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ORIGINAL RESEARCH

PspA Diversity, Serotype Distribution and Antimicrobial Resistance of Invasive Pneumococcal Isolates from Paediatric Patients in Shenzhen, China

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Introduction: To determine the phenotypes and genotypes of invasive *Streptococcus pneumoniae* (*S. pneumoniae*), 108 strains were isolated from paediatric patients with invasive pneumococcal diseases (IPDs) in Shenzhen from 2014 to 2018.

Methods: Serotype profiles were defined by multiplex PCR of the capsule gene. Pneumococcal surface protein A (PspA) classification was performed through *pspA* gene sequencing. Antimicrobial resistance was examined by broth microdilution. Multilocus sequence typing (MLST) was determined based on next-generation sequencing data.

Results: Eighty-one *S. pneumoniae* of 17 serotypes were finally collected. The coverage of the 13-conjugated polysaccharide vaccine (PCV13) was 88.9%. After the introduction of PCV13, the nonvaccine serotypes were added by serotypes 15b, 16F and 20. Vaccine serotype 3 increased by four serious cases. The pspA family 1 and pspA family 2 are predominant. The multiple drug resistance rate is 91.3%. None of the nonmeningitis isolates were resistant to penicillin, while 98.8% of all the isolates were resistant to erythromycin.

Discussion: This work characterizes the molecular epidemiology of invasive *S. pneumoniae* in Shenzhen. Continued surveillance of serotype distribution and antimicrobial susceptibility is necessary to alert antibiotic-resistant nonvaccine serotypes and highly virulent serotypes.

Keywords: *Streptococcus pneumoniae*, invasive pneumococcal disease, PspA family, serotype, antimicrobial resistance

Introduction

S. pneumoniae is a human respiratory tract pathogen that colonizes the nasopharynx of healthy carriers.[1,2] It can cause otitis media, sinusitis, bronchitis and invasive pneumococcal diseases (IPDs), such as sepsis and meningitis.[3] IPDs lead to high morbidity and mortality in young children and elderly people worldwide, posing a huge threat to public health.[4] The World Health Organization (WHO) reported that *S. pneumoniae* is the most common pathogen of pneumonia, accounting for 16% of all deaths in children under 5 years old globally.

S. pneumoniae is capsular Gram-positive diplococcus.[1] The capsule is an effective vaccine target. The currently available vaccines are based on pneumococcal capsular polysaccharides, of which 98 serotypes exist.[5] The 23-valent polysaccharide vaccine, which covers 23 serotypes, cannot stimulate efficient protective immunity in children under 2 years old.[6] The WHO recommended polyvalent

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conjugate vaccines, which include 7-conjugated polysaccharide vaccine (PCV7), 10-conjugated polysaccharide vaccine (PCV10) and 13-conjugated polysaccharide vaccine (PCV13). They show enhanced immunogenicity.[7] Since the introduction of PCVs, the incidence of IPDs caused by nonvaccine serotypes has increased drastically worldwide.[8–10] However, the PCVs can`t cover all serotypes. Serotype replacement in the postvaccine era stresses the need for the development of capsule-independent pneumococcal vaccines.

Surface proteins of *S. pneumoniae* are promising non-polysaccharide vaccine candidates.[11] PspA is able to elicit antibody-mediated protection against pneumococcal infection.[12–14] Multiple antigen vaccines containing PspA showed good immunogenicity and protection in a Phase 1 clinical trial.[15] PspA is exposed on all pneumococcal strains and highly variable in DNA and protein sequences. It consists of three domains: an α-helical domain (αHD) at the N-terminus, a proline-rich domain and a choline-binding domain with a short hydrophobic tail at the C-terminus.[16,17] The C-terminal 100 amino acids of αHD are immunogenic and protective,[18] forming a clade-defining region (CDR).[16] Based on CDRs, *S. pneumoniae* is distributed into three families and six clades: family 1 comprises clades 1 and 2; family 2 comprises clades 3, 4 and 5; and family 3 comprises only clade 6. PspA classification offers information about the antigenic/genetic diversity of PspA among prevalent serotypes.[19–22]

In this study, we will characterize the serotype distribution, PspA diversity and antimicrobial resistance pattern of clinical isolates from children with IPD. We aim to analyse the relevance of serotypes to the PspA family for successful selection of antigens of new vaccines and to monitor the antimicrobial resistance pattern to provide proof for choosing the appropriate antibiotic.

