# PNEUMOCOCCI: DRUG SUSCEPTIBILITIES AND PRELIMINARY EPIDEMIOLOGICAL STUDIES BY PENICILLIN BINDING PROTEIN GENOTYPING

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Abstract. A collection of 307 pneumococcal isolates form 84 children and 223 adults admitted to Siriraj Hospital were separated into two groups, penicillin-susceptible (PSSP) and penicillin-nonsusceptible (PNSP). Each group was tested for susceptibilities to 12 drugs (cefuroxime, amoxicillin, chloramphenicol, tetracycline, cefotaxime, ceftriaxone, imipenem, meropenem, ciprofloxacin, ofloxacin, erythromycin and co-trimoxazole). PSSP were susceptible to cefuroxime (87.5%), amoxicillin (100%), chloramphenicol (84.7%), tetracycline (45.8%), cefotaxime (99%), ceftriaxone (99%), imipenem (99%), meropenem (100%), ciprofloxacin (76%), ofloxacin (99%), erythromycin (94.8%) and co-trimoxazole (61.5%). PNSP were resistant to most drugs, except for amoxicillin (99%), ofloxacin (99%) and ciprofloxacin (86.3%). Twenty-two pneumococcal isolates belonging to the three most common serotypes (6, 19, 23) were randomly selected for studies of the *pbp2b* gene with RFLP. There were 7 distinct *pbp2b* RFLP patterns. RFLP pattern 1 was the most predominant resistant pattern. The RFLP pattern 2 was found only in PSSP.

## INTRODUCTION

*Streptococcus pneumoniae* is a leading cause of community-acquired illness, resulting in an estimated 3,000 cases of meningitis, 50,000 cases of pneumonia and 7 million cases of otitis media each year in the USA (Stein *et al*, 2003). Before 1967, it was uniformly susceptible to penicillin. In the early 1990s, some pneumococci were found to be resistant to penicillin and other β-lactam drugs (Hsueh and Luh, 2002). The spread of this resistance to many countries complicates treatment options and increases the likelihood of treatment failures (Appelbaum, 2002; Felmingham *et al*, 2002; Ulloa-Gutierrez *et al*, 2003).

We evaluated the differences between the drug susceptibilities of penicillin-susceptible (PSSP) and penicillin-nonsusceptible (PNSP) *S. pneumoniae*. A preliminary molecular epidemiological study was determined by penicillin-binding protein genotyping.

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## MATERIALS AND METHODS

A collection of 307 pneumococcal isolates from 84 children and 223 adults admitted to Siriraj Hospital with various infections caused by pneumococci between June 1997 and August 2001 were identified according to standard microbiological techniques (Kathryn *et al*, 2003). Drug susceptibilities to penicillin and 12 other drugs were performed by standard microbroth dilution using cation-adjusted Mueller Hinton broth supplemented with 3% lysed horse blood as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2000). *S. pneumoniae* ATCC 49619 was used for the quality control in each batch tested. Twenty-two pneumococcal isolates belonging to the three most common serotypes ( 6, 19, 23) were randomly selected for studies of the *pbp2b* gene with restriction fragment length polymorphism (RFLP). The two primers used in the polymerase chain (PCR) reaction were: Pn2B up (5′ GATCCTCATAATGATTCTCAGGTGG 3′) and Pn2B down (5′CAATTAGCTTAGCAATAGG TGTTGG 3′). The PCR product was then digested by restriction endonuclease *Hinf I* (Dejsirilert *et al*, 1999).

## RESULTS

### Drug susceptibility

Table 1 shows that the percent of PSSP during 1977-1999, was 48% (20.5% with intermediate and 31.5% with high resistance) with a $MIC_{50}$ and $MIC_{90}$ of 0.06 and 2 μg/ml, respectively. However, in 2000-2001, the percent of PSSP was 35.8% (with 19.5% intermediate and 44.7% high resistance) with a $MIC_{50}$ and $MIC_{90}$ of 1 and 4 μg/ml, respectively. For other drugs, 38.8% of the isolates were susceptible to cefuroxime and 58.2% were highly resistant. They were susceptible to amoxicillin (99%), chloramphenicol (63.2%), and tetracycline (25.4%).

