Vaccine 37 (2019) 7248–7255

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The changing phenotypes and genotypes of invasive pneumococcal isolates from children in Shenzhen during 2013–2017

Yanmin Bao [a,b,1], Qing Wang [a], Kaihu Yao [a], Gan Xie [a,b], Wei Gao [a], Lu Huang [b], Xiaoli Liu [b], Chunqin Zhu [b], Hongyu Chen [b], Heping Wang [b], Kungling Shen [a], Yuejie Zheng [b,*], Yonghong Yang [a,b,*]

[a] Beijing Children's Hospital Affiliated to Capital Medical University, Beijing, China
[b] Shenzhen Children's Hospital, Shenzhen, China

ARTICLE INFO

Article history:
Received 29 March 2019
Received in revised form 3 September 2019
Accepted 20 September 2019
Available online 18 October 2019

Keywords:
Streptococcus pneumoniae
Serotypes
Antibiotic resistance
Genotype
Invasive pneumococcal diseases

ABSTRACT

Background: The phenotypes and genotypes of *Streptococcus pneumoniae* isolated from invasive pneumococcal diseases (IPDs) were changing all the time. To monitor these changes of phenotypes and genotypes of *S. pneumoniae* isolates from children, we examined antibiotic susceptibility, serotype distribution and sequence types (STs) of *S. pneumoniae*, which were isolated before the 13-valent pneumococcal conjugate vaccine (PCV13) introduced into China.

Methods: Strains were isolated from children less than 14 years old between January 2013 and May 2017 from Shenzhen Children's Hospital. Serotypes, antibiotic resistance, and genotypes of these isolates were determined using capsular swelling, E-test, and multi-locus sequence typing, respectively.

Results: A total of 94 *S. pneumoniae* strains were isolated, which belonged to 15 serotypes. The five most prevalent serotypes were 19F (25.5%), 19A (19%), 14 (17%), 23F (7.5%), and 6B (9.6%). We found 42 STs for these isolates. The most abundant STs were ST271 (24.4%), ST876 (17%), and ST320 (10.6%), mainly related to 19F, 14, and 19A, respectively. The potential coverage of PCV13 was 87.2%. Among non-meningitis isolates, the resistance rates to penicillin and ceftriaxone were 0% and 2%. However, the meningitis isolates showed high resistance to penicillin (80%) and ceftriaxone (20%). Most of these isolates (95.7%) were resistant to erythromycin, and 66 (70.2%) strains carried the ermB gene and 24 (25.5%) strains carried both the ermB and mefA/E genes. Serotype 19A showed the highest mean minimum inhibitory concentration (MIC) for penicillin (MIC = 1.486) than the other serotypes, but no significant difference in penicillin MIC among the three main STs (ST271, ST320, and ST876).

Conclusions: The phenotypes and genotypes of invasive pneumococcal isolates from Shenzhen Children's Hospital have changed with the passage of time. Compared with PCV7, PCV13 can more effectively protect Chinese children from IPDs. To some extent, these changes are possibly related to the usage of antibiotics and vaccines.

1. Introduction

Streptococcus pneumoniae can cause invasive pneumococcal diseases (IPDs, for example, meningitis, sepsis, and bacteremic pneumonia) and noninvasive pneumococcal diseases (non-IPDs, for example, non-bacteremic pneumonia, otitis media, and rhinosinusitis), and can exert an enormous medical, social, and economic influence. It has been estimated that each year approximately one million children die of IPDs worldwide among infants and young children under five years of age, particularly in developing countries [1]. However, antibiotic-resistant *S. pneumoniae* and multidrug-resistant *S. pneumoniae* (MDRSP) have become a major problem worldwide since the 1990s [2,3]. Pneumococcal conjugate vaccines (PCVs) are the most effective strategies to reduce invasive pneumococcal infections and to control the spread of antibiotic-resistant *S. pneumoniae* [4].

China is also facing these serious problems. Recent data showed that *S. pneumoniae* is a leading cause of community acquired pneumonia and acute bacterial meningitis in Western China [5,6] Our previous research showed that the percentage of MDRSP isolated from IPDs in 2009–2012 was about 56.3% in Shenzhen, a city in Southern China [7]. We also found that when the penicillin

* Corresponding authors at: Beijing Children's Hospital Affiliated to Capital Medical University, No. 56 Nan-li-shi Road, Beijing 100045, China (Y. Yang); Shenzhen Children's Hospital, No. 7019, Yitian Road, Futian District, Shenzhen 518026, China (Y. Zheng).

E-mail addresses: yuejiez@sina.com (Y. Zheng), yyh628628@sina.com (Y. Yang).

[1] First author.

https://doi.org/10.1016/j.vaccine.2019.09.069
0264-410X/

breakpoint of Clinical and Laboratory Standards Institute (CLSI) 2007 (susceptible when MIC was ≤0.06 mg/ml and resistant when MIC was ≥2 mg/mL) was adopted, the penicillin intermediate rate of the non-meningitis isolates was up to 74.3%, and the resistant rate was 11.5% in our research [7].

To improve the serious situation in China, PCV7 was introduced in the Chinese mainland between 2008 and 2014, and many other measures have been adopted to reduce antibiotic application in China since 2012 [8,9]. PCV7 was replaced by the 13-valent pneumococcal conjugate vaccine (PCV13) in developed countries several years ago because of serotype replacement [10]. PCV13 was introduced in Shenzhen in June 2017. To further evaluate the effect of PCV13 in China and to provide the proof for choosing the appropriate antibiotic, it is necessary to monitor the changes in the phenotypes and genotypes of IPD isolates. This study was conducted to examine the serotype distribution and the antimicrobial resistance pattern of IPD isolates collected from children between January 2013 and May 2017 before the introduction of PCV13 in Shenzhen.

2. Materials and methods

2.1. Clinical isolate collection and culture

IPD isolates were defined as *S. pneumoniae* strains isolated from sterile body fluids, such as blood, synovial fluid, and cerebrospinal fluid (CSF). We collected 94 *S. pneumoniae* strains isolated from Shenzhen Children's Hospital between January 2013 to May 2017. The isolates were identified further by optochin sensitivity test (Oxoid, Basingstoke, Britain), bile solubility test, and Omni serum assay (Statens Serum Institute, Copenhagen, Denmark).

