Homology analysis of 51 penicillin-intermediate *Streptococcus pneumoniae* isolates from Wenzhou City, China

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Abstract

Objective: To investigate drug resistance features and homology among penicillin-intermediate *Streptococcus pneumoniae* isolates from Wenzhou City, China.

Methods: Fifty-one penicillin-intermediate *S. pneumoniae* isolates were obtained from respiratory samples of infants and children hospitalized with lung infections. An antimicrobial susceptibility test was used to assess drug resistance. Polymerase chain reaction and agarose gel electrophoresis were used to identify *S. pneumoniae* isolates and pulsed-field gel electrophoresis (PFGE) was used to analyze molecular subtypes. Hierarchical cluster analysis of PFGE fingerprints was used to compare genetic diversity and relatedness of *S. pneumoniae* isolates. The Quellung test was used for serotyping.

Results: Fifty-one penicillin-intermediate *S. pneumoniae* isolates showed evidence of multi-drug resistance and polyclonal origins. The isolates were classified into 25 subtypes through

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hierarchical cluster analysis of PFGE fingerprints. Three of these subtypes formed a supertype (15/51, 16/51 and 8/51 isolates), while the remaining subtypes occurred sporadically (12/51 isolates).

Conclusions: Transmission of penicillin-intermediate *S. pneumoniae* is mostly vertical and to a lesser extent horizontal. Effective prevention strategies, including respiratory tract management and contact isolation, are essential to control nosocomial *S. pneumoniae* infection. Once susceptibility is confirmed, vancomycin, high-dose penicillin or third-generation cephalosporins (cefotaxime and ceftriaxone) may be used to treat penicillin-intermediate *S. pneumoniae*.

Introduction

Streptococcus pneumoniae remains a major cause of morbidity and mortality worldwide, causing diseases such as otitis media, meningitis, sinusitis, septicemia, and pneumonia.[1,2] The emergence of penicillin-resistant and multi-drug resistant pneumococcal strains has become a global health problem.[3] Infections by resistant *S. pneumoniae* are more difficult to treat and have higher mortality.[4]

S. pneumoniae isolates that appear identical through conventional methods, such as antimicrobial susceptibility testing or serotyping, can be further distinguished through a variety of molecular subtyping techniques.[5] Molecular subtyping has revolutionized the field of epidemiology.[6] Several genetic analysis methods, including pulsed-field gel electrophoresis (PFGE), BOX PCR and multilocus sequence typing (MLST), have been used to assess genetic relatedness of pneumococcal isolates.[3,6,7] PFGE is considered the gold standard for typing many bacteria including *S. pneumoniae*.[8] PFGE allows large DNA fragments to be separated using agarose gel electrophoresis according to their molecular weights. In addition, PFGE is simple to perform, yields reproducible results, and produces high-resolution banding patterns.[5]

The aim of this retrospective study was to characterize the antibiotic susceptibility and molecular subtypes of 51 penicillin-intermediate *S. pneumoniae* isolates obtained from infants and children hospitalized with pneumonia.

Materials and methods

Strains and isolates

The *S. pneumoniae* reference strain ATCC49619 was provided by the Chinese National Center for Medical Culture Collections. Non-repetitive isolates of penicillin-intermediate *S. pneumoniae* were obtained from respiratory samples of infants and children patients hospitalized with pneumonia at the Second Affiliated Hospital of Wenzhou Medical University from January to August 2010. Pneumonia, an infective inflammation of the alveoli, distal airways or pulmonary interstitia, was diagnosed based on physical, imaging and etiological examinations. All cases were diagnosed by attending physicians. Samples were collected from the respiratory tract

