Original Article

Phenotypic and genotypic study of macrolide resistance of Streptococcus pneumoniae strains isolated in hospitals in Porto Alegre, in the state of Rio Grande do Sul, Brazil*

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ABSTRACT

Objective: The aim of this study was to determine the prevalence of macrolide-resistant S. pneumoniae and to identify its phenotypic and genotypic characteristics. **Methods:** Strains of S. pneumoniae isolated in the city of Porto Alegre between May 2002 and August 2004 from samples collected from different anatomical sites were analyzed. For the agar diffusion test, disks of erythromycin, clarithromycin, azithromycin and clindamycin were used. The minimum inhibitory concentrations of erythromycin were determined for macrolide-resistant isolates by the agar dilution method. Macrolide-resistant isolates were phenotyped by agar diffusion test and genotyped by polymerase chain reaction. **Results:** A total of 229 pneumococcal strains were evaluated, 12 (5.2%) of which were macrolide-resistant. Among the 12 resistant strains, 9 (75%) presented the MLSB phenotype, and 3 (25%) presented the M phenotype. Polymerase chain reaction testing indicated that 8 MLSB phenotype isolates harbored the ermB gene only, whereas the mefE gene was present in all 3 M phenotype isolates. One MLSB phenotype isolate presented both genes. **Conclusion:** In Porto Alegre, the S. pneumoniae resistance to macrolides is still low since such resistance is due primarily to the presence of the ermB gene expressing the MLSB phenotype.

Keywords: Pneumococcal infections/drug therapy; Respiratory tract infections/drug therapy; Streptococcus pneumoniae/isolation & purification; Drug resistance, bacterial; Macrolides/pharmacology; Anti-bacterial agents/pharmacology; Microbial sensitivity tests; Erythromycin/pharmacology; Genotype; Phenotype

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INTRODUCTION

With the growth of the Streptococcus pneumoniae resistance to penicillin over the last decades, the use of alternative drugs such as macrolides has become necessary.[1] However, a significant increase in pneumococcal resistance to this antimicrobial class has also recently been observed in various countries.[2-4]

Macrolide resistance in S. pneumoniae is due to two main mechanisms: that of modification of the binding site of the drug and that of active efflux. The first occurs due to the acquisition of the ermB gene, which confers resistance to the macrolides, lincosamides and streptogramin B. Therefore, this multiple-resistance phenotype is designated resistance to macrolides, lincosamides and streptogramin B (MLSB). The mechanism of resistance linked to the active efflux is associated with the mefE gene, which confers resistance only to 14- and 15-membered macrolides (erythromycin, clarithromycin and azithromycin), and is therefore designated the M phenotype.[5-7]

Variations in the prevalence of these genes and mechanisms of macrolide-resistance have been observed among isolated pneumococcal strains in different regions of the world.[8-9] The objective of this study was to investigate the occurrence, the molecular mechanisms and the phenotypic expression of macrolide-resistance in samples collected at a number of hospitals in the city of Porto Alegre, in the state of Rio Grande do Sul, Brazil.

METHODS

Initially, 259 samples of S. pneumoniae isolated from several clinical specimens were analyzed. These samples were collected between May 2002 and August 2004 at the hospitals involved in the study: Hospital Mãe de Deus, Hospital São Lucas of the Pontifícia Universidade Católica of Rio Grande do Sul, Hospital Nossa Senhora da Conceição, Irmandade Santa Casa de Misericórdia de Porto Alegre and Hospital de Clínicas de Porto Alegre.

The identification of the samples was confirmed by determining the type of hemolysis, colony morphology and susceptibility to optochin.[10]

All of the clinically isolated strains were submitted to the agar diffusion test (ADT), using 5-µg erythromycin disks (Oxoid Ltd., Basingstoke, England), 2-µg clarithromycin disks (Oxoid Ltd.), 15-µg azithromycin disks (Oxoid Ltd.) and 2-µg clindamycin disks (Oxoid Ltd.) a sterile swab was used to seed the bacterial suspension onto the surface of a Mueller-Hinton agar plate supplemented with 5% sheep blood (MHS; bioMérieux, Marcy l´Etoile, France). The erythromycin, clarithromycin, azithromycin and clindamycin disks were placed on the surface of the culture medium with the aid of sterilized forceps. The plates were incubated at 35°C for 20 to 24 h in an atmosphere of CO_2. The MLSB phenotype was characterized by no inhibition halos around the macrolide or clindamycin disks. When no inhibition halos were observed around the macrolide disks and there was no sensitivity halo around the clindamycin disk, the culture was classified as presenting the M phenotype.

