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Original article

Prevalence of *mefE*, *erm* and *tet*(M) genes in *Streptococcus pneumoniae* strains from central Italy

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Abstract

One hundred and seventy-three *Streptococcus pneumoniae* strains isolated from surveillance studies conducted in daycare centres were studied. The *mefE*, *erm* and *tet*(M) genes were detected in 16.2, 45.1 and 47.4% of isolates respectively. Agreement between PCR results and antibiotic susceptibility patterns was 100%. Macrolide resistance was due to the presence of *erm* in 73.6% of strains and to the presence of *mefE* in the remaining 26.4%. All tetracycline resistant strains carried the *tet*(M) gene. *erm* was associated with *tet*(M) in 98.7% of strains, whereas no isolate carrying *mefE* carried *tet*(M). A significant association was found between *mefE* and serogroup 6 ($P < 0.0005$) and between *erm* and *tet*(M) and serogroup 19 ($P < 0.00001$).

Keywords: *Streptococcus pneumoniae*; Antibiotic resistance genes; Italy

1. Introduction

Microbial resistance to antibiotics varies considerably throughout the world. Administration of inappropriate drugs, treatment failure, longer hospitalization, increased morbidity and mortality are consequences of the development of antibiotic resistance.

Increasing prevalence of resistance as well as resistance to multiple agents has become widespread in many countries [1–6]. To date many different genes coding for antibiotic resistance have been discovered in pathogenic microorganisms and several of these have been fully characterized. Resistance to macrolides frequently occurs in Gram-positive cocci and it has been shown that a widespread use of these antibiotics can induce a parallel increase in resistance rates in staphylococci and streptococci [7,8]. These antibiotics are frequently used as first line treatment of infections in children and prevalence of resistance varies widely geographically and temporally. For these reasons it is important to understand the genetic mechanisms through which resistance is developed and expressed.

Generally, four different resistance phenotypes to macrolides (M), lincosamides (L) and streptogramins type B (S_B) have been reported so far: MLS_B [8–12], ML [11,13], MS_B [8,12,14] and M [9,10,12] resistance phenotypes. To date neither the ML nor the MS_B phenotypes have been described in streptococci.

The MLS_B phenotype, in which pathogens are resistant to the three groups of antibiotics, is determined by a target modification mechanism, through methylation of the bacterial 23S rRNA. Methylases are encoded by the *erm* genes and have been reported in Gram-positive and -negative bacteria and in several classes of fungi [12,15]. The M-phenotype, in which organisms are resistant to macrolides but susceptible to lincosamides and streptogramins, has been recognised since 1994

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[8,9,14,16]. This resistance is due to the presence of an efflux pump encoded by the *mef* genes, named the *mefE* gene in *Streptococus pneumoniae* [17].

Resistance of *S. pneumoniae* to tetracycline, an antibiotic which was used very extensively at the beginning of the antibiotic era, is also very common and is often associated with *erm* macrolide resistance in Gram-positive and -negative organisms [4,18,19]. Tetracycline resistance is determined by the presence of the *tet* genes and is due to a mechanism which protects the bacterial ribosome. To date, at least fifteen different *tet* genetic determinants have been characterized. The *tet*(M) gene is the most common and has been found in Gram-positive and -negative organisms. Of the *tet* resistance genes only *tet*(M), and rarely *tet*(O), have been reported in *S. pneumoniae* [19,20].

In this surveillance study of antimicrobial susceptibility of *S. pneumoniae*, strains from children attending daycare centres in central Italy, we found erythromycin and tetracycline resistance to be common and the erythromycin-resistant strains to be either MLS_B or M phenotype. As these were the first reports of the presence of the M phenotype in *S. pneumoniae* in Italy, we decided to determine if this was due to the presence of *mefE* gene in these strains. In addition, we also tested for *erm* and *tet*(M) genes to determine the genetic basis of macrolide, lincosamide and tetracycline resistance.

2. Materials and methods

2.1. Source of strains and susceptibility testing

Strains of *Streptococcus pneumoniae* isolated from surveillance studies in children attending daycare centres in central Italy during 1996 and 1997 were used. Naso-pharyngeal specimens were plated onto Columbia agar plates supplemented with 5% sheep blood (Becton Dickinson, Italy). Pneumococci were identified by their inhibition by optochin and bile solubility. Strains were stored at –70°C and subcultured at least twice before further testing. Susceptibility testing based on the NCCLS [21] interpretative criteria was performed using Mueller–Hinton agar plates supplemented with 5% sheep blood. Susceptibility was determined by an agar diffusion method using disks of erythromycin (15 μg), clindamycin (2 μg) and tetracycline (30 μg). Results were read after incubation in 5% CO_2 atmosphere at 37°C for 24 h. Penicillin susceptibility was assessed in oxacillin resistant strains by the E test method (AB Biodisk, Sweden): strains with MICs $\leq$ 0.06 mg/l, 0.12–1 mg/l and $\geq$ 2 mg/l were considered susceptible, intermediate and fully resistant, respectively.

