Spread of Drug-Resistant *Streptococcus pneumoniae* in Asian Countries: Asian Network for Surveillance of Resistant Pathogens (ANSORP) Study

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Antimicrobial susceptibility of 996 isolates of *Streptococcus pneumoniae* from clinical specimens was investigated in 11 Asian countries from September 1996 to June 1997. Korea had the greatest frequency of nonsusceptible strains to penicillin with 79.7%, followed by Japan (65.3%), Vietnam (60.8%), Thailand (57.9%), Sri Lanka (41.2%), Taiwan (38.7%), Singapore (23.1%), Indonesia (21.0%), China (9.8%), Malaysia (9.0%), and India (3.8%). Serotypes 23F and 19F were the most common. Pulsed-field gel electrophoresis (PFGE) of 154 isolates from Asian countries showed several major PFGE patterns. The serotype 23F Spanish clone shared the same PFGE pattern with strains from Korea, Japan, Singapore, Taiwan, Thailand, and Malaysia. Fingerprinting analysis of *pbp1a*, *pbp2x*, and *pbp2b* genes of 12 strains from six countries also showed identical fingerprints of penicillin-binding protein genes in most strains. These data suggest the possible introduction and spread of international epidemic clones into Asian countries and the increasing problems of pneumococcal drug resistance in Asian countries for the first time.

Streptococcus pneumoniae has long been one of the most important bacterial pathogens causing pneumonia, meningitis, otitis media, and septicemia [1, 2]. For many years, pneumococci were uniformly susceptible to penicillin, until penicillin resistance was first reported in a clinical specimen in 1967 [3]. The extensive use of large numbers of antimicrobial agents, however, has fueled the crisis of antibiotic resistance in the era of modern chemotherapy [4]. During the past 3 decades, the resistance of *S. pneumoniae* to penicillin, other β-lactams, and non-β-lactam agents has been rapidly increasing in many parts of the world [5, 6]. Data on the prevalence of pneumococcal resistance to penicillin from some European countries and South Africa, which ranged from 44% to 59%, have raised global concern [7–9]. In the United States, the rate of resistance to penicillin among pneumococcal isolates rose steeply, up to 25%–46% in some areas [10, 11]. Emergence of resistance to multiple antimicrobial agents has further complicated the problem of antibiotic resistance [12]. Subsequent reports of treatment failure in pneumococcal meningitis caused by penicillin- or cephalosporin-nonsusceptible strains have made this emerging problem more significant in clinical practice [13, 14]. Despite these reports from Western countries, very little information has been available on the epidemiology of antibiotic-resistant pneumococci in Asian countries, where pneumococcal diseases are common and antibiotics are often used without prescription. Recent data from Korea and Hong Kong, however, have showed alarmingly high rates of penicillin resistance, which ranged from 68% to 77% [15–17] and 56% [18], respectively.

To investigate the prevalence of drug-resistant *S. pneumoniae* in Asia as well as to document the spread of pneumococcal drug resistance between Asian countries, the Asian Network for Surveillance of Resistant Pathogens (ANSORP) has performed a multicenter in vitro surveillance study of pneumococcal drug resistance in Asian countries as well as pulsed-field gel electrophoresis (PFGE) and fingerprinting analysis of penicillin-binding protein (PBP) genes of penicillin-nonsusceptible strains from Asian countries.

Materials and Methods

Organization of the ANSORP. ANSORP was first organized in 1996 (by J.-H.S.) to perform multicenter surveillance

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studies of antimicrobial resistance in Asia. ANSORP comprises 14 centers in 11 Asian countries: Korea (Samsung and Asan Medical Centers, Seoul), Japan (Nagoya and Nagasaki Universities), China (Shanghai Medical University), Thailand (Chulalongkorn University and Siriraj Hospital, Bangkok), Taiwan (Chang Gung Children's Hospital, Taipei), India (Christian Medical College, Vellore), Sri Lanka (University of Colombo), Singapore (National University of Singapore), Malaysia (Universiti Kebangsaan Malaysia, Kuala Lumpur), Indonesia (University of Indonesia, Jakarta), and Vietnam (Nhi Dong 2 Children's Hospital, Ho Chi Minh City).

