes and expressing erythromycin resistance due to *mef*-mediated active efflux. Unlike *erm*(B) in pneumococci with constitutive or inducible MLS resistance, the *mef* gene in M phenotype pneumococci is not known to be linked to tetracycline resistance, which in these strains could be due to a Tn*916*-like transposon. Similar findings—i.e., M phenotype

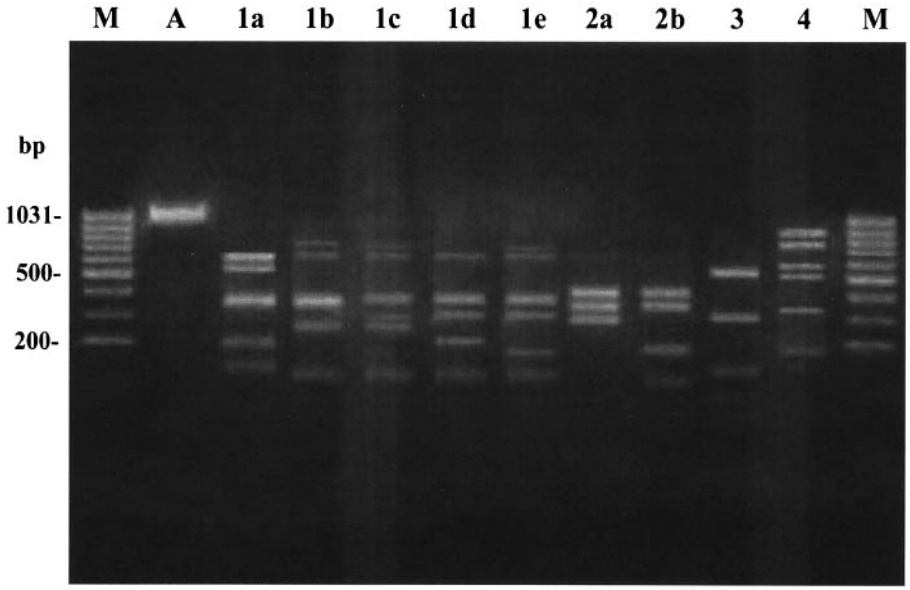

FIG. 1. Different fingerprinting profiles obtained by digesting the *tet*(M) amplicons from 64 *tet*(M)-positive pneumococci with four endonucleases. Lane M, molecular size marker (100-bp ladder). Lane A, undigested *tet*(M) amplicon. Lanes 1a to 1e, different *Aci*I profiles (*Aci*I_1 to *Aci*I_5). Lanes 2a and 2b, different *Rsa*I profiles (*Rsa*I_1 and *Rsa*I_2). Lane 3, *Mse*I profile. Lane 4, *Taq*I profile.

pneumococci carrying *mef*(A) or *mef*(E), *tet*(M), and *int-Tn*—have recently been reported in Spain (32) and Scotland (1). Interestingly, the same *tet*(M) allele (type II) was identified by HRRA in four of our five M phenotype strains carrying *mef*(A) or *mef*(E), *tet*(M), and *int-Tn*. In contrast, the prevalent *tet*(M) allele (type I) of the seven detected in the 64 *tet*(M)-positive pneumococci was identified in isolates of all phenotypes (cMLS, iMcLS, and iMLS) with *erm*(B)-mediated erythromycin resistance but in no strain with *mef*(A) or *mef*(E)-mediated resistance (M phenotype). The sixth M phenotype pneumococcus was the only test strain susceptible to kanamycin and the only one lacking *tet*(M). Since this strain was also negative for *tet*(O), *tet*(K), and *tet*(L), it is possible that some less common *tet* gene capable of conferring tetracycline resistance on streptococci (3, 5) was involved; however, its susceptibility to minocycline suggests an efflux mechanism (3).

