Antimicrobial resistance of *Streptococcus pneumoniae* isolated from children and genetic background of penicillin-resistant strains

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Original article

***Background*: *Streptococcus pneumoniae* (*S. pneumoniae*) is one of the most clinically significant pathogens with emerging antibiotic resistance, and most of the highly resistant strains to penicillin were clone related all over the world. This study was undertaken to investigate the antibiotic-resistant pattern and the genetic background of *S. pneumoniae* isolated from children in Hangzhou, China.**

***Methods*: The sensitivities of 323 strains of *S. pneumoniae* to 9 different antibiotics were determined *in vitro* with the Kirby-Bauer diffusion method. Minimal inhibitory concentrations (MICs) of penicillin, cefotaxime and erythromycin were determined with the E-test method. Genetic types were analyzed with BOX-PCR.**

***Results*: Among the 323 strains isolated from children between August 2001 and July 2002, 136 strains (42.1%) were sensitive to penicillin, while 57 strains (17.7%) were penicillin-resistant isolates. MICs for penicillin ranged from 0.012 μg/ml to 4.0 μg/ml. Three hundred and sixteen (97.8%) isolates were sensitive to cefotaxime with the MICs ranging from 0.008 μg/ml to 1.0 μg/ml. Seven isolates (2.2%) showed intermediate MICs with 2.0 μg/ml. Remarkably high levels of resistance were observed in 90.7% and 87.6% of the strains being resistant to erythromycin and tetracycline, respectively. Resistance to trimethoprim-sulfamethoxazole and choloromycetin was found in 48.6% and 14.9% of the strains. One hundred and ninety-seven strains (61.0%) were multi-resistant pneumococci, and most of them were cross-resistant to trimethoprim-sulfamethoxazole, erythromycin and tetracycline. Two strains (0.6%) were resistant to rifampin, and none was resistant to vacomycin and ofloxacin. On the basis of BOX-PCR typing of penicillin resistant *Streptococcus pneumoniae*, no dominant fingerprinting pattern could be identified among clinical isolates, whereas the banding patterns were always similar to or identical among the isolates from healthy individuals or from the same specimen / patient at different times.**

***Conclusions*: The antibiotic-resistance of pneumococci has been found to be high in Hangzhou, but third-generation cephalosporins are still the first option against penicillin-resistant *Streptococcus pneumoniae*. The penicillin-resistant pneumococci might be one of geographic origins in the Hangzhou region, and one child could be infected or colonized by more than one pneumococci clone at the same time or at different times.**

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***Key words*: *Streptococcus pneumoniae*;
children;
antibiotic-resistance;
BOX-PCR**

Introduction

The gram-positive bacterium *Streptococcus pneumoniae* (*S. pneumoniae*) is an important pathogen causing invasive and non-invasive infections in children. For many years, pneumococci were uniformly susceptible to penicillin, until the first clinical isolate resistant to penicillin was reported in 1967.[1] The extensive use of large numbers of antimicrobial agents fueled the crisis of antibiotic resistance in the era of modern chemotherapy. The resistance of *S. pneumoniae* to penicillin and other β-lactam agents has been rapidly increasing in many countries during the past decade, and it is now a worldwide problem. However, the frequency of penicillin resistant *S. pneumoniae* (PRSP) and the prevalence of PRSP among children have been

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shown to be dependent on the source of isolation and the geographic region.[2,3] Increases in the prevalence of PRSP have also been described.[2,4-6] The purpose of the present study was to determine the antibiotic-resistant types and epidemic characterization of *S. pneumoniae* isolated from children in Hangzhou, China.

Methods

Pneumococcal isolates

During the 12-month period between August 2001 and July 2002, a total of 323 isolates of *S. pneumoniae* (243 from inpatients and 80 from healthy children in a day care center) were consecutively identified at the Children's Hospital, Zhejiang University School of Medicine. All the 243 clinical isolates, one per patient, were recovered from respiratory tract secretions (sputum and bronchial washing fluid from 222 children with lower respiratory tract infections and nasopharynx swabs from 6 children with upper respiratory tract infections), conjunctival secretions (6 isolates), blood (5 isolates), vulvovaginal secretions (3 isolates) and urine (1 isolate).