(sputum, throat swabs and aspirates). Semi-quantitative methods were used for culture and isolation. *S. pneumoniae* isolates showing growth of two zones or more on blood agar plates were collected. All patients from whom isolates were obtained were diagnosed with pneumonia based on iconography and clinical syndrome. For the first culture, blood agar plates were incubated for 16 to 24 hours in a Thermo Forma 3111 CO_2 incubator (Thermo Fisher Scientific Inc., Rockford, IL, USA) under a 5% CO_2 atmosphere at 35°C.[9] Isolation was performed by aerobic cultivation at 35°C. Prior to inoculation, sputum was digested with Sputasol (Oxoid, Basingstoke, UK) for 15 minutes. This study was conducted with the approval of the Ethics Committee of Taizhou Municipal Hospital. Verbal informed consent was obtained from all participants. The *S. pneumoniae* isolates were identified using a VITEK-32 automatic microorganism analyzer (bioMérieux, Craponne, France). In addition, the *pbp2b* gene was also analyzed for each isolate.[3] Cultures were incubated for 16 to 24 hours in a Thermo Forma 3111 CO_2 incubator under a 5% CO_2 atmosphere at 35°C.[9]

A set of non-repetitive *S. pneumoniae* strains collected in 2010 from various samples (e.g., sputum, blood, cerebrospinal fluid and pus) at the Second Affiliated Hospital of Wenzhou Medical University served as a comparison. All strains were cultured and identified as described above.

Antibiotic susceptibility tests

For penicillin-intermediate strains from the respiratory tract, antimicrobial susceptibility testing was performed using the MicroSTREP Plus Antimicrobial Panel (Siemens Healthcare, Erlangen, Germany) according to the manufacturer's instructions. The test included the following antibiotics: chloramphenicol, cefaclor,

erythromycin, clindamycin, penicillin, cefuroxime, cefotaxime, tetracycline, ceftriaxone, cefepime, levofloxacin, gatifloxacin, trimethoprim, vancomycin, amoxicillin/clavulanic acid, azithromycin, and meropenem. For the larger comparison group of *S. pneumoniae* strains from various samples, ATB STREP 5 (bioMérieux, France) was used according to the manufacturer's protocol. The following antibiotics were included: chloramphenicol, erythromycin, clindamycin, penicillin, cefotaxime, tetracycline, levofloxacin, vancomycin, amoxicillin, and sulfamethoxazole/trimethoprim.

Polymerase chain reaction (PCR)

PCR reactions were performed using *S. pneumoniae* genomic DNA as template. DNA was extracted using a previously published method.[10,11] The *pbp2b* gene was amplified using the following primers: forward, 5′-CTGACCATTGATTTGGCTTTCCAA-3′ and reverse, 5′-TTTGCAATAGTTGCTACATACTG-3′ (Shinegene, Shanghai, China). The length of the PCR amplicon was 682 bp. Optimal reaction mix and PCR cycling conditions were used based on the results of a previous study.[10] Following PCR, 5 µL of each amplicon were mixed with 1 µL of 6× loading buffer. The samples were loaded on 2% agarose gels and electrophoresed at 150 V and 75 mA using a Power Pac 3000 electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). Gels were visualized using an ultraviolet gel imaging camera system (Bio-Rad). The *S. pneumoniae* reference strain ATCC49619 was used as a control.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed according to a previously published protocol.[1] *S. pneumoniae* chromosomal DNA was digested with *Sma*I (Takara Bio Inc., Shiga, Japan). Digested chromosomal DNA was separated on a CHEF-DRII apparatus (Bio-Rad) for

18 hours with pulse times ranging within 2 to 30 seconds at 14°C and 6 V/cm. Gels were photographed under ultraviolet light after staining with 0.5 µg/mL ethidium bromide. The *S. pneumoniae* reference strain ATCC49619 was used as a control.

Serotyping

The Quellung test was used for serotyping as described previously.[12] Standard typing antisera were from Statens Serum Institute (Copenhagen, Denmark). All penicillin-intermediate *S. pneumoniae* strains were tested.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 19.0 (IBM Corp., Armonk, NY, USA). For PFGE fingerprints, each band was converted into a binary matrix wherein 1 represents "Yes" and 0 represents "No". A dendrogram was generated using between-group linkage via hierarchical clustering. Differences among or between groups were assessed using two-tailed chi-square tests or Fisher's exact tests. Values of $P < 0.05$ were considered statistically significant.

