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ORIGINAL RESEARCH

Serotypes and Genotypes of *Streptococcus pneumoniae* in an Unvaccinated Population in Suzhou, China

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Background: *Streptococcus pneumoniae* is a significant etiological agent of infection and commonly inhabits the human nasopharynx, alongside other potentially pathogenic bacteria. In this study, *S. pneumoniae* strains were obtained from a community population and subjected to investigation of their phenotypes, genotypes, and vaccine coverage.

Methods: *S. pneumoniae* was isolated from nasopharyngeal swab samples of a healthy population in the Guangfu Community. Capsular serotypes and genotypes were identified using Quellung reaction and multilocus sequence typing (MLST), respectively. The antimicrobial susceptibility was tested using minimum inhibitory concentrations.

Results: In total, 500 unvaccinated people were sampled. Ninety-four *S. pneumoniae* strains were identified. Common serotypes were 19F, 6A, and 9V. The strain coverages of PCV13 and PPV23 were 61.7% and 58.5%, respectively. About 27.6% isolates were non-susceptible to penicillin, and over 80% were resistant to erythromycin and doxycycline. Among 27 novel sequence types (STs) identified in all strains, the most common STs were ST236 (6/94, 6.4%) and ST12669 (6/94, 6.4%). Nearly half of the strains were grouped into four clone complexes (CC12665, CC271, CC6011, and CC180), of which CC271 showed the highest resistance to PEN.

Conclusion: In our study, various drug-resistant clone complexes of *Streptococcus pneumoniae* were found in the healthy population, the elderly, and children. Consequently, pneumococcal vaccines should be included in the national immunization schedule to prevent disease spread.

Keywords: vaccine, *Streptococcus pneumonia*, serotype, antibiotics, STs

Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is a major cause of invasive pneumococcal diseases (IPD), including pneumonia, septicemia, and bacterial meningitis, particularly among pediatric and geriatric populations.[1,2] In 2017, an estimated 284000 children succumbed to pneumococcal disease annually, with the majority of cases occurring in developing countries.[3] The primary contributor to *S. pneumoniae* is human nasopharynx. Nasopharyngeal carriage can be a reservoir of lower respiratory tract infection and is a major prerequisite towards the development of pneumococcal diseases.[4,5] The Chinese children between 2 and 4 years of age have a higher carriage than other age groups, which was found to be associated with the administration of antibiotics.[6] Conversely, adults demonstrate lower rates of *S. pneumoniae* carriage.[7]

S. pneumoniae can be categorized into 90 serotypes based on capsular polysaccharide antigens, with 6 to 11 serotypes (1, 5, 6A, 6B, 14, 19F, and 23F) accounting for nearly 70% of IPD cases globally.[8] In China, 14, 19A, and 19F were common serotypes that led to pneumonia and meningitis among children under the age of five from 1980 to 2008.[9] Vaccination has proven to be highly effective in controlling pneumococcal disease. Currently available in China are two

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pneumococcal vaccines (PV): the 13-valent pneumococcal conjugate vaccine (PCV13) and the 23-valent pneumococcal polysaccharide vaccine (PPV23). According to the Expert Consensus on Immunoprophylaxis of Pneumococcal Disease, PCV13 is recommended for infants aged between 6 weeks and 15 months. For certain high-risk groups, it is suggested that children over 2 years of age should be inoculated with PPV23 to reduce the chance of pneumococcal infection.[10] China has not included any PV in its national immunization schedule, resulting in the majority of people needing to pay for the vaccine. Consequently, the corresponding inoculation rate is low across most areas of China. For instance, in 2017, the coverage of PPV23 among individuals over 2 years old in Hangzhou was only 2.98%, with an even lower coverage rate among elderly individuals.[11] On the other hand, after the promotion of PV, non-vaccine serotype (NVT) strains accounted for 42.2% of IPD isolates in children, but differences existed geographically.[12] Certain NVTs, such as serotypes 8, 12F, 24F, and 33F, are at the upper end of the invasiveness spectrum.[13]

The lack of an effective surveillance system has led to insufficient data on *S. pneumoniae* carriage across all age groups in China. In this study, we conducted an investigation into pneumococcal strains and analyzed both phenotypic and genotypic characteristics of isolates from children and adults within a community from November 2014 to January 2015. Our findings provide insight into the characteristics of the unvaccinated population and underscore the importance of PV vaccination efforts.