Patients and Methods

Collection of Clinical Isolates of *S. pneumoniae*

S. pneumoniae isolated from patients with IPDs from 2014 to 2018 was obtained from the biobank of a sentinel paediatric hospital in Shenzhen, China. The frozen samples were removed from the −80°C refrigerator and left at room temperature until completely dissolved. The bacterial solution was mixed and inoculated on a blood agar plate (Columbia agar supplemented with 5% sheep blood) and cultured at 37°C in 5% CO_2 for 18 hours to 24 hours. If the colony was small, round, moist, autolyzed with grass green lytic rings and positive in the optochin sensitivity test and biliary lysis test, it was determined to be *S. pneumoniae*. A total of 81 strains were successfully recovered.

Collection of Clinical Data

Clinical information was obtained from the medical records system of the hospital. Epidemiological information included age, sex, community infection, and antibiotic treatment. Clinical manifestations include underlying disease, pulmonary diseases, extrapulmonary diseases, length of hospital stay, admission to the intensive care unit (ICU), invasive mechanical ventilation and in-hospital death. Privacy of the patients was protected. All procedures performed in studies involving human participants were in accordance with the ethical standards of the ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Definitions

Immunosuppression was defined as cytotoxic chemotherapy or high-dose steroid therapy daily for ≥2 weeks and primary immunodeficiency disease. Pneumoniae were confirmed by clinical and chest CT manifestations. S. pneumoniae meningitis was diagnosed by neurological manifestations and cerebrospinal fluid (CSF) abnormalities accompanied by the growth of *S. pneumoniae* in CSF. *S. pneumoniae* osteomyelitis was diagnosed when a positive culture of *S. pneumoniae* from pyogenic fluids was combined with localized symptoms and imaging changes. Sepsis shock was defined by the 2005 International Pediatric Sepsis Definition Conference.

DNA Extraction, PCR and Sequencing

DNA of each strain was extracted using the QiAamp DNA Minikit. The upstream and downstream primers of PCR were LSM13 and SKH2, of which the sequences were 5-GCAAGCTTATGATATAGAAATTT GTAAC-3′ and 5′-CCACATACCGTTTTCTTGTTTC CAGCC-3′, respectively.[16] PCR was performed using a TaKaRa Taq™ Hot Start Version kit with a reaction volume of 50 μL. The PCR procedure was as follows: 95°C denaturation for 5 min, cycling consisted of 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds, repeated 40 times, extension at 72°C for 5 min, and cooling at 20°C for 10 seconds. The PCR product was obtained using the Axygen® AxyPrep

DNA Gel Extraction Kit. The DNA fragments were sent to Invitrogen (Shanghai) for Sanger sequencing.

PspA Sequence Analysis

The *pspA* gene sequences of 24 strains of *S. pneumoniae* reported in Hollingshead's publication were derived from the NCBI database.[16] The CDR and the proline-rich domain (PRD) were taken from the PspA amino acid sequence. CDR is the N-terminal 100 amino acids of αHD predicted by SMART.[23,24] PRD is considered to start at the first proline of a succession of prolines of succession of prolines interspersed with other amino acids. Mega-X was used to do multiple sequence alignment. Neighbour-joining trees were built from aligned sequences with a bootstrap of 1000. Based on the fasta and nwk files generated by mega-X, the phylogenetic tree was displayed using the ggtree package in R.

Serotyping

Pneumococcal serotypes were assigned using continuous multiplex PCR and PCR-based sequencing methods as described previously.[25] This process consists of seven rounds of sequential multiplex PCR. Each round of multiplex PCR includes four pairs of primers for the serotype-specific sequence of the capsule gene (CPS) and one pair for the serotype conservative sequence of CPS. Serotypes 6A and 6B were further distinguished by Sanger sequencing. For *S. pneumoniae* serotyped as 6A or 6B, PCR was carried out with the upstream primer 6A/B-F: AATTTGTATTTTATTCATGCCTATATCTGG and the downstream primer 6A/B-R: TTAGCGGAGATA ATTTAAAATGATGACTA. The PCR products were recovered and sent to Invitrogen for Sangar sequencing. Blastn was used to detect the single nucleotide polymorphism at codon 195 of the cps locus wciP gene as described previously.[26]

Antimicrobial Susceptibility Testing

Broth microdilution was used to measure the minimum inhibitory concentration (MIC) based on the MIC criteria of 17 antimicrobial agents (penicillin, cefotaxime, ceftriaxone, amoxicillin, ertapenem, meropenem, erythromycin, telithromycin, levofloxacin, moxifloxacin, ofloxacin, tetracycline, tetracycline, vancomycin, linezolid, chloramphenicol, and compound sulfamethoxazole). Multidrug resistance (MDR) is defined as resistance to three or more different antimicrobial classes.