Figs 1 and 2 show the cross-resistance of *S.pneumoniae,* which is separated into two categories, penicillin-susceptible (PSSP) and penicillin-nonsusceptible (PNSP). Most PSSP were also susceptible to cefuroxime (87.5%), amoxicillin (100%), chloramphenicol (84.7%), tetracycline (45.8%), cefotaxime (99%), ceftriaxone (99%), imipenem (99%), meropenem (100%), ciprofloxacin (76%), ofloxacin (99%), erythromycin (94.8%) and co-trimoxazole (61.5%). PNSP were resistant to most drugs except amoxicillin (99%), ofloxacin (99%), and ciprofloxacin (86.3%).

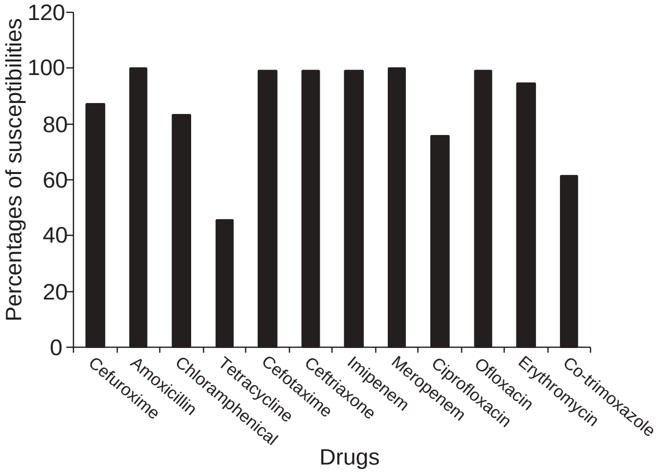


Fig 1–Cross-resistance of penicillin-susceptible pneumococci to other drugs.

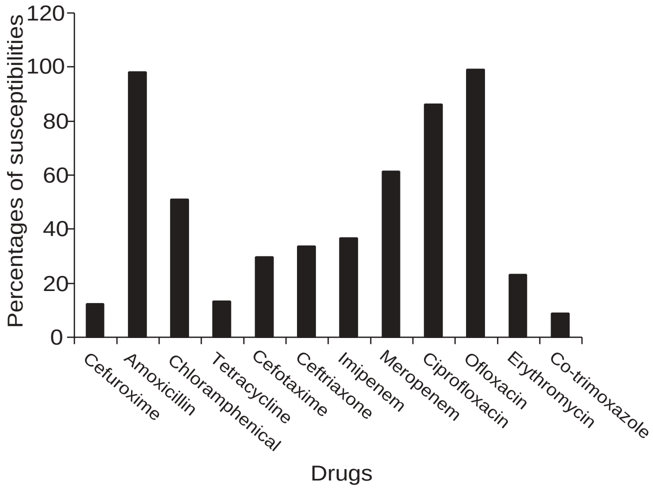


Fig 2–Cross-resistance of penicillin-nonsusceptible pneumococci to other drugs.

### PCR fingerprint of *pbp 2b* gene

The 1.5 kb *pbp 2b* gene fragment in chromosomal DNA was amplified by using the primers Pn2B up and Pn2B down. The 22 pneumococci showed 7 distinct RFLP patterns (Fig 3). Their *pbp 2b* fingerprint result and microbiological parameters, namely serotype, penicillin MIC, and resistance profile are shown in Table 2. The RFLP pattern 1 predominated and was found in 9 isolates. Eight of 9 isolates showing the RFLP pattern 1 had the same resistance profile, namely, resistance to penicillin, cefuroxime, chloramphenicol and tetracycline, but the other one isolate had a different resistance profile, namely, resistance to penicillin and cefuroxime. Nine isolates of RFLP pattern 1 were observed in 2 serotypes, 5 isolates of serotype 19 and 4 isolates of serotype 23. Seven of 9 isolates were highly-resistant to penicillin (MIC≥2 μg/ml) and the other 2 isolates had intermediate-resistance (MIC=0.5 and 1 μg/ml). The RFLP pattern 2 was observed in 3 PSSP (MIC ≤ 0.06 μg/ml) which showed different serotypes (6, 19, 23). This pattern was not observed in PNSP. The RFLP pattern 4 was found in 4 pneumococci which had the same resistance profile. Three of 4 isolates were serotype 6, but one isolate was serotype 19. Pattern 10 was observed in 2 isolates which had the same serotype (serotype 6) and resistant profile. Pattern 12 was observed in 2 isolates which had the same serotype. Both isolates were serotype 23, one isolate being highly-resistant (penicillin