Results

Antibiogram of S. pneumoniae isolates

Fifty-one non-repetitive isolates of penicillin-intermediate *S. pneumoniae* were obtained from respiratory samples of infants and children patients hospitalized with pneumonia. The ages of these patients ranged from 2 months to 5 years: 31 were <1 year old, 11 were between ≥1 but <3 years old, and 9 were ≥3 and ≤5 years old. In 2010, 515 non-repetitive *S. pneumoniae* strains were collected from various samples (e.g., sputum, blood, cerebrospinal fluid and pus) at the Second Affiliated Hospital of Wenzhou Medical University and these served as a comparison.

All 51 penicillin-intermediate *S. pneumoniae* isolates were tested for antibiotic susceptibility. According to the Clinical and Laboratory Standards Institute breakpoints, a minimal inhibitory concentration (MIC) of ≤0.06 µg/mL penicillin (oral penicillin V) was defined as susceptible, a MIC of 0.12 to 1.00 µg/mL was defined as intermediately susceptible, and a MIC of ≥ 2 µg/mL was defined as resistant.[13] However, the breakpoints for parenteral penicillin (non-meningitis indications) were ≤2.0, 4.0, and ≥8.0 µg/mL, respectively. According to the latter breakpoints, all isolates in this study (MIC: 4.0 µg/mL) were penicillin-intermediate and were totally resistant to cefaclor, erythromycin, clindamycin, cefuroxime, trimethoprim and azithromycin. All isolates were totally susceptible to levofloxacin, gatifloxacin and vancomycin. Among the 51 isolates, 5.9%, 19.6%, 54.9%, 54.9%, 62.7%, 68.6% and 96.1% were resistant to chloramphenicol, cefepime, cefotaxime, ceftriaxone, amoxicillin/clavulanic acid, meropenem and tetracycline, respectively. Furthermore, 21.6%, 23.5%, 76.5%, 35.3% and 31.4% of the isolates were intermediately susceptible to cefotaxime, ceftriaxone, cefepime, moxicillin/clavulanic acid and meropenem, respectively (Table 1). Table 1 also shows the susceptibilities of 515 *S. pneumoniae* isolates collected from various samples at our center in 2010 as a comparison. The MICs of antibiotics were used to define susceptibility, and multi-drug resistance (MDR) was defined as resistance to three or more antibiotic classes. All 51 respiratory *S. pneumoniae* isolates were totally MDR.

Molecular typing of penicillin-intermediate S. pneumoniae isolates by PFGE

PFGE was used to assess the genetic diversity of penicillin-intermediate *S. pneumoniae* isolates. Electrophoresis of *Sma*I-digested genomic DNA from the 51 *S. pneumoniae* isolates revealed diverse fingerprinting patterns. Most

Table 1. Antibiotic resistance patterns for 51 and 515 *S. pneumoniae* strains.

Antibiotic	Susceptible (%)		Intermediate (%)		Resistant (%)	
	n = 51	n = 515	n = 51	n = 515	n = 51	n = 515
Chloramphenicol[ns]	94.1	84.1	0	0	5.9	15.9
Cefaclor	0		0		100	
Erythromycin[ns]	0	0	0	0	100	100.0
Clindamycin[ns]	0	1.2	0	0	100	98.8
Penicillin (oral penicillin V)[ns]	0	4.3	0	33.0	100	62.7
Penicillin parenteral (nonmeningitis)[****]	0	95.0	100	4.3	0	0.8
Cefuroxime	0		0		100	
Cefotaxime[***]	23.5	52.0	21.6	33.4	54.9	14.6
Tetracycline[ns]	3.9	5.8	0	0	96.1	94.2
Ceftriaxone	21.6		23.5		54.9	
Cefepime	3.9		76.5		19.6	
Levofloxacin	100		0		0	
Gatifloxacin	100		0		0	
Trimethoprim	0		0		100	
Vancomycin	100		0		0	
Amoxicillin/clavulanic acid	2.0		35.3		62.7	
Azithromycin	0		0		100	
Meropenem	0		31.4		68.6	