All these strains were stored at −80 °C in 40% glycerol broth medium. Strains were grown on 5% horse blood agar and incubated at 37 °C in the presence of 5% CO2 for 12–15 h prior to the other biochemical and molecular assays.

2.2. Serotyping

All isolates were typed by a capsule-quelling test using type-specific antisera (Statens Serum Institute, Copenhagen, Denmark) against the serotypes. Typing was conducted by phase-contrast microscopy according to the previous described procedure [11]. Strains, which could not be typed, were denoted as untyped strains.

2.3. Detection of macrolide resistance genes

All erythromycin-resistant strains were tested for the ermB and mefA/E resistance genes [12]. Each PCR reaction contained 500 ng of template DNA, 50 mM potassium chloride, 10 mM Tris–hydrochloride (pH 8.3), 200 mM of each deoxynucleotide triphosphate, 2.5 U Taq DNA polymerase (Takara Bio, Dalian, China), 1.5 mM magnesium chloride, and 1.5 mM of each primer. The PCR products were visualized by 1.5% agarose gel electrophoresis and gold-view staining.

2.4. Multi-locus sequence typing (MLST)

Internal fragments, 450 bp long, from the aroE, gdh, gki, recP, spi, xpt, and ddl genes were amplified by PCR as previously described [13]. All of the sequence types (STs) absent in the pneumococcal MLST database were submitted to the MLST *S. pneumoniae* database for designation. The eBURST algorithm (http://eburst.mlst.net) was used to estimate the phylogenetic distance among isolates. STs that shared six identical alleles of the seven MLST loci with another ST in the group were subdivided into one group as a clone complex (CC). The software PHYLOViZ 2.0 was used to generate and visualize a complete minimum spanning tree (MST) based on the geoBURST distance [14].

2.5. Antimicrobial susceptibility testing

The MICs for penicillin (PG), amoxicillin (AC), ceftriaxone (TX), cefuroxime (XM), erythromycin (EM), vancomycin (VA), levofloxacin (LE), and imipenem (IP) were determined using E-test strips (AB Biodisk, Solna, Sweden) [11,15]. Breakpoints were based on the 2015 criteria of the CLSI [16]. *S. pneumoniae* strains ATCC 49,619 acted as standard control for antimicrobial susceptibility test. The isolates, which were resistant to three or more classes of antimicrobials, were considered MDRSP.

2.6. Statistical methods

We applied the WHONET software (version 5.6, WHO) to perform data analysis. The result of the X^2 test was calculated using SPSS version 10.0 (SPSS Inc., Chicago, USA), and the cutoff of the two-tailed test was $P < 0.05$.

3. Results

3.1. Characteristics of patients with IPDs

Among the 94 isolates, 23 were collected in 2013, 19 were collected in 2014, 22 were collected in 2015, 20 were collected in 2016, and 10 were collected in 2017. Fifteen (10.4%) strains were isolated from CSF samples, 68 (72.3%) strains were isolated from blood samples, and 11 (17.3%) strains were isolated from pleural fluid and joint cavity fluid (Table 1).

Sixty patients (63.9%) were under 2 years of age, and 29 (30.8%) patients were between 2 and 5 years of age. Bacteremia (53.1%) was the most common IPD. Seven patients suffered from several comorbidities. About 50 cases had a history of upper respiratory tract infections before IPDs In three cases, the comorbidities were primary immunological diseases; and in the other four cases, the comorbidities were malignancies and leukemia. Six patients died due to IPDs (septic shock and meningitis). Three patients had central nervous system sequelae (Table 1).

3.2. Distribution of serotypes and coverage of PCVs

Fig. 1 shows the serotype distribution of the 94 *S. pneumoniae* isolates. Among the 94 isolates, 19F (n = 24, 25.5%)), 19A (n = 18,

Table 1
Clinical characteristics of children with IPDs in Shenzhen during 2013–17.

Characteristics	No. of patients	%
Gender		
male	60	63.8
female	34	36.2
Age		
0–0.5	4	4.3
0.5–2	56	59.6
2–5	29	30.8
>5	5	5
Underlying diseases	6	7.6
Meningitis	15	16
Non-meningitis	79	84
Bacteremia	50	53.1
Severe pneumonia	9	9.6
Sepsis	9	9.6
Septic arthritis	7	7.4
other	4	4.3
Numbers of death	8	8.5

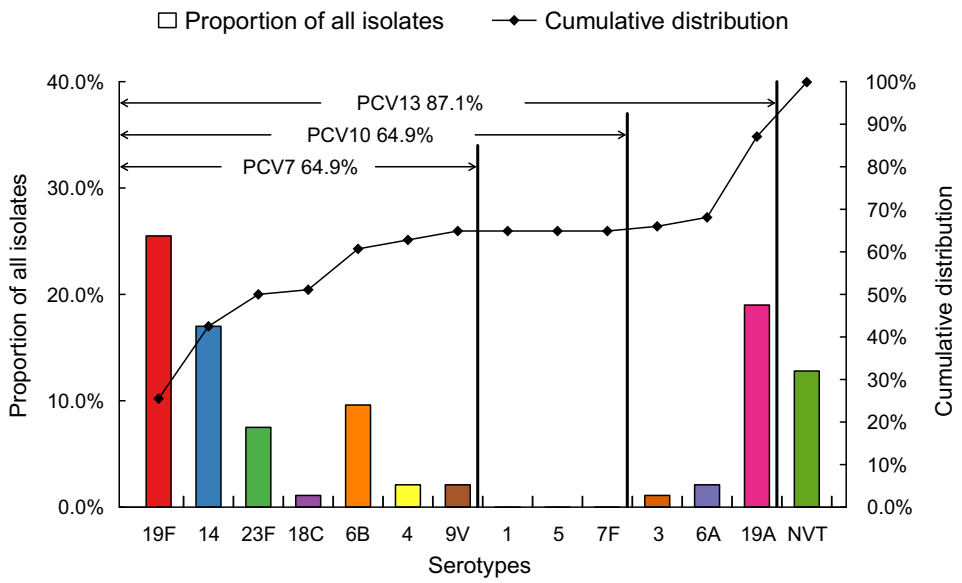

Fig. 1. Proportionate and cumulative serotype distributions of 94 *S. pneumoniae* isolates causing invasive infections among children in Shenzhen Children's Hospital from 2013 to 2017. NVT, non-vaccine serotypes not included in PCV13.

