

Received: 2015.11.19
Accepted: 2015.12.11
Published: 2016.08.02

Drug Resistance Characteristics and Macrolide-Resistant Mechanisms of *Streptococcus pneumoniae* in Wenzhou City, China

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Source of support: This work was supported in part by a grant from the Science and Technology Development Planning Funds (#10275; #153050) of Jiaojiang District, Taizhou City, China

Background: *Streptococcus pneumoniae* (SP) is a Gram-positive, alpha-hemolytic, facultative anaerobic member of the genus *Streptococcus*. The erythromycin-resistant methylase (*erm*) gene and macrolide efflux (*mef*) gene are the 2 main genes that can mediate SP. Transposon (*Tn*) also plays an important role in the collection and metastasis of the gene. In the present study we investigated the drug resistance characteristics and the macrolide-resistant mechanisms of SP in Wenzhou City, China.

Material/Methods: Sixty-eight strains of SP were isolated from sputum samples of hospitalized children in the Second Affiliated Hospital of Wenzhou Medical University. These strains were analyzed using antimicrobial susceptibility tests to determine their drug resistance to 10 kinds of antibacterials. Macrolide-resistant phenotypes were identified using K-B method. PCR method was used to analyze the *erm B* gene, *mef A* gene, and *int Tn* gene.

Results: Drug resistance rates of 68 strains of SP were 98.5%, 100.0%, 63.2%, 52.9%, 94.1%, 89.7%, 0.0%, 0.0%, 16.2%, and 14.7% for clindamycin, erythromycin, penicillin G, cefotaxime, tetracycline, sulfamethoxazole/trimethoprim, levofloxacin, vancomycin, chloramphenicol, and amoxicillin, respectively. Total detection rates of the *erm B* gene, *mef A* gene, and *int Tn* gene were 98.5%, 91.2%, and 100.0%, respectively.

Conclusions: SP shows significant multi-drug resistance in Wenzhou City, whereas there is no clinical value of macrolides antibiotics for SP. *cMLS_B* mediated by *erm B* gene is the most predominant phenotype among macrolide-resistant SP. The *int Tn* gene may play an important role in horizontal transfer and clonal dissemination of SP drug resistance genes in Wenzhou City.

MeSH Keywords: Drug Resistance, Multiple • Macrolides • *Streptococcus pneumoniae*

Full-text PDF: <http://www.medscimonit.com/abstract/index/idArt/896766>



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Background

Streptococcus pneumoniae (SP) is a Gram-positive, alpha-hemolytic, facultative anaerobic member of the genus *Streptococcus* [1]. SP was recognized as a major cause of pneumonia in 1881, and is a major causative organism of invasive and non-invasive diseases, such as community-acquired pneumonia, otitis media, meningitis, and hematosepsis, especially in infants and the elderly [2,3]. In developing countries, there are approximately 1 million deaths caused by SP every year in children under the age of 5 years [4]. The genome of SP is a closed, circular DNA structure that contains between 2.0 and 2.1 million base pairs, depending on the strain. It has a core set of 1553 genes, plus 154 genes contributing to virulence, and 176 genes maintaining a noninvasive phenotype. Genetic information can vary by up to 10% among strains [5].

In the 1980s and before, penicillin was the first-line drug for the treatment of SP. Penicillin antibiotics were among the first medications to be effective against many bacterial infections caused by staphylococci and streptococci [6]. In the 1990s, the first-line drug changed to erythromycin. However, with the wide clinical use of macrolides antibiotics, macrolide-resistant *Streptococcus pneumoniae* (MRSP) became more prevalent. According to the reports by the Asian Network for Surveillance of Resistant Pathogens (ANSORP), the drug resistance rate of SP to β -lactam or macrolides antibiotics in recent years continued at a high level [7,8]. Moreover, the proportion of multi-drug resistant (MDR) SP was nearly 60% [9,10]. These are challenges to the effective treatment and control of SP infection in children.