The detailed procedure of broth microdilution is as follows: The pure colonies of *S. pneumoniae* were suspended in sterile normal saline to prepare bacterial fluid of 0.5 McFarla. Sixty microlitres of the bacterial fluid was diluted into 12 mL CAMHB+LHB. Then, 100 μL of dilution was added to a 96-well plate for pneumococcal antimicrobial susceptibility testing and cultured at 37°C for 22 hrs. The 96-well plate was then read, and the MICs were recorded.

MLST

MLST of *S. pneumoniae* is based on sequences of internal fragments from the aroE, gdh, gki, recP, spi, xpt and ddl genes. The assembled genomes of four strains of serotype 3 in Shenzhen were gained through next-generation sequencing. MLST1.8 (https://cge.cbs.dtu.dk//services/MLST/) was used to analyse the STs. MLST data of *S. pneumoniae* serotype 3 from 13 foreign countries were collected from PubMLST (https://pubmlst.org/) and previous publications.[27,28] The Eburst algorithm was used to estimate the relationship

Table 1 Demographical and Clinical Characteristics of 81 Patients

Characteristics	n (%)/Median
Demographic characteristics	
Age (years)	1.08 (0.79, 3.20)
Gender	
Male	47 (58.0%)
Female	34 (42.0%)
Inpatients	69 (85.2%)
Length of hospital stay (days)	8 (5,18)
Underlying conditions	
Cytotoxic chemotherapy	1 (1.2%)
Primar immunodeficiency disease	1 (1.2%)
Hematologic malignancy	1 (1.2%)
Congenital heart disease	4 (4.9%)
Congenital meningocele	1 (1.2%)
IPD	
Pneumonia	17 (21.0%)
Severe pneumonia	5 (6.2%)
Meningitis	21 (25.9%)
Sepsis	41 (50.6%)
Sepsis shock	7 (8.6%)
Septic arthritis	4 (4.9%)
Osteomyelitis	4 (4.9%)
Intensive care unit admission	16 (19.8%)
Invasive mechanical ventilation	6 (7.4%)
In-hospital mortality	11 (13.6%)

among isolates. STs that share six identical alleles of the seven MLST loci were grouped as a clone complex (CC).

Statistical Analysis

The chi-square test was used to compare the rates, and Fisher's exact test was used if the expected value in the grid was less than 5. All statistical analyses were performed using SPSS 19.0. P value<0.05 is considered significant.

Results

Demographical and Clinical Characteristics of Patients with IPDs

Among the 81 isolates, three were collected in 2014, six were collected in 2015, 17 were collected in 2016, 20 were collected in 2017 and 35 were collected in 2018. Eight strains were isolated from CSF, 66 were isolated from blood, and 7 were isolated from pleural fluid and joint cavity fluid. The median age of patients was 1.08 (0.79–-3.20). The male-to-female ratio was 1.4:1. The median duration of hospitalization was 8(5–18) days. There were eight children with underlying diseases. The most common IPD was sepsis (41, 50.6%). Among all the patients, 16 were admitted to the ICU, 19.8% and 7.4% of them used invasive mechanical ventilation, and 13.6% of them died. The detailed information is seen in Table 1.

PspA Diversity

The PspA CDRs were sequenced, and each amino acid sequence was obtained. An NJ tree was generated from 81 Shenzhen isolates and 24 strains reported by Hollingshead et al (Figure 1). The tree supports the same family and clade divisions of the 24 strains as those in Hollingshead's study. The Shenzhen isolates fell into three families and six clades. There were 24 strains (29.6%) in PspA family 1, 56 (69.1%) strains in family 2 and only one (1.2%) in family 3.