Materials and Methods

Study Population

Subjects enrolled were from the Guangfu Community of Wuzhong District, 28 km west of Suzhou, which is a peninsula in Tai Lake. The permanent population of this community is over 60000 and shows low population mobility.

From healthy individuals in the Guangfu Community, 500 nasopharyngeal swab samples were randomly collected and assigned to five equal age groups of 100: Group 1(≤5), Group 2(6–17), Group 3(18–34), Group 4(35–64), and Group 5(≥65) from Nov 2014 to Jan 2015. A questionnaire survey was conducted to record each participant's health condition, vaccination, demographic data, and other relevant information.

Culture and Pneumococcal Serotype

All nasopharyngeal swabs were cultured overnight at 37°C in 5% CO_2 on 5% sheep blood agar (Detgerm, China) immediately in a nearby community healthcare center after sampling. *S. pneumoniae* was identified based on colony morphology, α-hemolysis, optochin sensitivity (OXOID, UK) and bile solubility (Fluka Biochemika, Switzerland). We further utilize Vitek 2 (BioMérieux, France) to identify bacterial strains. Pneumococcal serotypes/groups were determined by the Quellung reaction using type-specific antisera (Statens Serum Institut, Denmark) as previously described.[14] Strains that reacted negatively were defined as "unclassified".

Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) of 12 antimicrobial agents against *S. pneumoniae* isolates were determined using microbroth dilution method supplemented with 5% defibrinated sheep blood. The antimicrobials used in the present study were penicillin (PEN,0.06~8μg/mL), cefotaxime (CTX,0.25~4μg/mL), cefepime (FEP,0.125~4μg/mL), meropenem (MEM,0.06~2μg/mL), erythromycin (ERY,0.06~4μg/mL), clindamycin (CLI,0.015~32μg/mL), chloramphenicol (CHL,1~32μg/mL), doxycycline (DOX,0.06~2μg/mL), rifampicin (RD,0.03~4μg/mL), levofloxacin (LEV,0.5~16 μg/mL), sulfamethoxazole (STX, 0.25/4.8~8/152μg/mL) and vancomycin (VAN,0.5~16μg/mL). The sensitivity test pilot plate is commercial reagent (Fosun Diagnose, China). The results were interpreted according to non-meningitis breakpoints of the Clinical and Laboratory Standards Institute (CLSI) criteria (2022, M100). *S. pneumoniae* ATCC 49619 was used as the quality control strain. Multi-drug resistance (MDR) was defined as the resistance to three or more antibiotic classes.[15]

DNA Extraction and Multilocus Sequence Typing (MLST)

Pneumococcal strains were cultured on blood agar plates and incubated overnight at 37°C in 5% CO_2. Then, we use DNA mini-Kit (Qiagen, Germany) to extract bacterial DNA for further use. MLST analysis was performed according to

the description provided on the website (https://pubmlst.org/). *aroE, gdh, gki, recP, spi, xpt*, and *ddl* genes of *S. pneumoniae* strains were amplified and sequenced by Sanger sequencing. The sequence type (ST) of the strains was obtained from the MLST database based on the resulting allelic profile. The new STs were submitted for assignment to the curator of the MLST website. Bionumerics software (version 8.1, Applied-Maths, Belgium) was used to explore the relationships among isolates and to group STs sharing six identical alleles at seven loci into a clonal complex (CC).

Statistical Analysis

Statistical analyses were performed using SPSS (version 24.0, SPSS, USA). Associations between serotypes, age groups, antimicrobial susceptibility, and STs were analyzed using Fisher's exact test, and a Bonferroni correction for multiple testing was applied. Statistical significance was set at $P < 0.05$.

Ethics Statement

The National Health Commission of the People's Republic of China ruled that the collection of data from population was part of the public health investigation and thus, the investigation was exempt from institutional review board assessment. Adult subjects agreed and signed the informed consent, while the minors (under 18 years) agreed and signed by their guardians before participating in the study. The data set was not anonymized in the investigation but was anonymized before data analysis. All procedures were performed in accordance with the Declaration of Helsinki.