Erythromycin-resistant methylase (*erm*) gene and macrolide efflux (*mef*) gene are the 2 main genes that can mediate SP. Transposon (*Tn*) also plays an important role in the collection and metastasis of the gene. In order to understand the phenotype of SP and its characteristics, and the drug resistance mechanisms of SP in Wenzhou City, we collected 68 strains of SP and tested *erm B* gene [11], *mef A* gene [12], and integrase *Tn* (*int Tn*) [13] in this study.

Material and Methods

Material

The standard SP strain ATCC49619 was provided by the Chinese National Center for Medical Culture Collections (Beijing, China). Sixty-eight strains of SP were isolated from sputum samples of hospitalized children in the Second Affiliated Hospital of Wenzhou Medical University in 2009, none of which were repetitive strains. All 68 strains were identified to species level with the Gram-Positive Identification Card (GPI) by automatic

microbial analyzer VITEK-32 (bioMérieux Co., Marcy-Etoile, France). They were confirmed with specific PCR of SP species reported by du Plessis [14].

Methods

Antimicrobial susceptibility tests

Sixty-eight strains were analyzed using antimicrobial susceptibility tests to determine their resistance to 10 kinds of antibiotics. ATB STREP 5 were bought from bioMérieux (France) and used according to the manufacturer's protocol. Macrolide-resistant phenotypes were identified using the disc diffusion test (K-B method) and test papers (erythromycin and clindamycin) were bought from OXOID (UK). When 2 papers are both without inhibition zone, phenotype is considered as constitutive resistance to macrolide, lincosamide, and streptogramin B (cMLS_B). When erythromycin is without inhibition zone but clindamycin is with defective inhibition zone (D style), the phenotype is defined as induced resistance to macrolide, lincosamide, and streptogramin B (μ MLS_B). If erythromycin shows drug resistance and clindamycin is sensitive, it is M phenotype.

Extraction of DNA

SP strains stored at -70°C were placed into Columbia blood agar plates at 37°C for 18–24 h with 5% CO₂ (Thermo Electron, USA). After passage, whole bacterial colonies in the plate were collected, and then DNA was extracted with hexadecyl trimethyl ammonium bromide (CTAB) method.

Detection of *pbp2B* gene, *erm B* gene, *mef A* gene, and *int Tn* gene

Polymerase chain reaction (PCR) method was used to analyze the *erm B*, *mef A*, and *int Tn* genes. And primers of *pbp2B* gene, *erm B* gene, *mef A* gene, and *int Tn* gene were f 5'-CTGACCATTGATTGGCTTTCCAA-3', r5'-TTTGCAATAGTTGTACATACTG-3'; f 5'-GAA AAG GTA CTC AAC CAA ATA-3', r5'-AGT AAC GGT ACT TAA ATT GTT TAC-3'; f5'-TGGTTCGGTGCTACTATTGT-3', r5'-CCCCTATCAACATTCCAGA-3'; f 5'-GCG TGA TTG TAT CTC ACT-3', r 5'-GAC GCT CCT GTT GCT TCT-3', respectively. The PCR reaction system (Eppendorf, Germany) consisted of 0.125 μl 5 U/ μl rTaq enzyme, 2.5 μl 10 \times Buffer, 2.0 μl 2 mmol/L dNTP, 1.5 μl 25 mmol/L MgCl₂, 0.5 μl 20 $\mu\text{mol/L}$ primer each (Shanghai Shinegene Molecular Biotechnology Co., LTD., China), 14.875 μl ddH₂O, and 3.0 μl DNA template. Reaction conditions were 30 cycles with pre-denaturation at 94°C for 5 min, reduction to annealing temperature for 1 min, then heating to 72°C for 1 min and keeping that for 1 min. Finally, the temperature was kept at 72°C for 10 min. The annealing temperatures for each gene are shown in Table 1. Electrophoresis analysis was done on 2.0% agarose with 0.5 μl PCR products

Table 1. Annealing temperature and products of PCR.

Gene	Annealing temperature	Product length
<i>pbp2B</i>	55°C	682 bp
<i>erm B</i>	56°C	639 bp
<i>mef A</i>	52°C	348 bp
<i>int Tn</i>	50°C	1046 bp

and 1.0 µl 6×loading Buffer for 25 min by PAC3000 (BIO-RAD, USA). Voltage and electric current were set at 150V and 75mA, respectively. Pictures were using a gel photograph system WD-9413A (Beijing Liuyi Instrument, China) after electrophoresis.