Samples determined to be macrolide-resistant in the ADT (triage test) were tested for minimum inhibitory concentrations (MICs) using the agar dilution test for erythromycin. Aliquots of 2 mL of each antimicrobial dilution and 1 mL of defibrinated sheep blood were added to 17 mL of Mueller-Hinton medium. Mediums containing known concentrations of erythromycin (0.25-8 µg/mL) were prepared. The plates containing different concentrations of the antimicrobial agent were cultured with the aid of a Steers replicator using a S. pneumoniae suspension diluted to 1:10 in sterile saline solution. The final inoculate deposited on the surface of the plates was approximately 104 colony forming units/mL. The plates were incubated at 35°C for 20 to 24 h in an atmosphere of 5% CO2. The results related to the collected MICs were interpreted according to the guidelines established in 2004 by the National Committee for Clinical Laboratory Standards.[11]

The phenotypically-resistant samples were tested by polymerase chain reaction for ermB and mefE genes, according to the protocol introduced by Sutcliffe et al.[12] To confirm the specificity of the polymerase chain reaction, phenotypically-susceptible pneumococcal samples, randomly selected from among those found to be susceptible to macrolides in the ADT, were also tested.

The DNA extraction was carried out by removing S. pneumoniae colonies from the culture medium, resuspending them in 300 µL of phosphate-buffered saline and then centrifuging them at 3000 rpm for 15 min. The supernatant was set aside and the sediment was used for DNA extraction. The sediment

was resuspended in 50 µL of 1x Tris-EDTA (TE) buffer at pH 7.4, incubated for 10 min at 37°C and at 100°C for 3 min. The samples were stored at -20°C until use (one to three days).

Polymerase chain reaction was carried out in total volume of 50 L, containing 200 µM of deoxynucleotide triphosphates (dATP, dCTP, dTTP and dGTP), 1.4 µM of initiator (direct ermB: 5' - GAA AAG GTA CTC AAC CAA - 3'; reverse ermB: 5' - AGT AAC GGT ACT TAA ATT GTT - 3'; direct mefE: 5' - AGT ATC ATT AAT CAC TAG TGC - 3'; reverse mefE: 5 '-TTC TTC TGG TAC TAA AAG TGG - 3'), 0.2 U of Taq DNA polymerase, 10 mM of Tris-HCl (pH 8.3), and 1 µL of the extracted DNA. A 2-mM concentration of magnesium chloride was used for detection of the ermB gene, and a 4-mM concentration of the same was usedfor detection of the mefE gene.

RESULTS

Of the 259 samples initially received from the private hospitals participating in the study, 30 were excluded due to bacterial death. The remaining 229 S. pneumoniae samples came from diverse specimens such as blood (80 samples), sputum (75 samples), liquor (20 samples), pleural fluid (15 samples), tracheal aspirate, (12 samples), ocular fluid (11 samples) bronchial fluid (7 samples) and other (9 samples).

All of the clinically isolated strains were submitted to the ADT, using disks impregnated with antimicrobials (erythromycin, clarithromycin and azithromycin). Results of the macrolide susceptibility tests are presented in Table 1.

TABLE 1

Susceptibility of the clinically-isolated Streptococcus pneumoniae strains to erythromycin, azithromycin and clarithromycin in the agar diffusion test

Antibiotic	Number of samples (%)	
	Sensitive	Resistant
Erythromycin	217 (94.8)	12 (5.2)
Azithromycin	217 (94.8)	12 (5.2)
Clarithromycin	217 (94.8)	12 (5.2)

TABLE 2

Distribution of erythromycin minimum inhibitory concentrations in the twelve Streptococcus pneumoniae isolates found to be erythromycin resistant in the agar dilution test

Antibiotic	MIC (µg/ml)					
Erythromycin	0.25	0.5	1.0	2.0	4.0	>8.0
Number of samples	-	1	-	-	2	9

MIC: minimum inhibitory concentration

The clinically isolated strains presenting resistance in the ADT were also submitted to the agar dilution test in order to determine the erythromycin MIC. The results are presented in Table 2.

Of the 12 isolates that demonstrated resistance to erythromycin in the ADT for macrolides and clindamycin, 9 (75%) presented the MLSB phenotype, and 3 (25%) presented the M phenotype.