Bacterial isolates. Pneumococcal isolates were consecutively collected from clinical specimens at 14 centers in 11 Asian countries during the period from September 1996 to June 1997. With the exception of lower respiratory tract specimens, isolates were recovered from clinical specimens representative of normally sterile body sites, such as blood, CSF, ascites, pleural fluid, synovial fluid, and sinus aspirate. Pneumococcal isolates from throat or nasal swab or nasopharyngeal aspirate specimens were excluded from the analysis.

Antimicrobial susceptibility tests. Pneumococci were screened for susceptibility to penicillin with a 1-μg oxacillin disk (BBL Microbiology Systems, Cockeysville, MD) by the disk diffusion method according to the performance standards from the National Committee for Clinical Laboratory Standards (NCCLS). Isolates with a zone of inhibition of $\geqslant$20 mm were considered susceptible to penicillin. Antimicrobial agents tested by disk diffusion were oxacillin, tetracycline, erythromycin, chloramphenicol, trimethoprim-sulfamethoxazole, and vancomycin. Zone diameter interpretive standards were defined according to the 1997 NCCLS guideline [19]. For strains that were not susceptible to penicillin by the oxacillin disk diffusion test, MICs were determined by E-testing for penicillin, cefotaxime, cefuroxime, amoxicillin/clavulanate, and imipenem. MIC interpretive standards for these antimicrobials were defined according to the 1997 NCCLS breakpoints [20].

S. pneumoniae ATCC 49619, *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *Staphylococcus aureus* ATCC 29213 were used as control strains. All in vitro tests were done strictly according to the research protocol, and a 4-day quality control run-in period was followed by actual in vitro studies. A panel of 11 pneumococcal strains with known susceptibilities to different antimicrobials (from M.R.J.) was provided to all investigators for documentation of proficiency in susceptibility testing of these strains before the study was begun. Proficiency in in vitro testing was confirmed by the data submitted by each investigator.

Serotyping. Serotyping was done with 205 isolates from seven countries by the coagglutination method [21], and results were confirmed with the quellung reaction with use of type-specific pneumococcal antisera (Statens Seruminstitut, Copenhagen) (by M.K.L.). Among 205 isolates, 165 were not susceptible to penicillin.

PFGE. A total of 154 pneumococcal strains (127 penicillin-nonsusceptible isolates) from seven Asian countries, which were randomly selected, were subjected to PFGE analysis as previously described [22]. One penicillin-susceptible R6 strain and three international strains, including an internationally epidemic Spanish strain (serotype 23F), an Iceland strain (serotype 6B), and a French strain (serotype 14), which were obtained from Dr. Alexander Tomasz (Rockefeller University, New York), were also tested by the same method. For restriction endonuclease digestion, thin slices were cut off the agarose plugs, equilibrated in the appropriate nuclease buffer for 30 min, and digested overnight with 40 U of *Sma*I at 25°C. PFGE was done with a contour-clamped homogenous electric field apparatus (CHEF-Mapper system; BioRad Laboratories, Richmond, CA) at 14°C for 20 h at 5.4 V/cm. Pulse times ranged from 30 seconds to 1 second. Concatemers of DNA isolated from the bacteriophage λ were used as molecular size markers. The DNA fragment patterns generated by PFGE were interpreted according to recent criteria [23].

PCR fingerprinting of PBP genes. A total of 12 strains, obtained from six different Asian countries, that were not susceptible to penicillin and showed the same PFGE pattern and a penicillin-susceptible R6 strain were subjected to fingerprinting analysis of *pbp1a, pbp2b,* and *pbp2x* genes. *pbp1a, pbp2b,* and *pbp2x* genes were amplified from chromosomal DNA by PCR as described previously [24]. Briefly, amplification of the gene encoding PBP 2B yielded a 1.5-kb product that included the region encoding the transpeptidase domain of the enzyme. The PBP 2X gene product was a 1.9-kb fragment, whereas the gene encoding PBP 1A was amplified as a 2.4-kb product. Gene fingerprinting was done as previously described, with use of *Hin*fI or *Mse*I plus *Dde*I as restriction enzymes. pBR 322 DNA digested with *Hpa*II and labeled with [α-^{32}P]dCTP was used as a size marker. Fingerprinting patterns were compared visually.