Antimicrobial agents and other materials

Standard antimicrobial agent discs, including oxacillin (OX, 1 μg), erythromycin (E, 15 μg), chloromycetin (C, 30 μg), rifampin (RD, 5 μg), tetracycline (TE, 30 μg), vacomycin (VA, 30 μg), ofloxacin (OFX, 5 μg) and trimethoprim-sulfamethoxazole (SXT, 1.25-23.75 μg) were obtained from Oxoid Company (England). The E test strips of penicillin (P, 0.002-32 μg/ml), cefotaxime (CTX, 0.002-32 μg/ml) and erythromycin (E 0.016-256 μg/ml) were obtained from AB BioDisk Company (Solna, Sweden). Columbia agar plates containing 5% sheep blood, Mueller-Hinton agar supplemented with 5% sheep blood, sterile cotton-swab and slidex pneumo-Kit were manufactured by BioMériex Company (France) and optochin discs were produced by Tianhe Microbiotic Production Company (Hangzhou, China). Taq DNA polymerase and dNTP were obtained from Shanghai Sangon Company and the Touchgene Gradient PCR engine was obtained from TECHNE Company in England.

Methods

Culture of isolation

Isolates were cultured for 18 to 24 hours on plates supplemented with 5% sterile sheep blood at 35℃ in a 5%-10% CO_2 incubator. *S. pneumoniae* isolates were screened according to typical colony appearance, α-hemolysis, and gram staining, and were identified using the optochin sensitivity test and latex agglutination test.

Antibiotic susceptibility test

Antimicrobial susceptibility test was performed from a bacterial inoculum whose turbidity was equivalent to that of a McFarland standard of 0.5. From this suspension, the sensitivities to penicillin, erythromycin, tetracycline, trimethoprim-sulfamethoxazole, chloromycetin, rifampin, ofloxacin and vacomycin were determined with the Kirby-Bauer diffusion method according to recommendations of the National Committee on Clinical Laboratory Standards (NCCLS M100-S13). The MICs of penicillin, cefotaxime and erythromycin were determined with the E-test method on 5% sheep blood Mueller-Hinton agar following the manufacturer's instructions and interpreted according to NCCLS breakpoints (M100-S13). MICs falling between two marks on the E-test strip were rounded up to the next higher dilution, as recommended in the instructions. Penicillin susceptibility was determined using both the agar disc diffusion method with 1 μg oxacillin and the penicillin G E-test method. In all assays, the *S. pneumoniae* reference strain ATCC 49 619 was included as a quality control strain.

BOX-PCR

The DNA sequence of primers, 5'-ATA CTC TTC GAA AAT CTC TTC AAA C-3', was designed according to the stem-loop structure of the consensus BOX-A sequence. Selected penicillin-resistant isolates and the strains from the same patient at different time or from the same specimen with non-identical phenotype were characterized further by using the BOX-PCR method. Template DNA was extracted: an inoculum was prepared from an overnight agar plate by suspending some colonies in distilled water to a density equivalent to 1.0 MacFarland turbidity in a volume of 0.2 ml and afterwards boiled for 15 minutes. The resulting suspensions were centrifuged at 12 000 rpm for 15 minutes; the upper liquid layer was reserved as template DNA. Amplification was done in 50 μl total volume, which included 5 μl of 10 × PCR buffer, 6 μl of 25 mmol $MgCl_2$, 2 μl of template DNA, 1 μl (98 pmol) of primer, 2 μl of dNTP, and 3.5 U of Taq DNA polymerase. The PCR program was designed as follows: the incubation mixtures were predenatured for 4 minutes at 95℃, after which 30 cycles of repeated denaturation 1 minute at 94℃, primer annealing 1 minute at 53℃, and chain extension 2 minutes at 72℃ took place. Finally, a postcycling incubation of 5 minutes at 72℃ was performed. The amplified products were separated by length on 2% agarose gels run in 1 × Tris-borate-EDTA at a constant voltage of 80 v for 1.5 hours. The data were evaluated by visual inspection of the banding pat-

terns. DNA banding patterns were analyzed and pictured with the gel photograph system exposed for 40 seconds. The clones were identified according to BOX patterns. BOX typing was performed as described previously;[7,8] the quality control strain ATCC 49619 was defined as A type. BOX PCR patterns showing a single band difference in number were defined as nonidentical types (e.g., A, B and C). Identical patterns with the same bands varying in the distribution of two or more bands were defined as subtypes (e.g., 1, 2, and 3).

Statistical analysis

Antibiotic-resistance between clinical isolates and the isolates from healthy children was compared using the chi-square. P values less than 0.05 was considered statistically significant.