Results

Demographic Characteristics and Carriage of S. pneumoniae

Five hundred unvaccinated people from five age groups were sampled. The age range of the sampled population was from 1 to 86 years old. A total of 94 strains of *S. pneumoniae* were isolated (Table 1). The total carriage rate of the population reached 18.8% (95% CI%: 15.6, 22.4). Group 2 had a higher carriage (42.0%, 95% CI%: 32.8, 51.8) than Groups 3, 4 and 5. Furthermore, the carriage in minors (<18 years) was significantly higher than in adults ($P<0.001$). About 18.4% (41/223) male carried *S. pneumoniae*, while in female, it reached to 19.1% (53/277). There was no statistical difference in the carriage between the genders ($P=0.832$).

Serotype Distribution and Vaccines Coverage

Among the 94 isolates, 89 were divided into 31 serotypes, and the remaining five strains were unclassified. The most prevalent serotypes were 19F (12.8%), 6A (7.4%), 9V (7.4%), 18C (6.4%), 19A (6.4%), 3(5.3%), 15A (5.3%), and 23F (5.3%), accounting for 56.4% of all strains. In Group 1, 19F, 19A, and 6A were common serotypes, nevertheless in Group 2, 18C, 15A, and 9V (Figure 1).

There were 15 serotypes and 57 isolates were covered by PPV23, as well as 10 serotypes and 56 isolates were covered by PCV13. Fifteen serotypes and 30 strains were NVT, in which the most common serotypes were 15A (16.7%), 6C (13.3%) and 35F (6.7%). The coverage rate of PV in different age groups was distinction (Figure 2). Group 1

Table 1 General Characteristics of the Pneumococcal Carriage Survey Population

Age Group	Median Age (year)	Chronic Disease (Hypertension, Diabetes)	Gender		Carriage Rate,%	P
			Male	Female		
Group 1 (≤5)	3.0	0	56	44	27.0	0.069
Group 2 (6–17)	9.5	0	52	48	42.0.	Reference
Group 3(18–34)	26	0	37	63	10.0	<0.001
Group 4(35–64)	51.5	28	21	79	7.0	<0.001
Group 5(>65)	73	55	57	43	8.0	<0.001
Total	26	83	223	277	18.8	

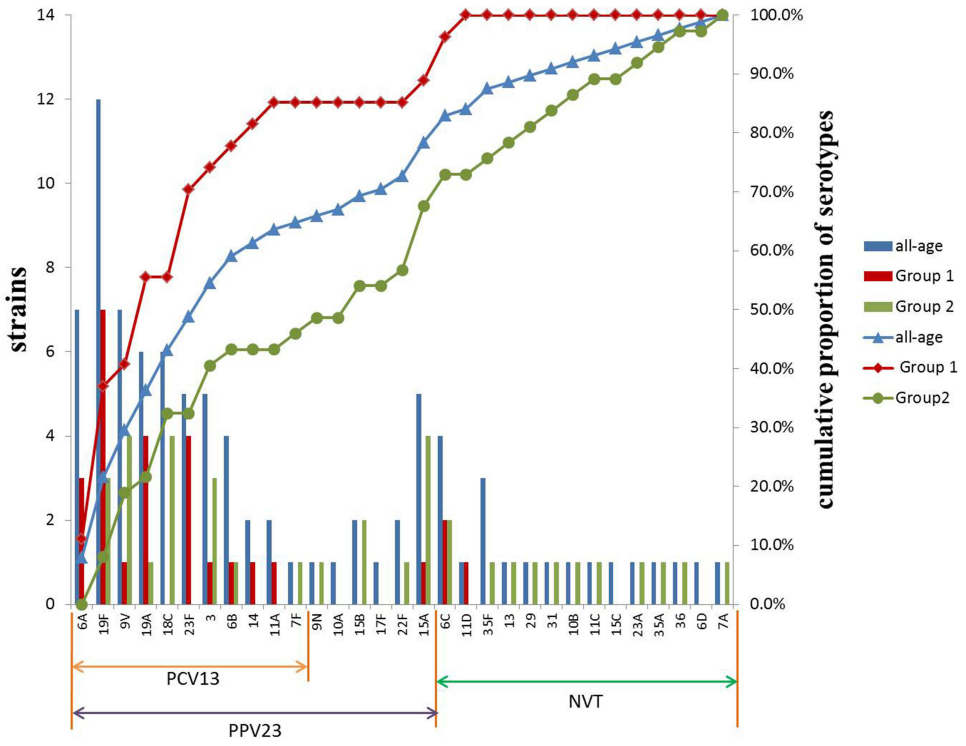

Figure 1 Serotypes distribution and cumulative proportion in all-age, Group 1 and Group 2.