We found 8 samples that tested positive for the ermB gene, 3 that tested positive for the mefE gene and 1 that tested positive for both. Figure 1 presents the results of the amplification of five S. pneumoniae isolates.

A correlation was found between phenotypes and

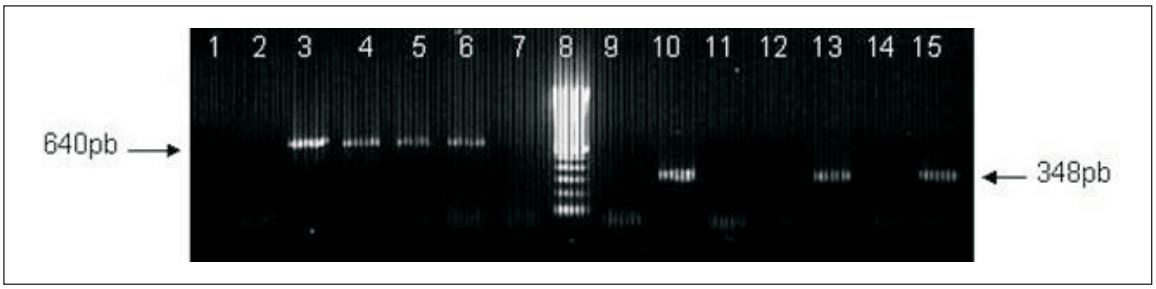

Figure 1 - Agarose gel electrophoresis of polymerase chain reaction-amplified products of the ermB and mefE genes in macrolide-resistant Streptococcus pneumoniae samples - Negative control: lanes 1 and 9; positive control for the mefE gene: lanes 2 and 10; positive control for the ermB gene: lanes 3 and 11; positive sample for the ermB gene alone: lanes 4 and 12; positive sample for ermB and mefE genes: lanes 5 and 13; positive sample for the ermB gene alone: lanes 6 and 14; positive sample for the mefE gene alone: lanes 7 and 15; 100-bp marker of molecular weight: lane 8.

resistance genotypes, that is, the mefE gene samples presented, in most cases, the M phenotype, and the ermB gene samples presented the MLSB phenotype.

DISCUSSION

The appearance and dissemination of penicillin-resistant and macrolide-resistant S. pneumoniae strains has caused increasing concern worldwide. Considerable geographic variations in this resistance, both genotypic and phenotypic, have been observed, and monitoring its local patterns is fundamental to providing the most specific antimicrobial treatment appropriate for use in each region.

In our study, phenotypical S. pneumoniae macrolide resistance, as determined through use of the ADT, was 5.2% for erythromycin, azithromycin and clarithromycin. This prevalence of resistance is similar to that determined in the only study previously carried out exclusively in Porto Alegre, in which 417 pneumococcal samples, isolated from 1995 to 1998, were analyzed, and a 4.5% prevalence of erythromycin resistance was found.[13] Similar results were found in another study, in which samples were collected in Porto Alegre, São Paulo (in the state of São Paulo) and Rio de Janeiro (in the state of Rio de Janeiro) from 1990 to 1999. In the 931 S. pneumoniae isolates, a 4.3% prevalence of resistance to erythromycin was observed.[14] In the PROTEKT study, carried out from 1999 to 2000, which included various Latin American countries (Argentina, Brazil and Mexico), the prevalence of erythromycin resistance found was 15.3%, 6.5% of which came from the Brazilian samples.[2] A more recent study, conducted during 2001 and 2002, demonstrated S. pneumoniae resistance rates of 9.5% for azithromycin and clarithromycin in samples collected in several Brazilian states.[15]

Studies carried out in other regions of the world have shown considerably higher resistance rates than that seen in Brazil. In the Asian Network for Surveillance of Resistant Pathogens project, which was carried out from 1998 to 2001, involving ten Asian countries and 555 isolates, erythromycin resistance was 59.3%.[8] Resistance to macrolides was also assessed in Germany from 2002 to 2003 in a study analyzing 241 pneumococcal samples, and 19.9% of strains were found to be resistant.[9] In another survey conducted as part of the PROTEKT study, 46 American states participated, a total of 10,102 samples were evaluated, and a similar (27.9%) prevalence of resistance was found.[4]

In our study, 9 (75%) of the 12 samples found to be macrolide resistant in the ADT presented the MLSB phenotype and 3 (25%) presented M phenotype. In a study of pneumococcal samples isolated in several Brazilian cities, 40 (4.3%) of the 931 samples analyzed were found to be erythromycin resistant.[14] Of those 40 samples, 37 (92.5%) presented the MLSB phenotype, and 3 (7.5%) presented the M phenotype. When the authors analyzed only the 13 samples isolated in Porto Alegre, 2 (15.3%) were found to present the M phenotype. Therefore, the Porto Alegre prevalence of the M phenotype found by those authors was similar to that found in our study.