19%), 14 (n = 16, 17%), 23F (n = 7, 7.5%), and 6B (n = 9, 9.6%) were the five most commonly found serotypes. These serotypes accounted for 78.6% of all of the isolates. The overall coverage rate of PCV13 was 87.2%, while that of PCV7 was only 64.9%. The percentage of non-vaccine types (NVTs) was 12.8, including 23A (n = 1), 19C (n = 4), 18F (n = 1), 15C (n = 2), 15A (n = 1), 8 (n = 1), 29 (n = 1), and 42 (n = 1). We further compared the frequency of common serotypes in this study with our previous study done between 2009 and 2012 in Shenzhen (Fig. 2) [7]. The frequency of 19F, 14, and 23F was decreased between 2013 and 2017, while the frequency of 19A was obviously increased. The percentage of the PCV7 serotype was reduced between 2013 and 2017.

3.3. Detection of macrolide resistance genes

Sixty-six (70.2%) isolates were only positive for ermB, but they were negative for mefA. Twenty-four (25.5%) pneumococcal isolates contained both the ermB and mefA/E genes. Thus, 90 (95.7%) isolates in our study carried macrolide resistance genes.

3.4. MLST

Forty-two STs were detected in these 94 *S. pneumoniae* isolates; and among them, 10 STs were newly assigned via MLST analysis. All of these new STs were novel combinations of known alleles.

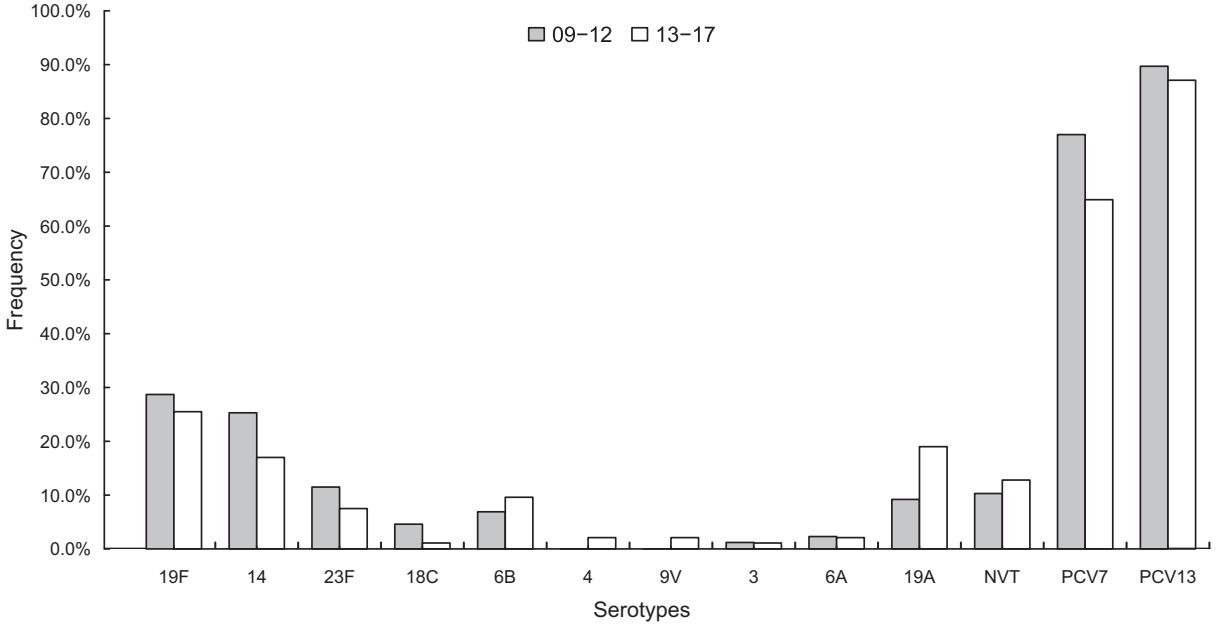

Fig. 2. Comparison of the frequency of common serotypes among different years in Shenzhen Children's Hospital. The bar colored in gray stands for the frequency of *S. pneumoniae* collected from 2009 to 2012, and the white bar indicates the frequency of *S. pneumoniae* collected from 2013 to 2017.

The predominant STs for all pneumococci were ST271 (24.4%, 23/94), ST876 (17.0%, 16/94), ST320 (10.6%, 10/94), ST902 (4.2%, 4/94), and ST81 (3.2%, 3/94), which were mainly related to serotypes 19F, 14, 19A, 6B, and 23F, respectively. The eBURST analysis showed seven clone complexes (CCs) and 11 singletons (Fig. 3). CC271 was the most frequent CC (including ST320 and ST271), followed by CC876 (including ST876 and ST902). The isolates of serotype 19F belonged to 5 STs; and among them, 20 isolates were from ST271. The isolates of serotype 19A had 8 STs; and 8 isolates were from ST320, and 3 isolates were from ST876. The isolates of serotype 19C belonged to ST320 and ST271.

3.5. Antimicrobial susceptibility testing

The antibiotic activities of these 94 *S. pneumoniae* isolates against 8 antimicrobials are presented in Table 2. According to the revised CLSI breakpoints for penicillin (resistant ≥ 8 mg/mL for non-meningitis isolates and ≥0.12 mg/mL for meningitis isolates), the prevalence rates of penicillin resistance were 0% and 80% in the non-meningitis and meningitis isolates, respectively. The percentage of isolates resistant to amoxicillin was 5.1% in the non-meningitis isolates. The proportions of isolates resistant to ceftriaxone were 2.5% in the non-meningitis isolates and 20% in the meningitis isolates. The cefuroxime resistance rate was 83%. All of the isolates were susceptible to vancomycin and levofloxacin. Also, 95.7% of these isolates showed high resistance to erythromycin. The non-susceptibility rate to imipenem was 64.9%. The mean MIC of penicillin, amoxicillin, and ceftriaxone for different serotypes is shown in Table 3. The serotypes 19F, 19A, and 14, especially 19F, had the high MIC for penicillin, amoxicillin, and ceftriaxone. The serotype 19A had the highest MIC for penicillin among all of the serotypes, while its MIC for ceftriaxone was relatively low. We further compared the difference in the antibiotic MIC among the main STs (ST271, ST320, and ST876;