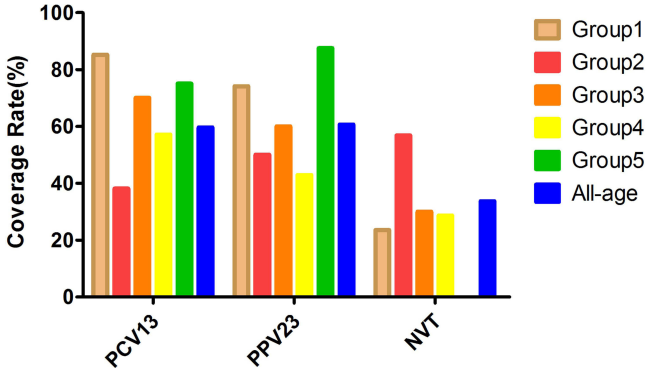

Figure 2 Coverage rates of PV and NVT in different age groups.

exhibited the highest PCV13 coverage at 85.2%, while Group 5 achieved the highest PPV23 coverage at 87.5%. NVT was not found in Group 5.

19A (9.8%) and 19F (9.8%) were common in male, but in female, they change to 19F (15.1%) and 9V (11.3%). The coverage rates of PCV13 and PPV23 strains were not different between male and female ($P_{PCV13\text{-}gender}$=0.147, $P_{PPV23\text{-}gender}$=0.877).

Antimicrobial Resistance in S. pneumoniae

The susceptibilities and MICs of *S. pneumoniae* strains to the 12 antibiotics are summarized in Table 2. All the strains were susceptible to RD and VAN. The percentage of resistant to ERY, CLI, DOX, and SXT was 93.6%, 79.8%, 83.0%, and 46.8%, respectively. Less than 10% strains were resistant to PEN, CTX, FEP, and CHL. The overall rate of MDR was 76.6%. The most common MDR pattern was ERY-CLI-DOX-SXT (n = 27, 28.7%) followed by ERY-CLI-DOX (n = 23, 24.4%).

Table 2 Antimicrobial Susceptibility and MICs of S. Pneumoniae Strains

Antibiotics	MIC (μg/mL)			No. of Strains (%)		
	50%	90%	Range	R	I	S
Penicillin (NM)	0.125	4	≤0.06–>8	8(8.5)	18(19.1)	68(72.4)
Rifampicin	≤0.03	≤0.03	≤0.03	0(0.0)	0(0.0)	94(100.0)
Cefotaxime	0.06	1	≤0.03–>4	5(5.3)	3(3.2)	86(91.5)
Cefepime	0.125	1	≤0.03–>4	2(2.1)	4(4.3)	88(93.6)
Meropenem	≤0.03	0.5	≤0.03–1	0(0.0)	15(16.0)	79(84.0)
Vancomycin	≤0.03	0.25	≤0.03–0.25	0(0.0)	0(0.0)	94(100.0)
Erythromycin	>4	>4	≤0.03–>4	88(93.6)	2(2.1)	4(4.3)
Clindamycin	>2	>2	≤0.03–>2	75(79.8)	2(2.1)	17(18.1)
Doxycycline	>2	>2	≤0.015–>2	78(83.0)	3(3.2)	13(13.8)
Levofloxacin	0.5	1	≤0.06–4	0(0.0)	1(1.1)	93(98.9)
SXT	2/38	>8/152	≤0.12/2.4–>8/152	44(46.8)	8(8.5)	42(44.7)
Chloramphenicol	2	4	1–>16	10(10.6)	84(89.4)	72(76. 6)

Further analysis revealed that age and serotypes were related to drug resistance, while gender was not. Among the different age groups, Group 1 and Group 2 showed higher resistance to PEN than other groups (P = 0.008).