Results

Antimicrobial susceptibility tests. A total of 996 isolates of *S. pneumoniae* were collected from 14 participating hospitals in 12 cities in 11 Asian countries during the period September 1996 to June 1997. The number of isolates contributed by each of the 14 participating centers varied from a low of 33 to a maximum of 183 isolates, with a mean of 71 isolates per center. The most common specimen source was sputum (66.8%), followed by blood (14.1%), CSF (3.6%), pleural fluid (0.7%), ascites (0.6%), and others, including sinus aspirates, synovial fluid, closed pus, and middle ear fluid (14.3%) (table 1). The number and percentage of pneumococcal isolates that were not susceptible to different antimicrobial agents varied by country (table 2). Overall, 588 isolates (59%) were penicillin-susceptible, 182 (18.3%) showed intermediate susceptibility to penicillin, and 226 (22.7%) were penicillin-resistant. With regard to penicillin resistance, Korea ranked first: 79.7% (95% CI, 73.0%–85.3%) of total isolates were not susceptible to penicillin (MIC $\geqslant$0.1 μg/mL). Korea was followed by Japan (65.3%; 95% CI, 54.3%–75.5%), Vietnam (60.8%; 95% CI, 45.3%–

Table 1. Specimen sources of pneumococcal isolates in Asian centers in a study of drug-resistant *Streptococcus pneumoniae*.

Country	Number of strains (%)						
	Sputum	Blood	CSF	Ascites	Pleural fluid	Other*	Total
Korea	146 (82.5)	8 (4.5)	5 (2.8)	1 (0.6)	0	17 (9.6)	177
Japan	62 (73.8)	0	0	0	0	22 (26.2)	84
Thailand	68 (54.0)	22 (17.5)	4 (3.2)	0	2 (1.6)	30 (23.8)	126
Vietnam	30 (65.2)	2 (4.3)	2 (4.3)	0	0	12 (26.1)	46
Sri Lanka	35 (85.4)	1 (2.4)	3 (7.3)	1 (2.4)	0	1 (2.4)	41
Taiwan	63 (46)	54 (39.4)	4 (2.9)	2 (1.4)	1 (0.7)	13 (9.5)	137
India	109 (59.6)	25 (13.7)	17 (9.3)	2 (1.1)	4 (2.2)	26 (14.2)	183
Singapore	56 (66.7)	12 (14.3)	1 (1.2)	0	0	15 (17.9)	84
Malaysia	12 (35.3)	16 (47.1)	0	0	0	6 (17.6)	34
Indonesia	33 (100)	0	0	0	0	0	33
China	51 (100)	0	0	0	0	0	51
Total no. (%)	665 (66.8)	140 (14.1)	36 (3.6)	6 (0.6)	7 (0.7)	142 (14.3)	996 (100)

* Synovial fluid, sinus aspirate, closed pus, and middle ear fluid.

74.9%), and Thailand (57.9%; 95% CI, 48.8%–66.7%). The percentages of nonsusceptible strains to penicillin in Sri Lanka, Taiwan, Singapore, and Indonesia were 41.2% (95% CI, 26.3%–57.9%), 38.7% (95% CI, 30.5%–47.4%), 23.1% (95% CI, 14.2%–33.1%), and 21% (95% CI, 8.9%–38.9%), respectively. However, China (9.8%; 95% CI, 3.3%–21.4%), Malaysia (9.0%; 95% CI, 1.9%–23.7%), and India (3.8%; 95% CI, 1.6%–7.7%) had a relatively low prevalence of penicillin resistance during the study period. MIC_{90}s of penicillin ranged from 0.06 μg/mL in China, India, and Malaysia to 8 μg/mL in Korea, Taiwan, and Indonesia (table 3). Among penicillin-nonsusceptible strains in Korea, >60% of isolates were not susceptible to other antimicrobial agents except chloramphenicol and vancomycin. Among isolates obtained from blood or CSF, 61.5% (Korea; 95% CI, 31.6%–86.1%), 46.2% (Thailand; 95% CI, 26.6%–66.7%), 29.3% (Taiwan; 95% CI, 18.1%–42.7%), 15.4% (Singapore; 95% CI, 1.9%–45.4%), and 4.7% (India; 95% CI, 0.6%–16.2%) were not susceptible to penicillin. Overall, penicillin nonsusceptibility was the highest in isolates from sputum (45.9%; 95% CI, 41.9%–49.8%), followed by blood (23.4%; 95% CI, 16.8%–31.5%) and CSF (16.7%; 95% CI, 6.4%–32.8%).