Strains were also serogrouped by capsular swelling using commercially available antisera (Statensserum Institute, Denmark).

2.2. DNA extraction

Isolated strains derived from single colonies were placed in 1 ml of saline and total DNA extracted as follows. Samples were centrifuged at 14 000 rpm at room temperature for 4 min. The pellet was then resuspended in 200 μl 5% Chelex resin (Perkin Elmer Applied Biosystems, UK) and heated at 60°C for 30 min. After vortexing, samples were heated at 100°C for 10 min and finally centrifuged for 4 min at 14 000 rpm. Ten μl of this supernatant was then used for each amplification.

2.3. Amplification protocols

All isolates were tested to detect the presence of the pneumolysin gene by means of PCR in order to confirm they were *S. pneumoniae* strains [22].

For the amplification of each of the three resistance genes we used primers described previously. Specific primers for the detection of the *tet*(M) gene were used [19]. Primers for amplification of both *mefA* and *mefE* genes and a set of conserved primers for simultaneous amplification of several classes of the *erm* gene were used [23,24].

An initial denaturing step at 94°C for 6 min and a final elongation step at 72°C for 12 min were performed for each PCR run. Amplifications for the *mefE* gene were carried out in 40 cycles of three steps: 94°C for 90 s, 52°C for 60 s and 72°C for 60 s: typical PCR products for *mefE* were sized 348 bp. The *erm* gene was amplified under the following conditions: 40 cycles of three steps: 94°C for 75 s, 40°C for 90 s and 72°C for 60 s: PCR products for *erm* genes were sized 530 bp. The conditions used for *tet*(M) were: 35 cycles of three steps: 94°C for 90 s, 56°C for 60 s and 72°C for 90 s. The *tet*(M) gene PCR products were 740 bp long. A negative control with no DNA was included in each PCR run. Amplifications were carried out in a final volume of 50 μl covered with mineral oil. PCR reaction buffers, AmpliTaq Gold DNA polymerase and oligonucleotide primers were provided by Perkin Elmer Applied Biosystems, UK. The final PCR mixture contained: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 200 μM of each of the deoxynucleotides dATP, dCTP, dGTP, dTTP, 30–50 pmol of each primer set, 1.25 U of AmpliTaq Gold. PCR for *erm* and *tet*(M) genes were performed in 1.5 mM $MgCl_2$, whereas amplifications for *mefE* were run in 4.0 mM $MgCl_2$.

Fifteen microlitres of each PCR reaction were electrophoresed on 1.7% agarose gels (Boehringer Mannheim, Germany) containing 0.5 μg/ml ethidium bromide in 0.5 × TBE buffer. PCR products were visualized under UV illumination. Their size was estimated using standard DNA molecular weight marker (φX174 RF DNA cut with *Hae*III, Boehringer Mannheim, Germany).

All isolates were tested twice or more in order to confirm results and all data were analysed using χ^2 and Fischer's exact tests; P values < 0.02 were considered statistically significant.

3. Results

A total of 173 strains were examined. All strains were positive for the pneumolysin gene, thus confirming that they were *S. pneumoniae* [22].

PCR detection of the three resistance genes was as follows (Table 1). *mefE* was detected in 28 strains (16.2%), *erm* in 78 strains (45.1%) and *tet*(M) in 82 strains (47.4%). No strains carrying both *mefE* and *erm* were found. Seventy-seven of seventy-eight strains (98.7%) found to have the *erm* gene had also the *tet*(M) gene, whereas no isolate carrying *mefE* carried also *tet*(M). Comparison of these genotypes with the susceptibility phenotypes of these strains showed 100% agreement between the presence of *erm* and resistance to erythromycin and clindamycin, *mefE* and resistance to erythromycin only and *tet*(M) and resistance to tetracycline.

Of the 78 strains carrying *erm*, 57 were penicillin-susceptible and 21 were -nonsusceptible (4 fully and 17 intermediate resistant), while only one of the 28 *mefE* strains was penicillin-nonsusceptible (intermediate resistant) ($P < 0.02$). Strains carrying *erm* and *tet*(M) genes were very diverse being found in many combinations of resistance to other classes of antimicrobials and serogroups. In contrast, 21 of the 28 strains carrying *mefE* belonged to serogroup 6 resistant only to erythromycin and trimethoprim-sulphamethoxazole (data not shown).