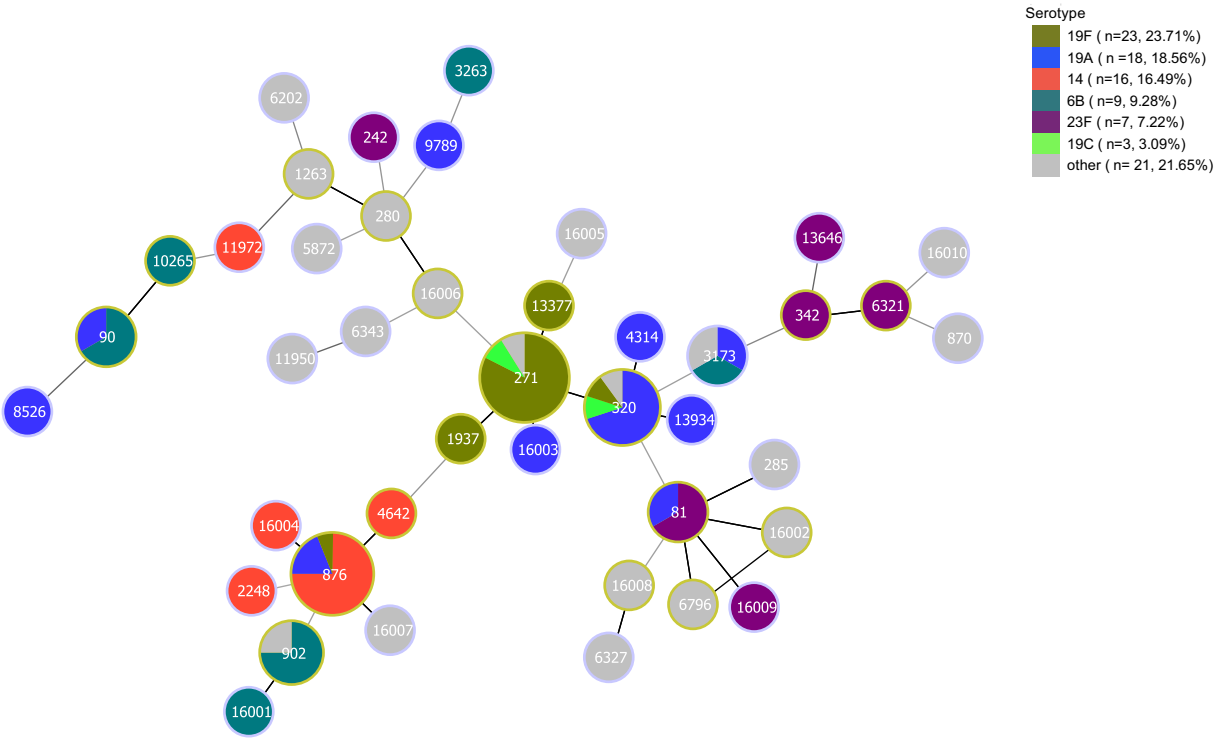

Fig. 3. Population snapshot of the 94 *S. pneumoniae* strains through eBURST analysis. One spot indicates one ST. The size of one spot corresponds to the number of pneumococcal isolates with the same ST. The lines indicate the presence of single locus variant (SLV) links among particular STs. Each spot is colored, the different colors represents that the spot is comprised of different serotypes.

Table 2
Susceptibility and MIC of antibiotics for IPD isolates in Shenzhen during 2013–17.

Antimicrobials	No. of isolates	Susceptibility			MIC (mg/ml)		
		Resistant	Intermediate	Susceptible	50%	90%	Range
Penicillin (P)	94						
P-Meningitis	15	12(80%)	0	3(20%)	1	1.5	0.016–2
P-non-meningitis	79	0	5(6.3%)	74(93.7%)	1	2	0.012–4
Amoxicillin	79	4(5.1%)	17(21.5%)	58(73.4%)	1.5	4	0.016–6
Ceftriaxone (C)	94						
C-meningitis	15	3(20%)	3(20%)	9(60%)	0.5	1.5	0.012–1.5
C-non-meningitis	79	2(2.5%)	18(22.8%)	59(74.7%)	0.75	2	0.012–3
Cefuroxime	94	78(83%)	2(2.1%)	14(14.9%)	3	6	0.016–48
Erythromycin	94	90(95.7%)	0	4(4.3%)	>256	>256	0.094∼>256
Vancomycin	94	0	0	94(100%)	0.5	1	0.25–1
Levofloxacin	94	0	0	94(100%)	1	1.5	0.002–2
Imipenem	94	2(2.1%)	59(62.8%)	33(35.1%)	0.19	0.25	0.06–1

Table 3
The mean MIC of antibiotics for different IPD isolates serotypes in Shenzhen, 2013–17.

	Penicillin	Amoxicillin	Ceftriaxone
19A	1.486	2.722	0.751
19F	1.397	3.063	1.479
14	1.313	1.25	0.728
23F	0.763	0.733	0.429
6B	0.962	1.057	0.644
NVT	0.889	1.479	0.496

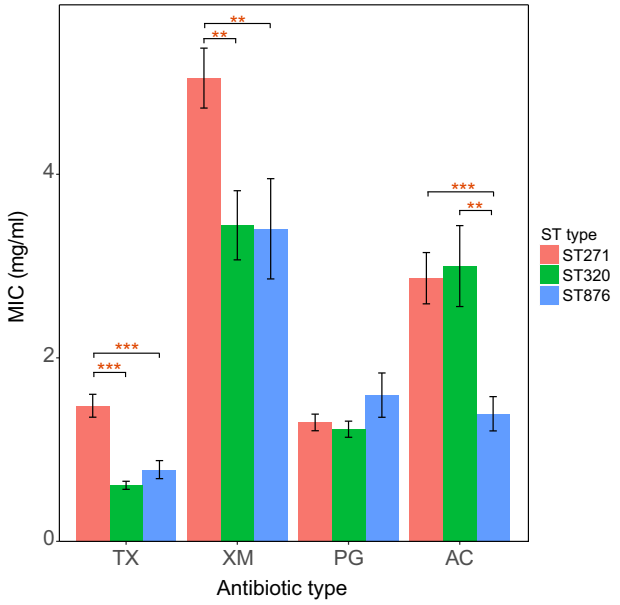

Fig. 4. The difference in the antibiotic MIC among the main STs (ST271, ST320, and ST876). The bars colored in red, green, and blue represent ST271, ST320, and ST876, respectively. ** and *** above the bars stand for significant differences with P-values ≤0.01 and ≤0.001, respectively. TX: ceftriaxone; XM: cefuroxime; PG: penicillin; AC: amoxicillin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4). There was no difference in the MIC for penicillin ($P > 0.05$). The MIC for cefuroxime and ceftriaxone in ST271 was the highest ($P < 0.05$). The MIC for amoxicillin in ST271 and ST320 was higher than that in ST876 ($P < 0.05$).