Serotype Distribution

PCV13 was introduced in Shenzhen in June 2017. We defined the period before June 2017 as the period before the introduction of PCV13, during which we collected 32 clinical strains. We defined the period after June 2017 as after the introduction of PCV13, during which we collected 49 strains of *S. pneumoniae*. Before and after the introduction of PCV13, the proportions of PCV13 serotypes were 87.5% and 89.8%, respectively, with no significant difference (Fisher's exact test, p=0.734). As shown in Figure 2,

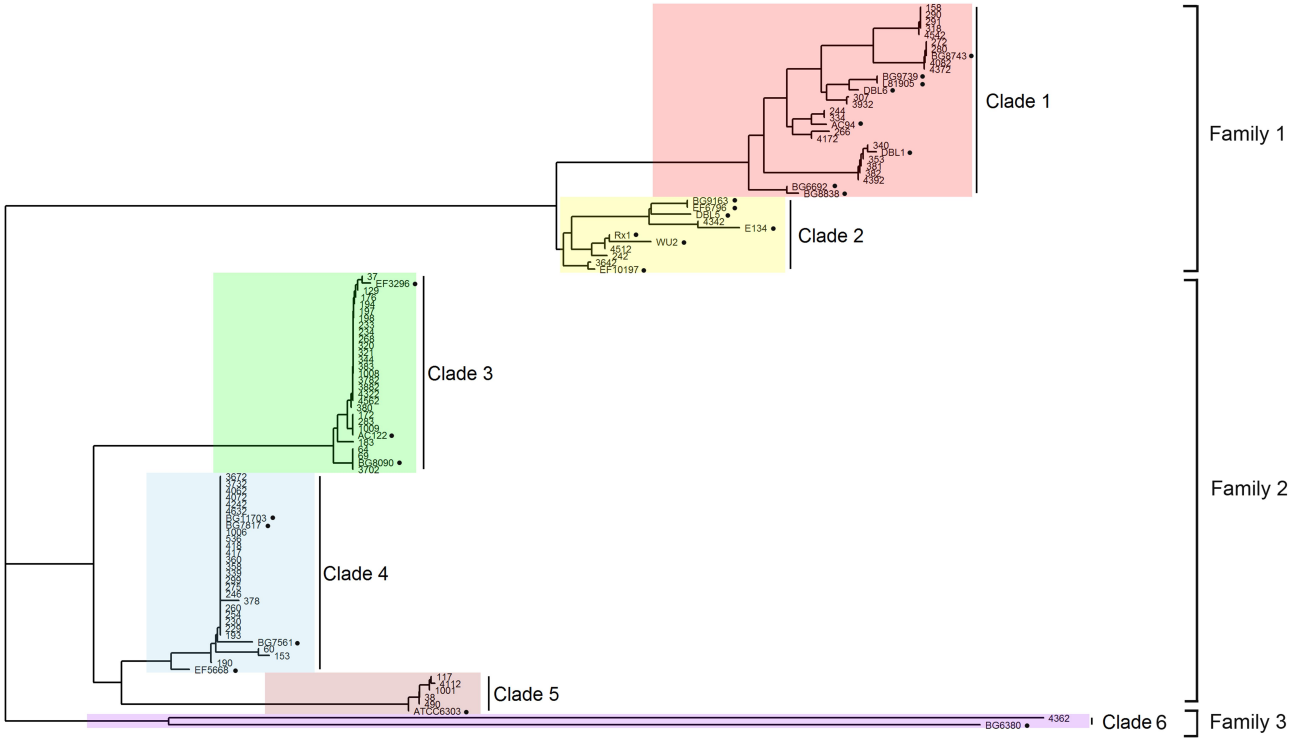

Figure 1 PspA families and clades of Shenzhen`s isolates. PspA clades 1–6 were highlighted by red, yellow, green, blue, pink and purple respectively. The black solid circles indicate the 24 strains reported previously, which were identified using their names.

before the introduction of PCV13, the nonvaccine serotypes were 35B, 33F, 34,15A. After the introduction of the vaccine, the nonvaccine serotypes were 35B, 33F, 15B, 16F, and 20. Isolates of serotypes 3,6A,34,15A,15B,16F and 20 only had PspA family 1. Isolates of serotypes 19F,14,18C,4, 9V,33F only had PspA family 2. Isolates of serotypes 6B, 19A, and 35B expressed either pspA family 1 or family 2. Isolates of serotype 23F expressed either PspA family 2 or family 3.