When the erythromycin MIC was assessed in the macrolide-resistant samples, 9 samples presented an MIC of 8 g/mL, 2 presented an MIC of 4 g/mL, and 1 sample presented an MIC of 0.5 g/mL. All of the samples with an MIC > 8 g/mL presented the MLSB phenotype, and the samples with MICs of 4 g/mL or 0.5 g/mL presented the M phenotype. The erythromycin MIC also correlated with the presence of the genes tested, that is, the ermB gene samples presented MICs of 8 g/mL, and the mefE gene samples had lower MICs, ranging from 0.5 g/mL to 4 g/mL. This correlation was also found in a study of 124 erythromycin-resistant samples from Christchurch, New Zealand, in which 117 (94.3%) presented high erythromycin-resistance (MIC of 128 g/mL), and 7 presented MICs between 4 g/mL and 8 g/mL.[16] When the authors determined the presence of genes in these samples and correlated this presence with the erythromycin MIC, 41 ermB gene samples presented a MIC of 128 g/mL, and 6 mefE gene samples presented MICs between 4 g/mL and 8 g/mL. Of the 77 isolates containing both genes, 76 presented a MIC of 128 g/mL. In our study, the isolate that contained both genes also presented a high level of resistance to erythromycin.

In a study carried out in Turkey, 45 (13.8%) of the 326 pneumococcal samples analyzed were found to be resistant to erythromycin.[17] Of those 45 samples, 39 (87.5%) contained the ermB gene, and 6 (12.5%) contained the mefE gene. In a survey conducted in the USA as part of the PROTEKT study, 2793 erythromycin-resistant pneumococcal samples were analyzed from 2001 to 2002.[4] The authors observed that 68.7% of the isolates

contained the mefE gene, 16.8% contained the ermB gene, and 12.2% contained both genes. In a study of samples collected in Brazil from 1990 to 1999, 40 erythromycin-resistant pneumococcal isolates were found, of which 92.5% contained the ermB gene, and 7.5% contained the mefE gene.[14]

Regarding the genotypic analysis carried out in the 12 isolates which were phenotypically resistant to macrolides, our results show that 8 samples (66.6%) contained the ermB gene, 3 (25%) contained the mefE gene, and 1 (8.33%) contained both genes.

Samples containing both genes (ermB and mefE) have been isolated in some countries. For example, in a study carried out in the USA between 1996 and 1997, a prevalence of 7% was found.[18] Serotype 19F, a multiresistant clone containing both genes, was found in 30.5% of the isolates from five laboratories in South Africa.[19] Of the 1043 macrolide-resistant isolates analyzed in the PROTEKT study between 1999 and 2000, most of them from South Korea, 71 (6.8%) were ermB- and mefE-positive.[20] In Brazil, the present study was the first to identify a S. pneumoniae isolate which contained both ermB and mefE genes.

In our study, we observed that there was a correlation between phenotypic and genotypic resistance, that is, the 8 isolates presenting the MLSB phenotype contained the ermB gene and the 3 isolates presenting the M phenotype contained the mefE gene. The isolate which tested positive for both genes presented the MLSB phenotype as evidenced by the clindamycin resistance conferred by the ermB gene. This was also observed in another study, in which the 37 samples containing the ermB gene presented the MLSB phenotype, and the 3 samples containing the mefE gene presented the M phenotype.[14]

The study of genotypic resistance is extremely relevant since, in the isolates containing the mefE gene, it is possible to use the macrolides clinically, even in the cases of samples presenting in vitro resistance, whereas, in the samples containing the ermB gene, due to the high level of in vitro resistance, treatment failure might occur. Therefore, continuous monitoring of the local resistance patterns, through epidemiologic surveillance studies, is essential in order to avoid the indiscriminate use of antimicrobial agents and the subsequent growth and dissemination of resistance.

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