Results

Macrolide-resistant phenotypes of the 68 strains of SP against 10 kinds of antibiotics are shown in Table 2, which shows that the 68 SP strains isolated in the clinic showed a high rate of MDR. Vancomycin, levofloxacin, and chloramphenicol were the only antibiotics with sensitivity higher than 70%. Drug resistance genotypes of the 68 SP strains are shown in Table 3.

Table 2. Macrolide-resistant phenotypes of 68 SP strains against 10 kinds of antibiotics.

Antibiotics	Resistant (%)	Intermediate (%)	Susceptible (%)
Clindamycin	98.5	0.0	1.5
Erythromycin	100.0	0.0	0.0
Penicillin G	63.2	32.4	4.4
Cefotaxime	52.9	32.4	14.7
Tetracycline	94.1	0.0	5.9
Sulfamethoxazole/Trimethoprim	89.7	10.3	0.0
Vancomycin	0.0	0.0	100.0
Levofloxacin	0.0	4.4	95.6
Chloramphenicol	16.2	0.0	83.8
Amoxicillin	14.7	36.8	48.5

Table 3. Drug resistance genotypes of 68 SP strains.

Phenotypes	Cases	Percent (%)	Resistant strains		Genotypes		
			Erythromycin	Clindamycin	<i>erm B</i>	<i>mef A</i>	<i>erm B + mef A</i>
cMLS _B	67	98.5	67	67	6	61	61
MLS _B	0	0	0	0	0	0	0
M	1	1.5	1	0	0	1	0

The *Int Tn* gene was tested in all 68 strains of SP and the total detection rate was 100.0%.

Table 2 shows the high-resistant rates of SP against several kinds of antibiotics. Vancomycin, levofloxacin, and chloramphenicol were the only antibiotics with sensitivity higher than 70%, but levofloxacin and chloramphenicol are not suitable for use in children.

Table 3 shows that phenotype cMLS_B is the predominant type for macrolide resistance of SP in Wenzhou City. Table 3 also shows the high rate of genotype *erm B+ mef A* and low rates of genotype *erm B* or *mef A* alone. The high rate of *mef A* gene and the low rate of M phenotype were because low-level drug resistance mediated by *mef A* gene was masked by high-level drug resistance mediated by *erm B* gene.

Discussion

Drug resistance is the reduction in effectiveness of a drug such as an antimicrobial, anthelmintic, or an antineoplastic in curing a disease or condition. When the drug is not intended to kill or inhibit a pathogen, the term is equivalent to dosage failure or drug tolerance [15]. Sometimes a combination of different classes of antibiotics may be used synergistically;

that is, they work together to effectively control bacteria that may be resistant to one of the antibiotics alone [16], but then MDR may appear. It has been reported that more than 30% of SP worldwide are MDR [17]. The data from the PROTEKT (Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin) study also showed that about 40% of SP strains collected from 38 countries in 2003 and 2004 appeared to have the MDR phenotype; among these, the percentage of MDR to erythromycin was as high as 89.2% [18]. Studies in 15 European countries during 2004–2005 reported that 15.8% of strains of SP were MDR, of which 40.8% were in France and 42.9% in Greece [19]. Compared to other parts of the world, MDR is more common in Asia. According to the ANSORP monitoring study, the total rate of drug resistance in Asia was 26.8% in 2000–2001 and 59.3% in 2008–2009. The highest was 83% in China, followed by 75.5% in Vietnam, 63.9% in South Korea, 62.2% in Hong Kong, and 59.7% in Taiwan [20]. From the results of this study, vancomycin, levofloxacin and chloramphenicol were the only antibiotics with sensitivity higher than 70%, but levofloxacin and chloramphenicol are unsuitable for use in children. However, for children infected with SP, the breakpoint of penicillin was often the same as that of oral preparations in China and elsewhere, and not injection preparation.