Strains of the five dominant serotypes (19F, 6A, 9V, 18C, and 19A) resistant to major antibiotics are shown in Figure 3A. Serotypes 19A showed higher resistance to PEN than serotypes 18C, 6A and 9V. There were no significant differences in the resistance to other antibiotics among the five main serotypes (Figure 3A). However, the five dominant serotypes had a higher MDR rate (81.6%) than the other serotypes (P<0.001). Among the vaccine-covered serotypes, PCV13 and PPV23 covered serotypes had higher resistance rate of PEN than NVT (Figure 3B).

MLST

These 94 strains belonged to 64 different STs, 27 of which were novel (ST12649–12671 and ST12676–12,678). Twenty-two STs were divided into four clone complexes (CC), named CC12665, CC271, CC6011 and CC180, representing for 47.9% of all isolates (Table 3), the rest 42 STs were ungrouped STs (UG-STs). The main STs were ST236 (6/94, 6.4%), ST12669 (6/94, 6.4%), ST320 (5/94, 5.3%) and ST6011 (5/94, 5.3%). ST236, ST12669, and ST230 strains were grouped into serotypes 19F, 9V, and 19A, respectively. Most ST6011 isolates were serotyped as 15A alone, except for one ST6011 strain. All STs of CC12665 were serotyped as 6A, but ST12652 was 23A (Figure 4A). Nine strains were resistant to PEN, eight of which belonged to CC271 (Figure 4B).

To compare the drug resistance rate between CCs, there was a statistical difference between CC180 and other CCs in SXT and CHL. In addition, CC271 had a higher resistance rate of PEN than any other CCs (Table 4).

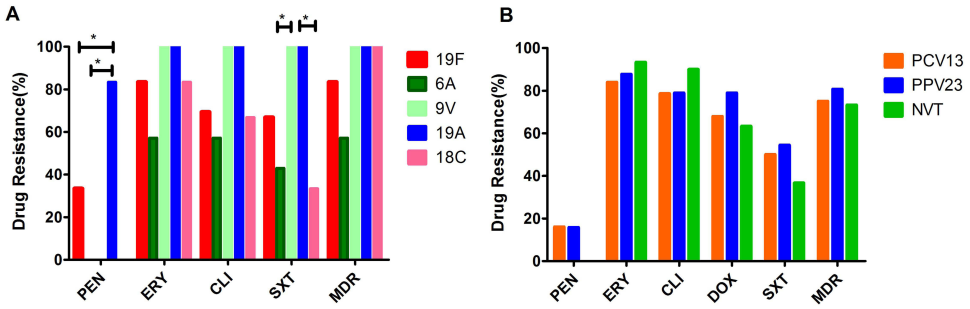

Figure 3 Resistant rate of major antibiotics in various serotypes. (**A**), five dominated serotypes. (**B**), PCV13, PPV23 covered serotypes and NVT,* indicates a statistical difference between two columns (P<0.05).

