Antimicrobial Susceptibility and Serotype Distribution of *Streptococcus pneumoniae* and Molecular Characterization of Multidrug-Resistant Serotype 19F, 6B, and 23F Pneumococci in Northern Thailand

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Penicillin-resistant *Streptococcus pneumoniae* is widely spread worldwide. Our study was undertaken to examine the susceptibility and serotypes of *S. pneumoniae* in northern Thailand. Ninety-three *S. pneumoniae* strains were isolated from 93 patients at Chiang Mai University Hospital, Chiang Mai, Thailand, from September 1999 to June 2000. The strains were isolated from sputum ($n = 51$), blood ($n = 15$), nasopharynxes ($n = 14$), and other sources (e.g., pus, ears, ascites, and cerebrospinal fluid) ($n = 13$). Of the 93 isolates, 29 (31.2%) were susceptible, 24 (25.8%) showed intermediate resistance (MIC, 0.12 to 1.0 $\mu\text{g/ml}$), and 40 (43.0%) were fully resistant (MIC, ≥ 2.0 $\mu\text{g/ml}$) to penicillin G. Seven (46.7%) from blood, 5 (35.7%) from nasopharynxes, 15 (29.4%) from sputum, and 2 (15.4%) from other sources were susceptible isolates. Serotyping with the use of antiserum revealed differences in the predominant types that were susceptible (6A, 11A, and 19A), intermediately resistant (6B and 23F), and fully resistant (6B, 19F, and 23F). Molecular typing by pulsed-field gel electrophoresis of multidrug-resistant pneumococci showed four patterns (A, B, C, and D) for 16 isolates of serotype 19F, with pattern B being predominant (12 isolates). This finding was different from that with the Taiwan multidrug-resistant serotype 19F clone. Eleven isolates of serotype 6B all showed pattern E, and nine isolates of serotype 23F showed two patterns (F and G), with pattern F being predominant (seven isolates). This finding was similar to that with the Spanish multidrug-resistant serotype 23F clone. Our results indicated that the resistance of pneumococci to antibiotics in northern Thailand is progressing rapidly and that effort should be intensified to prevent any spread of pandemic multidrug-resistant serotypes 19F, 6B, and 23F.

Streptococcus pneumoniae is a leading bacterial cause of pneumonia as well as otitis media, sinusitis, septicemia, and meningitis (17). *S. pneumoniae* used to be susceptible to penicillin, but penicillin-resistant *S. pneumoniae* is now widespread all over the world, and the resistance is not limited to penicillin but is expanding to include other antimicrobial agents (2, 15). A previous report from Thailand for the period from 1992 to 1994 showed that 37.2% of the pneumococci isolated from the nasopharynxes of children with acute respiratory tract infections were penicillin resistant (MIC, >0.1 $\mu\text{g/ml}$) (7). However, the present situation of penicillin-resistant *S. pneumoniae* in adults as well as children in Thailand is not clear. The aim of our study was to examine the antimicrobial susceptibility and serotype distribution of *S. pneumoniae* in Thailand. We also analyzed predominant multidrug-resistant serotype 19F, 6B, and 23F pneumococci by pulsed-field gel electrophoresis (PFGE) and compared these isolates to some pandemic pneumococcal isolates obtained from an international reference collection of the American Type Culture Collection.

MATERIALS AND METHODS

Bacterial strains. Ninety-three *S. pneumoniae* strains were isolated from 93 consecutive inpatients at Chiang Mai University Hospital, Chiang Mai, Thailand, from September 1999 to June 2000. The strains were isolated from sputum ($n = 51$), blood ($n = 15$), nasopharynxes ($n = 14$), and other sites or biological specimens (e.g., pus, ears, ascites, and cerebrospinal fluid) ($n = 13$). Culture plates were incubated overnight in a 5% CO_2 incubator. Optochin sensitivity and bile solubility tests were performed for confirmation of *S. pneumoniae*.