Antimicrobial Resistance Pattern and Multiple Drug Resistance

Due to test failure of one serotype 3 strain, the antibiotic activities of only 80 of the 81 *S. pneumoniae* isolates against 14 antimicrobials are presented in Table 2. According to the revised CLSI breakpoints for penicillin (resistance ≥ 8 mg/mL for nonmeningitis isolates and ≥0.12 mg/mL for meningitis isolates), the prevalence rates of penicillin resistance were 0% and 100% in the nonmeningitis and meningitis isolates, respectively. Antibiotic treatment before blood culture of eight children with pneumococcal meningitis is described as follows: no antibiotic (3), cephalosporins (2), cephalosporins and amoxicillin (1), amoxicillin and erythromycin (1), and meropenem (1). None of the isolates were resistant to amoxicillin/clavulanic acid. The cefuroxime resistance rate was 70%. The proportions of isolates resistant to ceftriaxone were 5.6% in the nonmeningitis isolates and 25% in the meningitis isolates. The cefepime resistance rate was zero for nonmeningitis isolates and 25% for meningitis isolates. As high as 8.8% of isolates were resistant to Meropenem. All of the isolates were susceptible to rifampicin, levofloxacin, linezolid and vancomycin. A total of 97.5% and 98.9% of these isolates were resistant to clindamycin and erythromycin, respectively. A total of 91.3% of these isolates were resistant to at least three classes of antibiotics. The MDR ratios were 94.4% and 66.7% in vaccine serotypes and nonvaccine serotypes,

