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Antimicrobial susceptibilities and distribution of resistance genes for β-lactams and macrolides in *Streptococcus pneumoniae* isolated between 2002 and 2004 in Tokyo

Norihisa Noguchi [a,*], Junko Tano [a], Yutaka Nasu [b], Masaharu Koyama [b], Koji Narui [a], Hajime Kamishima [c], Tetsuya Saito [c], Kazumitsu Tsuyuki [c], Masanori Sasatsu [a]

[a] *Department of Microbiology, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

[b] *Clinical Laboratory, Hachioji Medical Center, Tokyo Medical University, 1163 Tatemachi, Hachioji, Tokyo 193-0944, Japan*

[c] *Department of Pediatrics, Hachioji Medical Center, Tokyo Medical University, 1163 Tatemachi, Hachioji, Tokyo 193-0944, Japan*

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Abstract

Antimicrobial susceptibilities of 205 *Streptococcus pneumoniae* strains isolated between 2002 and 2004 in Japan were examined and the distribution of genes for resistance to penicillins and macrolides were investigated by polymerase chain reaction. The molecular epidemiology of 92 randomly selected isolates was also examined by pulsed-field gel electrophoresis (PFGE). The numbers of *S. pneumoniae* isolates resistant to benzylpenicillin, clarithromycin and tetracycline were, respectively, 39 (19%), 111 (54%) and 155 (76%), and the numbers increased annually. All isolates were susceptible to amoxicillin, fluoroquinolones, vancomycin and linezolid. Analysis of mutations in the genes for penicillin-binding protein showed that 92% of isolates had mutations in *pbp1a*, *pbp2b* and/or *pbp2x*. Susceptibility to benzylpenicillin decreased with increasing number of mutated *pbp* genes. The macrolide resistance genes *ermB* and *mefA* were found in 99 (48%) and 76 (37%) isolates, respectively. The presence of *ermB* was associated with high-level resistance to macrolides, and the percentage of isolates with *ermB* increased annually. The presence of *mefA* also increased with increasing number of mutated *pbp* genes. Although the 92 isolates belonged to 74 PFGE types, three groups with an 80% similarity in their PFGE patterns were found at high frequency. Two of the three groups contained no isolates susceptible to penicillin and/or tetracycline, and their percentages increased annually. Our results suggest that the number of *S. pneumoniae* isolates with reduced susceptibility due to accumulation of resistance genes has been increasing.

Keywords: *Streptococcus pneumoniae*; Susceptibility; Penicillins; Tetracyclines; Macrolides; Drug resistance gene; Penicillin-binding protein; Multidrug resistance

1. Introduction

Streptococcus pneumoniae is an indigenous bacterium found in the upper respiratory tract and is a common cause of community-acquired pneumonia, bronchitis and otitis media. The prevalence of penicillin resistance in *S. pneumoniae* among clinical isolates has increased worldwide, although penicillins were once effective in killing the bacterium [1,2]. In addition, many penicillin-resistant and partially resistant *S. pneumoniae* strains are resistant not only to β-lactams and macrolides used to treat respiratory tract infections but also to tetracyclines [3,4].

Resistance of *S. pneumoniae* to β-lactams is due to the reduced affinities of penicillin-binding proteins (PBPs) [5], particularly PBP1A, PBP2B and PBP2X [6,7]. *Streptococcus pneumoniae* contains the mosaic *pbp* sequence, which has high homology with *pbp* in oral streptococci [8], and resistance of *S. pneumoniae* to β-lactams results from homologous recombination of *pbp* with the *pbp* of β-lactam-resistant oral streptococci [9]. Macrolide resistance in *S. pneumoniae* occurs by two mechanisms [10–13]: efflux of drug out

* Corresponding author. Tel.: +81 42 676 5619; fax: +81 42 676 5647.
E-mail address: noguchin@ps.toyaku.ac.jp (N. Noguchi).

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of the cell and modification of the target site. Macrolide efflux is mediated by the product of the *mefA* gene [14,15], whereas target site modification occurs when a specific adenine residue on the 23S rRNA is dimethylated by an rRNA methylase [16].

Molecular epidemiological information on antimicrobial susceptibility and analysis of resistance genes is important in the prevention and treatment of infectious diseases such as drug-resistant *S. pneumoniae*. The present study was conducted to understand the mechanism associated with the development of multidrug-resistant *S. pneumoniae*. The antimicrobial susceptibilities of *S. pneumoniae* isolated between 2002 and 2004 in Tokyo, Japan, were investigated and those genes conferring resistance to penicillins and macrolides were analysed. Molecular typing of the isolates was also performed by pulsed-field gel electrophoresis (PFGE).