It is worth noting that in none of our tetracycline- and erythromycin-resistant *S. pneumoniae* isolates did we detect the *tet*(O) gene, whose presence in this species has occasionally been reported in limited numbers of South African (41), North

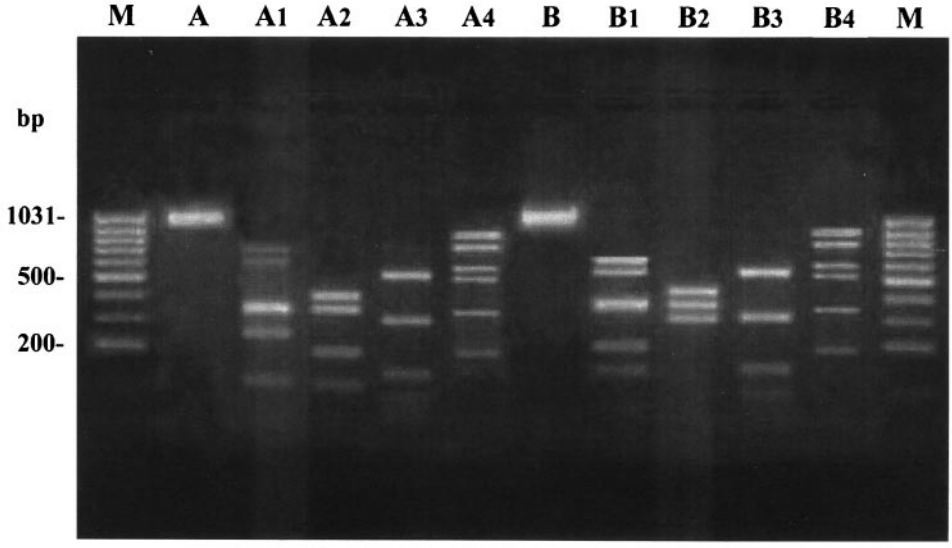

FIG. 2. HRRA patterns of two *tet*(M)-positive pneumococci with restriction types A and B. Lane M, molecular size marker (100-bp ladder). Lane A, undigested *tet*(M) amplicon of the strain exhibiting restriction type A; lanes A1 to A4, restriction profiles yielded by endonucleases *Aci*I, *Rsa*I, *Mse*I, and *Taq*I, respectively. Lane B, undigested *tet*(M) amplicon of the strain exhibiting restriction type B; lanes B1 to B4, restriction profiles yielded by endonucleases *Aci*I, *Rsa*I, *Mse*I, and *Taq*I, respectively.

American (20), and German (28) isolates. Conversely, it has very recently been shown that *tet*(O) largely accounts for tetracycline resistance in tetracycline- and erythromycin-resistant isolates of *S. pyogenes*, in which it is typically associated with *erm*(A) or *mef*(A) (E. Giovanetti, A. Brenciani, R. Lupidi, M. C. Roberts, and P. E. Varaldo, Program Abstr. 12th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. P472, p. 80, 2002). There are, however, few reports of *erm*(A) in *S. pneumoniae* isolates (2, 35); in particular, we have never found this gene in Italian pneumococci. As regards *mef*(A), while only true *mef*(A) is found in *S. pyogenes* isolates (E. Giovanetti and P. E. Varaldo, unpublished results), both *mef*(A) and the *mef*(E) variant can be found in M phenotype erythromycin-resistant pneumococci (9, 22). However, in the case of associated tetracycline resistance, whereas in *S. pyogenes* isolates the *mef* gene is associated with *tet*(O) (Giovanetti et al., 12th, ECCMID), this study shows that in pneumococci it is associated with *tet*(M).

The 65 tetracycline- and erythromycin-resistant *S. pneumoniae* strains tested were distributed over 16 serotypes and 49 PFGE types. These findings, in addition to the other phenotypic and genotypic differences, indicate that the test strains were substantially unrelated. This suggests a spread of resistance due to horizontal transfer of transposons or resistance determinants rather than to clonal dissemination. The most numerous serotypes (23F, 19A, 19F, 6B, 14, etc.) are essentially those that have more frequently been seen to combine macrolide and erythromycin resistance in other studies in Italy (18, 21) and in other European countries (1, 28). The rates of both penicillin-resistant (11%) and penicillin-intermediate (23%) isolates in our collection of tetracycline- and erythromycin-resistant pneumococci were higher than those recently reported among clinical *S. pneumoniae* isolates in Italy (13, 21).

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