Antimicrobial susceptibility test. MICs were determined by the agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (18). The susceptibilities of 93 *S. pneumoniae* isolates to the following 21 antibiotics were tested: penicillin G (Meiji Seika Kaisha, Tokyo, Japan), ampicillin (Meiji Seika Kaisha), cefazolin (Fujisawa Pharmaceutical Co., Osaka, Japan), cefotiam (Takeda Chemical Industries, Osaka, Japan), ceftazidime (GlaxoSmithKline, Tokyo, Japan), cefaclor (Shionogi Co., Osaka, Japan), cefixime (Fujisawa Pharmaceutical Co.), flomoxef (Shionogi Co.), imipenem (Banyu Pharmaceutical Co., Tokyo, Japan), meropenem (Sumitomo Chemical Co., Tokyo, Japan), fosfomycin (Meiji Seika Kaisha), chloramphenicol (Sankyo Co., Tokyo, Japan), minocycline (Lederle [Japan], Tokyo, Japan), tetracycline (Lederle), erythromycin (Dainippon Pharmaceutical Co., Osaka, Japan), clindamycin (Pharmacia K.K., Tokyo, Japan), gentamicin (Schering-Plough K.K., Osaka, Japan), levofloxacin (Daiichi Pharmaceutical Co.), ciprofloxacin (Bayer Yakuhin, Osaka, Japan), vancomycin (Shionogi Co.), and teicoplanin (Aventis Pharma, Tokyo, Japan). Serial twofold dilutions of each antibiotic (ranging from 0.008 to 128 $\mu\text{g/ml}$) were prepared. Mueller-Hinton agar was used as the culture medium. Approximately 0.01 ml (10^5 CFU/ml) of bacteria was inoculated onto antibiotic-containing medium and incubated overnight at 37°C. The MIC of each

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TABLE 1. Distribution of MICs of various antibiotics for 93 strains of *S. pneumoniae* in northern Thailand

Antibiotic	No. of isolates for which MIC ($\mu\text{g/ml}$) was:															
	≤ 0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128
Penicillin G	2	19	5	3		6	7	11	35	5						
Ampicillin			16	14		8	2	3	7	30	13					
Cefazolin				10	13	4	1	6	21	28	10					
Cefotiam				2	20	8	3	16	17	19	7	1				
Ceftazidime					1	22	6	2	6	4	13	26	11	2		
Cefaclor						3	17	12	4	3	2	3	2	19	15	13
Cefixime					10	16	5	2	3	6	8	15	23	3	2	
Flomoxef						1	19	11	4	5	13	30	10			
Imipenem	22	10	6	4	8	39	4									
Meropenem	4	25	4	8	2	2	31	17								
Fosfomycin										1	7	34	37	12	2	
Chloramphenicol								1	18	36	25	13				
Minocycline				2	9	7	4	8	8	15	24	9	6	1		
Tetracycline						9	7	1	1	3	4	13	32	19	4	
Erythromycin	1	2	8	23	4	8	5	12	3	8		3		2	1	13
Clindamycin		3	12	42	5	4	2	1				1	2	4	4	13
Gentamicin											51	38	4			
Levofloxacin							2	72	19							
Ciprofloxacin							1	37	31	18	4	2				
Vancomycin					2	65	26									
Teicoplanin		3	6	9	66	9										

antibiotic was defined as the lowest concentration of the antibiotic that prevented visible bacterial growth.

Serotyping. Pneumococci were serotyped on the basis of capsular swelling (quellung reaction) observed microscopically after suspension in pneumococcal diagnostic antisera (Statens Seruminstitut, Copenhagen, Denmark).

PFGE. PFGE of multidrug-resistant serotype 19F pneumococci was performed with 16 isolates of serotype 19F, 11 isolates of serotype 6B, 9 isolates of serotype 23F, the Spanish multidrug-resistant serotype 23F clone (ATCC 700669), and the Taiwan multidrug-resistant serotype 19F clone (ATCC 700905) (16). Those isolates were grown overnight in brain heart infusion broth at 35°C, and PFGE was performed to determine the genetic relatedness, as described previously (25). The DNA was digested with 10 U of *Sma*I (Takara Shuzo Co., Shiga, Japan) at 30°C overnight. CHEF Mapper pulsed-field electrophoresis systems (Bio-Rad Life Science Group, Hercules, Calif.) were used for electrophoresis, with a potential of 6 V/cm, switch times of 0.47 and 63 s, and a run time of 20 h and 18 min. After being stained with ethidium bromide, the band patterns were compared according to the criteria for bacterial strain typing described by Tenover et al. (24).