Results

Distribution and isolation rates

The rates of distribution and isolation of 222 strains of *S. pneumoniae* isolated from children with lower respiratory tract infection over 12 consecutive months are shown in Fig. 1. The highest rate of isolation was in December. The lower rates of isolation ranged from August to October.

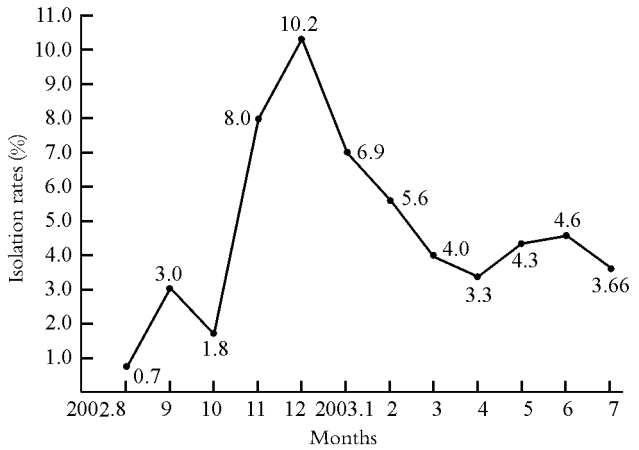

Fig. 1. The rates of distribution and isolation of 222 strains of *S. pneumoniae* isolated from 4238 children with lower respiratory tract infection.

Percentages of isolation susceptible, intermediate and resistant to penicillin

The percentages of isolates susceptible, intermediate and resistant to penicillin were 42.1%, 40.2% and 17.7%, respectively. The activities *in vitro* of 9 antimicrobial agents against *S. pneumoniae* were compared (Table 1). Of the 243 clinical isolates of *S. pneumoniae* collected in this study, 111 strains (45.7%) were found to be susceptible to penicillin (MIC≤0.064 μg/ml), whereas of the 80 strains of *S. pneumoniae* isolated from healthy children, only 25 strains (31.3%) were susceptible to penicillin. There was a higher rate of penicillin-susceptible strains in patients than in healthy children ($\chi^2=5.14$, $P<0.05$). Seventy-three (91.3%) of the 80 carrier isolates were sensitive to chloromycetin, whereas among the 243 clinical isolates, 197 strains (81.1%) were chloromycetin-susceptible ($\chi^2=4.55$, $P<0.05$). The susceptibility rates for the other 7 antibiotics did not show a statistically significant difference between clinical and carrier isolates. The sensitivity to 9 antimicrobial agents in the 243 clinical isolates and 80 carrier isolates was compared (Table 2).

Table 1. Activities of 9 antimicrobial agents against 323 isolates of *S. pneumoniae in vitro*

Antibiotics	Isolates (%)		
	Susceptible	Intermediate	Resistant
Penicillin	42.1	40.2	17.7
Cefotaxime	97.8	2.2	0.0
Erythromycin	9.0	0.3	90.7
Chloromycetin	83.6	1.5	14.9
Trimethoprim-sulfamethoxazole	44.0	7.4	48.6
Tetracycline	9.9	2.5	87.6
Ofloxacin	99.4	0.6	0.0
Rifampin	99.4	0.0	0.6
Vacomycine	100.0	0.0	0.0

Antibiotic resistant pattern

Of the 323 isolates of *S. pneumoniae*, only 11 (3.4%) strains were susceptible to all the 9 antibiotics, and 285 strains (88.2%) were resistant to more than one antibiotic. Of multi-resistant isolates, 197 (61.0%) strains were defined as resistant to 3 different classic antibiotics. The multi-resistant rate in PRSP (98.2%, 56/57) was higher than in penicillin sensitive *S. pneumoniae* (60.3%, 82/136), and the difference was statistically significant ($\chi^2=28.39$, $P<0.001$) (Table 3).

MICs tested with the E test method

Of the 323 isolates tested for MICs to penicillin, 42.1%, 40.2%, and 17.7% had an MIC 0.012-0.064 μg/ml, 0.094-1.0 μg/ml, and over 1.5 μg/ml, respectively. MIC_{50} and MIC_{90} of penicillin ranged from 0.012 μg/ml to 4.0 μg/ml, and MICs distribution was assessed with the E test method for isolates of *S. pneumoniae* (Fig. 2). MICs of cefotaxime ranged from 0.008 μg/ml to 2.0 μg/ml. About 97.8% of the isolates were cefotaxime-sensitive at levels from 0.008 μg/ml to 1.0 μg/ml, and 2.2% were cefotaxime-intermediate with reduced MICs at 2.0 μg/ml. None was resistant to cefotaxime. Erythromycin MICs were tested in 113 isolates of pneumococci strains and 73.9% of the isolates had MICs above 256 μg/ml. The distribution of

erythromycin MICs was detected (Fig. 3).