Note: Penicillin MICs were all 4.0 µg/mL for all the 51 strains. Comparison of resistance between the 51 and 515 strains: ns, non-significant; ***$P < 0.001$; ****$P < 0.0001$.
MIC, minimal inhibitory concentration.

PFGE profiles showed 11 to 16 discernible restriction fragments (Figure 1), and 18 different fragments were identified in total. The 51 *S. pneumoniae* isolates were classified into 25 subtypes. Table 2 shows the numbers of isolates for each subtype. The isolates were grouped into clusters or clones using the following criteria based on PFGE banding patterns: differences of 2 to 3 bands were defined as "closely related" with a single gene variation; differences of 4 to 6 bands were defined as "potentially related" with two independent gene variations; and differences of ≥7 bands were defined as "unrelated" with ≥3 gene variations.[14]

Dendrogram analysis of penicillin-intermediate *S. pneumoniae* PFGE fingerprints

A dendrogram based on PFGE fingerprints was generated through hierarchical cluster analysis. Based on criteria proposed by a previous study,[3] the 51 penicillin-intermediate *S. pneumoniae* isolates were divided into 25 subtypes (Figure 2). PFGE banding patterns were the same for strains 49 and 52, as well as for strains 29, 30 and 17. A difference of one band separated subtypes A and B, or subtypes C and D. A difference of two bands separated subtypes A+B from C+D. Thus, subtypes A, B, C, D and E could be classified as one supertype, potentially representing common descent from a single clone. Two other supertypes were identified as F–J and K–M. The remaining 25 subtypes occurred sporadically. Subtypes M–Q and T–Y showed susceptibility to ceftriaxone.

Serotypes of the 51 *S. pneumoniae* isolates

Among the 51 penicillin-intermediate *S. pneumoniae* isolates, 15 (29.4%), 9 (17.6%), 5 (9.8%), 5 (9.8%), 3(5.9%), 3 (5.9%), 2 (3.9%), 2 (3.9%), 2 (3.9%), 2
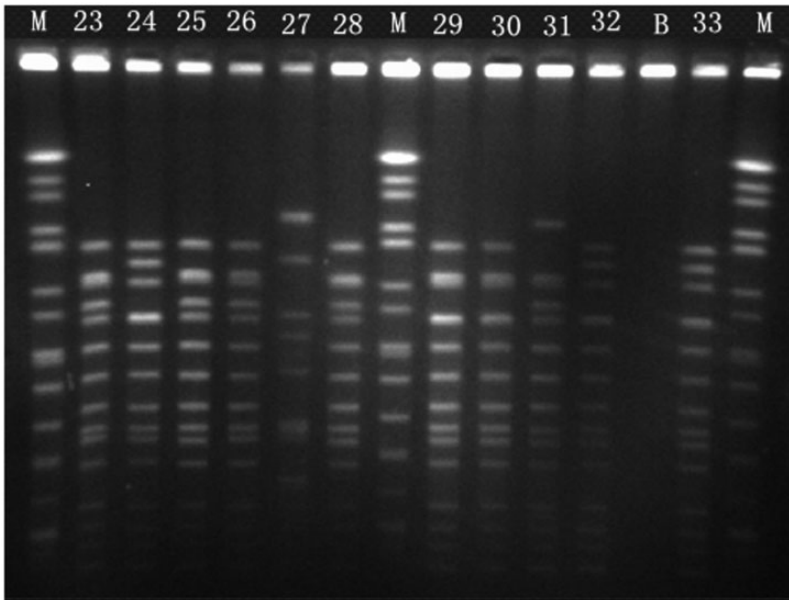
Figure 1. PFGE fingerprints of representative *S. pneumoniae* isolates. Lane M, DNA marker; Lanes 23–33, samples. Each lane comprised 11–16 visible bands. Lane B, blank.
PFGE, pulsed-field gel electrophoresis.