4. Discussion

Our previous research revealed that the rank order of serotypes of the isolates from IPDs in Shenzhen was 19F 28.7%), 14 (25.3%), 23F (11.5%), 19A (9.2%), and 6B (6.9%) between 2009 and 2012 [7]. Our study showed that the serotype distribution of the isolates from IPDs in Shenzhen has changed. Currently, the rank order of the most common serotypes of IPD isolates in Shenzhen is 19F (n = 24, 25.5%), 19A (n = 18, 19%), 14 (n = 16, 17%), 23F (n = 7, 7.5%), and 6B (n = 9, 9.6%). The coverage of PCV7 in our study is only 64.9%, which is lower than that reported earlier. In contrast, the coverage of PCV13 in Shenzhen is up to 87.2%. Our result is similar to that of the study performed by Si et al. between 2012 and 2017 in Beijing, which is a northern city in China [17]. Therefore, PCV13 can protect Chinese children from most of the pneumococcal diseases. Malaysia and India are the other Asian developing countries where PCVs have not been included in the national standard immunization program. The percentage of inoculation with PCV13 in these countries is also very low. In India, the most frequent pneumococcal serotypes causing invasive disease among children aged ≤5 years are 14, 1, 19F, 6B, 5, 6A, 9 V, and 23F [18]. In Malaysia, the most common serotypes are serotype 14, 6B, 19A, 6A, and 19F [19]. Thus, the serotype distribution of *S. pneumoniae* varies on the basis of geography and time.

Compared with the results obtained from Shenzhen during 2009–2012, the percentage of NVTs is slightly higher in our study [7]. Also, 15 (15A/B/C) and 19C are the main serotypes of NVTs. Shenzhen is bordered by Hong Kong and Taiwan, where PCV13 has been introduced since 2010. The study in Hong Kong showed that the non-susceptible serotype 15 (15A/B/C) has emerged and increased in the post-PCV13 era [20]. Taiwan also reported that 15A/B is the most commonly detected serotype in the isolates from IPDs in 2015 [21]. The serotype 19C was not found in our previous study. Currently, four isolates of 19C have been found. Therefore, we also need to pay more attention to the spread of NVTs in Shenzhen.

Since the first case of pneumococcal penicillin resistance was reported in the 1960s, emergence and spread of penicillin and multidrug-resistant pneumococci have become a serious concern worldwide [2,22]. The situation in China is also very serious. The rate of penicillin resistance of IPD isolates reported in China was about 42.6%–64.3% between 2005 and 2009 [23–25]. In our previous study performed in Shenzhen during 2009–2012, only three (3.9%) non-meningitis isolates were non-susceptible to penicillin-based on the 2010 CLSI criteria [7]. This rate was much lower than that reported earlier because of the change in penicillin breakpoints. But when the breakpoint of CLSI 2007 (susceptible when MIC was ≤0.06 mg/mL, but resistant when MIC was ≥2 mg/mL) was adopted, the corrected penicillin intermediate rate of the non-meningitis isolates was up to 74.3%, and the resistant rate was 11.5% [7]. In our study, the corrected penicillin intermediate rate of the non-meningitis isolates is 43% and the resistant rate is 34.6%, if the breakpoint of CLSI 2007 is used. The MIC50 and MIC90 of penicillin in the isolates were 1.0 mg/mL and 2.0 mg/mL, respectively. This result is similar to that in Beijing [17]. Therefore, compared with the previous results in China, the seriousness of the situation of pneumococcal penicillin resistance has not improved.

Although PCV7 was introduced in China several years ago, the percentage of inoculation was low. In our study, the three most commonly found serotypes are 19F, 19A, and 14; and they accounted for 52% of the isolates. The mean MIC of these three serotypes for penicillin is 1.400 mg/mL, 1.486 mg/mL, and 1.312 mg/mL, respectively, which is much higher than the mean MIC of 23F, 6B, and NVTs in our study. Therefore, the isolates from IPDs, which show high-level resistance to penicillin, are still prevalent in Shenzhen. Serotype 19A could not be found in Shenzhen in the study of IPD isolates between 2006 and 2008 [26]. But within the past decade, the percentage of serotype 19A has increased rapidly in Shenzhen. Currently, 19A is the most penicillin-resistant serotype in Shenzhen. Because of its high-level penicillin resistance, 19A will possibly spread further in Shenzhen under the pressure of antibiotics. However, the mean penicillin MIC of NVTs is 0.8888 mg/mL in our study. Although this value is much lower than that of the above-mentioned serotypes, it is very much higher than that reported in some countries, where the use of antibiotics is strictly restricted [27]. Therefore, there is a very urgent need to control the overuse of antibiotics to avoid the appearance of NVT pneumococcus that is highly resistant to penicillin. Otherwise, penicillin-resistant NVT pneumococcus would spread widely and make PCV13 less effective.