TABLE 2. Susceptibility patterns of 93 pneumococcal isolates according to specimen types and ages of infected patients

Specimen type or age range (yr)	No. susceptible ($n = 29$)	No. with intermediate resistance ($n = 24$)	No. resistant ($n = 40$)
Specimen types			
Sputum	15	15	21
Blood	7	3	5
Nasopharynx	5	4	5
Other	2	2	9
Age ranges			
0–5	3	2	9
6–15	5	5	5
16–25	0	1	4
26–35	4	3	2
36–45	7	3	3
46–55	1	0	2
56–65	5	3	5
66 and above	4	6	2
Unknown	0	1	8

RESULTS

Antimicrobial susceptibility test. MICs of 21 antibiotics for 93 *S. pneumoniae* isolates showed the tendency of these isolates to be resistant to various antibiotics except for imipenem, meropenem, vancomycin, and teicoplanin. For fluoroquinolones, the range of MICs of levofloxacin was 0.5 to 2 $\mu\text{g/ml}$ whereas that of ciprofloxacin was 0.5 to 16 $\mu\text{g/ml}$. Of the 93 isolates, 29 (31.2%) were susceptible, 24 (25.8%) showed in-

TABLE 3. Serotypes of susceptible, intermediately resistant, and fully resistant pneumococci

Serotype	No. susceptible ($n = 29$)	No. with intermediate resistance ($n = 24$)	No. resistant ($n = 40$)
1	2	0	0
3	2	1	1
4	1	0	0
5	2	0	0
6A	4	1	0
6B	0	3	11
9L	0	1	1
11A	4	0	0
13	2	1	0
14	0	2	0
15A	1	0	0
16F	1	1	0
18B	1	0	0
18C	1	0	0
19A	3	2	0
19B	0	1	0
19F	2	1	16
23B	1	0	0
23F	1	7	9
28A	1	0	0
29	0	0	1
35A	0	1	0
Nontypeable	0	2	1

FIG. 1. PFGE patterns of *Sma*I-digested DNA of 16 isolates of multidrug-resistant serotype 19F pneumococci isolated from 16 patients. Molecular typing by PFGE demonstrated that strains no. 2 and 5 showed pattern A, which was close to that of the Taiwan 19F clone, strains no. 1, 3, 4, 6, 7, and 10 to 16 showed predominantly pattern B, strain no. 8 showed pattern C, and strain no. 9 showed pattern D. M, molecular size markers.

FIG. 2. PFGE patterns of *Sma*I-digested DNA of 11 isolates of serotype 6B and 9 isolates of serotype 23F multidrug-resistant pneumococci isolated from 20 patients. Molecular typing by PFGE demonstrated that 11 isolates of serotype 6B all showed pattern E, strains no. 12 to 18 showed predominantly pattern F, which was close to that of the Spanish 23F clone, and strains no. 19 and 20 showed pattern G. M, molecular size markers.

intermediate resistance (MIC, 0.12 to 1.0 $\mu\text{g/ml}$), and 40 (43.0%) showed full resistance (MIC, ≥ 2.0 $\mu\text{g/ml}$) to penicillin G (Table 1). Seven (46.7%) from blood, 5 (35.7%) from nasopharynxes, 15 (29.4%) from sputum, and 2 (15.4%) from other sources were susceptible isolates. Further analysis showed that 9 of 14 strains (64.3%) isolated from children under age 5 were highly resistant to penicillin G compared to 23 of 70 strains (32.9%) from patients over age 5 (Table 2).

Serotyping. The 93 isolated pneumococci were classified into 23 different serotypes. The serotypes of susceptible isolates varied widely, and 6A (13.8%), 11A (13.8%), and 19A (10.3%) were relatively predominant. On the other hand, serotypes of fully resistant isolates were almost limited and included 6B (27.5%), 19F (40.0%), and 23F (22.5%). The serotypes of isolates with intermediate susceptibilities varied moderately, and 6B (12.5%) and 23F (29.2%) were predominant (Table 3).

Interpretation of PFGE results. Molecular typing by PFGE demonstrated four patterns (A, B, C, and D) for 16 isolates of multidrug-resistant serotype 19F pneumococci. Twelve (75.0%) of these isolates showed predominantly pattern B, which was different from the results with the Taiwan multidrug-resistant serotype 19F clone (ATCC 700905) (Fig. 1); 11 isolates of serotype 6B all showed pattern E, and 9 isolates of serotype 23F showed two patterns (F and G), with pattern F being predominant (seven isolates), which was similar to the results with the Spanish multidrug-resistant serotype 23F clone (ATCC 700669) (Fig. 2).