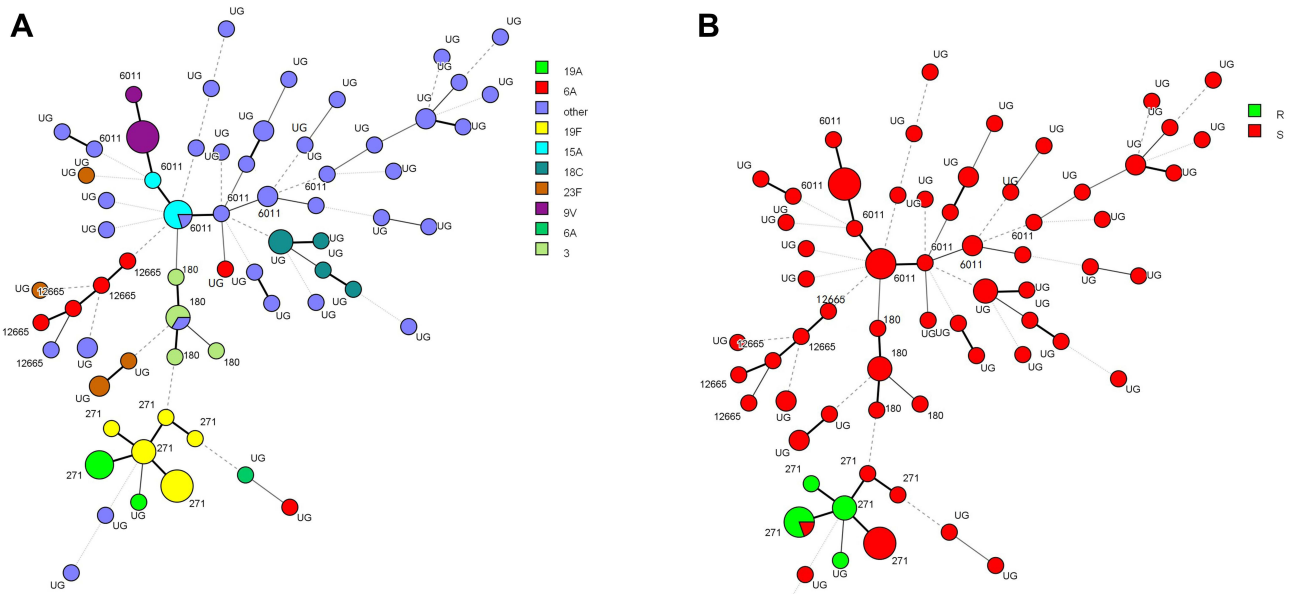

Figure 4 The cluster analysis with CCs and genotypic characters. (**A**) CCs and main serotypes, (**B**) CCs and PEN resistance. The size of the circle corresponds to the number of strains belonging to a ST. The lines indicate the presence of single locus variant links among particular STs.

Discussion

Data show that high carriage of *S. pneumoniae* is closely related to elevated incidence of pneumococcal disease. Through the nasopharyngeal swab sampling survey, we can directly understand the carriage of the population. In this study, the pneumococcal carriage in children ≤5 years is similar to a previous report in mainland China,[16] but higher than Hong Kong (13.5%) and Taiwan (14.1%).[17,18] It was greatly lower than in low-income countries, such as Bangladesh, Gambia and Kenya.[19–21] As known to all, children that constitute the main reservoir of *S. pneumoniae* are probably the vector for the spread of the bacterium to adults.[22,23] Out of the ordinary, the highest prevalence (42%) was between 6 and

Table 3 Distribution of Serotypes and STs in Different CCs

CC	ST	Serotype	NO. (%)
12,665	12,665, 3173, 12,646, 12,652, 12663	6A, 23A	5(5.3)
271	271, 2323, 236, 8592, 320	19A, 19F	17(18.1)
180	180, 297, 505, 12,677	3, 6C	6(6.4)
6011	6011, 99, 2647, 12,651, 12,657, 12,669, 12676	9V, 11A, 11C, 11D, 14, 15A	17(18.1)

Table 4 Drug Resistance of Different CCs

Antibiotics	CCs (%)			
	180	271	6011	12665
Penicillin (NM)	0(0.0)[a]	8(47.6)[b]	0(0.0)[a]	0(0.0)[a]
Erythromycin	6(100.0)[a]	15(88.2)[a]	15(88.2)[a]	3(60.0)[a]
Clindamycin	5(83.3)[a]	14(82.4)[a]	15(88.2)[a]	3(60.0)[a]
Doxycycline	4(66.7)[a]	12(70.6)[a]	11(64.7)[a]	4(80.0)[a]
SXT	0(0.0)[b]	11(64.7)[a]	10(58.8)[a]	2(40.0)[a]
Chloramphenicol	2(33.3)[b]	1(5.9)[a]	1(5.9)[a]	0(0.0)[a]

Notes: a, b, pair-to-pair comparison of drug resistance rate, different letters (a and b) indicate the existence of statistical differences.

17 years, which was different from other researches.[24,25] This indicates that adolescents may be a non-negligible spread source owing to their greater range of motion and mobility compared to children.

In the absence of PV introduction, healthy individuals can exhibit a wide range of serotypes. Amongst the 2063 villagers in Gambia, it was found that most serotypes accounted for 74 cases.[26] The dominant serotypes among all identified were found to be distinct from those observed in a healthy population residing in Xinjiang Uygur Autonomous Region of China,[27] namely, 19F, 6A and 9V. Serotype prevalence varies across low- and lower-middle-income countries, with types such as, 6A, 6B, 19A, 19F and 23F being commonly encountered.[28] In addition, the common serotypes in healthy children (19F, 19A, and 6A) in our study were similar to those in the sick population globally.[29,30] Moreover, a predominant number of serotypes (1, 5, 6A, 6B, 14, 19F, and 23F) are responsible for the majority of IPD cases worldwide. The inclusion of these specific serotypes in current PCV formulations accounts for approximately 49% to 88% of mortality rates in Africa and Asia.[8] This suggests that these particular serotypes may contribute to the occurrence of pneumococcal diseases in immunocompromised children.