The resistance rates of these isolates to cefuroxime and erythromycin are very high in China. The resistance rates to cefuroxime and erythromycin were 79.3% and 96.6% in our previous study

performed during 2009–2012 [7]. The resistance rates to cefuroxime and erythromycin in our study are 83% and 95.7%, respectively. Thus, there seems to be no improvement in these last few years. But ceftriaxone resistance rates have decreased. Ceftriaxone resistance rates during 2013–2017 were 2.5% and 20% in the non-meningitis and meningitis isolates, respectively, while these rates were 3.8% and 33.3% in the non-meningitis and meningitis isolates, respectively, during 2009–2012 [7]. According to our results, the non-meningitis isolates still show low resistance to amoxicillin. Therefore, it is recommended to choose amoxicillin in non-meningitis IPDs and ceftriaxone in pneumococcal meningitis as the first line therapies in China. Although resistance to levofloxacin in *S. pneumoniae* isolates has been reported [28,29], our results showed that all of the isolates from IPDs are susceptible to vancomycin and levofloxacin. As a type of Gram-positive cocci, the unsusceptibility of *S. pneumoniae* to imipenem is up to 64.9%. In our study, most of the isolates (95.7%) showed resistance and high MIC level for erythromycin (most of the isolates with a MIC of 256 mg/mL), which is similar to our previous results (96.4%), but the erythromycin resistance percentage was double than that reported in India and Malaysia [18,19]. We can infer that different preference to antibiotics in these countries is the reason for the difference in erythromycin resistance in *S. pneumoniae*

The predominant STs in our study were ST271, ST876, ST320, ST902 and ST81, which is basically similar with our previous study in 2009–2012. But we observed that the percentage of ST 320 related to 19A and CC271 (including ST271 and ST320) kept increasing. CC271 is the main high-level antibiotic-resistant clone from IPD isolates in Shenzhen. The MIC of penicillin in these STs is between 1 and 4 μg/ml. In USA, during the 1990s, the main CC identified among Spn19A strains was CC199 (MIC of penicillin, 0.12–1.0 μg/ml), which gradually decreased after the introduction of PCV7 [30,31]. After the use of PCV7 for eight years in the USA, high resistant ST320 (MIC of penicillin, 2.0 μg/ml) was predominant among almost half (49.3%) of all Spn19A strains in children aged <5 years [31] Since PCV10 was introduced into Brazil, 66.5% of Spn19A strains were ST320 in 2016–2017 [32]. Thus, the introduction of PCV7 and PCV10 caused an expansion of ST320 in Spn19A. On the other hand, reports from South Israel, some Asian countries and the northern of China also showed an increase in ST320 of Spn19A before the introduction of PCV7 [33–36], which means gene evolution caused high level resistant STs replacement of low level resistant STs. In Norway and Italy, where antibiotic prescription was restricted, PCV7 vaccination led to an increase in Spn19A related to CC199, and most of the strains were characterized as penicillin susceptible [37,38]. Thus, these results further demonstrated that besides vaccination genotype replacement and antibiotic pressure are important selective factors for the spread of Spn19A related to ST320. Except for penicillin, ST271 and/or ST320 showed higher resistance to amoxicillin, cefuroxime, and ceftriaxone in our study. This phenomenon is possibly related to the widespread use of broad-spectrum antibiotics in China. In addition, three NVT isolates of 19C were found in our study. Their STs were ST271 and ST320. It can be inferred that capsular transformation of 19A and 19F possibly resulted in the appearance of 19C. Because 19C is not included in PCV13, it is necessary to monitor whether 19C will spread in Shenzhen after PCV13 is introduced in Shenzhen.

Our study showed that high-level penicillin-resistant VT isolates from children with IPDs are dominant in Shenzhen. PCV13 coverage in Shenzhen is 87.2%. Many studies have shown that the introduction of PCV13 in the national immunization procedure is a very important step for prevention of IPDs and control of the spread of drug-resistant *S. pneumoniae.* [39,40]. Currently in Canada, 97.3%–99% of the isolates from IPDs are susceptible to penicillin (MIC $\leq$ 0.06 mg/ml), and the dominant serotypes (58.8%) are NVTs during 2011–2015 [27,40]. However, new problems have appeared after the introduction of PCV13. The penicillin-resistant NVTs in IPDs, such as 15A and 35B, have emerged and have increased gradually [41–44]. The NVT of serotype 12F has a significantly higher invasive potential in children aged 0–23 months and outbreaks in a local area [45]. Therefore, it is important to continuously monitor the serotype distribution and antibiotic resistance of the isolates from IPDs after PCV13 is introduced in China.

Our study has several limitations. For instance, all of the IPD isolates were obtained from one hospital and the situation of antibiotic usage was very unclear; thus, leading to bias and affecting the results of this study. In addition, only a small sample size of isolates was used in this study. Therefore, isolates from more pediatric patients should be included.

5. Conclusion

In summary, our current study provided updated information and changing trends in antimicrobial resistance, serotype distribution, and MLST of IPD isolates from children in Southern China before the introduction of PCV13 in China. The data showed a high prevalence of PCV13 serotypes in Shenzhen. Selective antibiotic pressure is one of the important factors that caused the spread of penicillin-resistant serotypes in China. Based on our results, the introduction of PCV13 and strict control of the use of antibiotics in China can reduce IPDs and the prevalence of penicillin-resistant *S. pneumoniae* in China. Because *S. pneumoniae* is a remarkably adaptable pathogen, we need to continuously monitor the trends in the isolates of IPDs after the introduction of PCV13 in China.

Author contribution

Conception and design: YB and YY; Specimens collection: QW and WG; Serotyping, sequencing typing and antibiotic resistance test: HL, XL, CZ and HC; Data acquisition and analysis: KY, GX and HW; Result interpretation and manuscript written: YB and KS; Result visualization and revision: GX and YZ; Final approval: YZ and YY; All authors reviewed this manuscript.

Roles of the funding source

This work was supported by Shenzhen Science and Technology Project (grant No. JCYJ20180228175330567) and Sanming Project of Medicine in Shenzhen (SZSM201512030).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors sincerely thank all members who took part in this study, especially co-workers in Shenzhen Children's Hospital, for their valuable assistance in collecting the isolates.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.09.069. These data include Google maps of the most important areas described in this article.

References

[1] Wahl B, O'Brien KL, Greenbaum A, Majumder A, Liu L, Chu Y, et al. Burden of Streptococcus pneumoniae and Haemophilus influenzae type b disease in children in the era of conjugate vaccines: global, regional, and national estimates for 2000–15. Lancet Glob Health 2018;6:e744–57. https://doi.org/10.1016/S2214-109X(18)30247-X.

[2] Whitney CG, Farley MM, Hadler J, Harrison LH, Lexau C, Reingold A, et al. Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States. New Engl J Med 2000;343:1917–24. https://doi.org/10.1056/NEJM200012283432603.

[3] Crook DW, Spratt BG. Multiple antibiotic resistance in Streptococcus pneumoniae. Br Med Bull 1998;54:595–610.