Table 1
Antimicrobial susceptibility pattern.

| Antimicrobial agents | MICs (μg/ml) | | | % of Pneumococci | | |
|---|---|---|---|---|---|---|
| | range | $MIC_{50}$ | $MIC_{90}$ | S | I | R |
| Penicillin | | | | | | |
| 1997-1999 | ≤ 0.06-4 | 0.06 | 2 | 48 | 20.5 | 31.5 |
| 2000-2001 | ≤ 0.06-4 | 1 | 4 | 35.8 | 19.5 | 44.7 |
| Cefuroxime | ≤ 0.12-8 | 4 | 8 | 38.8 | 3 | 58.2 |
| Amoxycillin | ≤ 0.06-8 | 0.5 | 2 | 99 | - | 1 |
| Chloramphenicol | ≤ 0.5-32 | 2 | 16 | 63.2 | - | 36.8 |
| Tetracycline | ≤ 0.25-16 | 16 | > 16 | 25.4 | 2 | 72.6 |

S = sensitive, I = intermediate, R = resistant

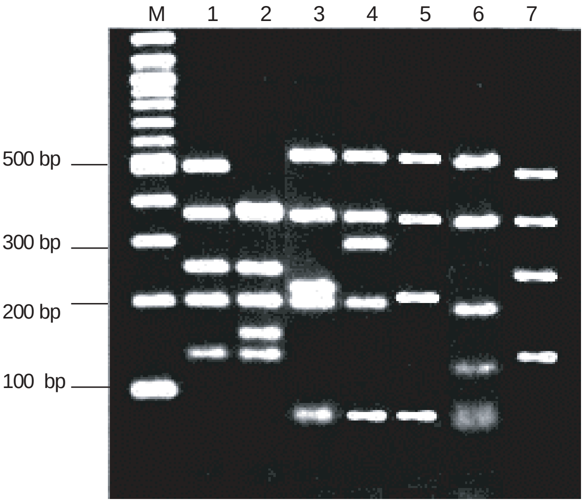


Fig 3–Lane M was the standard 100 bp DNA size marker. Lanes 1-7 were pbp2b gene fingerprint patterns 12, 1, 2, 4, 5, 10 and 6, respectively.

MIC=2 μg/ml) and the other isolate having intermediate-resistance (penicillin MIC=1 μg/ml ). Pattern 5 was observed in 1 isolate, which had intermediate-resistance to penicillin (MIC=0.25 μg/ml). Pattern 6 was observed in 1 isolate which was highly-resistant to penicillin (MIC=2 μg/ml). This isolate was serotype 6 and resistant to 4 drugs, namely, penicillin, cefuroxime, chloramphenicol and tetracycline.

## DISCUSSION

After increases in the MICs of pennicillin for pneumococci were recognized in 1965 (Kislak *et al*, 1965), PNSP was reported from many countries, including Spain, Hungary, France, and Romania. In contrast, penicillin resistance remains comparably low in some countries, such as the United Kingdom, Denmark, Sweden, Germany and Italy (Appelbaum, 1995; Baquero *et al*, 1991; Hedlund *et al*, 1995; Marton *et al*, 1992; Nielsen and Henrichsen , 1996; Sahm *et al*, 2000).

In Spain, PNSP has increased from 5% in 1980s to 35% in 1996 (Fenoll *et al*, 1998). It was reported that of pneumococcal isolates from 11 Asian countries during 1996-1997, Korea had the highest percentage of PNSP (79.7%), followed by Japan (65.3%) (Song *et al*, 1999). The percent of PNSP in Taiwan was higher than 70% (Chiou *et al*, 1998).