The PCV13 was introduced in China in 2017. None of the individuals were vaccinated in our study. Our findings indicate that PCV13 provided coverage for 77.8% and 62.5% of isolates from children and the elderly, respectively. In Chongqing, PCV13 could potentially cover 85.5% of serotypes in children with pneumonia.[31] It is important to note that despite not being included in the Chinese Expanded Program on Immunizations (EPI), the PCV13 vaccine is not currently available free of charge. PPV23 has been shown to increase anti-capsular antibody titers for specific serotypes in individuals over 2 years old;[32–34] however, it only covered 40.9% of isolates from seniors with influenza-like illness in the Netherlands.[35] Although data were limited, we observed that PPV23 covered 87.5% strains from aged individuals, which may explain why more cities are providing free PPV23 for people over 65 years old in China, particularly in Jiangsu Province such as Suzhou, Nantong and Wuxi.

Two newer pneumococcal conjugate vaccines, PCV15 and PCV20, were approved in 2021 leading to the discontinuation of PCV13 wherever these newer vaccines are available.[36] In our study, these two PCVs can cover approximately an additional 2–7% more serotypes than PCV13. So far, China has not yet introduced these new PCVs, but Hong Kong has done so. Certainly, the implementation of PVs is expected to result in alterations in the serotype distribution of S. *pneumoniae* and a rise in the prevalence of NVT. The proportion of 12F in IPD isolates increased from 4.4% to 24.6% during 2015 to 2017 in Japan.[37] Denmark observed an increase of IPD due to *S. pneumoniae* serotype 24F.[38] These serotypes were not found in this study. As vaccination rates continue to rise, it is imperative that NVT receives heightened attention and rigorous monitoring in order to ensure comprehensive public health management.

The resistance of *S. pneumoniae* to various antibiotics, including β-lactams and macrolides, is on the rise in China,[39] posing challenges for the treatment of pneumococcal infections. Approximately 10% of isolates were found to be resistant to PEN, with a higher prevalence among pediatric cases. This finding surpasses the rates observed in isolates causing invasive disease among Chinese children from multiple cities and south Brazil,[40,41] but aligns closely with those from Suzhou.[42] The variation may stem from differing strategies employed for managing pneumococcal infections and suggests that *S. pneumoniae* resistance in China has remained consistent over time.[43] Notably, our observations indicate that PCV13 serotype strains exhibit greater resistance to PEN and CFX compared to non-PCV13 serotype strains. These findings suggest that PCV13 vaccination may serve as an effective measure for controlling the escalation of drug resistance.

MLST analysis revealed significant diversity among all strains in terms of their STs. We confirmed that the STs (ST236, ST12669, ST320, and ST6011) differed from those identified in another study on children with respiratory infections in Suzhou.[44] The STs of *S. pneumoniae* were strongly associated with PEN resistance, with eight out of nine resistant strains belonging to the same clonal complex CC271 and having sequence types ST271, ST320, and ST8592. These resistant strains corresponded to serotypes 19A and 19F. This trend is consistent with previous studies, except for the susceptibility of ST236 and ST876 to PEN.[45,46] Notably, while ST320 has emerged as a major ST among 19A *S. pneumoniae* strains from North America and France,[47,48] it accounted for only 6% of our collections. In China, serotypes 23F and high β-lactam antibiotic-resistant sequence type (ST81) have replaced serotype 342 as predominant;[49] however, we only identified two susceptible-to-PEN isolates belonging to ST81.

Conclusions

The strains of the population from an unvaccinated community showed high resistance to erythromycin and clindamycin between 2014 and 2015. Moreover, the specific clone complex (CC271) of penicillin-resistant strains isolated from children should not be ignored. Due to the low vaccination rate to date, PCV13 and PPV23 could be included in EPI not only to protect children and seniors because of the high coverage of frequent serotypes but also to curb the rapid spread of resistant strains.

Disclosure

The authors report no conflicts of interest in this work.

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