Table 2. Number of isolates associated with each subtype.

Isolates per subtype	Subtypes
1	I, J, M–Y
2	A, C, E, F, G, K
3	B
5	L
6	D
10	H

(3.9%), 2 (3.9%) and 1 (2.0%) were typed as 19F, 19A, 6A, 23F, 6B, 15C, 14, 3, 15B, 6D, 7F and 19B, respectively. The 19F, 19A, 6A and 23F isolates were the most common serotypes, accounting for 66.7% of isolates. The correspondence between serotypes and subtypes was as follows: serotype 19F (1, 2, 3, 7, 14, 18, 23, 25, 26, 28, 32, 33, 35, 36 and 41), serotype 19A (5, 6, 8, 9, 10, 15, 16, 24, 31), serotype 6A (11, 12, 19, 21, 47), serotype 23F (13, 37, 39, 42, 43), serotype 6B (27, 50, 51), serotype 15C (17, 29, 30), serotype 14 (44, 46), serotype 3 (49, 52), serotype 15B (22, 45), serotype 6D (40, 48), serotype 7F (4, 34) and serotype 19B (38).

Discussion

S. pneumoniae is the most common bacterial pathogen responsible for community-acquired infections or pneumococcal diseases in children.[15] Infections caused by *S. pneumoniae* continue to be a problem. In this study, pneumococcal isolates from respiratory samples of hospitalized infants and children with lung infections were collected and cultured. Fifty-one non-repetitive strains of penicillin-intermediate *S. pneumoniae* were confirmed using a VITEK-32 automatic microbial analyzer and tested for drug resistance using a MicroSTREP Plus antimicrobial panel. *S. pneumoniae* isolates were further confirmed by PCR of the *pbp2b* gene. We investigated the molecular subtypes and genetic diversity of the *S. pneumoniae*
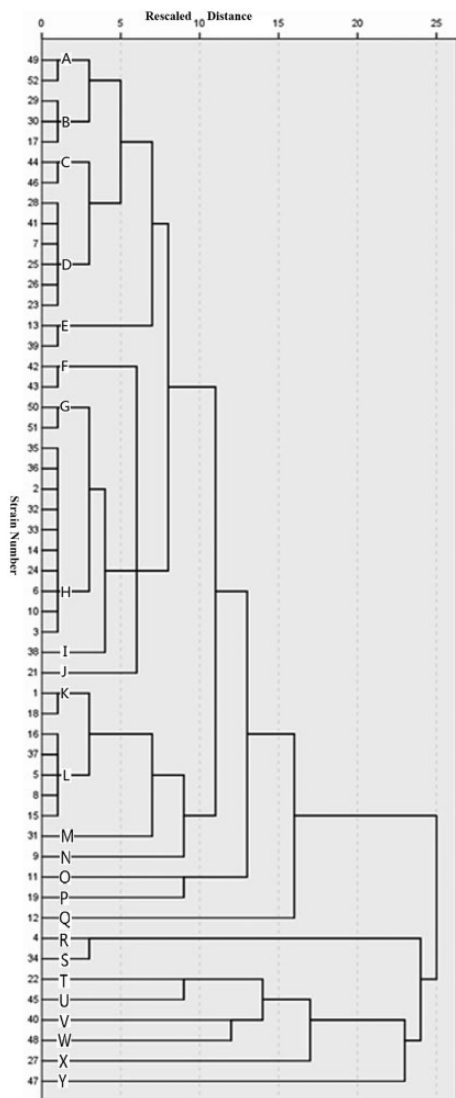
Figure 2. Dendrogram of PFGE fingerprints for 51 pneumococcal isolates generated using hierarchical cluster analysis.
PFGE, pulsed-field gel electrophoresis.

isolates by PFGE and hierarchical cluster analysis.