2. Materials and methods

2.1. Bacterial strains

Isolates of *S. pneumoniae* ($n = 205$) were collected from outpatients (children, $n = 129$; adults, $n = 69$; unknown, $n = 7$) seen at Hachioji Medical Center of Tokyo Medical University from 2002 to 2004. Clinical samples were intranasal (125), sputum (47), otorrhea (7), pharynx (6) and other (20). *Streptococcus pneumoniae* strains ATCC 49619, IDD553 and R6 were used as controls. Clinical isolates were grown on 5% sheep blood brain heart infusion (BHI) agar. *Streptococcus pneumoniae* was identified by the α-haemolytic and Walk/Away system and was confirmed by susceptibility to optochin as well as polymerase chain reaction (PCR) amplification of the autolysin gene (*lytA*) [17]. Isolates were stored in 15% glycerol at −80 °C.

2.2. PCR analysis

The DNA fragment containing *lytA* and the PBP genes *pbp1a*, *pbp2b* and *pbp2x* were amplified by PCR using the primers designed by Ubukata and co-workers [2,18]. Presence of the macrolide resistance genes *ermB* and *mefA* was assessed by PCR using the following sets of primers [14,16]: 5′-GATTCTACAAGCGTACCTTGGA and 5′-TCTGGAACATCTGTGGTATGG for *ermB*; and 5′-TGTGCTAGTGGATCGTCATGA and 5′-TGCAATCACAGCACCCAATA for *mefA*. Isolates were grown at 35 °C for 24 h. PCR assay was carried out as described previously [19]. PCR conditions were as follows: for *lytA*, *pbp1a*, *pbp2b* and *pbp2x*, 5 min at 94 °C, 35 cycles of 15 s at 94 °C, 15 s at 53 °C and 15 s at 72 °C; and for *ermB* and *mefA*, 2 min at 94 °C, 30 cycles of 20 s at 94 °C, 20 s at 55 °C and 30 s at 72 °C. The amplified DNA fragments were analysed by electrophoresis on 2% agarose gels.

2.3. Antimicrobial susceptibility test

Susceptibility testing was performed using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Breakpoints for antimicrobial agents were as defined by the CLSI [20]. Amoxicillin (Astellas Pharma Inc., Tokyo, Japan), clarithromycin (Taisho Pharmaceutical Co. Ltd., Tokyo, Japan), azithromycin (Pfizer Inc., New York, NY), ciprofloxacin (Bayer AG, Leverkusen, Germany), sparfloxacin (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), levofloxacin (Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan), gatifloxacin (Kyorin Pharmaceutical Co. Ltd., Tokyo, Japan), ulifloxacin (an active metabolite of prulifloxacin; Meiji Seika Kiasha Ltd., Tokyo, Japan), moxifloxacin (Bayer), sitafloxacin (Daiichi Pharmaceutical Co. Ltd.), pazufloxacin (Toyama Chemical Co. Ltd., Tokyo, Japan), cefotiam (Takeda Pharmaceutical Co. Ltd., Osaka, Japan), ceftazidime (Tanabe Seiyaku Co. Ltd., Tokyo, Japan), ceftriaxone (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan), cefpirome (Shionogi & Co. Ltd., Osaka, Japan), cefcapene (Shionogi & Co. Ltd.), cefdinir (Astellas Pharma Inc.), cefditoren (Meiji Seika Kiasha Ltd.), faropenem (Daiichi Suntory Pharma Co. Ltd., Tokyo, Japan), vancomycin (Shionogi & Co. Ltd.) and linezolid (Pfizer Inc.) were kindly provided by their respective manufacturers. Benzylpenicillin, norfloxacin, tetracycline and minocycline were purchased from Wako-Pure Chemical Industries (Osaka, Japan).

2.4. PFGE typing

Streptococcus pneumoniae grown on 5% blood BHI agar at 35 °C for 24 h were collected in 4 mL of BHI broth until turbidity at 600 nm = 0.4. PFGE of *Sma*I-digested chromosomal DNA from *S. pneumoniae* was performed using 1 mL of the cell suspension and the Gene Path group 1 kit (Japan Bio-Rad Laboratories, Inc., Tokyo, Japan), according to the manufacturer's instructions [19,21,22]. The DNA patterns obtained by PFGE were analysed using BioNumerics software version 4 (Applied Maths) using the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) with 2% tolerance and 1% optimisation [23]. PFGE-based clusters were defined as isolates with ≥80% genetic relatedness on the dendrograms.

3. Results

3.1. Annual change in antimicrobial susceptibility

Table 1 shows the annual change in antimicrobial susceptibility from 2002 to 2004. All isolates from this period were susceptible to amoxicillin, fluoroquinolones, vancomycin and linezolid. The minimum inhibitory concentrations (MICs) at which 50% of the isolates were inhibited (MIC_{50}) by β-lactams, macrolides and tetracyclines were