BOX-PCR typing

BOX-PCR typing was carried out to define the types or the subtypes of A, B, B_1, B_2, C, C_1, C_2, C_3, D, D_1, D_2, D_3, D_4, E, E_1, E_2, E_3, F, F_2, F_3, G, H, I, J, and L. No dominant fingerprinting pattern was found in PRSP of patients, whereas more than half of the banding patterns were type C in PRSP of healthy children. Six couples of *S. pneumoniae* with different phenotypes (including 4 couples with different dome and 2 couples with different drug-resistant types) isolated from the same specimen were typed with BOX-PCR, and 5 couples were identified as having the same BOX patterns except one couple with different patterns of 2 bands. *S. pneumoniae* were isolated more than one time from the same 5 patients with recurrent infections (including 5 times in one patient), only 1 of the 5 groups of pneumococci had different DNA banding patterns. Some common BOX-PCR types are shown in Fig. 4.

Table 2. A comparison of the sensitivity to 9 antimicrobial agents in 243 clinical isolates and 80 carrier isolates

Antibiotics	Clinical isolates (243 strains) (%)			Carrier isolates (80 strains) (%)			χ^2 value	*P* value*
	S	I	R	S	I	R		
Penicillin	45.7	38.7	15.6	31.3	45.0	23.7	5.14	<0.05
Cefotaxime	97.5	2.5	0.0	98.8	1.2	0.0	0.01	>0.05
Erythromycin	9.5	0.4	90.1	7.5	0.0	92.5	0.09	>0.05
Chloromycetin	81.1	2.1	16.8	91.3	0.0	8.7	4.55	<0.05
SXT	44.0	8.2	47.8	43.8	5.0	51.2	0.001	>0.05
Tetracycline	8.6	2.9	88.5	13.7	1.3	85.0	1.78	>0.05
Ofloxacin	99.2	0.8	0.0	100.0	0.0	0.0	-	-
Rifampin	99.6	0.0	0.4	98.8	0.0	1.2	-	-
Vacomycine	100.0	0.0	0.0	100.0	0.0	0.0	-	-

SXT: trimethoprim-sulfamethoxazole; *: sensitivity rate.

Table 3. Antibiotic-resistant patterns in 285 strains of *S. pneumoniae*

Antimicrobial agents	No. of isolates	PSSP	PISP	PRSP	MRSP
E, TET	105	26	53	27	27
E, SXT	14	2	7	5	5
E, TET, SXT	119	55	49	15	119
E, TET, C	11	8	1	2	11
E, SXT, C	3	2	0	1	3
E, TET, SXT, C	29	17	8	4	29
E, TET, RD	2	0	1	1	2
TET, C	1	0	0	1	1
TET	1	-	-	1	0
Total	285	110	119	57	197

E: erythromycin; TET: tetracycline; SXT: trimethoprim-sulfamethoxazole; C: Chloromycetin; RD: rifampin; PSSP: penicillin-susceptible *S. pneumoniae*; PISP: penicillin-intermediate *S. pneumoniae*; PRSP: penicillin-resistant *S. pneumoniae*; MRSP: multi-resistant *S. pneumoniae*.

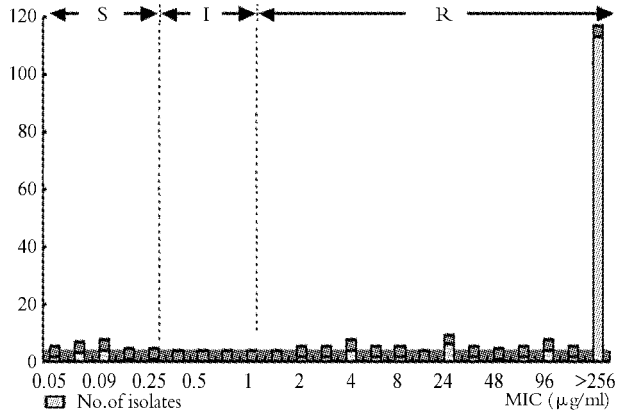

Fig. 3. MIC distribution of erythromycin with the E test method for 113 isolates of *S. pneumoniae*. S: susceptible; I: intermediate; R: resistant.