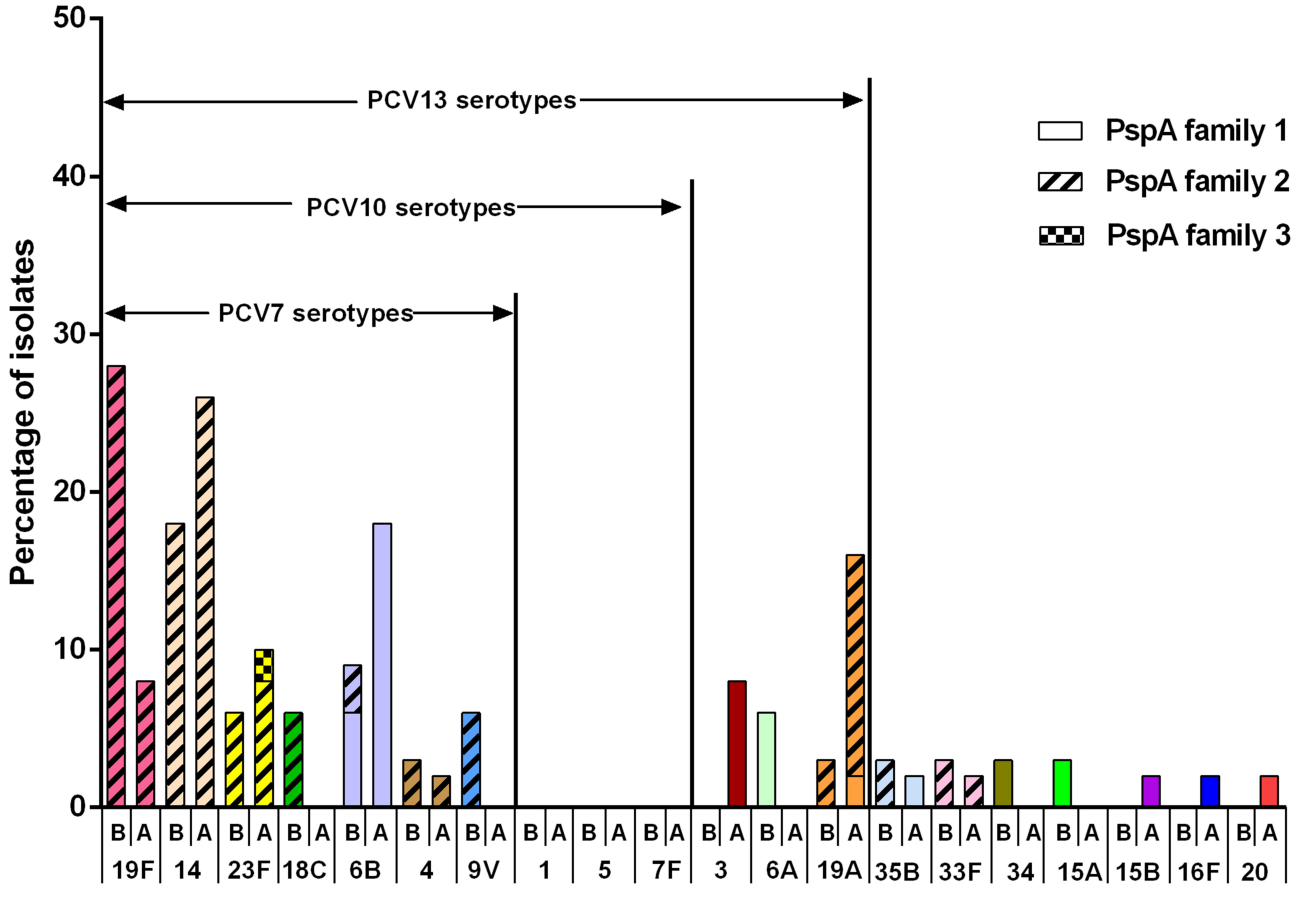

Figure 2 Serotype distribution before and after PCV13 introduction. B represents before introduction of PCV13, and A represents after introduction of PCV13. Each serotype is colored specifically. The height of the column indicates the composition ratio of the serotype in either period. The proportion of PspA family 1, 2 and 3 are expressed by different filling patterns.

Table 2 Susceptibility and MIC of Antibiotics for IPD Isolates in Shenzhen

Antimicrobials	No. of Isolates	Susceptibility			MIC mg/mL		
		Resistant (%)	Intermediate (%)	Susceptible (%)	50%	90%	Range
Penicillin G(P) –non-Meningitis	72	0	27.8	72.2	2	4	≤0.032–4
Penicillin G(P) –Meningitis	8	100	0	0	2	4	0.125–4
Amoxicillin/clavulanic acid	72	0	22.2	77.8	≤0.5	4	≤0.5–4
Cefuroxime	80	70	3.8	26.2	2	>8	≤0.25–>8
Cefatriaxone-non-Meningitis	72	5.6	13.9	80.6	0.5	2	≤0.25–4
Cefatriaxone-Meningitis	8	25	12.5	62.5	0.5	2	≤0.25–2
Cefepime-non-Meningitis	72	0	16.7	83.3	0.5	2	≤0.25– 2
Cefepime-Meningitis	8	25	12.5	62.5	0.5	2	≤0.25– 2
Meropenem	80	8.8	31.2	60	0.25	0.5	≤.064– 1
Rifampicin	80	0	0	100	≤0.25	≤0.25	≤0.25 – ≤0.25
Levofloxacin	80	0	0	100	≤0.5	1	≤0.5– 1
SMZ-TMP	80	15	41.2	43.8	1	4	≤0.25– 4
Clindamycin	80	97.5	0	2.5	>2	>2	≤0.064–>2
Erythromycin	80	98.8	0	1.2	>2	>2	0.25–>2
Linezolid	80	0	0	100	0.25	0.5	≤0.125–0.5
Vancomycin	80	0	0	100	≤0.125	0.25	≤0.125– 0.25
Tetracycline	80	82.5	12.5	5	16	>16	0.5–>16

respectively. The MDR ratio of each serotype is shown in Figure 3.

MLST of Serotype 3

Serotype 3 of *S. pneumoniae* is highly virulent[29] and not protected against by immunization with PCV13,[30,31] even though serotype 3 polysaccharide is in the vaccine. Serotype 3 is widely spread in Europe, Japan, North America and South America. The majority of this serotype is a single clonal complex 180CC.[32] Serotype 3 was rarely isolated from children in China before 2017.[33] We isolated serotype 3 *S. pneumoniae* from four inpatients admitted in 2018, two of whom developed necrotizing pneumonia. MLST results are ST505 (1), ST4655(1) and ST12902(2). ST4655 with serotype 3 was reported in Shanghai in 2019.[34] ST12902 is a novel ST. PHYLOViZ showed that ST505 belongs to CC180 (Figure 4).

Discussion

IPDs in infants and children lead to high mortality. Vaccination is an effective preventive measure. PCVs, which combine capsular polysaccharides of epidemic serotypes and protein vectors, can cause an effective immune response in infants younger than 2 years old. *S. pneumoniae* has 98 serotypes, and the proportion of serotypes changes with the passage of time and use of vaccines. The present vaccine based on capsular polysaccharides cannot cover all serotypes. Therefore, the recombinant protein vaccine is a better choice.