Serotype and antimicrobial susceptibility (table 4). The most common serogroup in Asian isolates was serogroup 23 (30.2%), followed by 19 (24.9%) and 6 (15.1%). Among the strains that were serotyped, serotype 23F (61/62 isolates) and 19F (40/51 isolates) were the most common. Among 165 isolates that were not susceptible to penicillin, serogroup 23 (34.5%) was the most common, followed by 19 (29.7%) and 6 (14.5%).

Table 2. Antimicrobial susceptibility data on a total of 996 pneumococcal isolates from 11 Asian countries.

Country	% intermediately susceptible/% resistant to antimicrobial agent								
	Pen	Ctax	Cfur	Amox/Clv*	Imi/Cil	Tet	Em	Chl	TMP-SMZ
Korea (n = 177)	24.3/55.4	21.7/33.1	5.2/66.5	26/34.1	61.7/—	5.7/71.6	6.2/74.6	35.8	3.4/63.6
Japan (n = 84)	38.4/26.9	19.5/11.6	14/36.6	23.3/2.7	30.6/1.3	—/81.1	7.7/67.9	42.3	7.8/36.3
Thailand (n = 126)	35.7/22.2	9.5/32.5	6.3/45.2	16.7/7.1	22.2/—	7.2/52.3	8.7/32.5	26.9	6.3/61.1
Vietnam (n = 46)	28.2/32.6	47.9/17.3	NA	23.9/28.2	NA	4.3/32.6	8.7/65.2	36.9	6.6/84.7
Sri Lanka (n = 41)	41.2/—	—/—	17.6/5.9	2.9/—	5.9/—	9.8/43.9	9.8/17	22.0	7.3/51.2
Taiwan (n = 137)	9.3/29.4	14.7/17.0	—/37.2	18.6/11.6	30.1/1.6	2.1/94.2	1.4/89.1	54.3	10.9/57.9
Indonesia (n = 33)	2.8/18.2	3.0/3.0	3.0/—	—/—	18.2/—	21/46	12/36	6.0	13/42
Singapore (n = 84)	4.9/18.2	13.5/6.0	—/20.7	15.8/14.6	18.2/—	4.9/45.1	—/28	17.0	17.1/42.6
Malaysia (n = 34)	6.0/3.0	—/—	—/—	—/6.0	3.0/—	6.1/27.2	—/3.0	9.0	6.1/15.1
India (n = 183)	3.8/—	—/—	0.6/1.1	—/—	—/—	3.4/21.2	—/—	5.5	5.5/33.3
China (n = 51)	9.8/—	—/—	—/—	—/—	—/—	5.9/68.6	—/35.2	23.5	7.9/54.9

NOTE. Amox = amoxicillin; Cfur = cefuroxime; Chl = chloramphenicol; Cil = cilastatin; Clv = clavulanate; Ctax = cefotaxime; Em = erythromycin; Imi = imipenem; NA = not available; Pen = penicillin; SMZ = sulfamethoxazole; Tet = tetracycline; TMP = trimethoprim. All strains tested were susceptible to vancomycin.

* Amoxicillin/clavulanate was tested in a 2:1 ratio, and data reflect the amoxicillin component.

Table 3. MIC_{90}s for penicillin-nonsusceptible pneumococcal strains from Asian countries.