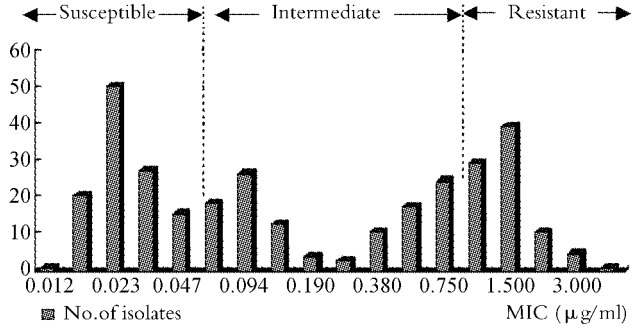

Fig. 2. MIC distribution of penicillin with the E test method for isolates of *S. pneumoniae*.

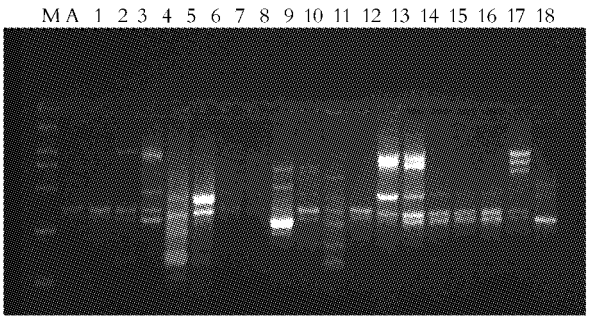

Fig. 4. Examples of BOX-patterns in pencillin-resistant *S. pneumoniae*, A: ATCC49619; M: marker ladder 2000.

Discussion

S. pneumoniae is one of the most significant pathogens responsible for a variety of infections in children. In the present study, *S. pneumoniae* clinical strains were isolated from samples of secretion from the lower respiratory tract, indicating that *S. pneumoniae* cause lower respiratory tract infection such as bronchitis and bronchopneumonia. The constituent ratio of the strains isolated from blood was low and no meningitis strain was found in the study presumably because of the low incidence of the 2 diseases and the extensive use of antibiotics in the Hangzhou area. *S. pneumoniae* infections were found to show seasonal peaks of prevalence. More strains and higher isolation rates were found in November, December, January, and February than in other months. This finding was consistent with the clinical observation of a particularly high incidence of respiratory tract infections in Hangzhou in these months. The results of drug susceptibility tests showed that 40.2% and 17.7% of the strains were penicillin-intermediate and penicillin-resistant, respectively. These rates were higher than those reported from Beijing (15.4%),[9] and similar to those reported from neighboring countries.[10] Penicillin MIC distribution showed 2 peaks at 0.023 μg/ml and 1.5 μg/ml respectively, a pattern being different from the normal distribution reported by Wang et al.[11] Thirty isolations (9.3%) had penicillin MICs at 1.0 μg/ml, which was the penicillin breakpoint between the intermediate and resistant, indicating that the total average sensitivity to penicillin was low in the areas. Contrary to other studies,[9,12] we found in this study a higher sensitivity in clinical strains than in carrier strains. We assumed that this sensitivity may be caused by the different sources of isolations and different geographic regions. Patients with clinical strains came from a vast geographic catchment area, whereas carrier strains were collected from only the one-day care center by nasopharyngeal swabs culture. Although all strains of *S. pneumoniae* were susceptible to vacomycin, this substance was not used in pediatrics because of its toxic side-effect. Based on recent NCCLS-approved breakpoints, 316 isolates (97.8%) were susceptible to cefotaxime, and 7 isolates (2.2%) showed reduced MICs and no resistant strain was found. It is suggested that third generation cephalosporins should be the first choice in the management of infections known or suspected to be caused by pneumococci, and PRSP in particular.

Macrolide resistance in *S. pneumoniae* has remained at a low level in most countries, despite its variable geographic distribution. In the United States, the rate of resistance to erythromycin was reported as 25.7%.[13] In the present study, our isolates demonstrated a high incidence of resistance to erythromycin, and 90.7% of the strains were resistant, in contrast to 72.1% in Beijing,[11] and 53% in Shanghai.[14] This incidence probably reflects the wide use of macrolide in Hangzhou. The microbial resistance is thought to respond to different antibiotic pressures in the community. In Spain, for example, one of the areas with a high prevalence of penicillin-nonsusceptible *S. pneumoniae*, the low incidence of erythromycin resistance is probably due to the infrequent use of erythromycin in this country. Whereas macrolides are often prescribed by physicians as first-line antibiotics in Hangzhou and are readily available over the counter in drugstores. Of the 113 consecutive isolates tested for MICs, 83 isolates (73.9%) were highly resistant to erythromycin with an MIC above 256 μg/ml. Thus, it is reasonable to suggest that erythromycin may not be considered as a routine option in the treatment of *S. pneumoniae* infections in Hangzhou.