PspA is the most promising candidate vaccine antigen. Both CDR and PRD sequences in PspA can induce cross-reactive antibodies and confer protective immunity of *S. pneumoniae* with PspA of the family. The families of PspA in adult isolates are mainly family 1 and family 2 in the United States, Japan and other foreign countries,[16,19–21] while the data from children are

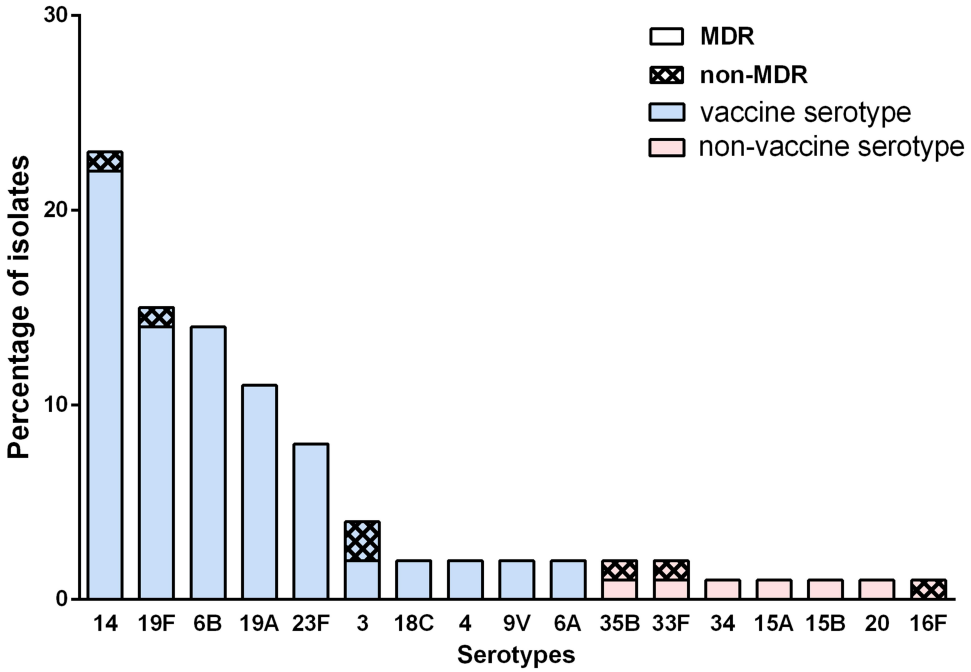

Figure 3 The multiple drug resistance rate of prevalent serotypes in Shenzhen. Vaccine serotypes refers to the serotypes covered by PCVs, which are colored by blue. The non-vaccine serotypes were not covered by PCVs and are colored by red. The proportion of MDR and non-MDR are distinguished by different filling patterns.

limited. In our study, invasive isolates of children fell into PspA family 1, family 2 and family 3. Accordingly, in the preparation of a successful recombinant PspA vaccine, both family 1 and family 2 should be incorporated.

In addition, associations between particular serotypes and the PspA family were observed in our work (eg, serotype 3, PspA family 1; serotype 19F, PspA family 2), as previously reported in another study with a small sample size.[22] However, a study including 251 isolates of *S. pneumoniae* from upper respiratory tract infections drew a different conclusion that serotypes were generally not exclusively associated with certain PspA families, although some capsular types showed a much higher proportion of either family 1 or 2.[35] Therefore, more strains are needed to determine the association.

Antibiotic resistance of *S. pneumoniae* is a worldwide concern.[36,37] Penicillin is the first-line antibiotic for treating *S. pneumoniae* infection.[38] According to the revised CLSI breakpoints for penicillin, the penicillin nonsensitivity rate of nonmeningitis strains was 27.8%, and the penicillin resistance rate of meningitis strains was 100%. The resistance rate of meropenem was 8.8%. One meningitis isolate (No. 320, serotype 19A) showed extensive resistance to the spectrum of common antibiotics, including penicillin and meropenem. In addition, we paid attention to the antimicrobial resistance of nonvaccine serotypes. The MDR rate of 9 nonvaccine serotypes was lower than that of vaccine serotypes. This is possibly because vaccine serotypes are dominant in prevalence and are receiving antibiotic screening. We also found that a serotype 34 strain was resistant to penicillin, and we sequenced the whole genome to further study its resistance mechanism. The limitation of this study is the small sample size. We will perform continuous surveillance in Shenzhen to draw a comparative analysis of the molecular epidemiological characteristics and drug resistance trends of invasive *S. pneumoniae*.