Automatic bacterial identification platforms in clinical laboratories provide rapid and reliable diagnosis of most pathogens.[16] Previous studies reported that PCR was a useful tool for rapid identification of *S. pneumoniae* from both clinical samples and bacterial isolates.[17] Intermediate sensitivity to penicillin in *S. pneumoniae* may be mediated by altered penicillin binding protein (pbp) genes present in genomic and not plasmid DNA.[15,17,18] Our results clearly showed that isolates from clinical samples were *S. pneumoniae*.

It is important to rapidly identify antibiotic resistance to enable clinical therapy of bacterial infections.[19] Our results indicated that penicillin-intermediate *S. pneumoniae* isolates were totally MDR (Table 1). In infants and children, vancomycin, high-dose penicillin or third-generation cephalosporins such as cefotaxime and ceftriaxone (once susceptibility is confirmed) could be used to treat penicillin-intermediate *S. pneumoniae* (Table 1).

Pneumococcal isolates can be characterized by epidemiological typing methods such as serotyping, amplified fragment length polymorphism, MLST, PFGE as well as by antibiotic susceptibility testing.[3,20] These phenotyping and genotyping methods have been used to distinguish *S. pneumoniae* isolates.[21]

PFGE has recently become an important tool for assessing microbial genetic relatedness. Molecular subtyping of *S. pneumoniae* using high-quality gel images from PFGE can be accomplished with minimal time and effort.[5] PFGE provides a potentially universal method for fingerprinting and comparing isolates based on banding patterns in PFGE fingerprints.[22] In the present study, the PFGE clustering patterns indicated that the 51 penicillin-intermediate *S. pneumoniae* isolates originated from 25 clones (Figure 2). The data also revealed that representative isolates of the 25 clones had PFGE fingerprints consisting of 11 to 16 DNA fragments (Figure 1). In addition, three clones were classified as supertypes (15/51, 16/51 and 8/51 isolates), while the remaining clones occurred sporadically

(12/51 isolates). Sporadically occurring clones showed greater susceptibility to third generation cephalosporins than the three supertypes. Members of the three supertypes may derive from three different clones. Transmission of penicillin-intermediate *S. pneumoniae* is mostly vertical and to a lesser extent horizontal. Thus, in addition to proper use of antibiotics, effective prevention measures such as respiratory tract management and contact isolation are essential for control of nosocomial infection.

The 19F, 19A, 6A and 23F isolates were the four most common serotypes, accounting for 66.7% of all isolates. This was partially consistent with other reports,[23,24] and differences may have arisen from different sample sources, strain definitions and isolation areas. Several previous studies analyzed clonal distribution using serotyping. These studies suggested that genetic diversity varied by serotype and that there was a close connection between serotypes, PFGE clones, and distribution of pneumococcal isolates. In addition, PFGE clones and antibiotic MICs were correlated.[25] The genotype patterns were correlated with previously reported antibiograms.[1] In future studies, antimicrobial resistance, serotyping and molecular subtyping of *S. pneumoniae* should be combined in a single study to provide stronger clinical evidence to guide drug administration.

A limitation of this study was that the source of isolates was limited to respiratory samples, and all samples were collected from infants and children with lung infections. Thus, our data may not be representative of the true epidemiological situation of *S. pneumoniae* in China.

In summary, our data showed that subtyping methods such as PFGE are essential for understanding the epidemiological characteristics of *S. pneumoniae*. Our results also provide a valuable reference for the selection of alternative antimicrobial agents for Chinese children with *S. pneumoniae*

infection, especially those infected with penicillin-intermediate isolates.

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