Resistance to tetracycline (87.6%) is observed at a remarkably high level, too, but the cause of high resistance was obscure because tetracycline has not been administered to pediatric patients in this hospital for decades. Compared with the results from other studies, the proportions of resistance to chloromycetin and trimethoprim-sulfamethoxazole are 14.9% and 48.6% respectively, in contrast to 51.0% and 62.4% in Beijing, whereas those of resistance to tetracycline, rifampin and ofloxacin are similar in the two areas. Significant difference or similar activities of antibiotics against pneumococci might be due to the different or the identical use of antibiotics in those areas.

Multi-resistant *S. pneumoniae* (MRSP), defined as resistant to 3 or more different classic antibiotics, is a phenotype of high clinical significance in chemotherapy. In this study, 197 isolates (61%) exhibited a multi-resistant pattern, and the most frequently observed pattern of cross-resistance included erythromycin, tetracycline and trimethoprim-sulfamethoxazole. As in other studies,[15] the proportion of multi-resistant *S. pneumoniae* was significantly greater in penicillin-resistant pneumococci than in penicillin susceptible *S. pneumoniae*. Being relatively more resistant to other non β-lactam drugs, the PRSP compromised the choice of antibiotics available for empiric treatment of infections caused by these organisms.

The *S. pneumoniae* genome contains a group of highly conserved DNA sequences called BOX elements, which are located within intergenic regions of the chromosome and are composed of 3 subunits (boxA, boxB, and boxC).[16] The BOX elements, indicating the genetic background of clones, are highly conserved in persistence and sequence even over a long time. A previous study showed that the BOX repeat

element-based PCR was a rapid and highly reproducible method in identifying genotypic variation among pneumococcal isolations that did not rely on phenotypic characteristics. Its capacity of discriminating between similar, but not identical, isolates reaches or exceeds that of other available molecular typing approaches.[8] Thus, on the basis of this study,[8] the DNA primer sequence was designed according to the stem-loop structure of the consensus BOX-A sequence and a BOX PCR was performed in this study. Because BOX-A primer generated satisfactory amounts of amplicons in highly variable numbers and sizes, the clonally related *S. pneumoniae* strains were identified successfully according to the band patterns. The more common the bands, the closer relation between isolates. And strains with an identical banding pattern belong to the same clone. Thus, this method could be used in investigations of pneumococcal outbreaks and carriage in molecular epidemiology and could differentiate reinfection from recurrence in clinical medicine.[17] In the present study, 12 isolates, 2 of each patient with different phenotype, were studied with the BOX PCR method. The identical BOX patterns of the 5 couples indicated the same clone, except that the pattern of 1 couple only differed by 2 bands, indicated a nonidentical clone. Both mucoid and non-mucoid dome-shaped pneumococci were isolated after culture of a sputum specimen, and later they were identified to have the same BOX pattern, indicating that some pneumococci isolates had acquired an outside source DNA encoding for the synthesis of the new capsule or some generation of the clone had lost some DNA sequence encoding for the synthesis of the original capsule. It is known that this sort of gene transfer events is common among pneumococci species.[18,19] In 5 patients of this group, *S. pneumoniae* strains were isolated more than once. Four of the 5 patients could be characterized on the basis of an identical band pattern as suffering from recurrent infection. One patient with 5 clinical episodes of infection showed 4 recurrent infections with an identical band pattern and a community-acquired reinfection with a different pattern in 1 band.

Mechanisms influencing the prevalence of PRSP, especially that of highly resistant strains, include mostly the geographic spread of PRSP genetic lineages (clones), whereas another minor cause is the acquisition of penicillin resistance genes from other species of pneumococci or streptococci.[7,20] According to the fact that high-level PRSP showed a little variation in their genetic background, and no dominant fingerprinting pattern was found among PRSP isolated from patients in this study, with combination of the high proportion of penicillin-intermediate in the Hangzhou area, we conclude that the PRSP clones are presumably of Hangzhou origin. In contrast to the clinical isolates, more than half of the carrier PRSP had the same BOX pattern, showing that horizontal spread is common in children in crowded environments and may cause a high prevalence of PRSP.

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