Conclusion

Our work demonstrates that PCV13 is more effective in protecting Chinese children than PCV7 and PCV10. After the introduction of PCV13, the proportion of serotypes of invasive *S. pneumoniae* changed. Existing PspA family 3 indicates diverse antigens of Shenzhen's isolates. Pneumococcal antibiotic-resistant nonvaccine serotypes and highly virulent serotype 3 are likely to be an expanding threat to children with IPD.

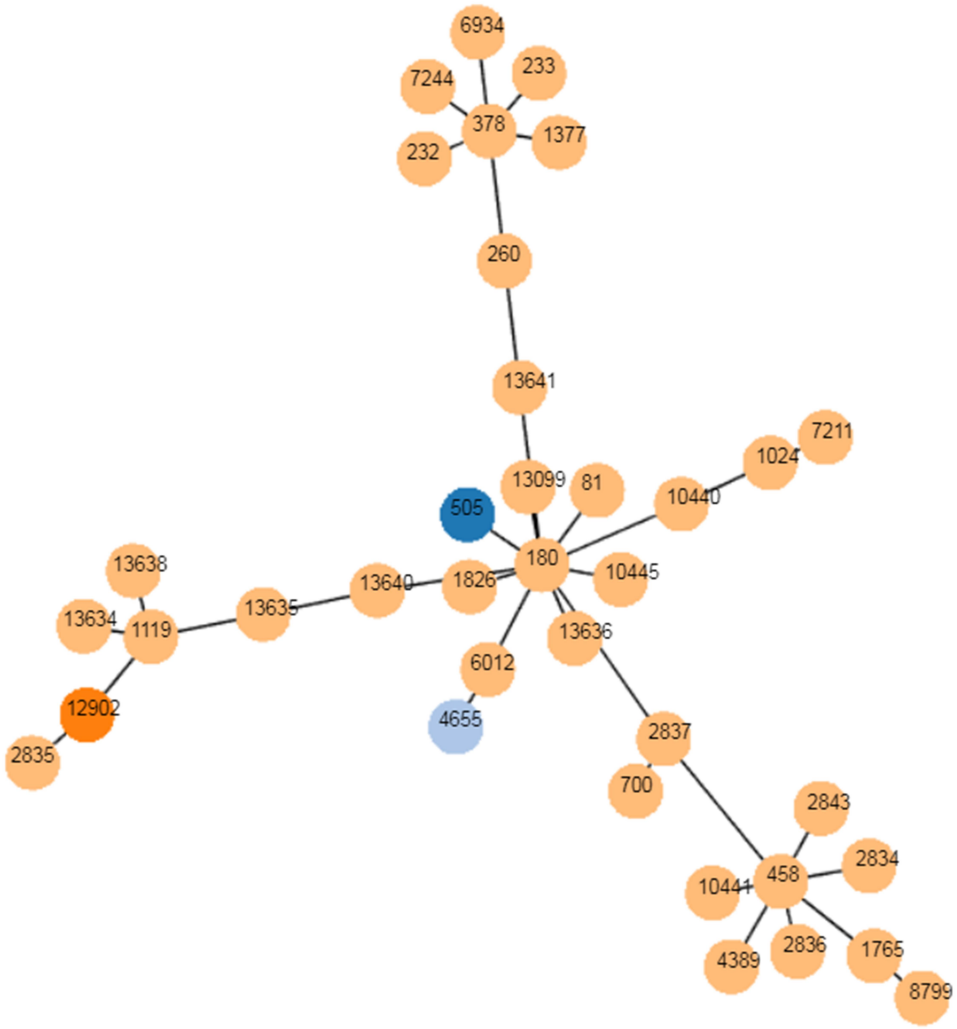

Figure 4 The goeBURST analysis of serotype 3. PHYLOViZ was used to identify the relationship of clonal complexes (CCs) of serotype 3 globally. Each node represents a ST. STs found in Shenzhen are colored with dark blue (ST 505), sky blue (ST 4655) and orange (ST 12902), respectively. STs colored with yellow are from foreign countries.

Ethics Statement

All procedures performed in studies involving human participants were approved by the ethics committee of Shenzhen Children's hospital and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent for using the clinical isolates of *S. pneumoniae* isolated in this study was signed by a parent and/or legal guardian of each participant.

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Disclosure

The authors declare that there is no conflict of interest regarding the publication of this paper.

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