DISCUSSION

Penicillin-resistance *S. pneumoniae* is at present distributed worldwide, and the distribution appears to be increasing rapidly. Furthermore, resistance seems to be expanding to include multiple antimicrobial agents (2, 15, 20). In Thailand, the proportion of penicillin-resistant *S. pneumoniae* (MIC, >0.1 $\mu\text{g/ml}$) was 6.7% in 1978 (23) but has progressively increased to 10.6% in 1987 (12), 37.2% in the period from 1992 to 1994 (7), 57.9% in the period from 1996 to 1997 (22), and as high as 68.8% in the period from 1999 to 2000, as demonstrated in our study. Previous reports of the frequency of penicillin-resistant *S. pneumoniae* for the period from 1996 to 1997 indicated that the proportion of penicillin-resistant *S. pneumoniae* was 79.7% in Korea, 65.3% in Japan, 60.8% in Vietnam, 41.2% in Sri Lanka, 38.7% in Taiwan, 23.1% in Singapore, 21.0% in Indonesia, 9.8% in China, 9.0% in Malaysia, and 3.8% in India (22). Thus, it seems that the frequency of penicillin-resistant *S. pneumoniae* in Thailand is among the highest in Asia.

In our study, pneumococci tended to be more resistant to ciprofloxacin than to levofloxacin. The cause of discrepancy between these two antibiotics is unknown in detail, although ciprofloxacin was commonly used in this area rather than levofloxacin. Recently, it has been reported that *parC* and/or *gyrA* mutations were related to ciprofloxacin resistance in pneumococci, and this finding is noteworthy (8).

Pneumococcal disease is a major cause of morbidity and mortality in infants and young children worldwide (14). However, it has been reported that immunization by polysaccharide vaccines is associated with poor results in children under the age of 2 years (4). On the other hand, pneumococcal conjugate

vaccines seem to be effective for prevention of invasive pneumococcal disease in young children (5, 14, 26). In our study, serotype coverage of a 7-valent pneumococcal conjugate vaccine against penicillin-resistant *S. pneumoniae* was 76.6% (49 of 64). Therefore, introduction of conjugate vaccines for infants in Thailand should be considered.

In addition, we have witnessed a marked increase in the incidence of infection caused by human immunodeficiency virus (HIV), which appeared in Thailand in the late 1980s and has exploded since then (6). It has been reported that among patients with HIV infection, the incidence of invasive pneumococcal disease is high, bacteremia is a common complication of pneumonia, and relapses occur frequently (11). Recently, in countries where the majority of the population can access highly active antiretroviral therapy, such treatment has already resulted in a marked decrease in morbidity and mortality of HIV-infected individuals (1, 3). However, such therapies are not available in Thailand and the proportion of HIV-infected patients remains high (13, 19). Therefore, prophylaxis by pneumococcal vaccine is important, especially among patients with HIV infection. Since the efficacy of 23-valent pneumococcal polysaccharide vaccines in HIV-infected patients is controversial (9, 10, 21), the introduction of such an effective vaccine is worth considering.

Serotypes 19F, 6B, and 23F of fully resistant pneumococci were of the pandemic type in our study, and molecular typing by PFGE demonstrated that 12 (75.0%) of 16 isolates of multidrug-resistant serotype 19F pneumococci showed predominantly pattern B, 11 isolates of serotype 6B all showed pattern E, and 9 isolates of serotype 23F showed two patterns (F and G), with pattern F being predominant (seven isolates), which was similar to the results with the Spanish multidrug-resistant serotype 23F clone. Previous studies suggested the possible introduction and spread of the Spanish pandemic clone 23F in Asian countries, including Thailand (7, 22). However, our results demonstrated that not only the Spanish 23F clone but also serotype 19F pneumococci, which were different from the Taiwan 19F clone, and serotype 6B may have spread recently as fully resistant pneumococci in Thailand.

In conclusion, our results indicated that the resistance of pneumococci in northern Thailand to various antibiotics is progressing rapidly and care should be exercised to prevent the spread of pandemic multidrug-resistant serotypes 19F, 6B, and 23F.

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