[4] Principi N, Esposito S. Development of pneumococcal vaccines over the last 10 years. Expert Opin Biol Ther 2018;18:7–17. https://doi.org/10.1080/14712598.2018.1384462.

[5] Jiang H, Su M, Kui L, Huang H, Qiu L, Li L, et al. Prevalence and antibiotic resistance profiles of cerebrospinal fluid pathogens in children with acute bacterial meningitis in Yunnan province, China, 2012–2015. PLoS ONE 2017;12:. https://doi.org/10.1371/journal.pone.0180161e0180161.

[6] Zhang Q, Guo Z, MacDonald NE. Vaccine preventable community-acquired pneumonia in hospitalized children in Northwest China. Pediatr Infect Dis J 2011;30:7–10. https://doi.org/10.1097/INF.0b013e3181ec6245.

[7] Ma X, Zhao R, Ma Z, Yao K, Yu S, Zheng Y, et al. Serotype distribution and antimicrobial resistance of Streptococcus pneumoniae isolates causing invasive diseases from Shenzhen Children's Hospital. PLoS ONE 2013;8:. https://doi.org/10.1371/journal.pone.0067507e67507.

[8] Zhang ZG, Chen F, Ou Y. Impact of an antimicrobial stewardship programme on antibiotic usage and resistance in a tertiary hospital in China. J Clin Pharm Ther 2017;42:579–84. https://doi.org/10.1111/jcpt.12544.

[9] Bao L, Peng R, Wang Y, Ma R, Ren X, Meng W, et al. Significant reduction of antibiotic consumption and patients' costs after an action plan in China, 2010–2014. PLoS ONE 2015;10:. https://doi.org/10.1371/journal.pone.0118868e0118868.

[10] Tin Tin Htar M, Christopoulou D, Schmitt HJ. Pneumococcal serotype evolution in Western Europe. BMC Infect Dis 2015;15:419. https://doi.org/10.1186/s12879-015-1147-x.

[11] Sorensen UB. Typing of pneumococci by using 12 pooled antisera. J Clin Microbiol 1993;31:2097–100.

[12] Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother 1996;40:2562–6.

[13] Enright MC, Spratt BG. A multilocus sequence typing scheme for Streptococcus pneumoniae: identification of clones associated with serious invasive disease. Microbiology 1998;144(Pt 11):3049–60. https://doi.org/10.1099/00221287-144-11-3049.

[14] Nascimento M, Sousa A, Ramirez M, Francisco AP, Carrico JA, Vaz C. PHYLOViZ 2.0: providing scalable data integration and visualization for multiple phylogenetic inference methods. Bioinformatics 2017;33:128–9. https://doi.org/10.1093/bioinformatics/btw582.

[15] Kelly LM, Jacobs MR, Appelbaum PC. Comparison of agar dilution, microdilution, E-test, and disk diffusion methods for testing activity of cefditoren against Streptococcus pneumoniae. J Clin Microbiol 1999;37:3296–9.

[16] Wayne P. Performance standards for antimicrobial susceptibility testing: Twenty Fifth International Supplement M100–S25. Clinical and Laboratory Standards Institute; 2015.

[17] Shi W, Li J, Dong F, Qian S, Liu G, Xu B, et al. Serotype distribution, antibiotic resistance pattern, and multilocus sequence types of invasive Streptococcus pneumoniae isolates in two tertiary pediatric hospitals in Beijing prior to PCV13 availability. Expert Rev Vacc 2019;18:89–94. https://doi.org/10.1080/14760584.2019.1557523.

[18] Singh J, Sundaresan S, Manoharan A, Shet A. Serotype distribution and antimicrobial susceptibility pattern in children</=5years with invasive pneumococcal disease in India - A systematic review. Vaccine 2017;35:4501–9. https://doi.org/10.1016/j.vaccine.2017.06.079.

[19] Arushothy R, Ahmad N, Amran F, Hashim R, Samsudin N, Azih CRC. Pneumococcal serotype distribution and antibiotic susceptibility in Malaysia: A four-year study (2014–2017) on invasive paediatric isolates. Int J Infect Dis: IJID: Off Publ Int Soc Infect Dis 2019;80:129–33. https://doi.org/10.1016/j.ijid.2018.12.009.

[20] Liyanapathirana V, Nelson EA, Ang I, Subramanian R, Ma H, Ip M. Emergence of serogroup 15 Streptococcus pneumoniae of diverse genetic backgrounds following the introduction of pneumococcal conjugate vaccines in Hong Kong. Diagn Microbiol Infect Dis 2015;81:66–70. https://doi.org/10.1016/j.diagmicrobio.2014.09.028.

[21] Cho YC, Chiu NC, Lu CY, Huang DT, Huang FY, Chang LY, et al. Redistribution of *Streptococcus pneumoniae* Serotypes After Nationwide 13-valent Pneumococcal Conjugate Vaccine Program in Children in Northern Taiwan. Pediatr Infect Dis J 2017;36:e334–40. https://doi.org/10.1097/INF.0000000000001664.

[22] Lee NY, Song JH, Kim S, Peck KR, Ahn KM, Lee SI, et al. Carriage of antibiotic-resistant pneumococci among Asian children: a multinational surveillance by the Asian Network for Surveillance of Resistant Pathogens (ANSORP). Clin Infect Dis: Off Publ Infect Dis Soc Am 2001;32:1463–9. https://doi.org/10.1086/320165.

[23] Liu Y, Wang H, Chen M, Sun Z, Zhao R, Zhang L, et al. Serotype distribution and antimicrobial resistance patterns of Streptococcus pneumoniae isolated from children in China younger than 5 years. Diagn Microbiol Infect Dis 2008;61:256–63. https://doi.org/10.1016/j.diagmicrobio.2008.02.004.

[24] Ho P-L, Chiu SS, Ang I, Lau Y-L. Serotypes and antimicrobial susceptibilities of invasive Streptococcus pneumoniae before and after introduction of 7-valent pneumococcal conjugate vaccine, Hong Kong, 1995–2009. Vaccine 2011;29:3270–5.

[25] Liu C, Xiong X, Xu W, Sun J, Wang L, Li J. Serotypes and patterns of antibiotic resistance in strains causing invasive pneumococcal disease in children less than 5 years of age. PLoS ONE 2013;8:e54254.