Table 2 shows the distribution of genes among serogroups: of all strains studied, the most frequent serogroups detected were serogroups 6 (43.3%) and 19 (15%); *mefE* was strongly associated with serogroup 6 ($P < 0.0005$), while both *erm* and *tet*(M) were associated with serogroup 19 ($P < 0.00001$).

Table 1
Genotypes and antimicrobial resistance phenotypes detected in 173 *Streptococcus pneumoniae* isolates

Resistance phenotype[a]	Genotype[b]			Number of isolates
	tet(M)	*erm*	*mefE*	
T E C	+	+	−	77
E C	−	+	−	1
T	+	−	−	5
E	−	−	+	28
Susceptible[c]	−	−	−	62

[a] T, tetracycline; E, erythromycin; and C, clindamycin.
[b] +, Presence of the gene; and −, absence of the gene.
[c] Strains susceptible to tetracycline, erythromycin and clindamycin.

Table 2
Association between antibiotic resistance genetic determinants and serotypes

Serotypes	*mefE*	*erm*	*tet*(M)	Total (%)
6	21	38	39	75 (43.3)
9	1	0	0	11 (6.4)
19	0	23	24	26 (15)
23	1	4	4	12 (7)
Other serotypes*	5	13	15	49 (28.3)
Total	28	78	82	173 (100)

* Strains whose serotype was either 11, 14, 33, A, B, D, or pool G; strains in which all tested antisera gave negative results are also included.

4. Discussion

In this investigation molecular tests showed that 16.2, 45.1 and 47.4% strains carried the *mefE*, *erm* or *tet*(M) genes, respectively and these findings were in agreement with the phenotypic characteristics of these strains. Association between genotype and serogroup was also found: serogroup 6 strains were associated with presence of *mefE*, while serogroup 19 was associated with isolates carrying both *erm* and *tet*(M) genes.

Resistance to macrolides has been described during the last decade in many countries. Prevalence of resistance ranged from 2.3 to 6.7% in Austria, Finland, Israel and United Kingdom [5,25–27] to 20–24.5% in Greece, Spain and Hong Kong [4,18,28]. In Hungary macrolide resistance in *S. pneumoniae* rose to 48.5% in the late 1980s [6]. Resistance to tetracycline varied from 3.4 to 8.5% in United Kingdom, Israel, Norway, Finland and Austria [5,25–27,29] to 26% in Greece [18]; much higher rates were reported in Hungary 66.5% [6] and Hong Kong 78.9% [4].

A multicentre study conducted in Italy on *S. pneumoniae* during 1993 showed that only 6% of strains were erythromycin resistant [30]. Increase in macrolide resistance was not confined only to *S. pneumoniae* but was also detected in *S. pyogenes* in Italy, with prevalence rising from 5.1% in 1993 to 25.2% in 1995 [31].

Our findings of 61.3% resistance to macrolides and 47.4% to tetracycline were among the highest ever reported. In this regard it is important to emphasize that our strains were collected exclusively from children attending daycare centres. Prevalence of antimicrobial resistant *S. pneumoniae* has been reported to be significantly higher in children than in adults with 40 versus 24% of strains resistant to penicillin in Hong Kong ($P < 0.05$) [4] and 54.9 versus 30% and 72.2 versus 50% resistant to erythromycin and tetracycline respectively in Hungary ($P < 0.01$) [6].

In our study, the presence in central Italy of strains of *S. pneumoniae* were documented with the new macrolide efflux resistance mechanism which is encoded

for by the *mefE* gene. In particular we found that 73.6% of the 106 macrolide resistant strains was due to the *erm* gene which determines an MLS_B phenotype, whereas only 26.4% carried the *mefE* gene and were M phenotype. These data were similar to those recently reported in South Africa (MLS_B 89%) [32] but very different from those reported by Sutcliffe et al. [16] who found 85% of macrolide resistant *S. pneumoniae* strains to be of the M phenotype. Tetracycline resistance was always due to the presence of the *tet*(M) gene. In agreement with previously reported data we also found that the *erm* gene was almost always (98.7%) associated with the *tet*(M) gene [19,33].

Although in our survey a 100% correlation between phenotypic and genetic characteristics of the strains was found, the confirmation of in vitro microbiology patterns of resistance through detection of their genetic determinants could result in better understanding of the mechanisms of selection and spread of multiresistant pathogenic strains and contribute to the discovery of new resistance mechanisms.

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