Minimum inhibitory concentrations (MIC) of oral preparations were ≤ 0.06 , 0.12–1, and ≥ 2 $\mu\text{g}/\text{mL}$ for susceptible, intermediate, and resistant, respectively; MICs of injection preparations were ≤ 2 , 4, and ≥ 8 $\mu\text{g}/\text{mL}$, respectively. The percentage of cerebrospinal fluid infection was less than 1% in infected types of SP strains isolated clinically. The percentage of MIC ≥ 4 $\mu\text{g}/\text{mL}$ was less than 5%, whereas that of MIC ≥ 8 $\mu\text{g}/\text{mL}$ was less than 1% in SP strains isolated in the clinic, which is almost the same as that reported by Kim et al. [7]. This explains the contradiction between the laboratory results and the clinical efficacy. However, for patients infected with SP, penicillin can still be used as a first-line medication in the clinic, but often with injection preparation. Clinically, the dosage of penicillin has a great range of adjustment (3 times), allowing physicians to easily cope with intermediate patients.

For 20 years, the incidence of MRSP has increased due to the wide application of macrolides. The results of Table 2 show that macrolides antibiotics have almost no value in clinical control of SP infection. According to degree of resistance of SP to erythromycin, lincomycin, and streptogramin B, resistance phenotype can be divided into M, MLS_B , MSB, and ML, of which M and MLS_B are the most common. M type means resistance to erythromycin and susceptibility to lincosamide and streptogramin B, which results from the *mefA* gene alone. MLS_B type means resistance to erythromycin, lincosamide, and streptogramin B, which results from the *ermB* and/or *mefA* gene. The main mechanisms of drug resistance of SP to

macrolides antibiotics includes the change of ribosome target sites, enhancement of active efflux mechanism, and mutations of genes coding ribosomal protein L4, L22, and (or) rRNA 23S [21]. The *ermB* gene codes methylase of rRNA 23S, which results in MLS_B . According the difference in the upper regulation sequence of the methylase gene, MLS_B phenotype is divided into cMLS_B and rMLS_B . MLS_B shows high-level resistance to macrolide (MIC ≥ 64 mg/L).

Mef was discovered in 1996, and includes *mefA* and *mefE*. The macrolide-resistant phenotype mediated by *mefA* was M, while *mefA* can also transfer in SP [22]. M shows low-level resistance to macrolide (MIC: 1–32 mg/L). There are obvious geographical differences in the prevalence of *mef* genes and *ermB* gene [23–25]. In this study, the total detection rate of *ermB* gene was 98.5%, which indicates that cMLS_B phenotype of MRSP coded by *ermB* gene was the main phenotype in Wenzhou City. In addition, the total detection rate of *mefA* gene was 91.2%, with only 1.5% M phenotype. This is because low-level drug resistance mediated by *mefA* gene was masked by high-level drug resistance mediated by the *ermB* gene. The results of this study were significantly different from the reports in the cities of Shanghai and Guangzhou [26,27]. Mutations of rRNA 23S and (or) genes coding ribosomal protein L4, L22, which were reported in the literature [25], were not found in our study.

Some studies suggested that the drug resistance gene of SP is located at conjugative *Tn* and not at plasmid. *Tn1545* can bring *ermB* gene, *tetM* gene, *catpc194* gene and be efficient to transfer drug resistance genes among strains. *intTn* gene is the integrase gene of *Tn1545*. Studies have shown that the existence of *Tn1545* can be judged through detecting *intTn* gene [25,28]. The detection rate of 100.0% for *intTn* gene shows its important role in horizontal transfer and clonal dissemination of SP drug resistance genes in Wenzhou City.

Conclusions

The total detection rate of *intTn* was 100.0% in this study, indicating that *Tn* bringing drug resistance genes may be extremely widespread among MRSP strains in Wenzhou City. It also can partly confirm the important status of *Tn* in the development of SP drug resistance from the side.

Acknowledgements

All data and experiments were done by our team. We thank professor Zhao and Ren P for their energetic support and help, and we greatly appreciate the months of guidance and support they provided to us in this study.

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