[26] Xue L, Yao K, Xie G, Zheng Y, Wang C, Shang Y, et al. Serotype distribution and antimicrobial resistance of Streptococcus pneumoniae isolates that cause invasive disease among Chinese children. Clin Infect Dis: Off Publ Infect Dis Soc Am 2010;50:741–4. https://doi.org/10.1086/650534.

[27] Karlowsky JA, Adam HJ, Golden AR, Baxter MR, Nichol KA, Martin I, et al. Antimicrobial susceptibility testing of invasive isolates of Streptococcus pneumoniae from Canadian patients: the SAVE study, 2011–15. J Antimicrob Chemother 2018;73:vii5-vii11. https://doi.org/10.1093/jac/dky156.

[28] Baek JY, Kang CI, Kim SH, Ko KS, Chung DR, Peck KR, et al. Emergence of multidrug-resistant clones in levofloxacin-nonsusceptible Streptococcus pneumoniae isolates in Korea. Diagn Microbiol Infect Dis 2018;91:287–90. https://doi.org/10.1016/j.diagmicrobio.2018.02.010.

[29] Schmitz J, van der Linden M, Al-Lahham A, Levina N, Pletz MW, Imohl M. Fluoroquinolone resistance in Streptococcus pneumoniae isolates in Germany from 2004–2005 to 2014–2015. Int J Med Microbiol: IJMM 2017;307:216–22. https://doi.org/10.1016/j.ijmm.2017.04.003.

[30] Hulten KG, Kaplan SL, Lamberth LB, Barson WJ, Romero JR, Lin PL, et al. Changes in Streptococcus pneumoniae serotype 19A invasive infections in children from 1993 to 2011. J Clin Microbiol 2013;51:1294–7. https://doi.org/10.1128/JCM.00058-13.

[31] Beall BW, Gertz RE, Hulkower RL, Whitney CG, Moore MR, Brueggemann AB. Shifting genetic structure of invasive serotype 19A pneumococci in the United States. J Infect Dis 2011;203:1360–8. https://doi.org/10.1093/infdis/jir052.

[32] Cassiolato AP, Almeida SCG, Andrade AL, Minamisava R, de Cunto Brandileone MC. Expansion of the multidrug-resistant clonal complex 320 among invasive Streptococcus pneumoniae serotype 19A after the introduction of a ten-valent pneumococcal conjugate vaccine in Brazil. PLoS ONE 2018;13:e0208211.

[33] Dagan R, Givon-Lavi N, Leibovitz E, Greenberg D, Porat N. Introduction and proliferation of multidrug-resistant Streptococcus pneumoniae serotype 19A clones that cause acute otitis media in an unvaccinated population. J Infect Dis 2009;199:776–85.

[34] Shin J, Baek JY, Kim SH, Song J-H, Ko KS. Predominance of ST320 among Streptococcus pneumoniae serotype 19A isolates from 10 Asian countries. J Antimicrob Chemother 2011;66:1001–4.

[35] Xue L, Yao KH, Yu SJ, Liu ZJ, Qian J, Shen XZ, et al. Molecular epidemiology of serotype 19A Streptococcus pneumoniae isolated from children in Beijing, 1997–2006. Chin Med J 2011;124:1769–74.

[36] Ma X, Yao KH, Yu SJ, Zhou L, Li QH, Shi W, et al. Genotype replacement within serotype 23F Streptococcus pneumoniae in Beijing, China: characterization of serotype 23F. Epidemiol Infect 2013;141:1690–6. https://doi.org/10.1017/s0950268812002269.

[37] Del Grosso M, Camilli R, D'Ambrosio F, Petrucci G, Melchiorre S, Moschioni M, et al. Increase of pneumococcal serotype 19A in Italy is due to expansion of the piliated clone ST416/CC199. J Med Microbiol 2013;62:1220–5.

[38] Vestrheim DF, Steinbakk M, Aaberge IS, Caugant DA. Postvaccination increase in serotype 19A pneumococcal disease in Norway is driven by expansion of penicillin-susceptible strains of the ST199 complex. Clin Vaccine Immunol 2012;19:443–5.

[39] Ubukata K, Takata M, Morozumi M, Chiba N, Wajima T, Hanada S, et al. Effects of Pneumococcal Conjugate Vaccine on Genotypic Penicillin Resistance and Serotype Changes, Japan, 2010–2017. Emerg Infect Dis 2018;24:2010–20. https://doi.org/10.3201/eid2411.180326.

[40] Golden AR, Adam HJ, Karlowsky JA, Baxter M, Nichol KA, Martin I, et al. Molecular characterization of predominant Streptococcus pneumoniae serotypes causing invasive infections in Canada: the SAVE study, 2011–15 vii20–vii31. J Antimicrob Chemother 2018;73. https://doi.org/10.1093/jac/dky157.

[41] Olarte L, Kaplan SL, Barson WJ, Romero JR, Lin PL, Tan TQ, et al. Emergence of multidrug-resistant pneumococcal serotype 35B among children in the United States. J Clin Microbiol 2017;55:724–34.

[42] Janoir C, Lepoutre A, Gutmann L, Varon E. Insight into resistance phenotypes of emergent non 13-valent pneumococcal conjugate vaccine type pneumococci isolated from invasive disease after 13-valent pneumococcal conjugate vaccine implementation in France. Open forum infectious diseases: Oxford University Press; 2016.

[43] Kawaguchiya M, Urushibara N, Kobayashi N. Multidrug Resistance in Non-PCV13 Serotypes of Streptococcus pneumoniae in Northern Japan, 2014. Microbial Drug Resist 2017;23:206–14. https://doi.org/10.1089/mdr.2016.0054.

[44] Sheppard C, Fry NK, Mushtaq S, Woodford N, Reynolds R, Janes R, et al. Rise of multidrug-resistant non-vaccine serotype 15A *Streptococcus pneumoniae* in the United Kingdom, 2001 to 2014. Euro Surveill 2016;21(50). https://doi.org/10.2807/1560-7917.ES.2016.21.50.30423.

[45] Ikuse T, Habuka R, Wakamatsu Y, Nakajima T, Saitoh N, Yoshida H, et al. Local outbreak of Streptococcus pneumoniae serotype 12F caused high morbidity and mortality among children and adults. Epidemiol Infect 2018;146:1793–6.