	MIC_{90} (μg/mL)				
Country*	Pen	Ctax	Cfur	Amox/Clv†	Imi/Cil
Korea	8	4	8	8	0.5
Japan	4	2	8	1	0.5
Thailand	1	4	8	1	0.25
Sri Lanka	0.5	0.25	1	0.25	0.25
Taiwan	8	2	16	2	0.5
Indonesia	8	4	4	8	0.5
Singapore	2	1	8	1	0.25
Malaysia	0.06	0.25	0.25	0.5	0.12
India	0.06	<0.5	<0.5	<0.5	<0.12
China	0.06	<0.5	<0.5	<0.5	<0.12

NOTE. Amox = amoxicillin; Cfur = cefuroxime; Cil = cilastatin; Clv = clavulanate; Ctax = cefotaxime; Imi = imipenem; Pen = penicillin.

* Data from Vietnam were not available.

† Amoxicillin/clavulanate was tested in a 2:1 ratio, and MICs represent the amoxicillin component.

PFGE. A total of 154 penicillin-nonsusceptible strains from seven Asian countries showed 60 different PFGE patterns (table 5). Several major PFGE patterns with *Sma*I restriction were noted to which many penicillin-nonsusceptible pneumococcal isolates from different Asian countries belonged. For example, the type A pattern on PFGE was the most common, which was found in 35 strains from six countries, including Korea (6 isolates), Japan (1), Singapore (9), Taiwan (14), and Thailand (4). PFGE patterns A, B, C, D, F, and I were also shared by strains from more than one country. All strains that were tested by PFGE showed penicillin MICs of $\geq$0.12 μg/mL (0.12–8 μg/mL), with varying cefotaxime, amoxicillin/clavulanate, cefuroxime, and imipenem MICs. PFGE patterns were not related to serotype, pattern of antimicrobial resistance, or specimen source, although most strains with the type A pattern belonged to serotype 23F or 19F.

The PFGE pattern of a serotype 23F strain from Spain was identical to the type A pattern that was seen in many Asian strains, whereas those of strains from Iceland and France were quite different from any PFGE patterns of Asian strains.

PCR fingerprints of PBP genes. All of 12 pneumococcal strains, obtained from six Asian countries, that were not susceptible to penicillin and showed the type A PFGE pattern had *pbp1a, pbp2b,* and *pbp2x* gene fingerprints that differed from those of the penicillin-susceptible strain R6. With *Hin*fI or *Dde*I plus *Mse*I digestion, 8 penicillin-nonsusceptible strains among 11 strains (exceptions, 2 Japanese strains and 1 Malaysian strain) showed the same fingerprints for the *pbp2b* gene. One Japanese strain and a Malaysian strain had fingerprints for the *pbp1A* and *pbp2x* genes that differed from those of other strains.

Discussion

This is the first organized surveillance study of the prevalence of drug-resistant *S. pneumoniae* in Asian countries. Despite the global concern about the increase in pneumococcal drug resistance in various parts of the world during the past decades, it has not been recognized as a major issue in Asia. ANSORP was organized as the first multicountry collaborative study group in Asia for the surveillance of specific antimicrobial resistance.

Our data from the ANSORP study revealed potentially important information on the epidemiology of pneumococcal drug resistance in Asia. First, some Asian cities may be some of the most serious problem areas worldwide with regard to pneumococcal resistance. Among 11 Asian countries, Korea (Seoul), Japan (Nagasaki), Vietnam (Ho Chi Minh City), and Thailand (Bangkok) showed alarmingly high prevalence of penicillin resistance (>50%). The figures in these countries were certainly higher than those from Eastern European countries [7, 8]. As noted from previous reports, penicillin resistance in Korea, almost 80% of all isolates, is at the highest level in the world. In 1998, more than 80% of clinical isolates were not susceptible to penicillin in some Korean tertiary-care hospitals. Pneumococcal drug resistance in Nagasaki, Japan, may be as serious as in Korea. The ANSORP study revealed significantly

Table 4. Serotype distribution of pneumococcal isolates from 7 Asian countries.