In this study, it is obvious that PNSP at Siriraj Hospital is increasing, but less than in Korea. Pneumococci from normally sterile body fluids (blood and CSF) were more susceptible to penicillin than those from non-sterile sites (Lee *et al*, 1995; Doern *et al*, 1996; Sahm *et al*, 2000). In this study, PNSP from normally sterile sites (blood, CSF, bronchial washings, pus from sinusitis) was similar to PNSP from non-sterile sites (64.1% *versus* 64.2%). In addition, pneumococcal isolates from patients ≤5 years were more resistant to penicillin than isolates from patients >60 years.

The increasing incidence of PNSP has been paralleled by an increase in resistance to other classes of drugs. Thus penicillin resistance may serve as a marker of resistance to other drugs. This study provides important information for the clinician. Amoxicillin is a reliable choice even for PNSP.

Table 2
The *pbp 2b* gene fingerprint result and microbiological parameters of 22 pneumococci.

| Strain no. | Specimen source | Serotype | Fingerprint pattern of *pbp2b* | Penicillin MIC (μg/ml) | Resistant profile[a] |
|---|---|---|---|---|---|
| 1 | Sputum | 19 | 1 | 2 | $PC_f$ |
| 2 | Bronchial washing | 19 | 1 | 2 | $PC_fCT$ |
| 3 | Sputum | 19 | 4 | 2 | $PC_fCT$ |
| 4 | Sputum | 23 | 12 | 2 | $PC_fCT$ |
| 5 | Sputum | 23 | 1 | 2 | $PC_fCT$ |
| 6 | Bronchial washing | 19 | 1 | 2 | $PC_fCT$ |
| 7 | Blood | 6 | 6 | 2 | $PC_fCT$ |
| 8 | Sputum | 19 | 1 | 2 | $PC_fCT$ |
| 9 | Sputum | 6 | 4 | 2 | $PC_fCT$ |
| 10 | Pus | 6 | 4 | 2 | $PC_fCT$ |
| 11 | Pus | 23 | 1 | 2 | $PC_fCT$ |
| 12 | Blood | 23 | 1 | 2 | $PC_fCT$ |
| 13 | Sputum | 6 | 4 | 1 | $PC_fCT$ |
| 14 | Blood | 23 | 12 | 1 | $PC_fT$ |
| 15 | Sputum | 19 | 1 | 1 | $PC_fCT$ |
| 16 | Blood | 6 | 10 | 1 | $PC_fCT$ |
| 17 | Sputum | 23 | 1 | 0.5 | $PC_fCT$ |
| 18 | Sputum | 6 | 10 | 0.25 | $PC_fCT$ |
| 19 | CSF | 23 | 5 | 0.25 | $PC_fCT$ |
| 20 | Gastric washing | 6 | 2 | 0.06 | CT |
| 21 | Blood | 23 | 2 | 0.06 | - |
| 22 | Sputum | 19 | 2 | 0.06 | - |

[a]P = penicillin, $C_f$ = cefuroxime, A = amoxicillin, C = chloramphenicol, T = tetracycline

PCR may provide rapid estimates of drug resistance and in specific bacterial strains. To distinguish between PSSP and PNSP, PCR protocols using oligonucleotides specific to susceptible and nonsusceptible penicillin-binding protein (PBP) gene allele sequence have been developed (Ubukata *et al*, 1996; Dowell and Schwartz, 1997; Plessis *et al*, 1998). Another rapid method under development is based on the observation that DNA sequence of genes associated with penicillin-binding proteins are highly uniform in PSSP, whereas they vary with PNSP. The RFLP strategy has been valuable in molecular epidemiological studies at national and hospital levels (Gillespie *et al*, 1997; Dejsirilert *et al*, 1999). In Korea, the molecular characteristics of 22 isolates of multidrug-resistant pneumococci highly-resistant to penicillin (MIC≥ 2 μg/ml) were investigated by the PCR-RFLP method (Song *et al*, 2000). Four distinct *pbp2b* RFLP patterns (1, 2, 3, 4) were observed. RFLP pattern 1 was the most predominant pattern and consisted of three distinct serotypes (6, 19, 23). In this study, an interesting finding is that PSSP had a unique pbp2B pattern that might be used as a rapid diagnostic tool, as has been observed by others (Oneill *et al*, 1999 ).

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