	No. of strains of serogroup												
Country	23	19	6	14	3	11	10	15	33	29	38	Other	Total no. of strains
Korea	16	13	8	5	3	4	...	1	...	2	1	12	65
Japan	8	15	4	...	3	...	5	...	...	...	...	...	35
Singapore	5	7	1	2	...	...	...	...	...	...	...	...	15
Taiwan	20	10	1	...	1	...	...	...	...	...	...	...	32
Thailand	7	4	11	...	1	...	...	3	...	...	...	3	29
China	5	1	6	1	1	...	...	1	1	...	...	11	27
Malaysia	1	1	...	...	...	...	...	...	...	...	...	...	2
Total no. of strains	62	51	31	8	9	4	5	5	1	2	1	26	205

Table 5. Pulsed-field gel electrophoresis (PFGE) patterns of penicillin-nonsusceptible pneumococcal isolates from Asian countries.

Country	No. of isolates with PFGE pattern										Total no. of patterns	No. of isolates tested
	A	B	C	D	E	F	G	H	I	Other		
Korea	6	5	5	4	...	...	...	...	2	6	11	28
Japan	1	2	15	...	...	...	7	2	...	10	15	39
Thailand	4	...	...	...	...	15	...	...	1	10	13	32
Taiwan	14	...	5	...	9	2	...	...	...	6	10	36
Singapore	9	...	...	1	...	1	...	...	...	4	7	15
Malaysia	1	1	...	...	...	...	...	...	...	...	2	2
Sri Lanka	...	...	...	...	...	...	...	...	...	2	2	2
Total	35	8	25	5	9	18	7	2	3	38	60	154

increasing penicillin resistance in Nagasaki, from 8.7% in 1988 and 36.6% in 1992 [25] to 65.3% in 1996–1997. The ANSORP study also revealed a 40-fold increase in penicillin resistance in Singapore, from 0.5% in 1977–1986 [26] to 21% in 1996–1997. Data from Shanghai in the current study were similar to recent data from Beijing [27].

However, since only a limited number of referral centers, mainly in urban areas, were involved in the study, with relatively few isolates tested in some centers, current data may not reflect the overall resistance status in a whole country. Prevalence of drug resistance might also be affected by the age of the patients, history of antimicrobial usage, type of hospitals, and type of specimens.

Differences in the prevalence of antimicrobial resistance in Asian countries may be due to several factors. Different patterns of antimicrobial usage, which lead to variable selective pressure on resistance, might be one of the primary factors [28]. Other factors could be the distribution of specific serotypes and the spread of resistant clones within certain regions. Serotype distribution of penicillin-nonsusceptible strains from Asian countries was investigated with limited numbers of isolates from seven countries. Although only a small number of isolates were serotyped, serotype 23F and 19F were the most common serotypes in Asian countries.

There have been many reports on the spread of pneumococcal drug resistance between different countries or within a certain region since 1991 [29–31]. Our data documented the genetic relationship between penicillin-nonsusceptible strains from Asian countries by PFGE typing. About 70% of strains from Asian countries belonged to seven major PFGE patterns. This suggests the genetic relatedness between Asian strains and potential spread of resistant clones between different Asian countries. Interestingly, the Spanish 23F resistant strain, which is an international epidemic clone, showed a type A PFGE pattern, which was identical to that of Korean and other Asian strains. Recent data documented that PFGE patterns of serotype 19F and 23F multidrug-resistant pneumococcal isolates from Korea were indistinguishable from those of representative multiresistant 23F clones from Croatia, Portugal, and New York City [32]. These findings suggest that the Spanish 23F clone had been introduced to Asian countries as it had been to many other parts of the world.

The genetic relationship between Asian strains, which was documented by PFGE, was reassessed by fingerprinting analysis of PBP genes. Given the fact that the PBP genes of resistant pneumococci are very highly variable in sequence, the existence of identical fingerprints of PBP genes by use of multiple restriction enzymes strongly suggests the high likelihood of similarity in DNA sequences of PBP genes, which means clonality of Asian resistant strains.

From the first multicenter surveillance of pneumococcal drug resistance in Asian countries by the ANSORP Study Group, increasing resistance to penicillin and other antimicrobial agents in many Asian countries was documented, which was more serious than expected. Data from molecular epidemiologic studies indicated that it may be partly due to the spread of resistant clones within and between Asian countries as well as to injudicious use of antimicrobial agents. Continuous surveillance of pneumococcal drug resistance is strongly warranted in this region in the future.

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